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## Manuscript

**Title:** A stress-induced rice  $\beta$ -glucosidase represents a new subfamily of glycosyl hydrolase family 5 containing a fascin-like domain

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## SYNOPSIS

The cDNA for a stress-induced glycosyl hydrolase family 5 (GH5)  $\beta$ -glucosidase, *GH5BG*, was cloned from rice seedlings. The *GH5BG* cDNA encodes a 510 amino acid precursor protein that comprises 19 amino acids of prepeptide and 491 amino acids of mature protein. The protein was predicted to be extracellular. The mature protein is a member of a rice-specific subfamily of GH5 proteins that contain two major domains, a  $\beta$ -1,3-exoglucanase-like domain and a fascin-like domain, which is not commonly found in plant enzymes. The *GH5BG* mRNA is highly expressed in the shoot during germination and in leaf sheaths of mature plants. The *GH5BG* was up-regulated in response to salt stress, submergence stress, methyl jasmonate, and abscissic acid in rice seedlings. A thioredoxin fusion protein produced from the *GH5BG* cDNA in *Escherichia coli* hydrolyzed various *p*-nitrophenyl-glycosides, including  $\beta$ -D-glucoside,  $\alpha$ -L-arabinoside,  $\beta$ -D-fucoside,  $\beta$ -D-galactoside,  $\beta$ -D-xyloside and  $\beta$ -D-cellobioside, as well as  $\beta$ -(1,4)-linked glucose oligosaccharides and  $\beta$ -(1,3)-linked disaccharide (laminaribiose). The catalytic efficiency ( $k_{cat}/K_m$ ) for hydrolysis of  $\beta$ -(1,4)-linked oligosaccharides by the enzyme remained constant as the degree of polymerization (DP) increased from 3 to 5. This substrate specificity is significantly different from fungal GH5 exoglucanases, such as *Candida albicans* exo- $\beta$ -(1,3)-glucanase, which may correlate to a marked reduction a loop that makes up the active site wall in the *Candida* enzyme.

**Keywords:**  $\beta$ -glucosidase, rice, glycosyl hydrolase family 5, fascin-like domain, recombinant protein expression, environmental stress

**Abbreviations:** ABA, abscissic acid; *C. albicans* Exg, Exo- $\beta$ -(1,3)-glucanases of *C. albicans*; DP, degree of polymerization; GH, Glycosyl hydrolases; GH5, glycosyl hydrolase family 5; IMAC, immobilized metal affinity chromatography; ORF, open reading frame; *p*NP, *p*-nitrophenyl; *p*NPG, *p*NP- $\beta$ -D-glucoside; PGO, peroxidase/glucose oxidase

## INTRODUCTION

Glycosyl hydrolases (GH), enzymes that catalyze the hydrolysis of glycosidic bonds between sugars and other moieties, can be classified into more than 100 families [1] (for up-to-date information see Carbohydrate-Active Enzymes database (CAZY) at <http://www.cazy.org/CAZY/index.html>). Based on their 3-dimensional structures, GH can be grouped into clans of related structures [1]. Clan A is the largest group and contains 17 families, the structures of which contain a core ( $\beta/\alpha$ )<sub>8</sub> barrel with two catalytic amino acid residues, an acid/base and a nucleophile, on the ends of strands 4 and 7 of the barrel, respectively [2-4]. GH family 5 (GH5) is one clan A family that contains enzymes with a wide range of catalytic activities, including cellulases, chitosanases, endoglucanases, exoglucanases, exoxylanases, endoxylanases, and mannanases, with those that have investigated coming primarily from microorganisms [5-10], though a  $\beta$ -mannanase from a plant was recently described [11]. Although there is much variation in the protein sequences and enzyme activities of the family members, they all possess eight conserved residues (including two glutamate residues acting as catalytic acid/base and nucleophile) around the active site, which distinguish GH5 from other GH families [8, 12].

There are now twenty-one known GH5 3-D crystal structures: endoglucanases from *Acidothermus cellulolyticus*, *Bacillus agaradhaerens*, *Bacillus sp.*, *Clostridium thermocellum*, *Erwinia chrysanthemi*, *Pseudoalteromonas haloplanktis*, *Thermobifida fusca*, and *Thermoascus aurantiacus*; exo- $\beta$ -(1,3)-glucanases (Exg) from *C. albicans* and *Saccharomyces cerevisiae*; mannanases from *Bacillus sp.*, *Cellvibrio mixtus*, *Thermobifida fusca*, *Thermotoga maritima*, *Hypocrea jecorina*, *Lycopersicon esculentum*, and *Mytilus edulis*; and xylanase from *Hypocrea jecorina* ([4]; <http://www.CAZY.org/CAZY/index.html>). This abundance of structural data is necessary, since the similar overall structure of GH5 has resulted in several distinct activities, as indicated. Though these enzymes may have similar ( $\beta/\alpha$ )<sub>8</sub> barrel structures, differences in the loops at the ends of the  $\beta$ -strands of this barrel result in active site clefts ranging from long grooves to slot-like pockets [8, 13].

Exoglucanases are generally secreted enzymes with both hydrolase and transferase activities on  $\beta$ -glucans [14]. Exo- $\beta$ -(1,3)-glucanases may act in the metabolism of cell wall glucan by cleaving a single glucose from the nonreducing end of  $\beta$ -1,3-glucans [15]. Most GH5 exoglucanases that have been studied are fungal exo- $\beta$ -(1,3)-glucanases, including those from *C. albicans* [8, 16], *S. cerevisiae* [17], *Agaricus bisporus* [18], *Lentinula edodes* [19], and *Pichia pastoris* [20]. Cutfield et al. [8] reported the structure of *C. albicans* Exg to be a distorted ( $\beta/\alpha$ )<sub>8</sub> barrel structure with a deep active-site pocket. The geometry of the pocket fits

for cleavage of  $\beta$ -1,3- but not  $\beta$ -1,4-glycosidic linkages. According to active site labeling and mutagenesis experiments, Glu-192 and Glu-292 in the mature *C. albicans* Exg protein were identified as the proton donor and nucleophile, respectively [14, 16].

There has been no previous report of characterization of a GH5 exoglucanase from a plant. However, several genes encoding proteins similar to fungal  $\alpha$ -1,3- $\beta$ -glucanases are found in the genomic sequences from rice. In this study, we cloned cDNA of one of a putative GH5 glucan-1,3- $\beta$ -glucosidase containing a fascin-like domain near its N-terminus from germinating rice based on genomic data, and assessed its function by recombinant protein expression and its expression by northern blot. The catalytic activity indicated the enzyme is a  $\beta$ -glucosidase. This is the first report of a GH5  $\beta$ -glucosidase from a plant that contains a fascin-like domain.

## EXPERIMENTAL

### Plant materials and growth conditions

Rice (*Oryza sativa* L. spp. *indica* cv. KDML105 and spp. *japonica* cv. Yukihihikari) seeds were germinated in the dark from day 0 to day 3 and in 12 h light-12 h dark from day 4 to day 7 at 28°C on germinating paper moistened with sterile distilled water. For expression analysis whole Yukihihikari seedlings were harvested and some were dissected into separate parts (shoot, root, and endosperm) and kept at -70°C. Some 14-d-old rice seedlings were transferred to soil and grown for an additional 4 weeks. Rice plants were harvested and separated to six parts (flower, stem, root, node, leaf blade, and leaf sheath). Some 7-d-old rice seedlings were exposed to abiotic stresses and plant hormones for an additional 2 days under the following conditions: salt stress (0.3 M NaCl), osmotic stress (0.3 M sorbitol), drought (no water), flooding (full submergence of seedlings 1 cm below surface of distilled water), cold stress (5 and 12°C), heat stress (37°C),  $10^{-4}$  M methyl jasmonate,  $10^{-4}$  M ABA (abscissic acid), and 1 mg/mL ethephon. All plant samples were kept at -70°C for RNA isolation.

### Cloning of GH5BG cDNA

Total RNA was isolated from 100 mg 5-6-d-old rice cv. KDML105 seedlings with Trizol Reagent, and 5  $\mu$ g of total RNA was used as the template to synthesize the first-strand cDNA with SuperScript II reverse transcriptase according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). The GenBank rice genome contig accession number AC107314 (deduced protein sequence GenBank AC AAM08614) and AK065000 cDNA sequences [21] were used to design the primers to amplify a full-length coding sequence (CDS) cDNA and a

cDNA encoding the mature protein of rice glycosyl hydrolase family 5  $\beta$ -glucosidase (designated *GH5BG*). The 5' sense primer AK065000f (5'-GCTGAAAAATCTTCGTCTTCATC-3') and the antisense primer AAM08614EcoRIr (5'-CCATCCAACCTGGAATTCTCACTG-3') were used to amplify a 774 bp-5' PCR fragment. The 5' sense primer AAM08614EcoRIr (5'-CGCAGTGAGAATTCCAGTTG-3') and the antisense primer AK065000r (5'-CTTCACAAGAGAAAGTTACACTC-3') were used to amplify a 1016 bp-3' PCR fragment. The amplification for 5' and 3' PCR fragments was done with *Pfu* DNA polymerase (Promega, Madison, WI) with the first-strand cDNA as the template. Finally, the AK065000f and AK065000r primers were used to amplify a full CDS cDNA by using the 5' and 3' PCR cDNA fragments as template in overlapping PCR. A full-length product was cloned into the *EcoR* V site of pBlueScript II SK+ (Stratagene, La Jolla, CA), and sequenced.

Protein sequence alignments were done with the ClustalX implementation of ClustalW [22, 23] and manually adjusted with the Gendoc program [24]. Protein analyses were done at the ExPASy proteomics server (<http://www.expasy.org>), and the signal sequence and cellular location were predicted with SignalP [25] and PSORT [26], respectively.

### **Recombinant protein expression in *E. coli***

The cDNA encoding the predicted mature protein of the *GH5BG* was PCR amplified with the cloned full-length cDNA as the template, the AAM08614matNcoIf (5'-CACCATGGTCTCCGATGGGAGGACG-3') and AAM08614XhoIstopr (5'-CCCTCGAGCTAGCTTTTGAGAGAGATGATCC-3') primers and *Pfu* DNA polymerase to introduce an *NcoI* site at the 5' end and an *XhoI* site at the 3' end. The amplification was done as described above, but with 45°C annealing temperature. The cDNA product was digested with *NcoI* and *XhoI*, cloned into pENTR4 Gateway entry vector that had been digested with the same restriction enzymes, and subcloned into the pET32a+/DEST Gateway expression vector [27] by LR Clonase recombination by the recommended protocol (Invitrogen) and thoroughly sequenced. The recombinant pET32a+/DEST-*GB5BG* plasmid was transformed into *E. coli* strain OrigamiB (DE3) [28], and positive clones were selected on 15  $\mu$ g/ml kanamycin, 12.5  $\mu$ g/ml tetracycline and 50  $\mu$ g/ml ampicillin LB-agar.

To produce the protein, selected clones were grown in the selection media at 37°C until the optical density at 600 nm reached 0.5-0.6, then induced with 0.5 mM IPTG at 20°C for 12 h. Induced cultures were harvested by centrifugation at 3000  $\times$  g at 4°C for 10 min. The cell

pellets were resuspended in freshly prepared extraction buffer (50 mM sodium phosphate, pH 8.0, 200  $\mu$ g/ml lysozyme, 1% Triton-X 100, 1 mM phenylmethylsulfonyl- fluoride, 40  $\mu$ g/ml DNase I), and incubated at room temperature for 30 min. The soluble protein was recovered by centrifugation at 12,000 x g at 4°C for 10 min. The expressed thioredoxin-GH5BG fusion protein was purified by immobilized metal affinity chromatography (IMAC) with BD TALON cobalt resin according to the manufacturer's instructions (Clontech, Palo Alto, CA). The fractions with *p*NPG hydrolysis activity were pooled and concentrated with 10 kD-cut off centrifugal ultrafiltration membranes (YM-10, Amicon, Beverly, MA). All of the protein samples were analyzed by SDS-PAGE by standard methods [29].

### Enzyme assays and kinetic analysis

Kinetic parameters were calculated from triplicate assays of 5-7 substrate concentrations done at 37°C in 50 mM sodium acetate, pH 5.0. The purified thioredoxin-GH5BG recombinant protein was tested against *p*NP derivatives of monosaccharides and cellobioside to determine sugar specificity. In a 100  $\mu$ L reaction assay volume, 1.47-2.94 pmol enzyme was incubated with substrate in 50 mM sodium acetate, pH 5.0, at 37°C, except for the assay with *p*NP- $\beta$ -D-cellobioside, in which 29.4 pmol enzyme was used. At the end of the reaction time, 70  $\mu$ L of 0.4 M sodium carbonate was added to stop the reaction, and the absorbance of the liberated *p*NP was measured at 405 nm. The enzyme was tested with oligosaccharides including cello-oligosaccharides with DP of 2-6, laminari-oligosaccharides DP 2-5 and gentiobiose. In a 50  $\mu$ L reaction volume, 0.74 pmol enzyme was incubated with substrate in 50 mM sodium acetate, pH 5.0, for 5 min at 37°C, except for the assay with cellobiose, in which 14.7 pmol enzyme was used. The reactions were stopped by boiling, and the glucose released was quantified by the PGO (peroxidase/glucose oxidase) assay method [30, 31].

The enzyme was also tested for hydrolysis of polysaccharides. In the assay, 1-5  $\mu$ g enzyme was incubated separately with 0.5% (w/v) laminarin and barley  $\beta$ -glucans in 50 mM sodium acetate, pH 5.0, at 37°C for 30-60 min. The reaction was stopped by the addition of *p*-hydroxybenzoic acid hydrozide reagent and the increase in reducing sugars was measured colorimetrically, as described by Lever [32]. Protein assays were performed by the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA) using bovine serum albumin as a standard. Kinetic parameters,  $K_m$  and  $V_{max}$  (at pH 5.0 and 37°C), were calculated by linear regression of Lineweaver and Burk plots with the Enzfitter computer program (Elsevier Biosoft, Cambridge, U.K.). Note that the activity values for disaccharides were determined by dividing the amount

of glucose released by two, since two glucose molecules are released per molecule of disaccharides hydrolyzed. The amount of products for oligosaccharides is given in terms of total glucose released, since release of more than one glucose per substrate molecule due to sequential cleavage should be negligible for  $V_0$ .

The pH optimum was determined by measuring the release of *p*NP from *p*NPG in different 50 mM buffers ranging in pH from 3.5 to 10 in 0.5 pH unit increments for 10 min (formate, pH 3.5-4.5; sodium acetate, pH 4.0-5.5; sodium phosphate, pH 5.5-8; Tris, pH 7.5-9.0; CAPS, pH 9.0-10).

### Northern blot analysis

*GH5BG* gene-specific probe was amplified using rice genomic DNA as the template with the AAM08614\_Cterf (5'-GAATGTGCAGGGAGCATC-3') and AAM08614\_3UTRr (5'-CTTTAATTCAGCTTCAC-3') primers derived from the C-terminal part of the CDS and 3'-untranslated region of the gene, respectively. The rice *18S rRNA* probe (342 bp) was PCR amplified using first-strand cDNA synthesized from RNA extracted from 6-d-old rice seedlings as template with the *18Sricef* (5'-AAGTTTGAGGCAATAACAG-3') and *18Sricer* (5'-CCTCTAAATGATAAGGTTC-3') primers, derived from the GenBank accession number AF069218 sequence. The amplification for both probes was done with *Taq* DNA polymerase (Roche Diagnostics, Indianapolis, USA).

Total RNA was isolated from different parts of rice (*Oryza sativa* cv. Yukihihikari) plants at various developmental stages and environmental conditions by the method of [33]. Twenty micrograms of total RNA from each sample was denatured and electrophoresed on a 1.2% formaldehyde-agarose gels and transferred onto Hybond N<sup>+</sup> nylon membrane (Amersham-Pharmacia, Uppsala, Sweden) by standard procedures [28]. RNA blots were hybridized separately with the  $\alpha$ -[<sup>32</sup>P]dCTP-labeled gene-specific probe for *GH5BG* (396 bp), and *18S rRNA* (342 bp) for 16 h at 65°C. The blots were then washed once in 0.1% SDS, 2 x SSC for 20 min at 65°C and twice in 0.1% SDS, 0.1 x SSC for 20 min at 65°C. The membranes were then exposed to x-ray film for signal detection for 4 to 6 days at -80°C.

## RESULT AND DISCUSSION

### GH5BG cDNA cloning and sequence analysis

With the completion of high quality drafts of the rice genome, analysis of GH5 members in rice has been reported in the CAZY homepage (<http://www.cazy.org/CAZY/>). Twenty GH5 genes putatively encoding 7 cellulases, 9 endo- $\beta$ -mannanases, 3 glucan 1,3- $\beta$ -glucosidases, and one 1,3- $\beta$ -glucanase have been identified in rice databases (see CAZY). The putative glucan 1,3- $\beta$ -glucosidases encoded by these genes include Genbank accession numbers (AC) AAM08614, AAM08620, and AAV43969. A BLAST comparison of AAM08614 with the others showed that AAM08620 contains 3 repeats of homologous sequences, each of which has 71% identity to AAM08614. The AAV43969 sequence is 69% identical to the AAM08614 sequence. The amino acid sequence of the putative 1,3- $\beta$ -glucanase BAD10703 is also 49% identical to AAM08620, which has only 28-33% identity with glucan-1,3- $\beta$ -glucosidases of fungi, so BAD10703 is more closely related to AAM08620, despite its different annotation. Therefore, there appear to be four putative rice glucan 1,3- $\beta$ -glucanase genes, of which AAM08614 was chosen for investigation.

A GH5 glucan-1,3- $\beta$ -glucosidase cDNA, designated GH5BG, was cloned from rice seedlings by RT-PCR with KDML105 rice seedling RNA and primers derived from the GenBank accession number AC107314 (rice genomic contig from which AAM08614 is derived) and AK065000 (full-length cDNA, [21]) sequences. A specific PCR product of 1680 bp was produced, and its sequence overlapped those of AC107314 and AK065000. The full-length cDNA sequence contains a 1530-nucleotide open reading frame (ORF) encoding a 510 amino acid precursor protein. The protein sequence was predicted to contain a 19 amino acid long prepeptide and a 491 amino acid long mature protein, and to be secreted out of the cell. Its predicted pI is 5.28. The mature protein includes two domains, a fascin-like domain (amino acids 70-180) and a glucan-1,3- $\beta$ -glucosidase domain (amino acids 37-60 and 208-496) (Figure 1). The fascin-like domain found at the N-terminus of this enzyme is not commonly found in plant enzymes, but it aligned well with the N-terminus of fascin found in sea urchin, *Drosophila*, *Xenopus*, mouse, and human [34-38]. BLAST search analysis in GenBank revealed the fascin-like domain was found in 5 plant enzymes, at the N-terminus of a putative *Medicago truncatula* endoglucanase, AC ABE91799, and the previously described putative rice GH5 glucan-1,3- $\beta$ -glucosidases, AC AAM08614 (GH5BG), AAM08620, AAV43969, and BAD10703. In human, fascin contributes to the bundle of F-actin [39]. However, this action requires a cytoplasmic location, whereas GH5BG is predicted to be extracellular, so it is

unlikely to interact with intracellular actin molecules. In fact, all the plant sequences with the fascin-like domain, except AAM08620, contain signal sequences for secretion, so the fascin-like domain may have been adapted for binding other molecules or to bind actin in the case of cell lysis.

### Functional expression of recombinant GH5BG and substrate specificity

The *GH5BG* cDNA was expressed in redox-deficient, Origami (DE3), as a catalytically active thioredoxin fusion protein. Induced cultures of *E. coli* Origami (DE3) expressing GH5BG thioredoxin fusion proteins had intense bands at 68 kD in total cell lysates in SDS-PAGE (Figure 2). The fusion protein was purified and found to hydrolyze *p*-nitrophenyl  $\beta$ -D-glucopyranoside (*p*NPG) with an optimal pH of 5.0. The activity of the purified GH5BG towards artificial glycosides and oligosaccharides is summarized in Table 1. The GH5BG hydrolyzed *p*-nitrophenyl (*p*NP)-glycosides of the monosaccharides,  $\beta$ -D-glucoside,  $\beta$ -D-fucoside,  $\beta$ -D-galactoside,  $\beta$ -D-xyloside, and  $\alpha$ -L-arabionoside, but it could not hydrolyze *p*NP- $\beta$ -D-mannoside. Among *p*NP-glycosides, *p*NP- $\beta$ -D-fucoside was hydrolyzed twice as efficiently as *p*NPG, whereas *p*NP- $\beta$ -D-galactoside and *p*NP- $\alpha$ -L-arabionoside were hydrolyzed with 27% and *p*NP- $\beta$ -D-xyloside with 15% the efficiency of *p*NPG. These results indicate there is low stringency at the -1 subsite of GH5BG, in which the non-reducing glycosyl moiety is bound, which is somewhat similar to many GH1 and GH3  $\beta$ -glucosidases, such as rice BGlu1 [32, 40]. The enzyme could hydrolyze glycosides of  $\beta$ -D-glucose,  $\beta$ -D-fucose,  $\beta$ -D-galactose,  $\beta$ -D-xylose, and  $\alpha$ -L-arabionoside, which are epimers with equatorial OH-2, but not  $\beta$ -D-mannoside, which has an axial projection at OH-2. Therefore, the epimerization of OH-2 is critical for binding of the sugar residue to the -1 subsite. However, the equatorial or axial projection at OH-4 and the conversion of CH<sub>2</sub>OH-6 of D-glucose to H- in D-xylose or CH<sub>3</sub>- in D-fucose are not critical to the capacity of the substrates to bind to the active site.

For disaccharides, GH5BG hydrolyzed laminaribiose ( $\beta$ -1,3) with a relatively high efficiency of 5.05 mM<sup>-1</sup>s<sup>-1</sup>, but it was 20 fold less efficient with cellobiose ( $\beta$ -1,4). Hydrolysis of gentiobiose ( $\beta$ -1,6-linked) was not detectable. It also hydrolyzed cello-oligosaccharides with degrees of polymerization (DP) of 3-6 at relatively high rates, with a catalytic efficiency of 9-10 mM<sup>-1</sup>s<sup>-1</sup> for DP 3-5 and a drop to half this for cellohexaose, due to a drop in the *k*<sub>cat</sub> for this substrate. On the other hand, GH5BG could not hydrolyze laminari-oligosaccharides with DP 3-5, laminarin, or barley 1,3-1,4- $\beta$ -glucans. Having rates of hydrolysis of oligomeric substrates which remain approximately constant or decrease with increasing DP length is a characteristic

often observed with  $\beta$ -glucosidases, unlike polysaccharide exohydrolases, in which the hydrolytic rate increases with oligosaccharide length [41].

The marked preference for  $\beta$ -1,4-linked oligosaccharides and  $\beta$ -1,3-linked disaccharide of rice GH5BG is different from fungal GH5 exo- $\beta$ -1,3-glucanases, which prefer to hydrolyze 1,3-glucans (laminarin) [8, 19, 20]. This difference in substrate specificity must be the result of differences in the structures and/or positions of amino acid residues in the active site between rice GH5BG and the fungal enzymes. The geometry of the pocket of *C. albicans* Exg allows hydrolysis of longer  $\beta$ -1,3-linked oligosaccharides and is not well suited for cleavage of 1,4-glycosidic linkages [8]. In contrast, GH5BG exhibited a marked preference for  $\beta$ -1,4-linked oligosaccharides and cannot hydrolyze extended  $\beta$ -1,3-linked oligosaccharides, thus the geometry of the pocket may be quite different from *C. albicans* Exg.

The sequence alignment between GH5BG with Exg from *C. albicans* and a GH5 endo-1,4- $\beta$ -glucanase (cellulase) from *C. thermocellum* [13] in Figure 1 shows that though rice GH5BG is somewhat more similar to *C. albicans* Exg, it shares some features more similar to the cellulase. In addition to being more similar at some residues near the active site, such as Tyr407, Asn409, and Tyr488, it lacks the extended loop at the end of strand 7 of the  $\beta$ -barrel, which forms a wall along one side of the active site in the fungal exoglucanases. Nevertheless, the unique structure of the rice GH5BG and the related rice exoglucanase-like genes, along with GH5BG's distinct substrate preferences, suggest that GH5BG and its three closely related rice homologues should be considered a separate subfamily of GH5.

Comparison of the deduced amino acid sequence of rice GH5BG with those of fungal GH5 exoglucanases revealed that Glu-347, which lies in the conserved NEP motif, is likely to be the catalytic acid/base and Glu-450, which lies in the conserved GEW motif, is likely the catalytic nucleophile [8, 19, 20]. Similar to other GH5 members, rice GH5BG contains eight invariant residues, these being Arg-247, His-291, Asn-346, Glu-347, His-406, Tyr-408, Glu-450, and Trp-486. These residues contribute hydrogen-bond interactions to the nonreducing terminal sugar residue at the -1 subsite found in *C. albicans* Exg. Since these residues are conserved, the geometry of the -1 subsite is not likely to account for the differences in substrate specificity between GH5BG and the fungal enzymes. However, differences are seen at the +1 subsite, in which amino acid residues Trp-229, Leu-304, and Asn-305 of *C. albicans* Exg are not conserved in GH5BG. *C. albicans* Exg residues Leu-304 and Asn-305 are located in the extended loop after strand 7 of the  $\beta$ -barrel and surround Glu-292 (the catalytic nucleophile) together with Ala-296, Asp-299, and Gly-306 [14]. These residues are conserved among fungal

GH5 exo-  $\beta$ -1,3- glucanases and many GH5 members with this extended loop, but not GH5BG. However, GH5BG does contain Phe-300 and Phe-411, corresponding to Phe-144 and Phe-258 in *C. albicans* Exg, which were found to be located at the +1 subsite near the entrance to the active-site pocket [8]. Indeed, these residues are found in the same position when a homology model of GH5BG was built with the *C. albicans* Exg structure (1CZX) as template. Cutfield et al [8] suggested that the role of the aromatic side chains of these two Phe residues is to direct substrates into the pocket and that it acts as a clamp for acceptor molecules participating in transfer reactions. The geometry of the +1 subsite might have a high stringency requirement for the stereochemistry of the linkage and the orientation of a second sugar. Hrmova et al. [42] suggested that the narrow phenyl groups would restrain the position of the second sugar in productive binding for hydrolysis more than the larger tryptophan indole rings found in a similar position with respect to the active site in barley GH family 3 exo-glucanase, which can accept  $\beta$ -1,3- and  $\beta$ -1,4- linkages. If the orientation of the nonreducing glucose residue in the -1 site is the same in each case, the orientation of the sugar in the +1 site should be different, depending on whether it is linked through its 3, 4, or 6 oxygen. So, the  $\beta$ -1,4- and  $\beta$ -1,6- linked disaccharides and longer  $\beta$ -1,3-linked oligosaccharides may not maintain proper binding geometry.

However, this does not explain how the rice GH5BG can hydrolyze longer  $\beta$ -1,4- oligosaccharides and 1,3-linked disaccharides. The fact that the cellotriose is hydrolyzed better than cellobiose implies that GH5BG has 3 subsites for binding  $\beta$ -1,4-linked glucosyl residues in the active site. Although GH5BG was designated a putative glucan 1,3- $\beta$ -glucosidase based on sequence homology, its catalytic activity is somewhat like GH1  $\beta$ -glucosidases, which show similar oligosaccharide preferences. Rice BGlu1 GH1 exoglucanase/ $\beta$ -glucosidase [40] and rice Os4bglu12 GH1  $\beta$ -glucosidase [27] are enzymes that prefer to hydrolyze  $\beta$ -1,4-linked oligosaccharides and a broad range of *p*NP-glycosides, but with differences in catalytic efficiency compared to GH5BG. Similar to rice BGlu1, GH5BG had cleaved the  $\beta$ -glucosidic bond between the 2 glucose residues in *p*NP- $\beta$ -D-cellobioside, thereby releasing glucose and *p*NPG, which was then rapidly hydrolyzed (data not shown), but the catalytic efficiency of GH5BG for hydrolysis of *p*NP- $\beta$ -D-cellobioside is about 10 times lower than that of rice BGlu1 [31, 40]. Although GH5BG could hydrolyze both cellotriose and *p*NP- $\beta$ -D-cellobioside, it hydrolyzed *p*NP- $\beta$ -D-cellobioside about 26 times less efficiently than cellotriose. This suggests that the *p*NP- $\beta$ -D-cellobioside, unlike cellotriose, cannot bind well to the third subsite in the active site cleft of GH5BG.

### **Expression of *GH5BG* in rice tissues and in response to environmental conditions**

The transcript level of *GH5BG* was high in the shoot of 7-d-old seedlings and very low in the root and endosperm. In 6-week-old mature plant, *GH5BG* transcripts were detected at significant levels in leaf sheaths, while low signal was seen in other mature plant parts (Figure 3).

To determine the effects of environmental conditions on rice *GH5BG* gene expression during seedling growth, the transcript levels of *GH5BG* were compared between 9-d-old seedlings that had been exposed to various conditions for 2 days to 9-d-old rice seedlings grown at 28°C (control) (Figure 3). The *GH5BG* transcript level was up-regulated in response to salt stress, submergence stress,  $10^{-4}$  M methyl jasmonate, and  $10^{-4}$  M abscissic acid in rice seedlings and increased slightly in response to ethephon. The mRNA transcripts were detected at levels similar to the control when the seedlings were treated with sorbitol, drought, cold, and heat stresses. The up-regulation of *GH5BG* in response to various environmental conditions may correlate with a need to recycle cell wall oligosaccharides in these processes, or to the function of other, as yet unidentified, substrates.

In summary, the cDNA of a putative GH5 glucan-1,3- $\beta$ -glucosidase containing a fascin-like domain at the N-terminus was cloned from rice seedlings. A recombinant thioredoxin-GH5BG produced in *E. coli* showed high hydrolytic activity toward various kinds of *p*NP-glycosides and exhibited a marked preference for  $\beta$ -(1,4)-linked oligosaccharides and laminaribiose ( $\beta$ -(1,3)-linked disaccharide). The substrate specificity of GH5BG is different from fungal GH5 exo- $\beta$ -(1,3)-glucanases, which is likely due to differences in the structures of the loops and types of amino acids around the active site, indicating GH5BG along with 3 closely related rice enzymes could be considered a new subfamily of GH5. GH5BG is expressed rice leaves and seedling shoots, while its expression is induced by stress-related hormones, submergence and salt in whole seedlings. The protein appears to be secreted outside the cell, where it may be involved in release of glucose from cell wall derived oligosaccharides in these conditions.

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## Figure legends

**Figure 1** Alignment of the protein sequences of rice GH5BG with exo- $\beta$ -1,3-glucanases and endo- $\beta$ -1,4-glucanase. GH5BG is rice GH5BG, *Candida* is exo- $\beta$ -1,3-glucanase from *Candida albicans* (AC CAA39908), *Lentinula* is exo- $\beta$ -1,3-glucanase from *Lentinula edodes* (AC AB192344), *Pichia* is exo- $\beta$ -1,3-glucanase from *Pichia pastoris* (AC AY954499), and *Clostridium* is endo- $\beta$ -1,4-glucanase from *Clostridium thermocellum* (AC AAA23220). The alignment was generated with the ClustalX implementation of ClustalW [22, 23] and analyzed and manually adjusted with Gendoc [24]. Alignment of the *C. thermocellum* sequence relied on the structural alignment of the 1CEC structural model with the *C. albicans* Exg 1CZ1 structure. The positions of the  $\beta$ -strands of the central  $(\beta/\alpha)_8$  barrel are indicated by arrows above the alignment. Red bars mark invariant GH family 5 residues and the black and grey shading highlight other identities between sequences, the asterisks identify the two catalytic glutamate residues, the blue bars indicate the two phenylalanine found at the +1 subsites of *C. albicans* Exg. The region of rice GH5BG homologous to fascin is indicated by blue text.

**Figure 2** SDS-PAGE of GH5BG-thioredoxin fusion protein expressed in *E. coli* strain OrigamiB (DE3) after incubation in the presence of 0.5 mM IPTG, at 20°C for 12 h. Lanes: 1, standard marker (Bio-RAD); 2, total protein of *E. coli* cells containing pET32a(+)/DEST-GH5BG; 3, soluble fraction of *E. coli* cells containing pET32a(+)/DEST-GH5BG; 4, purified thioredoxin-GH5BG. The arrow points to the thioredoxin-GH5BG.

**Figure 3** Northern-blot analysis of GH5BG transcript levels in (A) 7-d-old rice seedlings and 6-wk-old mature plant tissues and (B) 7-d-old rice seedlings grown a further 2 days with various abiotic stresses and plant hormones. GH5BG, RNA blots were probed with  $\alpha$ - $^{32}$ P-labeled GH5BG gene-specific probe, 18SrRNA indicates the same blot probed with an  $\alpha$ - $^{32}$ P-labeled 18S rRNA cDNA probe. Twenty micrograms of total RNA from the appropriate tissues were loaded in each lane.

**Table 1** Kinetic parameters of rice GH5BG in the hydrolysis of *p*NP-glycosides, disaccharides and oligosaccharides

| Substrate                            | $k_{cat}$<br>( $s^{-1}$ ) | $K_m$<br>(mM)     | $k_{cat}/K_m$<br>( $s^{-1} \text{ mM}^{-1}$ ) |
|--------------------------------------|---------------------------|-------------------|-----------------------------------------------|
| <i>p</i> NP- $\beta$ -D-glucoside    | $36.1 \pm 0.7$            | $0.47 \pm 0.03$   | $77 \pm 4$                                    |
| <i>p</i> NP- $\beta$ -D-fucoside     | $24.5 \pm 0.5$            | $0.17 \pm 0.07$   | $144 \pm 3$                                   |
| <i>p</i> NP- $\beta$ -D-galactoside  | $27 \pm 3$                | $1.30 \pm 0.10$   | $20.7 \pm 0.5$                                |
| <i>p</i> NP- $\beta$ -D-xyloside     | $3.2 \pm 0.3$             | $0.27 \pm 0.05$   | $11.9 \pm 1.5$                                |
| <i>p</i> NP- $\alpha$ -L-arabinoside | $2.88 \pm 0.08$           | $0.14 \pm 0.02$   | $21 \pm 3$                                    |
| <i>p</i> NP- $\beta$ -D-cellobioside | $2.07 \pm 0.09$           | $6.23 \pm 0.17$   | $0.34 \pm 0.01$                               |
| <i>p</i> NP- $\beta$ -D- mannoside   | n.d. <sup>a</sup>         | n.d. <sup>a</sup> | n.d. <sup>a</sup>                             |
| Cellobiose                           | $4.3 \pm 0.8$             | $16.4 \pm 1.9$    | $0.27 \pm 0.02$                               |
| Cellotriose                          | $41 \pm 5$                | $4.53 \pm 0.01$   | $9.1 \pm 1.2$                                 |
| Cellotetraose                        | $38 \pm 2$                | $4.09 \pm 0.17$   | $9.3 \pm 0.9$                                 |
| Cellopentaose                        | $35.5 \pm 0.4$            | $3.4 \pm 0.4$     | $10.4 \pm 0.4$                                |
| Cellohexaose                         | $9.7 \pm 0.8$             | $2.2 \pm 0.5$     | $4.5 \pm 0.5$                                 |
| Laminaribiose                        | $36 \pm 5$                | $7.0 \pm 1.1$     | $5.05 \pm 0.07$                               |
| Laminaritriose                       | n.d. <sup>a</sup>         | n.d. <sup>a</sup> | n.d. <sup>a</sup>                             |
| Laminaritetraose                     | n.d. <sup>a</sup>         | n.d. <sup>a</sup> | n.d. <sup>a</sup>                             |
| Laminaripentaose                     | n.d. <sup>a</sup>         | n.d. <sup>a</sup> | n.d. <sup>a</sup>                             |
| Laminarin                            | n.d. <sup>a</sup>         | n.d. <sup>a</sup> | n.d. <sup>a</sup>                             |
| Barley 1,3, 1,4- $\beta$ -glucans    | n.d. <sup>a</sup>         | n.d. <sup>a</sup> | n.d. <sup>a</sup>                             |

<sup>a</sup> means not detectable

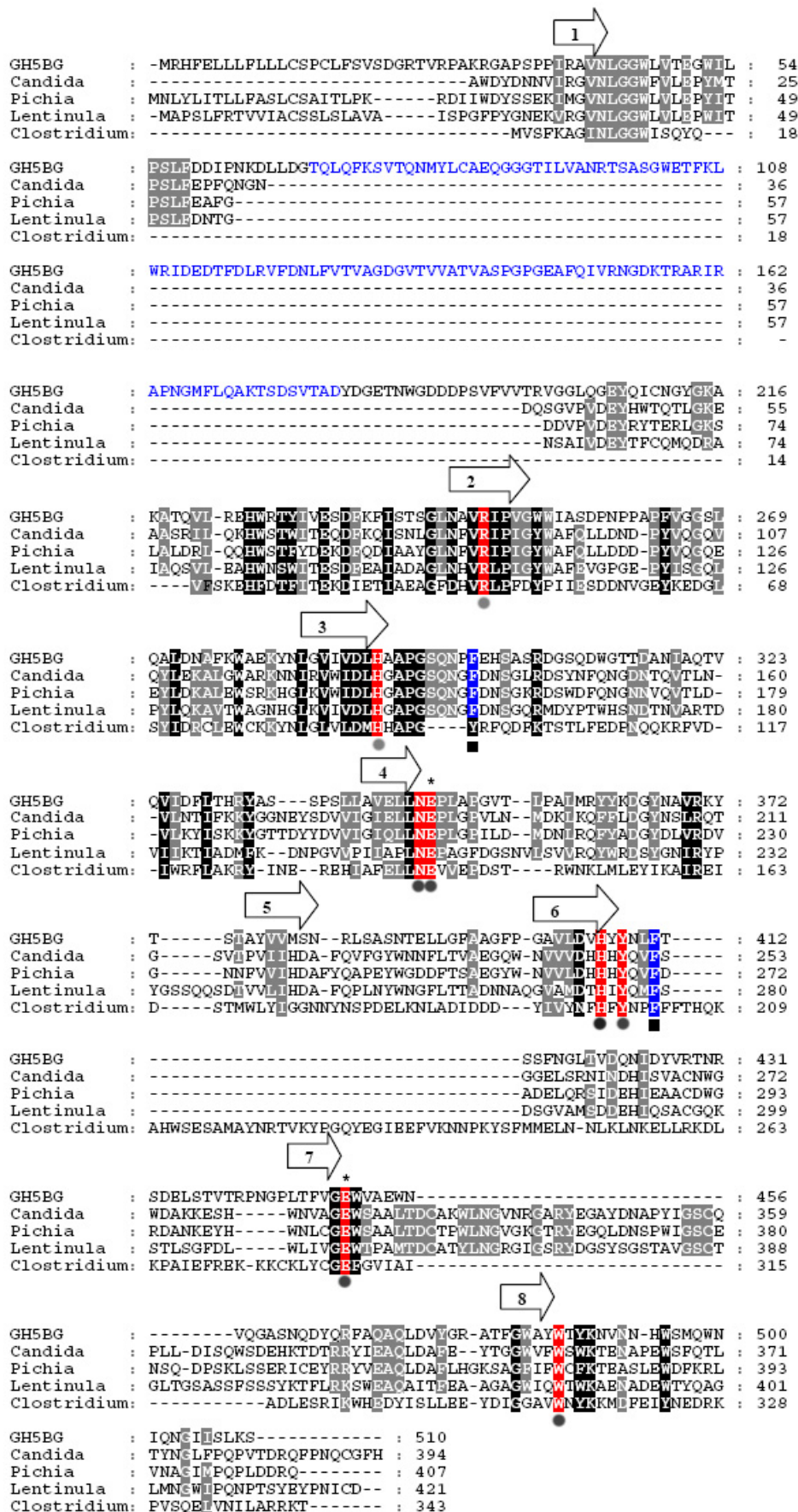


Figure 1

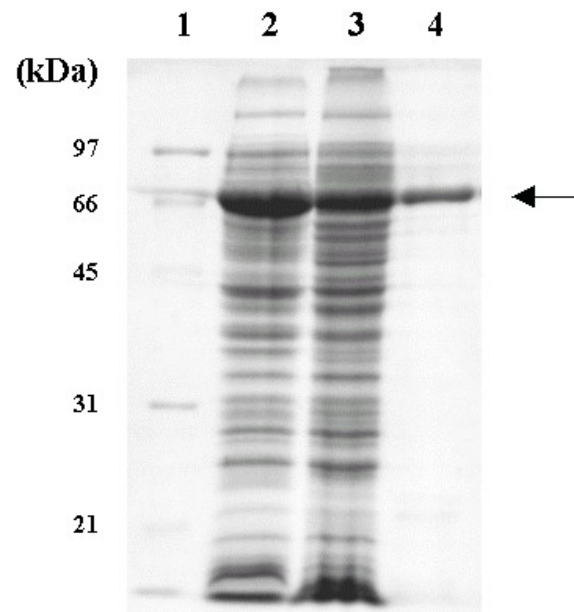


Figure 2

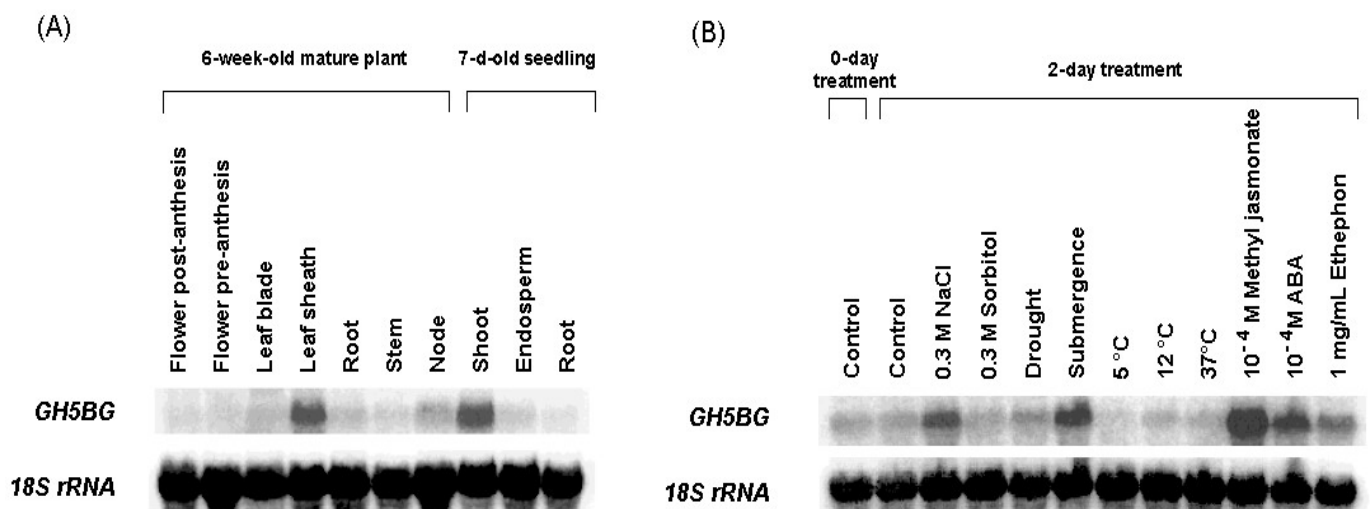


Figure 3