

Structure and expression of a barley acidic β -glucanase gene

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Abstract

A barley acidic β -1,3-glucanase gene was recovered from a barley genomic library by homology with a partial cDNA of barley basic β -1,3-glucanase isoenzyme GII. The gene, *Abg2*, is homologous to the PR2 family of pathogenesis-related β -1,3-glucanase genes. The ABG2 protein has 81% amino acid similarity to barley basic β -1,3-glucanase GII. The ABG2 protein is encoded as a preprotein of 336 amino acids including a 28 amino acid signal peptide. A 299 bp intron occurs within codon 25. The mature ABG2 protein has a predicted mass of 32642 Da and a calculated isoelectric point of 4.9. The second exon of the *Abg2* gene shows a strong preference for G + C in the third position of degenerate codons. The *Abg2* gene was functionally expressed in *Escherichia coli*. *Abg2* mRNA is constitutively expressed in barley root; leaf expression of *Abg2* mRNA is induced by mercuric chloride and infection by *Erysiphe graminis* f. sp. *hordei*. Southern blot analysis indicates that *Abg2* is a member of a small gene family.

Introduction

Pathogenesis-related (PR) proteins were originally described as extracellular acidic proteins induced in tobacco by tobacco mosaic virus [3]. Related PR proteins have since been observed in a wide variety of monocot and dicot plant species, and diverse conditions induce their expression including infection, wounding, chemical and environmental stresses [3, 5, 29]. Several classes of PR proteins have a postulated antifungal role, and their synergistic antifungal effect has been

demonstrated *in vitro* [28, 33]. Fungal inhibition by endochitinases and endoglucanases is thought to result from hydrolysis of fungal cell wall components; fungal inhibition by thaumatin-like PR proteins has been proposed to result from hydrophobic interaction with fungal membranes [44, 57]. PR2-class proteins include acidic and basic isoenzymes of β -1,3-glucanase (1,3- β -D-glucan 3-glucanohydrolase; EC 3.2.1.39). In addition to their well characterized response to pathogen infection and stress, glucanases are also expressed in response to a variety of hormonal

The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number M91814.

and developmental signals in healthy plants. The basic glucanase of tobacco accumulates intracellularly in roots of healthy plants [36], and is expressed during floral induction of explants [38]. The rice *Gns1* β -glucanase gene is also constitutively expressed in roots, and is induced in shoots in response to ethylene, salicylic acid, cytokinin, and fungal elicitors [49]. A glycosylated acidic glucanase accumulates heavily during anthesis in the extracellular matrix of tobacco styles, where an enzymatic or physiochemical function during pollen tube elongation has been proposed [42]. In addition to being a component of pollen cell walls, β -1,3-glucan (callose) is deposited by plant cells in vascular tissue and around wound sites in response to hormonal and stress signals [17, 58]. The role of β -1,3-glucanases in modulating deposition of callose is unclear.

A number of β -1,3-glucanase isoenzymes have been purified from barley [1, 18], but their physiological function (beyond a potential antifungal activity [28]) remains to be elucidated. The barley basic β -1,3-glucanase isoenzyme GII has been purified from germinating barley [18], and from 12-day old barley seedlings [28] supporting a possible physiological role in healthy plants. The GII β -1,3-glucanase has also been purified from the medium of barley suspension cells cultured in the presence of 2,4-D [24], indicating that this protein is secreted. Furthermore, message for GII β -1,3-glucanase has been identified among the infection-related (IR) mRNAs preferentially induced in barley cultivars resistant to powdery mildew [23]. Further supporting a defensive role for the GII β -1,3-glucanase is the finding that the GII protein isolated from 12-day old seedlings synergized with other barley seed proteins to inhibit fungal growth [28].

Closely related barley β -1,3-1,4-glucanases are expressed in scutellar and aleurone tissue of germinating barley seed [35, 51, 60]; these enzymes function in hydrolysis of endosperm cell walls, facilitating starch mobilization [14, 52]. Gibberellic acid and indole acetic acid stimulate expression of β -1,3-1,4-glucanase isoenzyme EII in aleurone [50]; these treatments also induce expression of barley isoenzyme EI in leaves. Reg-

ulation by gibberellic acid was reproduced using EII promoter-GUS fusions in barley aleurone protoplasts [56]. While the GII β -1,3-glucanase isoenzyme has been purified from commercial barley malt [1], the role of the GII enzyme in germinating barley seed may be distinct from that of the β -1,3-1,4-glucanases. Expression of GII mRNA was observed not only in aleurone tissue but also in rootlets, coleoptiles and shoots of germinating seeds; In addition, GII mRNA expression in aleurone was not affected by gibberellic acid [28].

We have characterized a barley gene which encodes an acidic homolog to the basic β -1,3-glucanase GII found in germinated barley. The gene encodes a functional glucanase enzyme as demonstrated by the expression of active enzyme in *E. coli*. This gene is expressed in barley in a manner consistent with pathogenesis related proteins: constitutive expression is observed in roots, and leaf expression is induced in response to biotic and abiotic stresses.

Materials and methods

Plant material and treatments

Barley variety B458 (gift of W. Johnson, Northrup-King Co.) was grown in Metromix-350 (Grace-Sierra Co., Malpitas, CA) at 22 °C with a 12 h photoperiod for five days prior to treatments. Mercuric chloride (0.2%) or water were sprayed to runoff on leaves of 5-day old barley seedlings, which were then sealed in bags for overnight incubation in the dark. Bags were then removed and plants were grown for an additional 2 days until harvest. For fungal infection, 5-day old plants were heavily inoculated over their leaf surface with spores of *Erysiphe graminis* f. sp. *hordei* (gift of A. Otal, Monsanto Co.), and maintained in a growth chamber for 5 additional days at 21 °C, 80% relative humidity, and 10 h photoperiod. Root tissue was obtained from untreated 5-day old seedlings grown in soil, or from surface-sterilized seeds aseptically germinated 5 days on filter paper.

Genomic library screening

The partial cDNA pRP7-25 was isolated from *Hordeum vulgare* (L.) 'Psaknon 4*/ (F14) Man. (R)' of USDA Accession CI 16146, from among infection-related mRNA species induced in response to powdery mildew infection [9, 23].

The genomic library of *H. vulgare* L. var. NK1558 DNA was purchased from Clontech (Palo Alto, CA). Approximately 96000 phage were plated and transferred to nitrocellulose filters for *in situ* plaque hybridization according to standard procedures [45]. The 225 bp cDNA insert of pRP7-25 was ³²P-labelled by nick translation (Amersham, Arlington Heights, IL). The filters were hybridized in 40% formamide, 5 × SSC, 5 × Denhardt's solution, 0.2% SDS and 200 µg/ml *E. coli* tRNA at 42 °C for 16 h and washed in 1 × SSC at 50 °C. Eight strongly hybridizing phage were picked and purified through an additional round of screening. DNA was isolated from the eight clones for further characterization.

Restriction analysis and Southern blot hybridizations

Restriction enzymes were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Digestions of phage DNA were electrophoresed in agarose gels, capillary blotted onto nitrocellulose (Hybond-C Extra; Amersham, Arlington Heights, IL) and hybridized with the pRP7-25 probe. Hybridization and rinsing conditions were as described for plaque screening. Restriction fragments identified as containing hybridizing regions were subcloned into pUC118 [54].

Barley genomic DNA was prepared from etiolated seedling leaves using the method of Federoff [48]. Genomic DNA was digested twice sequentially for 16 h with 20 units of various enzymes per microgram DNA. DNA was ethanol precipitated from digestions, and resuspended in 10 mM Tris pH 8, 1 mM EDTA prior to electrophoresis. A 5 µg portion of DNA was loaded per lane. After electrophoresis, DNA was transferred

to Hybond-N plus membrane (Amersham, Arlington Heights, IL) according to manufacturer's directions. The probe for Southern analysis was the entire second exon of the *Abg2* gene, recovered from the procaryotic expression construct (see below) and labeled using a nick translation kit (Amersham). Hybridization was conducted at 65 °C in 5 × SSPE, 5 × Denhardt's solution, 20 µg per ml salmon sperm DNA, 0.5% SDS. Blots were rinsed twice at ambient temperature for 15 min in 2 × SSPE, 0.1% SDS, and once at 55 °C in 1 × SSPE, 0.1% SDS. For high stringency rinsing, filters were rerinsed once at 68 °C for 15 min in 0.1 × SSPE, 0.1% SDS.

DNA sequence analysis

Plasmid DNA isolated on CsCl gradients was denatured with alkali [16] and sequenced using the dideoxynucleotide chain termination method [46] using the Sequenase Version 2.0 kit (United States Biochemical Corp., Cleveland, OH). Universal and reverse sequencing primers were used for appropriate plasmid subclones. Oligonucleotide primers (Midland Certified Reagent Company, Midland, TX) were utilized to complete sequencing of both strands. Formamide (40%) was incorporated into urea/acrylamide sequencing gels to relieve sequencing compressions. DNA sequence analysis was facilitated by using the Genetics Computer Group Sequence Analysis Software Package [12].

Northern analysis

Total RNA was prepared from leaf and root tissue using the RNAid kit (Bio-101, La Jolla, CA) according to manufacturer's directions. Total RNA (25 µg per lane) was electrophoresed through agarose gels containing formaldehyde according to established procedures [45]. Transfer, hybridization, and rinsing conditions were as described for genomic Southern blots.

Construction of *E. coli* expression vector

A 'sense' oligonucleotide primer (5'-GGG-AGATCTCCATGGCGCACTCCATCGGCG-TGTGCAACGG-3') and an 'antisense' primer (5'-CCCAGATCTCAGAAGCGCATGTAGT-AGG-3') were used to clone the second exon of the *Abg2* gene, encoding the mature protein. These oligonucleotides were used in a polymerase chain reaction using the GeneAmp kit (Perkin-Elmer Cetus, Norwalk, CT). The template for the reaction was supercoiled plasmid DNA containing the subcloned genomic copy of the *ABG2* gene. Reaction conditions included 1 min denaturing at 94 °C, 2 min annealing at 45 °C, 3 min extension at 72 °C, repeated for 30 cycles, followed by 15 min extension at 72 °C. Reaction product was blunted with Klenow polymerase (Promega, Madison, WI) and cloned into the *Sma* I site of pUC118 to give pMON8975 (placing the stop codon of the gene proximal to the *Hind* III site of the multiple cloning site). Due to an internal *Nco* I site, the gene was transferred to an *E. coli* expression vector in two steps. The expression construct utilized was derived from pMON5743 [41] except that the *Ptac* promoter [10] was substituted for the *PrecA* promoter. To reconstruct the *Abg2* gene, a carboxyl-terminal 0.3 kb *Nco* I-*Hind* III fragment was transferred to *Nco* I-*Hind* III sites of the expression vector to give pMON8976. The remainder of the gene was transferred as a 0.6 kb *Nco* I fragment to the *Nco* I site of pMON8976 to give pMON8977. As a negative control plasmid, a construct was retained with the 0.6 kb *Nco* I fragment inserted in the antisense orientation.

Expression of recombinant *ABG2* protein

E. coli JM101 cells bearing plasmid pMON8977 or the negative control plasmid were grown to low density ($A_{600} < 0.5$) in M9 medium supplemented with $2 \times$ casamino acids, 1 M sorbitol, and 25 μ g/ml ampicillin at 37 °C. These cultures were diluted with fresh medium 1:100, transferred to 30 °C and grown to low density ($A_{600} < 0.5$). IPTG was added to 0.5 mM and cultures were

incubated overnight at 30 °C. Cells were recovered by centrifugation, rinsed once in 12 mM Tris pH 6.8, 1 mM EDTA, 1 mM PMSF, then resuspended in the same buffer. Cells were disrupted using a Branson sonifier, and debris was removed by centrifugation. Extracts were desalted by gel filtration in PD-10 columns (Pharmacia, Piscataway, NJ) equilibrated with 12 mM Tris pH 6.8, 1 mM EDTA. Extracts were concentrated by ultrafiltration in Centricon-10 units (10 kDa MW cutoff; Amicon, Beverly, MA) and dialyzed against 12 mM Tris pH 6.8, 1 mM EDTA. Glucanase enzyme activity in fractions was assessed by detection of reducing sugar (glucose equivalents) released upon incubation with laminarin (Sigma Chemical Co., St. Louis, MO) using the neocuproine colorimetric assay of Nelson [39].

Enrichment of recombinant *ABG2* protein

The induced protein was partially purified by preparative native polyacrylamide gel electrophoresis using a discontinuous buffer system [43]. Approximately 500 μ g of total protein from the sonicated extracts was loaded onto a 8 cm $\times$ 10 cm, 1.5 mm thick 15% polyacrylamide nondenaturing gel (stacking gel 125 mM Tris pH 6.8; resolving gel 375 mM Tris pH 8.8). After 6 h of electrophoresis at 80 V, the gel was cut into strips, proteins were eluted by diffusion and concentrated by lyophilization. Fractions were assayed for glucanase activity as above, and for protein content by Bradford assay [4] (Biorad, Richmond, CA). After SDS-PAGE [25], proteins were visualized by silver staining (Enprotech, Hyde Park, MA). The content of *ABG2* protein in the most enzymatically active fraction was determined by laser densitometry using an LKB model XL enhanced laser densitometer (Pharmacia-LKB, Piscataway, NJ).

Results

Cloning and sequencing

Eight clones hybridizing strongly with the pRP7-25 cDNA insert were isolated from the ge-

omic library of *H. vulgare* cv. NK1558. The *Eco* RI restriction analysis of the phage DNAs revealed four clones (8-2, 8-3, 8-4 and 9) with unique *Eco* RI restriction patterns. A Southern blot of the *Eco* RI and *Hind* III digests of the four clones was hybridized with the pRP7-25 cDNA insert. Two *Eco* RI fragments and two *Hind* III fragments of clone 9 hybridized with the cDNA insert. Subsequent cloning and sequencing of the 2.8 kb *Eco* RI fragment revealed a glucanase-related pseudogene. Homology extended roughly 900 bp over the region representing the mature portion of glucanases, but many frame shifts were evident and no first exon could be found within 2 kb upstream of the second exon-like region (data not shown). The 4.5 kb *Hind* III fragment of clone 9 was subcloned into the *Hind* III site of pUC118. A number of internal restriction enzyme fragments of this plasmid were assessed for hybridization by Southern analysis. In this fashion the hybridizing region was narrowed to a 2.3 kb region within the 4.5 kb *Hind* III fragment.

Sequencing of this region revealed an intron-containing open reading frame (exons indicated with a heavy arrow in Fig. 1). The 2300 bp sequence includes 590 bp of sequence 5' to the initiation codon, 73 bp of open reading frame, 299 bp of intron, 938 bp of open reading frame, and 400 bp of sequence 3' to the stop codon (Fig. 2). While the overall G + C content of the *Abg2* gene is 63%, with 92% G + C in the third ('wobble') position of codons, the coding portion of the first exon is only 53% G + C, with 50% G + C in the wobble position. The G + C content of the intron is 35%. The observation of an intron within the *Abg2* gene is supported by its reduced

G + C content, conserved splice junction sequences, and by the observation of introns in analogous positions within the genes for barley β -1,3-1,4-glucanases, tobacco acidic and basic β -1,3-glucanases [7, 30, 31, 55], and rice β -glucanase [49].

The *Abg2* gene (Fig. 2) encodes a preprotein of 336 amino acids, molecular mass 35 453 Da, with a predicted isoelectric point of 5.2. The first 73 bp of open reading frame, upstream of the intron, encodes the majority of the inferred 28 residue signal peptide. The ABG2 signal peptide has characteristic features of barley glucanase signal peptides: a length of 28 residues; a positively charged residue near the amino terminus; a central hydrophobic core beginning with val-ala-ser, and ending with a proline; and a serine residue at the presumed cleavage site. The 19 amino acid hydrophobic core of the signal peptide represents the longest hydrophobic region of the ABG2 protein, consistent with it functioning as an amino-terminal membrane translocation signal for entry of the ABG2 protein into the secretory system. Presence of an amino terminal signal peptide is a feature shared by all characterized vacuolar and secreted pathogenesis-related proteins [29].

If the 28 amino acid signal peptide is cleaved as predicted, the molecular weight of the mature ABG2 protein is 32 642 Da and the predicted isoelectric point is 4.9. One consensus site (asn-gly-thr) for asparagine N-linked glycosylation is present, at residue 290 (nucleotide 1167). Because native ABG2 protein has not been purified, the utilization of this site *in planta* is unknown. Each of the barley β -1,3-1,4-glucanases has a glycosylation site, but these are observed at an earlier

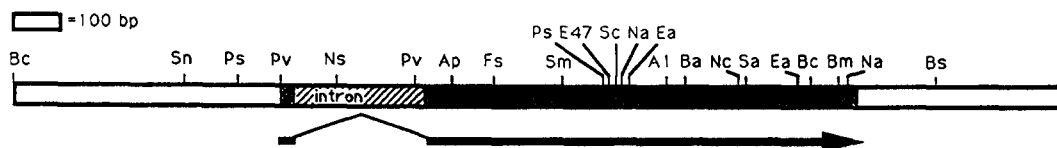


Fig. 1. Restriction map of the *Abg2* gene. A 2.3 kb genomic fragment harboring the *Abg2* gene was completely sequenced. The two exons of the open reading frame are indicated by dark hatched areas; the intron is indicated as a light hatched area. The position of the two exons and the direction of the open reading frame are indicated beneath the restriction map with a large arrow. Restriction sites are: A1, *Apa* I; Ap, *Apa* LI; Ba, *Bal* I; Bm, *Bam* HI; Bc, *Bcl* I; Bs, *Bst* BI; Ea, *Eag* I, E47, *Eco* 47III; Fs, *Fsp* I; Na, *Nae* I; Nc, *Nco* I; Ns, *Nsi* I; Ps, *Pst* I; Pv, *Pvu* I; Sa, *Sal* I; Sc, *Sac* II; Sn, *Sna* BI.

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CATATGCAGCAAGAAAAAAGACACTGTTGACTGGCTATCTGAGTCCCCGGCAGCATGGCTGCATCTACAGCGCCGAGGAAGGACTCACAACCTCGA -491
TTGAGCAGCTAGTGGACTACGATCGGGTGTGAACCACTGCATAGAAATTAACAAGTCCATGAGACAAGTAGCACGCTCGCACACAAGAGACAAGTAGC -391
TTCGTTAGTGGCGGGAAGCAAAAGAGCTGTCCACCAGGCCACCATTCATCCAGCCATCGATTGATACCCGTTCAACAAAGAGCCGGTCTGGACTGGA -291
TGGTGACACCGTTCAACGGGTTTCCACGGGCCAACGAACAAGTTGCGCGTGCCAGCACTAGCTTGGCACTTGGCACGTACGTACAGTAGCCGCTATGTTA -191
GTGGTGAAAAATGGAACGGATAAAGGATAAAGAGACTCATGGTGGACTCGATGGACCATCAACCAACGTCGGAACGTGTATTGCGCCAAACTGCAGCGCG -91
ATAGTGCTGAGAATGGCTTATAAAGATACGTGTGGTGGCTGGTATTATATCATGCAAAATCGAAGATCGGACAGAACAATCGACGATCGATGGCGAAGC +10
                                     M A K Q
AAGGTGTTGCTTCCGTGCTTGAATGGCGTGGTCTTGTCTTCTGGCAGCATTCCCTACAGGTATCATATATATGATATCTAATATGATTAAGATGC +110
G V A S V L A M A L V L V F L A A F P T G/intron.....
ATCTGCATCAACAAATTTCTTGAAGTGTCTCTGGAAAATTTATTTAATGCACGATATTTTGCATAAAAGAGTGCAGTTCTCACCGGGCAGCTAACCCCA +210
.....
GCGGACATACAAAAGCAGGTTCACTATCTCATCTGTGTTAAATAACATGTATTTCGAGGAACATAATTTTCGCTGATTGTATTGATCGACAACCTCAAAATC +310
.....
TCTAATTTGAGACGCAACGAAACTGTTTTTTTTTATATATTGGAACGTGTTCCATCGTAACAGGGGTGCACTCCATCGGCGTGTGCAACGGCGTGATCGGC +410
.....intron/ V H S I G V C N G V I G
AACAACCTGCCCGCGCCAGCGACGTAGTCAAGCTCTACAATCCAAGGGCATCAACGCCATCGGCATCTACGCGCCGAGAGCAACGTCCTCAAGGCGC +510
N N L P A P S D V V K L Y K S K G I N A M R I Y A P E S N V L K A L
TCAGTGGCAGCGGCATCGCGCTCTCATGGACGTGGGCAACGGCGTCTCCCCAGCCTCGCAACGACCCCTCCGCGCGGGCCGCTGGGTCAAGGCCAA +610
S G T G I G V L M D V G N G V L P S L A N D P S A A A A W V K A N
CGTGACGCCCTACCCGGGCGTCTCTTCCGCTACATCGCCGTCGGNAACGAGGTCTGGACAGCGAGGGCCAGAAGACCATCTCCACCCCTCAAGAAC +710
V Q P Y P G V S F R Y I A V G N E V M D S E G Q K T I L P A M K N
CTGCAGGGAGCGCTCGCCGCGGCGGCTCGGCGGCGCGTCAAGGTGTCCACGTGCGGTACGGTTGACGTGGTCACCGACACCTTCCACCCCTCAACG +810
L Q G A L A A A G L G G R V K V S T S V R F D V V T D T F P P S N G
GCGTGTTCGCGGACCTTGACTACATGGGCCCCATCTCGGACTTCTGGTGGACACCGATGCGCGCTGCTGGCCAACGTGTACCCCTACTTCGCTACAA +910
V F A D L D Y M G P I L D F L V S T D A P L L A N V Y P Y F A Y K
GGGTGACCCGAAGACATCAAGCTCAACTACGCCACCTTCGCGCGGGCACCACCGTGAACGACGACGGCAACGGCCTGACCTACACTAACCTCTTCGAC +1010
G D P K N I K L N Y A T F A P G T T V N D D G N G L T Y T N L F D
GCCATGGTGCAGTCCATCTACGCCGCGCTGGAGGACCCGAGACGCGGGCGTGAAGTTCGTGTCGGAAGCGGGTGGCGCTCGGACGGCGGGTTTG +1110
A M V D S I Y A A L E D A E T P G V K V V V S E S G W P S D G G F G
GAGCGACGGCCGAGAACGCGGGCGGTACAACAGGGGTGATCAACACGTCGGTAATGGCACACCAAGAGGTCCGGTCCGTTGGAGACGTATGTCTT +1210
A T A E N A R A Y N Q G L I N H V G N* G T P K R S G P L E T Y V F
CGCCATGTTCAATGAGAACGAGAAGAGGGGATCCGACGGAGAACCACTTTGGGTGTTCAATCCGACAAAGTCGCGGCTACTACATGCGCTTCTGA +1310
A M F N E N E K K G D P T E N H F G L F N P D K S P A Y Y M R F stop
CAAGTGCGAGAAACCCCTGAATATTTCTGTTCATAAGCTGCAATGCAAGTCAACACCCCTGTGTGTGTTTAAAGTTCTAACTTATTTTAACTCCAA +1410
TAAACAAGTCATTTTTTAAATTAATTCACCTTTCTATATGCCATATTTCAACAAGAGGGATCTTTCGAACCTGGATGTATACACCCCTATATGCAAAA +1510
TAAATAGCAATTAACGAAAGTCAAAAACGCTGAAAATACTTTTTAAATTAATTTACCTTTTATTGTACTTGTAAAGAAAAGTTCCAGAAAAGAAA +1610
ATGATACTTTCCTTCTTTTCAAAAAAGACAATTTTTAACCAAAATAATGTGAATAGTGGCCTATATTGCAAGTAATTTTCTATTTTTTGCCCAAGAT +1710

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Fig. 2. Nucleotide sequence of the *Abg2* gene. Nucleotide positions are given with respect to the first nucleotide of the initiation codon. The inferred amino acid sequence of the ABG2 protein is given, with single letter amino acid abbreviations beneath the first nucleotide of each codon. The 299 bp intron is indicated with a dotted underline. The inferred amino terminus of the mature protein is boxed. An asterisk denotes a consensus N-linked glycosylation site. Two potential TATA boxes, and one inverted CCAAT box, are underlined. Consensus polyadenylation sequences are double-underlined.

position in the protein sequence, in a context which is poorly conserved in the ABG2 protein. The more homologous barley β -1,3-glucanase

isoenzyme GII has no predicted N-linked glycosylation site. The tobacco basic β -1,3-glucanase has been shown to be glycosylated within a car-

boxyl terminal propeptide which is proteolytically removed during maturation and transport [47]. Such C-terminal propeptides have been shown to serve as vacuolar targeting signals for tobacco chitinase and barley lectin [2, 40]. The ABG2 protein is not encoded with a C-terminal propeptide, but N-terminal or internal regions of the protein could direct intracellular retention [8].

The ABG2 amino acid sequence shows extensive homology to barley glucanases (Fig. 3). The strongest homology is 64% identity/81% similarity to barley basic β -1,3-glucanase isoenzyme GII [18]. Of the nonconservative differences between the two proteins, 37 positions involve charged residues: ABG2 has a net increase of 2 positive and 10 negatively charged residues, accounting in part for its lower predicted isoelectric point. The ABG2 amino acid sequence is 52% identical/74% similar to barley basic β -1,3-1,4-glucanase isoenzyme EII, and to the less basic isoenzyme EI [14, 31, 55]. The ABG2 amino acid sequence is 50% identical/70% similar to a rice β -glucanase

[49] and 45% identical/65% similar to acidic or basic β -1,3-glucanase isoenzymes of tobacco [30]. An internal self-homology noted for the GII isoenzyme [1], perhaps indicative of a domain reiteration, is not preserved in the ABG2 sequence.

At the DNA level, the *Abg2* gene is most similar (78% identical, over the coding region of the second exon) to the pseudogene on the 2.8 kb *Eco* RI fragment of clone 9. Over this same region, the *Abg2* gene shows 75% identity to the cDNA encoding β -1,3-glucanase isoenzyme GII [18], and 65% identity to the *Glb2* gene encoding barley basic β -1,3-1,4-glucanase isoenzyme EII [55].

The 299 bp intron within the *Abg2* gene occurs after the first nucleotide of codon 25. The intron preserves invariant splice junction consensus sequences at the 5' (GT) and 3' (AG) ends. The intron can be classified as having a 'purine-rich' 3' splice region [15] by virtue of four consecutive purine residues 18–21 bp upstream of the 3' splice

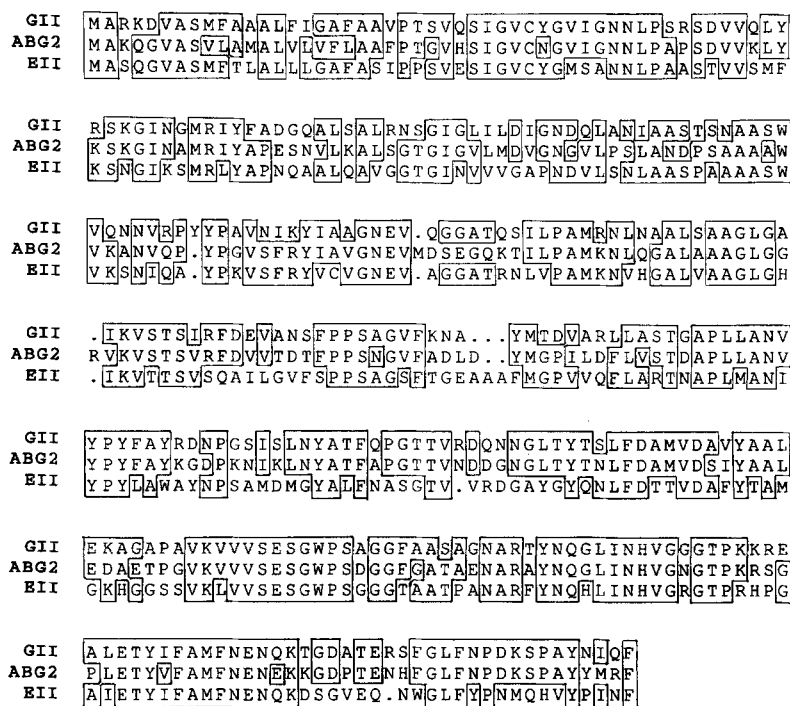


Fig. 3. Comparison of the amino acid sequence of ABG2 with two similar barley glucanases. Positions of amino acid identity are boxed. GII is barley β -1,3-glucanase isoenzyme GII [18]. EII is barley β -1,3-1,4-glucanase isoenzyme EII [14, 55]. Spaces, denoted by dots, have been introduced to optimize the alignment.

junction; however, a poly-thymidine tract can be observed another 10 bp upstream [6, 15]. A number of inverted repeats and palindromes are present within the intron sequence. Two are observed in the vicinity of the 3' splice junction, TGGAAC(t)GTTCCA, which is located 10 to 22 bp upstream of the splice junction; and CTGTTcC(atc)GtAACAG, which terminates at the splice junction itself. Inverted repeats can also be found near the 3' splice junctions of the introns within the genes for barley β -1,3-1,4-glucanase isoenzymes EI and EII [31, 55]. Specific recognition of these sequences or any role in secondary structure formation is unknown.

The first 590 bp of the *Abg2* gene promoter have been sequenced, and two potential TATA-box like elements, TTATAAA and TATTATA, are located at positions -71 and -45 (relative to first base of the methionine codon). An inverted CCAAT-box motif is observed between -74 and -78. The methionine codon itself occurs immediately following a naturally occurring *Pvu* I site, which does not maintain adenosine at -3, a characteristic of animal translation initiation sites which is generally maintained in plant genes. The dinucleotide GC following the methionine codon does conform to the strongly conserved context for plant translation initiation sites [21, 32]. No obvious homology is seen to the proposed gibberellic acid responsive elements or G-boxes which are observed upstream of barley amylase and β -1,3-1,4-glucanase genes [19, 31]. In addition, no similarity is seen to conserved barley promoter elements shared by the rice *Gns1* β -glucanase gene [49]. In the 3'-flanking region, consensus polyadenylation sites (AATAAA; [22]) are observed 98, 120, and 198 bases downstream of the stop codon.

Southern analysis

The entire *Abg2* second exon was used as a probe of a genomic Southern blot; the four restriction enzymes shown (Fig. 4) do not cut within the 4.5 kb *Hind* III genomic fragment harboring the *Abg2* gene. Five to seven hybridizing fragments

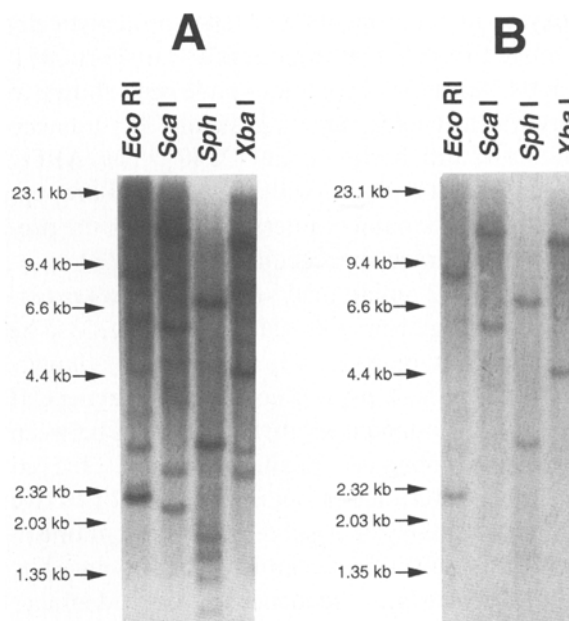


Fig. 4. Barley genomic Southern analysis. Barley genomic DNA was extensively digested with *Eco* RI, *Sca* I, *Sph* I, or *Xba* I. Five μ g DNA was loaded per lane. After electrophoresis and transfer to nylon membrane, the filter was hybridized with the *Abg2* second exon, radiolabelled by nick translation (see Materials and methods). A. Intermediate stringency of rinsing. The filter was rinsed in $1\times$ SSPE, 0.1% SDS at 55°C . Autoradiographic exposure time was 3 days. B. High stringency of rinsing. The filter was rinsed in $0.1\times$ SSPE, 0.1% SDS at 68°C . Autoradiographic exposure time was 7 days. The migration of molecular weight standards, given in kb are indicated with arrows.

were observed (Fig. 4A) in each of the four restriction digestions under conditions of intermediate rinsing stringency ($1\times$ SSPE at 55°C). The 2.8 kb *Eco* RI fragment observed in genomic DNA under intermediate stringency may represent the 2.8 kb *Eco* RI fragment from clone 9, which harbored a pseudogene with 78% DNA identity to *Abg2*. When rinsing stringency was increased to $0.1\times$ SSPE at 68°C (Fig. 4B) this and other weakly hybridizing fragments were no longer observed. Two hybridizing fragments remained in each of four digests. Digestion with *Eco* RI produced 8.1 kb and 2.1 kb fragments; *Sca* I produced 0.5 kb and 5.9 kb fragments; *Sph* I produced 7.0 kb and 2.9 kb fragments; and *Xba* I produced 9.8 kb and 4.5 kb fragments. Be-

cause all of these enzymes cut outside the 4.5 kb *Hind* III fragment bearing the characterized *Abg2* gene, fragments smaller than 4.5 kb may be inferred to carry the gene from the alternate locus encoding ABG2 or a closely related isoenzyme.

Northern analysis

Total RNA was prepared from leaf and root tissue of untreated barley seedlings, and from leaf tissue of barley seedlings exposed to mercuric chloride or infection by *Erysiphe graminis* f. sp. *hordei* (powdery mildew). At the time of harvest, mercuric chloride had produced small necrotic lesions on leaf surfaces; fungal infection had produced chlorosis underlying regions of heavy fungal growth.

A northern blot was hybridized with the entire *Abg2* second exon (nick-translated), and were rinsed at high stringency (68 °C in 0.1 × SSPE, 0.1% SDS). Whereas control healthy leaf tissue exhibited no hybridizing species (Fig. 5; 'Lf'), a 1200 nucleotide message was observed in healthy root tissue (Fig. 5; 'Rt'). This message was induced in seedling leaves in response to mercuric chloride (Fig. 5; 'Hg') which is an established inducer of PR proteins of monocots and dicots [11, 37]. The *Abg2* message was induced to the greatest extent in leaves infected with powdery mildew (Fig. 5; 'Eg'), which has also been shown to induce expression of barley basic β -1,3-glucanase isoenzyme GII [9, 23]. Induction of pathogenesis response by both mercuric chloride and fungal infection in these experiments was also supported (data not shown) by detection of class 5 PR protein induction on western blots using antiserum against a *Zea mays* thaumatin-like protein [20]. Because Southern blots indicated two closely related members of the *Abg2* gene family exist (Fig. 4B), the *Abg2* mRNA expression observed may be a composite of two distinct patterns of regulation from both gene copies. Treatments of seedlings with various phytohormones (5 mM salicylate; 0.1% 2-chloroethylphosphonic acid; 10 μ M each gibberellic acid, kinetin, or 2,4-dichlorophenoxyacetic acid) were

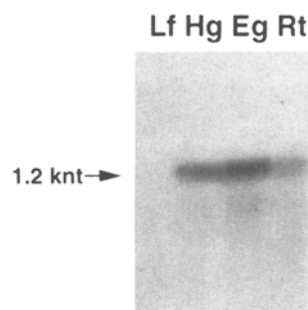


Fig. 5. Northern analysis for *Abg2* expression. Leaves of 5-day old barley seedlings were sprayed with water ('Lf') or 0.2% mercuric chloride ('Hg'), or heavily inoculated with spores of powdery mildew (*Erysiphe graminis* f. sp. *hordei*; 'Eg'). Sprayed leaves were collected 3 days after treatment; fungus-infected leaves were collected 5 days after inoculation. Total RNA was prepared from affected leaf tissue, as well as from roots of 5-day old untreated seedlings ('Rt'). Per lane 50 μ g total RNA was loaded and electrophoresed through an agarose gel containing formaldehyde. After transfer to nylon membrane, the blot was hybridized and rinsed as described for Southern blots (see Materials and methods). The migration of the 1200 nucleotide *Abg2* mRNA is indicated with an arrow. Samples which gave no detectable signal under these conditions included desiccated whole seed, germinated whole seed, and leaves sprayed with either: 5 mM salicylate, 0.1% 2-chloroethylphosphonic acid, 10 μ M gibberellic acid, 10 μ M kinetin, or 10 μ M 2,4-dichlorophenoxyacetic acid.

ineffective in inducing expression of *Abg2* (data not shown).

Procaryotic expression of recombinant ABG2

For procaryotic expression, the mature portion of the ABG2-coding sequence was engineered by PCR to have the amino-terminal sequence Met-Ala-His-Ser-Ile-Gly..., where the methionine and alanine residues result from the introduction of an *Nco* I restriction site. The next two residues may in fact be part of the signal peptide of the ABG2 protein: most barley β -1,3- and β -1,3,4-glucanases begin with the conserved sequence Ile-Gly-Val-Cys... [1, 18, 59]. However, more amino terminal cleavage sites are known such as the tobacco basic β -1,3-glucanase enzyme (Gln-Ser-Ile-Gly-etc. [47]). The product of the PCR reaction was completely verified by sequencing. The

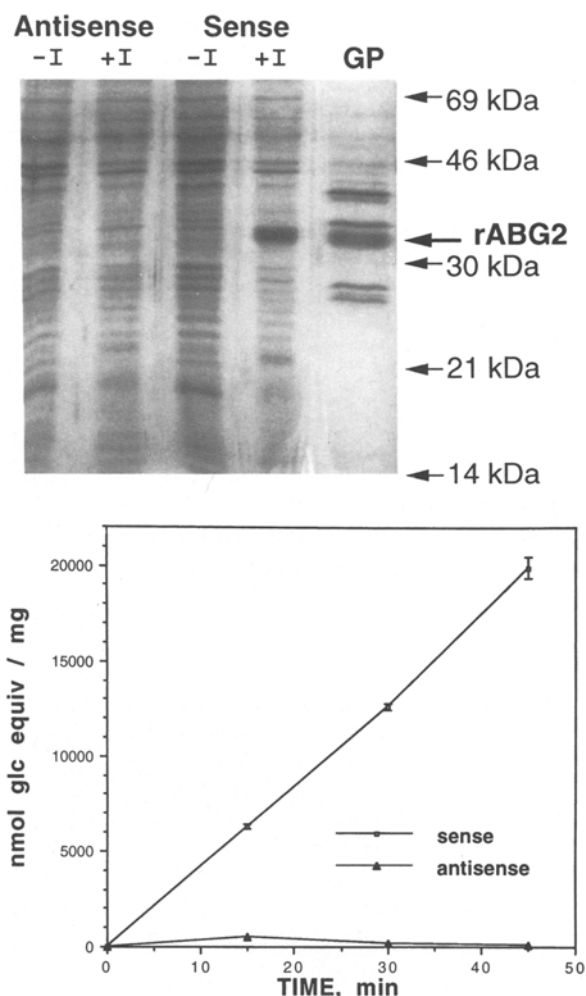


Fig. 6. Expression of ABG2 in *E. coli*. A (top). ABG2 protein expressed in *E. coli*. *E. coli* JM101 cells bearing pMON8977 ('Sense') and a negative control plasmid ('Antisense'; see Materials and methods) were incubated for 4 h in the absence ('-I') or presence ('+I') of 1 mM IPTG. After induction, aliquots of whole cells were boiled in sample buffer and analyzed by silver-stained SDS-PAGE. 'GP' denotes anodic native PAGE fraction enriched for ABG2 protein (see Materials and methods). The migration of the recombinant ABG2 protein ('rABG2') is indicated with an arrow. B (bottom). β -1,3-glucanase activity in sonicated extracts of induced cells. Sonicated extracts were prepared and assayed for β -1,3-glucanase activity by digestion of laminarin (see Materials and methods). Activity is expressed as nanomoles glucose equivalents released per milligram of total protein in the extracts. Error bars for the 'sense' construct represent standard deviation for three independent trials.

gene was transferred to an *E. coli* expression vector behind the *Ptac* promoter and bacteriophage

T7 gene 10 5'-untranslated leader [10, 41]. A cloning byproduct with the majority of the *Abg2*-coding sequence inserted in an antisense orientation was retained as a negative control plasmid.

The 'sense' construct in *E. coli* JM101 gave rise to a 33 kDa protein in response to the IPTG induction (Fig. 6A; 'sense' '+I'). Whereas whole-cell lysates revealed a major induction of this protein, sonicated extracts of induced cells contained less than 0.1% ABG2 protein; much of the induced protein was irretrievably lost to refractile inclusion bodies. The sonicated extracts still retained significant soluble glucanase activity (Fig. 6B). The soluble ABG2 protein was enriched by preparative native PAGE, followed by elution from gel slices. The 33 kDa ABG2 protein was determined by laser densitometry to comprise 19% of the total protein in the most enzymatically active fraction (Fig. 6A, 'GP'). The specific activity of the *E. coli*-expressed recombinant ABG2 protein was determined to be 2400 nkat (nanokatal) per mg protein, at its optimal pH of 4.3. This is nearly equivalent to the reported specific activity of barley β -1,3-glucanase isoenzyme GII [18, 28]; however, as the native ABG2 protein has not yet been characterized, its specific activity is not known. The substrate specificity for the recombinant ABG2 protein has not yet been determined.

Discussion

In very general terms, dicot PR proteins consist of extracellular acidic proteins induced in response to infection, and intracellular basic isoforms induced in response to developmental signals and to infection. The barley basic β -1,3-glucanase isoenzyme GII has been identified in a number of experimental systems as an extracellular protein which is induced in response to pathogenesis-related as well as developmental signals [14, 18, 23, 24, 28, 60]. The mounting implication of the GII protein as a defense-related extracellular protein raised the question of whether barley has acidic PR-2 class β -1,3-glucanases which serve as extracellular PR pro-

teins. Acidic β -1,3-glucanases may have been present in the extracellular fluids of mildew-infected barley [23], where at least five serologically related proteins were observed; whether any of these proteins were in fact acidic isoenzymes has not been reported.

A β -glucanase gene has been recovered from a barley genomic library by screening with a partial cDNA of the infection-related basic β -1,3-glucanase isoenzyme GII. The complete *Abg2* gene resided on a 4.5 kb *Hind* III fragment. A linked pseudogene recovered in the same clone was evidently not an artifact of rearrangement during cloning. Numerous point deletions and insertions suggest that the pseudogene is a vestigial second exon of a glucanase gene. A vestigial first exon may exist farther upstream than the 2 kb characterized; introns as large as 2.9 kb have been reported in barley β -1,3-1,4-glucanase genes [55]. Southern blots of genomic digests indicated that two authentic members of the *Abg2* gene family reside in the barley genome. Several additional hybridizing fragments observed after intermediate rinses are presumed to represent the pseudogene (a 2.8 kb *Eco* RI fragment in genomic DNA as well as in clone 9) and other β -1,3- and β -1,3-1,4-glucanase genes, which share 65–78% nucleotide identity with *Abg2*.

The ABG2 protein sequence is most homologous (64% identical/81% similar) to the barley basic β -1,3-glucanase GII, which has a pI greater than 10 [18]. The ABG2 protein is slightly less similar to the barley β -1,3-1,4-glucanases [31, 55] with still more distant relationship to the rice *Gns1* β -glucanase gene [49] and the acidic and basic tobacco β -1,3-glucanases [30]. Though the ABG2 protein is most homologous to a basic β -1,3-glucanase of barley, the *Abg2* primary sequence predicts a protein with an isoelectric point under 5. However, as the native ABG2 protein has not yet been characterized, the true isoelectric point, as well as the mature amino terminus and the glycosylation state of the ABG2 protein remain to be determined.

The reduced G + C content of the first *Abg2* exon, relative to the rest of the gene, was also noted for the barley β -1,3-1,4-glucanase isoen-

zyme EI gene [31]. This may reflect a possible common ancestral lineage for the barley genes [18], but this difference has also been observed in the rice *Gns1* β -glucanase gene [49]. The coding region of the *Abg2* second exon has a greater than 90% preference for G + C in the third position of degenerate codons. High G + C content in the wobble position has been postulated to be a potential mechanism for rapid translation during germination, if isoaccepting tRNA species are correspondingly abundant [14]. However, northern blots of dry and germinated barley seed RNA (data not shown) indicated that *Abg2* is not induced during germination, in contrast to barley β -1,3-1,4-glucanases which serve to degrade glucans of barley endosperm cell walls [13, 14].

Abg2 exhibits hallmarks of a PR protein gene, for its expression is induced by stress, infection, and developmental signals. *Abg2* mRNA was induced in leaf tissue in response to mercuric chloride, which is a known inducer of pathogenesis response in monocots and dicots [11, 37]. Expression of *Abg2* mRNA was most highly induced in response to infection by *Erysiphe graminis* f. sp. *hordei*, a demonstrated inducer of barley β -1,3-glucanase isoenzyme GII [9, 23]. Therefore, at least at the transcriptional level, *Abg2* can be considered a genuine component of pathogenesis-response. Because the abundance and distribution of native *Abg2* protein have not yet been determined, posttranscriptional regulation of *Abg2* expression is possible. Evidence for such regulation has been demonstrated for tobacco basic PR5 protein osmotin [27].

Expression of *Abg2* mRNA is constitutive in healthy roots, as has been reported for certain other dicot basic PR proteins [3, 29], and the rice *Gns1* β -glucanase gene [49]. *Abg2* root expression was also observed in aseptically germinated seedlings, and did not require exposure to soil for induction. Despite the root-specific expression of *Abg2*, no homology to the cauliflower mosaic virus 35S root specificity motif [26] is observed in the available promoter sequence. The signals mediating developmental *Abg2* expression are unknown; various phytohormone treatments (auxin, cytokinin, ethylene, gibberellic acid, salicylic acid)

applied as foliar sprays were ineffective in inducing *Abg2* mRNA expression. An *Abg2* promoter-reporter gene fusion may ultimately be required to more sensitively assess response of *Abg2* to phytohormone treatments. Likewise *Abg2* promoter analysis may prove similarly useful in identifying regulatory elements responsible for the observed developmental, stress and disease induction.

Because two copies of *Abg2* were shown to exist by Southern blots using the same high stringencies of northern experiments, the *Abg2* mRNA may represent a composite of developmental and pathogenesis-induced expression from either or both copies of *Abg2*. As has been shown for barley β -1,3-1,4-glucanases [50, 51] nearly identical isoenzymes may have distinct patterns of regulation.

To verify that the *Abg2* gene encoded a functional glucanase enzyme, the gene was engineered by PCR for procaryotic expression. The amino terminal four residues of recombinant ABG2 were assigned arbitrarily for the purposes of cloning. Nevertheless, induction of *E. coli* cells bearing the ABG2 expression construct gave rise to a protein of the expected molecular weight. Much of the induced protein was irretrievably lost to refractile bodies, but sonicated extracts of these cells retained detectable soluble glucanase activity. Specific activity of the recombinant ABG2 protein in an enriched sample was comparable to native barley β -1,3-glucanase isoenzyme GII, suggesting that the ABG2 protein does not require eucaryotic posttranslational modification for activity. However, results from procaryotic expression cannot be definitively extrapolated to the native ABG2 protein, which has not yet been characterized. The pH optimum for recombinant ABG2 activity was 4.3. Should native ABG2 prove also to have an acidic pH optimum, this could reflect the pH of the compartment where the enzyme exerts its effect. Typically, acidic PR2-class β -1,3-glucanases are induced in response to infection and exported to the intercellular space [3, 5]. Here they may serve a defensive role through hydrolysis of glucan in fungal hyphae, and glucanase enzymatic activity may have been optimized for function in the acidic extracellular milieu. If

retained intracellularly in the vacuole, a glucanase with an acidic pH optimum might serve to degrade internalized glucans derived from extracellular callose. However, retention in the vacuole might also be a means of sequestering defensive antifungal hydrolases until cells are lysed and vacuolar contents liberated [34, 53]. Therefore, though the localization of ABG2 protein is unknown, a defensive role could be served from either an intracellular or extracellular destination.

The array of barley pathogenesis-related proteins is less well defined than those of tobacco. The identification of an acidic β -1,3-glucanase gene in barley will provide a useful tool for future investigations into the expression of PR proteins in barley. The observation of developmental, stress, and disease induction of *Abg2* expression has verified the biological function of the *Abg2* gene. The ease of procaryotic expression of active ABG2 enzyme will facilitate purification, antibody production, antifungal testing, and possible structure and mechanism studies. The availability of both acidic and basic PR2-class β -1,3-glucanase genes now makes it possible to address questions of the differential induction and localization of these proteins. The answers to these questions will further elucidate the role of β -1,3-glucanases in plant physiology and plant defense.

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