Theor Appl Genet (2001) 102:353–362

W.L. Li · J.D. Faris · S. Muthukrishnan · D.J. Liu
P.D. Chen · B.S. Gill

Isolation and characterization of novel cDNA clones of acidic chitinases and β-1,3-glucanases from wheat spikes infected by *Fusarium graminearum*

Received: 19 April 2000 / Accepted: 15 May 2000

Abstract Chitinases and β-1,3-glucanases are important components of plant defense in response to attack by pathogens. To identify specific chitinases and β-1,3-glucanases, we constructed a cDNA library using mRNA from wheat spikelets inoculated with conidia of *Fusarium graminearum*. Two chitinase and two β-1,3-glucanase clones were isolated using a rice chitinase Ia gene and barley cDNA clones for chitinase II and β-1,3-glucanase as probes. Sequence analysis showed that the cDNA clone SM194 encodes an acidic isoform of class-VII chitinase, the cDNA clone SM383 encodes a class-IV chitinase and the cDNA clones SM289 and SM638 encode two different acidic isoforms of β-1,3-glucanases. Nulli-tetrasomic analysis indicated that SM194 and SM383 were located on all of the group-2 chromosomes of wheat. Genetic mapping showed that at least three copies of class-IV and/or class-VII chitinase genes were clustered on the long arm of chromosome 2D of *Aegilops tauschii* and that they mapped genetically close to the centromere. SM289 and SM638 were located on all of the group 3 chromosomes of wheat by nulli-tetrasomic analysis, and to the β-1,3-glucanase clusters in the 3BL and 3DL chromosome arms of wheat by genetic mapping. Northern blot hybridization showed that the expression of these genes is induced upon infection with *Fusarium graminearum*. The accumulation of transcripts for these PR-proteins was more rapid in the resistant variety Sumai 3 than in its susceptible mutant during the first 24 h. This is the first report of the induction of class-IV and class-VII chitinases in cereals by a fungal pathogen.

Keywords Chitinase · β-1,3-Glucanase · PR proteins · cDNA cloning · Genetic mapping · Induced expression · *Triticum aestivum* · *Fusarium graminearum*

Communicated by G. Wenzel

Contribution No. 00–389-J from the Kansas Agricultural Experiment Station, Kansas State University, Manhattan, Kansas, USA. The sequences reported in this paper have been deposited in GenBank database with accession numbers AF112963 for SM194, AF112965 for SM289, AF112966 for SM383 and AF112967 for SM638.

W.L. Li · D.J. Liu · P.D. Chen
Cytogenetics Institute, Department of Agronomy,
Nanjing Agricultural University, Nanjing 210095,
The People's Republic of China

J.D. Faris · B.S. Gill (✉)
Wheat Genetics Resource Center, Department of Plant Pathology,
Kansas State University, Manhattan, KS 66506, USA
e-mail: bsg@ksu.edu

S. Muthukrishnan
Department of Biochemistry, Kansas State University,
Manhattan, KS 66506, USA

Present address:
W.L. Li, Wheat Genetics Resource Center,
Department of Plant Pathology, Kansas State University,
Manhattan, KS 66506, USA

Introduction

Plants express a wide variety of genes in response to pathogen/pest infection. Such genes are referred to as pathogenesis-related (PR) genes (Bowles 1990). The best characterized genes belonging to this group are those that encode the hydrolytic enzymes known as chitinases (EC 3.2.1.14) and β-1,3-glucanases (EC 3.2.1.39). These hydrolytic enzymes inhibit the growth of many fungi *in vitro* by hydrolyzing the chitin and β-glucan of fungal cell walls. Furthermore, oligomeric products of digested chitin and β-glucan can act as signal molecules to stimulate further defense responses. These lytic enzymes have attracted much attention and have become very important resources in the genetic engineering of crop plants for disease resistance (Muthukrishnan et al. 2000).

Chitinase and β-glucanase genes exist as families: seven classes of chitinases and at least three classes of glucanases have been recognized (Collinge et al. 1993; Meins et al. 1992, 1994; Neuhaus 1999; Leubner-Metzger and Meins 1999). Individual members within each class can reside at different chromosomal locations (Li et

al. 1999), and some exhibit differential expression depending on the pathogen. In addition to the pathogen-inducible PR proteins, other PR proteins that are developmentally regulated have also been identified in almost all plant species (Van Loon 1999). In polyploid species such as hexaploid wheat (*Triticum aestivum* L., 2n=6x=42, AABBDD), which carry paralogous sets of PR genes on homoeologous chromosomes, there might be more opportunities for structural and functional evolution of PR genes with respect to pathogen induction, developmental regulation and recruitment for new function. To analyze the role of specific PR proteins in the defense process, it is desirable to induce their expression by a given pathogen, identify the specific chitinase and glucanase genes induced and determine the positions of the expressed genes in the plant genome.

Wheat scab, caused by *Fusarium graminearum*, can be a devastating disease which not only lowers grain yield but has an adverse effect on grain quality as well. We are studying the interaction between wheat and *F. graminearum* using the resistant variety Sumai 3 and its susceptible mutant. A cDNA library of mRNA from scab-infected Sumai 3 was constructed, and cDNA clones encoding chitinases and glucanases were isolated using a rice and barley clones for chitinase and β-1,3-glucanase as probes. In the present paper, we report the isolation and characterization of these clones.

Materials and methods

Plant material

Sumai 3 was selected from the cross Funo × Taiwan Wheat at the Agricultural Institute of Suzhou Prefecture, China, and has been grown at the Experimental Station of Nanjing Agricultural University for many years. The susceptible mutant was first recognized by its green anther color and later by direct screening for reaction to *F. graminearum* (W.L. Li et al. unpublished). Nulli-tetrasomic (NT) lines of cv. Chinese Spring (Sears 1966), where nullisomy for a specific chromosome is compensated for by two extra copies of a homoeologue, were used to locate the cDNA clones on specific wheat chromosomes. N2AT2B and N4BT4D were identified cytologically as these plants are maintained as mono-tetrasomic lines. For genetic mapping, a population of recombinant inbred lines (RILs) derived from a cross between a synthetic hexaploid wheat, W-7984, and the common wheat variety Opata 85, produced as described in Nelson et al. (1995a), was provided by Dr. M.E. Sorrells, Cornell University, Ithaca, New York. An *Aegilops tauschii* Coss. [syn. *Ae. squarrosa* L., syn. *Triticum tauschii* (Coss.) Schmal. 2n = 14, DD] mapping population described by Gill et al. (1991) consisting of 56 F_2 plants from the cross of *Ae. tauschii* accessions TA1691 and TA1704 was used when polymorphism was not detected between W-7984 and Opata 85.

Pathogen

GZ3639, a highly virulent isolate of *F. graminearum*, was collected and kindly provided by Dr. R.L. Bowden, Department of Plant Pathology, Kansas State University. The isolate was grown in mung bean broth and cultured (at room temperature and 200 rpm) for 4–7 days to produce conidia. The conidia was collected by centrifugation for 5 min at 5000 rpm, washed twice by resuspending in fresh mung bean broth media and adjusted to a final concentration of 1.8×10^5 ml^{-1}.

Clones

Chi11, a genomic clone of rice that encodes a class-Ia chitinase, was isolated by Huang et al (1991). Two barley cDNA clones – HvChtN12, which encodes a class–II chitinase, and BH72-I1, which encodes β-1,3-glucanase – were kindly supplied by Dr. D.B. Collinge of the Department of Plant Pathology, Royal Veterinary and Agricultural University, Denmark.

Inoculation

About ten spikelets per spike of Sumai 3 and the susceptible mutant were inoculated with the conidium suspension of *F. graminearum* (GZ3639) and kept in a mist chamber for up to 72 h. The inoculated spikelets were collected for RNA isolation 24 h after inoculation (hai), 48 hai and 72 hai. A mock-inoculation was made with fresh mung bean broth.

RNA isolation, cDNA synthesis, cDNA cloning and sequencing

Total RNA was isolated from the non-inoculated, inoculated, and mock-inoculated spikelets using Trizol™ reagent from Gibco BRL Life Technologies (Grand Island, N.Y.). For the 24 hai treatment of Sumai 3, mRNA was purified using the PolyATract mRNA Isolation System from Promega (Madison, Wis.); cDNA was synthesized and amplified using the Smart™ polymerase chain reaction (PCR) cDNA synthesis Kit from CLONTECH (Palo Alto, Calif.). All experiments were conducted following the manufacturer's recommendations. After 16 cycles of PCR amplification, the cDNA sample of Sumai 3 was size-fractionated by electrophoresis on a 1.2% agarose gel. Using the molecular weight standard as a guide, 1- to 2-kb fragments were eluted from the gel using the PCR-Pure kit from CLONTECH, these were then ligated to T-vector (Promega) and used to transform competent cells of *E. coli* (JM109). Recombinants were picked from LB/Amp/X-gal plates and grown in LB/Amp broth. A 1-µl aliquot of the culture of each recombinant clone was used as the template for PCR amplification, and the remaining culture was stored in 15% glycerol at –80°C. A 5-µl sample of heat-denatured PCR product was dotted onto Hybond N^+ membrane (Amersham, UK) and baked at 80°C for 2 h. The membranes were hybridized with [^{32}P]-labeled coding region probes of the chitinase and glucanase clones and washed under a stringent condition (0.2 × SSC, 1% SDS at 65°C).

The positive clones were grown in LB liquid culture, and the plasmids were prepared using the Plasmid Miniprep kit from Qiagen (Valencia, Calif.) and used as templates for sequencing. Open reading frames (ORFs) and amino acid sequences were deduced using the ORF FINDER program, and homology searches were conducted using the BLAST 2.0 program of the National Center of Biotechnology Information (NCBI), available at the website http://www.ncbi.nlm.nih.gov. The theoretical isoelectrical points, molecular weights and cleavage sites of signal peptides were analyzed with ''DNA & Protein Analysis Toolkit'', available at the website http://www.rockefeller.edu/rucs/toolkit/toolkit.html.

Mapping analysis

The mapping population consists of 114 RILs and has been the subject of an extensive genome mapping effort by investigators of the International Triticeae Mapping Initiative (ITMI) (Marino et al. 1996; Nelson et al. 1995a, b, c; Van Deynze et al. 1995). The first 60 RILs of the ITMI population and 56 F_2 plants of the *Ae. tauschii* population were used for genetic mapping. Base maps were constructed (Li et al. 1999) for the placement of chitinase and glucanase genes. Linkage relationships were evaluated with MAPMAKER (Lander et al. 1987) using a minimum LOD of 2.0 and the Kosambi mapping function (Kosambi 1944).

Genomic DNA isolation from Chinese Spring NT lines, W-7984, Opata 85 and the RILs, restriction enzyme digestion, gel electrophoresis, Southern blotting, probe labeling and hybridization were done as described in Faris et al. (2000).

Northern blot hybridization

Total RNA (10 µg) was denatured, separated by agarose/formaldehyde gel electrophoresis, blotted onto nitrocellulose membrane (Schleicher & Schuell) with 10 × SSC buffer, cross-linked with UV, and hybridized to [^{32}P]-labeled probes following the manufacturer's instructions.

Results and discussion

Isolation and sequences of chitinase cDNA clones

Four clones, SM169, SM194, SM233 and SM383, were identified when 400 clones of the cDNA library were screened using a mixture of the inserts of clones Chi11 and HvChtN12 as a probe. SM194 and SM383 are similar to SM169 and SM233 in insert sizes, respectively. DNA sequencing showed that the latter two were slightly shorter forms of the former two. Single-pass sequencing of the entire library revealed two additional chitinase clones, SM98 and SM471, that showed high sequence similarity to SM194 and SM383 (J. Fellers, personal communication). We will focus on SM194 and SM383 in this report.

SM194 and SM383 contain cDNA inserts of 956 bp and 1088 bp, respectively. The ORFs extend from position 67 to position 759 of SM194, and from position 62 to 880 of SM383. SM194 encodes a polypeptide of 230 amino acids with a theoretical molecular weight (MW) of 24.7 kDa and a pI of 5.00. SM383 encodes a polypeptide of 272 amino acids with a MW of 29.03 kDa and a pI of 4.96. The first 26 amino acids of SM194 and SM383 showed characteristics of signal peptides of secretory proteins, *i.e.* a positively charged residue near the amino terminus and a hydrophobic core. SM194 and SM383 have 37 and 55 charged amino acid residues, and 2 and 7 net negatively charged residues in preproteins, respectively. After cleavage of the signal peptides, SM194 is predicted to have a charge of –4 and a pI of 4.87, and SM383 to have a charge of –8 and a pI of 4.62, indicating that they are acidic chitinases. Both clones contain the glutamate residues implicated in catalysis (Neuhaus 1999) (positions 94 and 103 in SM194, 135 and 144 in SM383). They also possess a conserved amino acid sequence NYNYG (at positions 103–107 in SM194 and positions 134–138 of SM383). This motif is present in most PR-3 chitinases (Neuhaus 1999), and the first Y is implicated in substrate binding in a maize chitinase (Verburg et al. 1992) to which SM194 and SM383 are homologous. These data imply that these genes encode active chitinases.

BLAST searches with the nucleotide sequences of SM194 and SM383 indicated that they have low degree of sequence similarity to some chitinase genes from maize and rice. BLAST searches using the deduced amino acid sequences of SM194 and SM383 indicated that they have high sequence similarity to genes or cDNAs encoding class-IV and class-VII chitinases of diverse plant species including class-VII chitinase of rice and class-IV chitinases of lambsquarters (*Chenopodium amaranticolor*), grape (*Vitis vinifera*) and maize (Fig. 1). In contrast, SM194 and SM383 showed very low sequence similarity to class-I and class-II chitinases including Chi11 (accession: X54367) (Huang et al. 1991) and HvChtN12 (accession: X78672; Collinge DB, unpublished), which were the probes used in their isolation. Alignment with class-I and -II chitinases indicated that deletions had occurred in the hinge region, loops 1 and 3 of SM194 and SM383, but loop 2 remained intact. Compared with SM383, SM194 lacked the chitin binding domain (CBD) of 41 amino acids near the N-terminus which contains 8 cysteine residues characteristic of class-I and class-IV chitinases and other chitin-binding proteins such as wheat-germ agglutinin (Neuhaus 1999) (Figs. 1 and 2). SM383 had a deletion in the N-terminal chitin binding region diagnostic of the class-IV chitinases. According to the chitinase classification and their structural features, SM194 and SM383 can be assigned to class-VII and class-IV chitinases, respectively. The chitinases encoded by SM194 and SM383 share 80% amino acid sequence identity.

Several chitinase genes or cDNAs have been isolated from the major cereals. Most of them are class-I and class-II chitinases (Muthukrishnan et al. 2000). A class-III chitinase and a class-VII chitinase have been isolated from rice (accessions: BAA23807 and BAA19793) and two closely related class-IV chitinases have been isolated from maize seeds (Huynh et al. 1992). Only one genomic clone for class-Ib chitinase has been reported in wheat. SM194 is only the second class-VII chitinase gene isolated from a plant, and SM383 is the first class-IV chitinase gene isolated from wheat. According to Neuhaus (1999), the deletion in loop 1, which extends into the catalytic cleft of these chitinases, might result in the loss of a sugar-binding sub-site. Furthermore, loss of loops 3 and 4 in class-IV and -VII chitinases reduces the overall surface area of the chitinases. The functional differences between the chitinases encoded by SM194 and SM383 and class-I and class-II chitinases in the defense response are unknown. It is interesting to note that even though we used probes for class-I and class-II chitinases to screen our library, only class-IV and -VII chitinases were identified. This might suggest the greater importance of these chitinases over other classes of chitinases in the defense response.

Isolation and sequence analysis of β-1,3-glucanases

Two cDNA clones, SM289 and SM638, were isolated by screening 880 clones of the cDNA library using the barley β-1,3-glucanase cDNA clone BH72-I1 as a probe. The cDNA inserts in SM289 and SM638 are 1269 bp

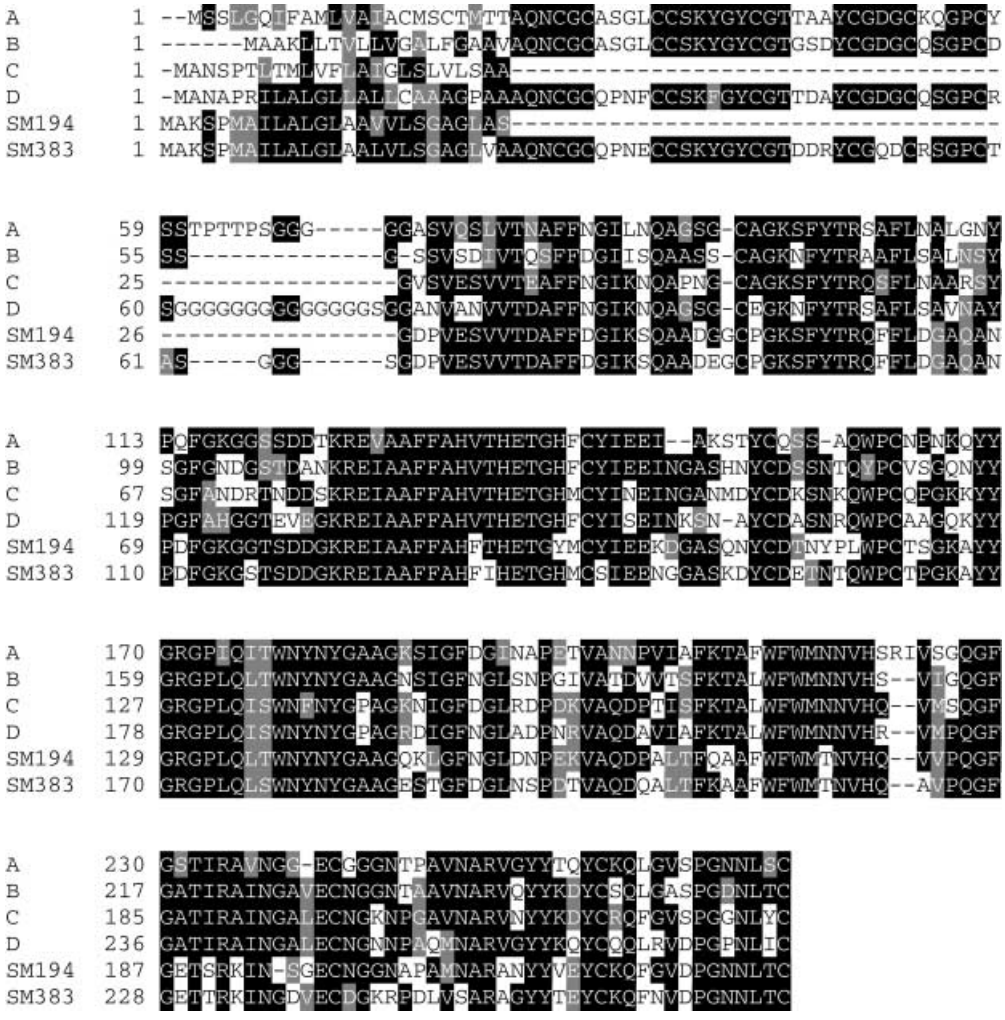

Fig. 1 Alignment of the amino acid sequences of SM194 and SM383 with known class-IV and -VII chitinases. *A* Chitinase of *Chenopodium amaranticolor* (gi|2570162|dbj|BAA22966.1), *B* class-IV endochitinase of *Vitis vinifera* (gi|2306811|gb|-AAB65776.1), *C* rice chitinase VII (gi|2055262|dbj|BAA-19793.1), *D* maize chitinase A (gi|283037|pir||A42424). Dark and/or gray backgrounds were applied to highlight the positions where more than half of the accessions show identical and/or similar residues, respectively

and 1439 bp, respectively. The longest ORFs in SM289 and SM638 encode proteins of 334 amino acids in length with theoretical pI/MW of 5.70/34.88 kDa and 4.35/34.66 kDa, respectively. After cleavage of the signal peptides (the first 27 residues in SM289 and the first 29 residues in SM638), SM289 is predicted to have a pI/MW of 5.46/32.20 kDa and SM638 of 4.22/31.79 kDa. Neither of them contained the C-terminal extension, although SM638 showed high sequence similarity with several β-1,3-glucanases that do possess the C-terminal extensions (Fig. 3).

BLAST searches revealed extensive sequence similarity with previously reported β-1,3-glucanase genes at the DNA and amino acid levels. At the nucleotide sequence level, SM289 showed the highest sequence similarity with the barley β-1,3-glucanase genes AGB2 (accession: M91814) and HV13GEIII (accession: X67099) with about 85% identities (Malehorn et al. 1993; Wang et al. 1992). SM638 is homologous to a rice β-1,3-glucanase cDNA (AB027428) and a rice β-1,3-glucanase gene (U72251) with an identity of 87% (617 and 445 bits of score, respectively). Amino acid sequences of proteins encoded by SM289 and SM638 were compared and aligned with β-1,3-glucanases in the databases. Similar to the result of the nucleotide sequence comparison, the predicted SM289 protein had the highest sequence similarity to three barley β-1,3-glucanases with 76%/83% of identities/similarities (Fig. 4). Similarly, the predicted SM638 protein showed over 65%/71% of identities/similarities with two rice and two maize β-1,3-glucanases (Fig. 3). Although BLAST searches indicated that SM289 and SM638 were related to several previously reported wheat β-1,3-glucanases, the sequence identity was much lower. The highest sequence similarity to SM289 was observed with the wheat β-1,3-glucanase cDNA clone of accession Y18212, with a score of 224 bits in nucleotide sequence and an identity of 61.2% in amino acid sequence. Therefore, SM289 and SM638 represent new types of β-1,3-glucanases in wheat that have not been reported before.

SM289 and SM638 showed very high sequence similarity to the β-1,3-glucanases of barley and maize, respectively. However, the sequence identity/similarity between them is only 55%/69% (Table 1). The β-1,3-glucanases, like chitinases, have been grouped into various classes (Meins et al. 1992; Xu and Fincher 1992). Ac-

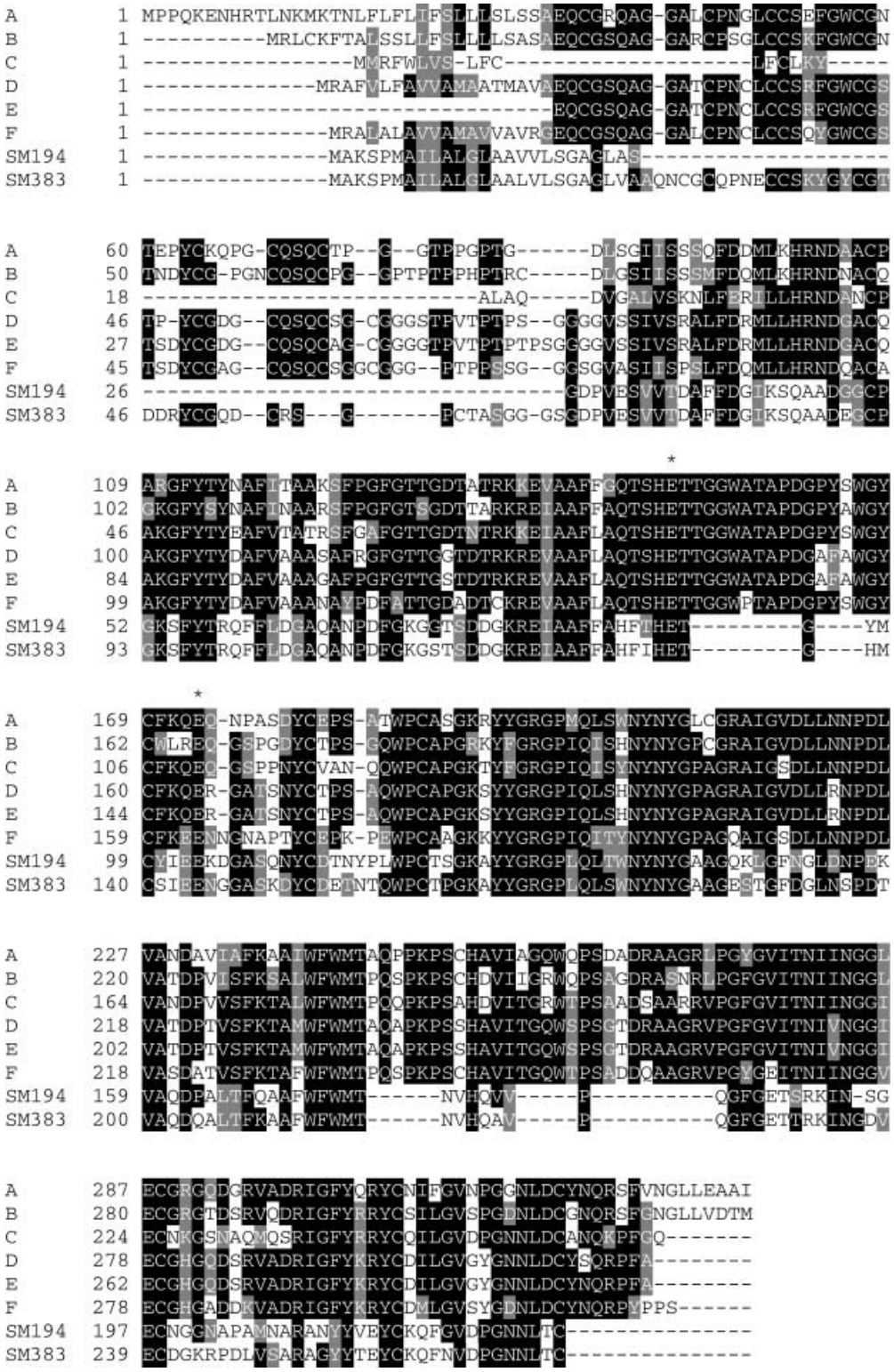

Fig. 2 Alignment of the amino acid sequences of SM194 and SM383 with known class-I chitinases. *A* Basic chitinase precursor of *Arabidopsis thaliana* (gi|320556|pir||B45511), *B* tobacco basic chitinase (gi|256133|gb|AAB23374.1), *C* tobacco chitinase 134 (gi|3790355|dbj|BAA33971.1), *D* barley endochitinase precursor (gi|2506281|sp|P11955), *E* rye chitinase a (gi|542173|pir||JC2071), *F* rice endochitinase (gi|500617|dbj|BAA03751.1). Dark and gray backgrounds were applied to highlight the positions where more than half of the accessions show identical and/or similar residues, respectively. The asterisks indicate the two catalytic glutamates

cordingly, SM289 and SM638 might belong to different classes of glucanases.

Chitinases and β-1,3-glucanases are included in the subclass of defense response genes which encode PR proteins. Most PR proteins have isoforms with different isoelectric points, *i.e.* acidic and basic types. Generally, basic PR proteins are intracellularly targeted to the vacuole, and the acidic proteins are located extracellularly (Meins et al. 1992). In the present study, we isolated two chitinase cDNA clones and two β-1,3-glucanase cDNA clones, all of which are predicted to have acidic pIs. They all have typical signal peptides characteristic of se-

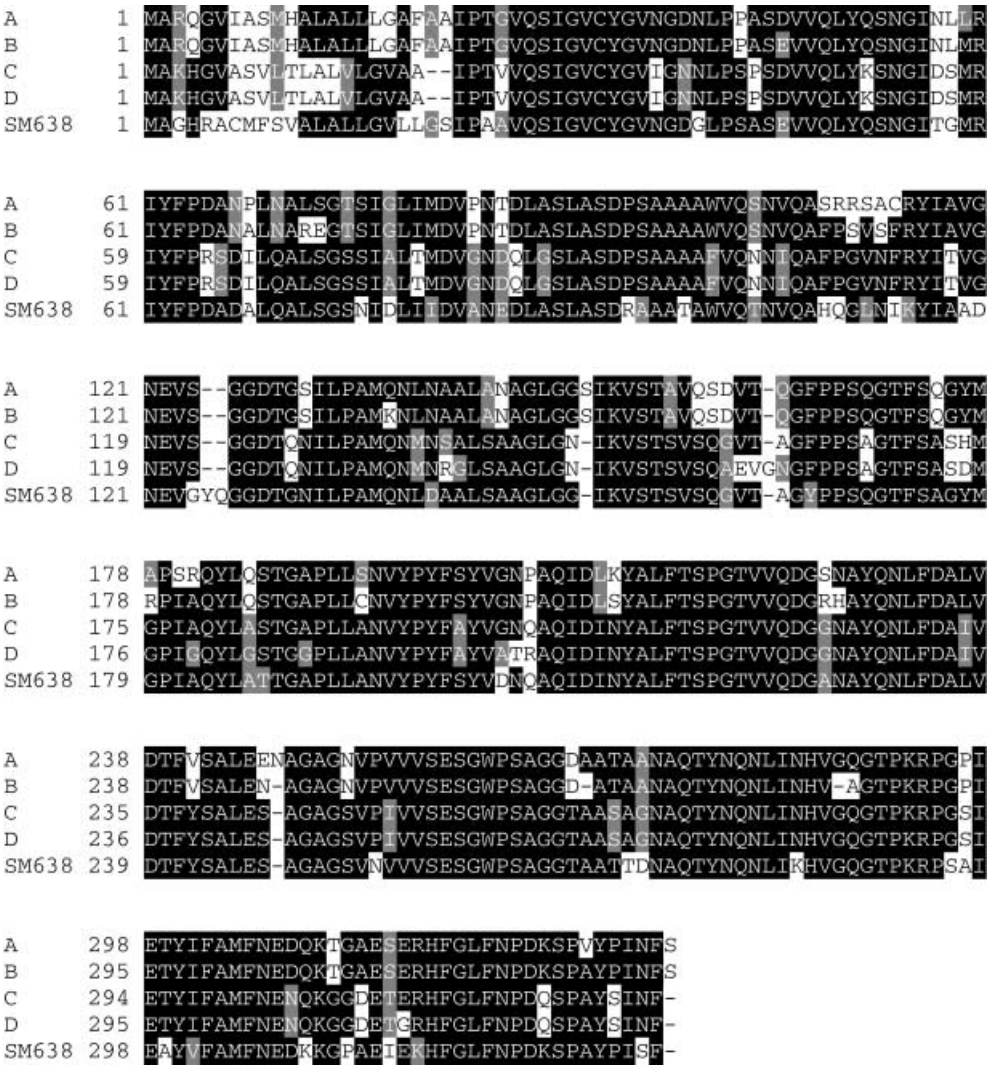

Fig. 3 Alignment of the amino acid sequence of SM638 with known β-1,3-glucanases. *A* Maize acidic β-1,3-glucanase precursor (gi|1352327|sp|-P49237), *B* maize β-1,3-glucanase PRm 6b (gi|1839591-|gb|AAB47177.1), *C* β-1,3-glucanase (gi|4884526|dbj|-BAA77783.1), *D* gi|4097940-|gb|AAD10382.1 β-1,3-glucanase precursor. Dark and/or gray backgrounds were applied to highlight the positions where more than half of the accessions show identical and/or similar residues, respectively

cretory proteins. The presence of the signal peptides together with low pI suggests that SM194, SM383, SM289 and SM638 encode proteins that are targeted to extracellular locations.

Genetic mapping of chitinase and β-1,3-glucanase cDNA clones

Southern analysis of NT lines with cDNA inserts of the chitinase clones SM194 and SM383 gave identical hybridization patterns and produced approximately ten bands (*Eco*RI digest). All the bands were localized to group-2 chromosomes of wheat. Both clones were monomorphic between Opata 85 and the synthetic hexaploid wheat W7984. Four polymorphic and two monomorphic bands (data not shown) were detected by SM194 and SM383 in the *Ae. tauschii* parents. SM194 and SM383 detected co-segregating loci *Xksu932* (Cht7) and *Xksu931* (Cht4), respectively. They were mapped to the long arm of chromosome 2D at a position between markers *Xwg405* and *Xpsr102*, which appear to be genetically close to the centromere (Boyko et al. 1999) (Fig. 5). This result indicates that there are probably at least three members of class-IV and/or class-VII chitinase genes organized into a cluster in chromosome 2D of *Ae. tauschii*. The same scenario is predicted in hexaploid wheat because *Ae. tauschii* is a diploid progenitor of the hexaploid. Restriction digestion and mapping data confirm the high sequence similarity of the two clones. In contrast, Chi11 and HvChtN12, the probes used for their isolation, detected several additional loci besides those in group-2 chromosomes. Polymorphic loci of Chi11 mapped to chromosomes 2B and 3 A, and ChtN12 detected fragments on chromosome 1B of wheat and chromosome 5D of *Ae. tauschii* (Li et al. 1999). These data are consistent with the extent of sequence divergence between the two chitinase clones reported here and other previously reported cereal chitinase genes.

Analysis of NT lines assigned the β-1,3-glucanase genes SM289 and SM638 to group-3 chromosomes just as BH72-I1, the probe used for their isolation. Genetic mapping localized SM289 to 3BL and 3DL [*Xksu933* (Glb3)], and SM638 to 3DL [*Xkus934* (Glb3)] of wheat

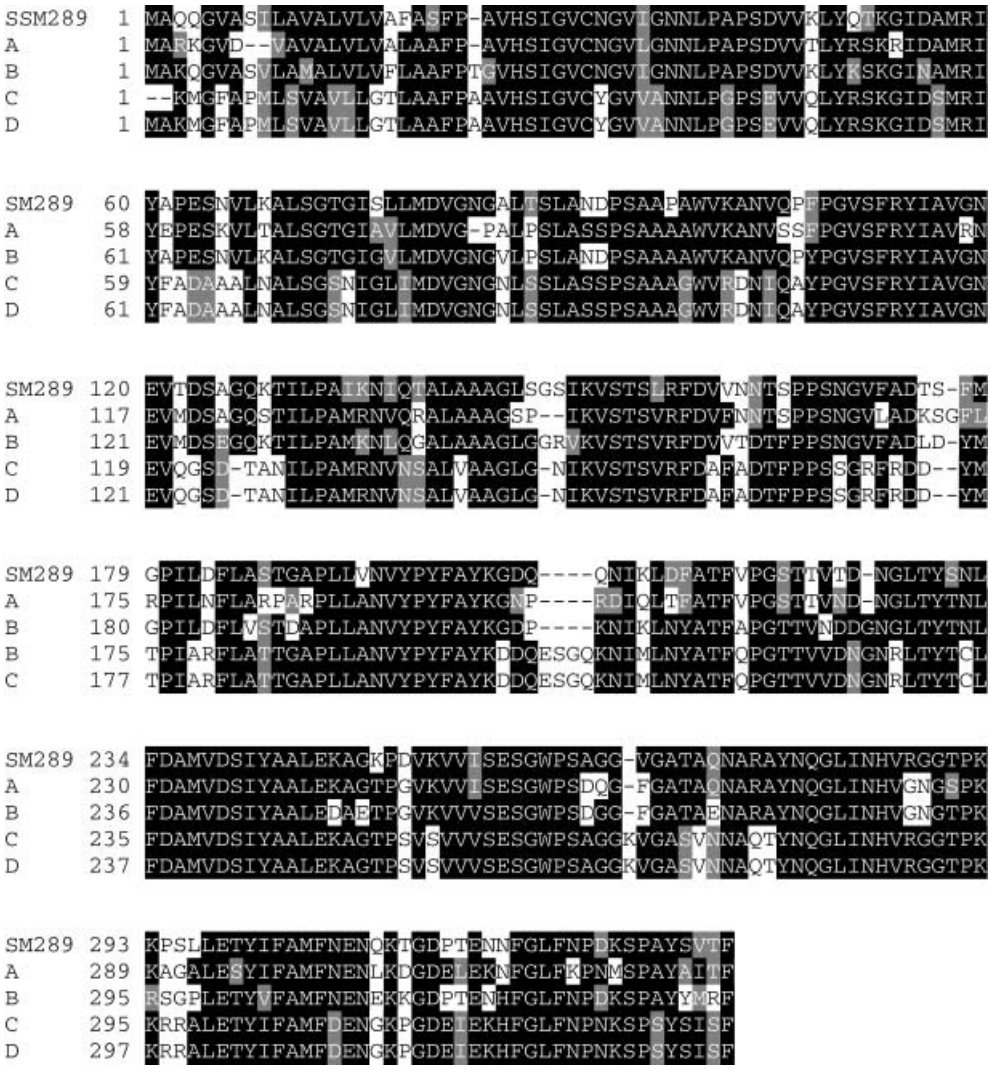

Fig. 4 Alignment of the amino acid sequence of SM289 with known β-1,3-glucanases. *A* Barley β-1,3-endoglucanase GIII (gi|461980|sp|Q02126), *B* barley β-1,3-glucanase (gi|479687|pir||S35156), *C* rice β-1,3-glucanase (gi|4884530-|dbj|BAA77785.1), *D* rice β-1,3-glucanase (gi|4884528-|dbj|BAA77784.1). Dark and/or gray backgrounds were applied to highlight the positions where more than half of the accessions show identical and/or similar residues, respectively

Table 1 Comparison of the predicted SM289- and SM638-encoded proteins and published β-1,3-glucanase protein sequences. Proteins are from published clones and accession numbers: A, barley genomic clone AGB2 (M91814); B, barley β-1,3-glucanase GIII genomic clone (X67099); C, wheat cDNA clone (Y18212); D, barley β-1,3-glucanase GII cDNA clone (X15205); E, maize cDNA clone (S82315); F, maize cDNA clone (M95407)

Glucanase	Percentage of positions with identical/similar amino acids							
	A	B	C	D	E	F	SM289	SM638
SM289	80/88	76/83	63/77	63/77	60/72	58/71	100	55/69
SM638	56/69	51/68	59/72	60/72	74/82	74/82	55/69	100

(Fig. 5). These loci map very close to the position of β-1,3-glucanase loci (Glb3) detected by BH72-I1 (Li et al. 1999). This result not only confirmed the presence of a β-1,3-glucanase gene cluster in 3BL, but also revealed another cluster of β-1,3-glucanase genes in 3DL of wheat. The β-1,3-glucanase gene cluster was also observed in the 3HL chromosome arm of barley, where seven β-1,3-glucanase genes are closely linked within 20 cM (Li et al. 1996). The formation of this gene cluster might have been an early event in *Triticeae* evolution.

Induced expression

Northern blot hybridization showed that the expression of the chitinase and β-1,3-glucanase genes characterized in this study is induced in wheat spikelets upon infection with *F. graminearum*. In contrast, transcripts for all of these genes were very low in the non-inoculated control. In mock-inoculated (mung bean broth) control plants, there was a moderate increase in chitinase and β-1,3-glucanase transcripts, presumably as a response to the inoc-

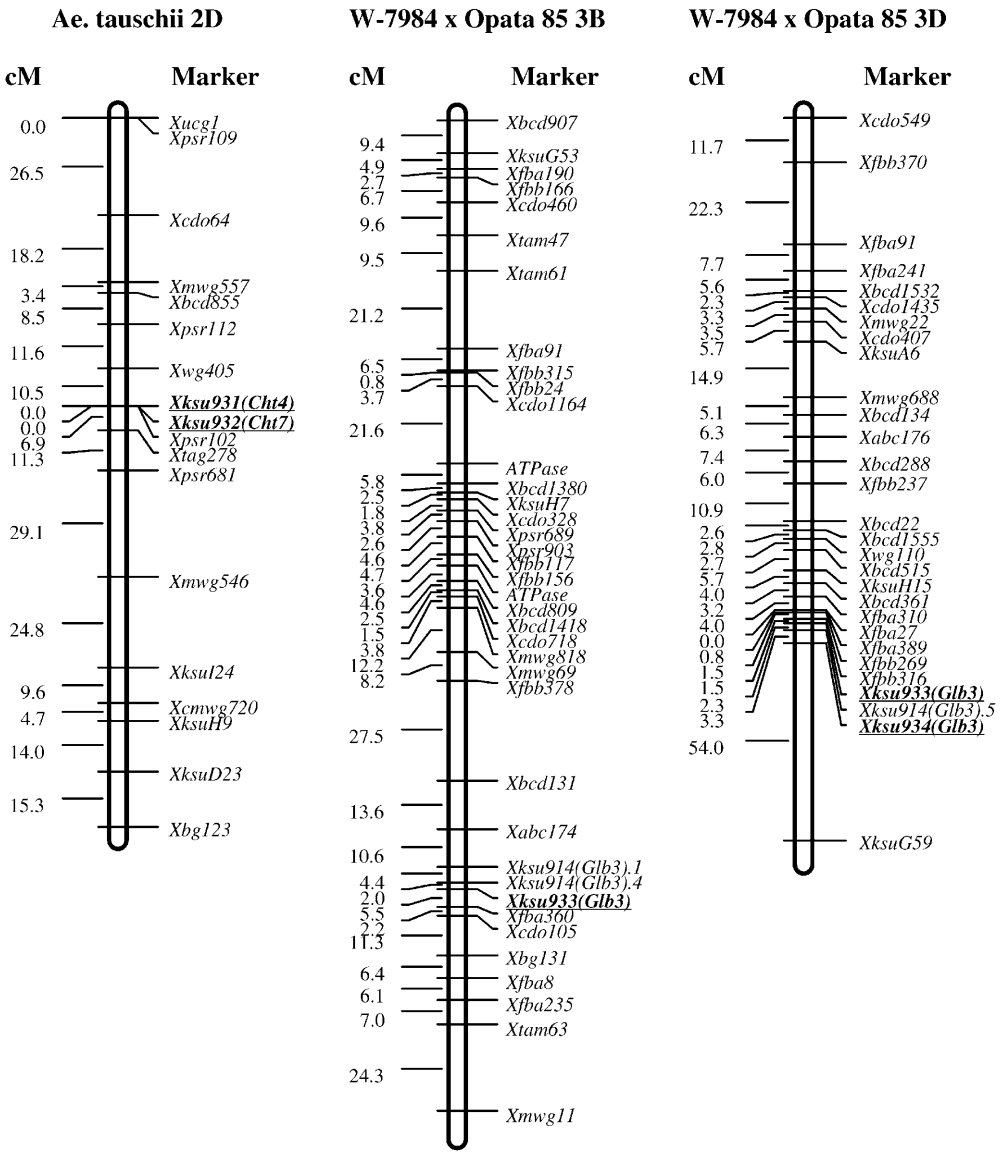

Fig. 5 Linkage maps of the 2D chromosome of *Ae. tauschii* and of the 3B and 3D chromosomes of common wheat. CentiMorgan (cM) distances are indicated at the *left* of the chromosomes and marker loci to the *right*. The map positions of clones mapped in this experiment are indicated in *bold face*. The symbols for the marker loci detected by clones SM194, SM289, SM383 and SM638 are *Xksu932* (Cht7), *Xksu933* (Glb3), *Xksu931* (Cht4) and *Xksu934* (Glb3), respectively

ulation protocol. The chitinase and β-1,3-glucanase mRNA levels were much higher in spikes inoculated with a conidial suspension of *F. graminearum* than in the mock-inoculated controls (Fig. 6), indicating that these genes encode *bona fide* PR-proteins.

The transcription profiles of these genes were different in the scab-resistant cultivar Sumai 3 and the scab-susceptible mutant, indicating their role in disease resistance. Although the peak expression levels are similar in Sumai 3 and the susceptible mutant, there are differences in the kinetics of induction of transcripts between the two lines. Expression levels at the three time points studied followed the pattern 24 hai ≥ 48 hai > 72 hai in Sumai 3; but in the mutant the pattern generally was 24 hai < 48 hai > 72 hai. The transcripts of both chitinase and β-1,3-glucanase genes reached the maximum point at or before 24 hai in Sumai 3. In the mutant, the peak values were not reached until 48 hai or later, indicating a slower defense response to infection by *F. graminearum* in the mutant. For SM194, a larger transcript was detected clearly at 24 hai in Sumai 3, but it was still very weak at 48 hai in the mutant. This messenger might be transcribed from another member of the class-VII chitinase genes. These results suggest that the mutant may be defective in a common step leading to the induction of both chitinases and β-1,3-glucanases. They are in agreement with results reported recently by Pritsch et al. (2000) who showed that several PR-protein genes were induced earlier in Sumai 3 than the susceptible (non-isogenic) genotype, Wheaton.

In conclusion, we have isolated and characterized several new chitinase and β-1,3-glucanase cDNA clones that have not been reported previously. In particular, the importance of class-IV and class-VII chitinases in defense response to pathogen infection has not been demonstrated for any cereal. This is the first report of the pathogen-inducible nature of a class-VII chitinase in any plant. Their greater abundance (in 880 cDNA clones)

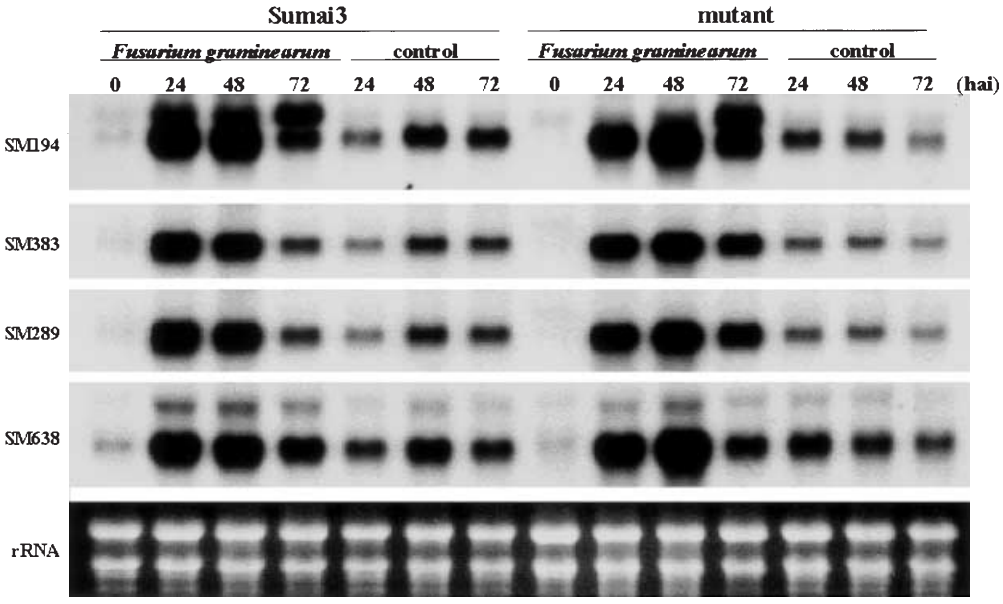

Fig. 6 Northern blot hybridization analysis of the expression of chitinase (SM194 and SM383) and β-1,3-glucanase (SM289 and SM638) genes in scab resistant wheat variety Sumai 3 and its susceptible mutant. The spikelets of Sumai 3 and the mutant were inoculated with conidia of *Fusarium graminearum* and with medium (mung bean broth) as control. The transcript levels were measured at 0, 24, 48 and 72 hai. The ribosomal RNA (rRNA) amounts show that the same level of total RNA was loaded in each lane

compared to other chitinases (class-I and class-II) suggest that these chitinases may be important components of plant defense against fungal pathogens.

Acknowledgments We thank Dr. D.B. Collinge for supplying the DNA clones, Dr. R.L. Bowden for providing fungus isolate and Dr. M.E. Sorrells for providing the mapping population. This research was funded by the Cooperative Crop Research Program of the McKnight Foundation.

Reference

Bowles DJ (1990) Defense related proteins in higher plants. Annu Rev Biochem 59: 873–907

Boyko EV, Gill KS, Mickelson-Young L, Nasuda S, Raupp WJ, Ziegle JN, Singh S, Hassawi DS, Fritz AK, Namuth D, Lapitan NLV, Gill BS (1999) A high-density genetic linkage map of *Aegilops tauschii*, the D-genome progenitor of bread wheat. Theor Appl Genet 99: 16–26

Collinge BD, Kragh KM, Mikkelsen JD, Nielsen KK, Rasmussen U, Vad K (1993) Plant chitinases. Plant J 3: 31–40

Faris JD, Haen KM, Gill BS (2000) Saturation mapping of a gene-rich recombinant hot spot region in wheat. Genetics 154: 823–835

Gill KS, Lubbers EL, Gill BS, Raupp WJ, Cox TS (1991) A genetic linkage map of *Triticum tauschii* (DD) and its relationship to the D genome of bread wheat (AABBDD). Genome 34: 362–374

Huang J-K, Wen L, Swegle M, Tran H-C, Thin TH, Naylor HM, Muthukrishnan S, Reeck GR (1991) Nucleotide sequence of a rice genomic clone that encodes a class-I endochitinase. Plant Mol Biol 16:479–480

Huynh QK, Hironaka CM, Levine EB, Smith CE, Borgmeyer JR, Shah DM (1992) Antifungal proteins from plants. Purification, molecular cloning, and antifungal properties of chitinases from maize seed. J Biol Chem 267: 6635–6640

Kosambi DD (1944) The estimation of map distances from recombination values. Ann Eugen 12: 172–175

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1: 174–181

Leuber-Metzger G, Meins F Jr (1999) Functions and regulation of plant β-1,3-glucanases (PR-2). In: Datta SK, Muthukrishnan S (eds) Pathogenesis-related proteins in plants. CRC Press, Boca Raton, Fla., pp 49–76

Li CD, Langridge P, Lance RCM, Xu P, Fincher GB (1996) Seven members of the (1→3)-β-glucanase gene family in barley (*Hordeum vulgare*) are clustered on the long arm of chromosome 3 (3HL). Theor Appl Genet 92: 791–796

Li WL, Faris JD, Chittoor J, Leach JE, Liu DJ, Chen PD, Gill BS (1999) Genomic mapping of defense response genes in wheat. Theor Appl Genet 98:226–233

Malehorn DE, Scott KJ, Shah DM (1993) Structure and expression of a barley acidic β–1,3-glucanase gene. Plant Mol Biol 22: 347–360

Marino CL, Nelson JC, Lu YH, Sorrells ME, Leroy P, Tuleen NA, Lopes CR, Hart GE (1996) Molecular genetic maps of the group 6 chromosomes of hexaploid wheat (*Triticum aestivum* L. em Thell). Genome 39: 359–366

Meins F Jr, Neuhaus JM, Sperisen G, Ryals G (1992) The primary structure of plant pathogenesis-related glucanohydrolases and their genes. In: Boller T, Meins F Jr (eds) Genes involved in plant defense. Springer, Berlin Heidelberg New York. pp 245–282

Meins F Jr, Fritig B, Linthorst HJM, Mikkelsen JD, Neuhaus JM, Ryals J (1994) Plant chitinase genes. Plant Mol Biol Rep 12: S22-S28

Muthukrishnan S, Liang GH, Trick HN, Gill BS (2000) Enhnaced disease resistance in cereals by transformation with genes for pathogenesis-related proteins. Annu Rev Plant Biotech (in press)

Nelson JC, Van Deynze AE, Autrique E, Sorrells ME, Lu YH, Merlino M, Atkinson M, Leroy P (1995a) Molecular mapping of wheat. Homoeologous group 2. Genome 38: 516–524

Nelson JC, Van Deynze AE, Autrique E, Sorrells ME, Lu YH, Negre S, Bernard M, Leroy P (1995b) Molecular mapping of wheat. Homoeologous group 3. Genome 38: 525–533

Nelson JC, Sorrells ME, Van Deynze AE, Lu YH, Atkinson M, Bernard M, Leroy P, Faris JD, Anderson JA (1995c) Molecular mapping of wheat. Major genes and rearrangements in homoeologous group 4,5 and 7. Genetics 141: 721–731

Neuhaus JM (1999) Plant chitinases (PR-3, PR-4, PR-8, PR-11). In: Datta SK, Muthukrishnan S (eds) Pathogenesis-related proteins in plants. CRC Press, Boca Raton, Fla., pp 77–105

Pritsch C, Muehbauer GJ, Bushnell WR, Somers DA, Vance CP. (2000) Fungal development and induction of defense response gene during ear infection of wheat spikes by *Fusarium graminearum*. Mol Plant-Microbe Interact 13:159–169

Sears ER (1966) Nullisomic-tetrasomic combinations in hexaploid wheat.In: Riley R, Lewis KR (eds) Chromosome manipulation and plant genetics. Oliver and Boyd, Edinburgh, pp 29–45

Van Deynze AE, Dubcovsky J, Gill KS, Nelson JC, Sorrells ME, Dvorak J, Gill BS, Lagudah ES, McCouch SR, Appels R (1995) Molecular-genetic maps of group 1 chromosomes of Triticeae species and their relation to chromosomes in rice and oat. Genome 38: 45–59

Van Loon LC (1999) Occurrence and properties of plant pathogenesis-related proteins. In: Datta SK, Muthukrishnan S (eds) Pathogenesis-related proteins in plants. CRC Press, Boca Raton, Fla., pp 1–19

Verburg JG, Smith CE, Lisek CA, Huynh QK (1992) Identification of an essential tyrosine residue in the catalytic site of a chitinase isolated from *Zea mays* that is selectively modified during inactivation with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. J Biol Chem 267: 3886-3893

Wang J, Xu P, Fincher GB (1992) Purification, characterization and gene structure of (1→3)-β-glucanase isoenzyme GIII from barley (*Hordeum vulgare*). Eur J Biochem 209: 103–109

Xu P, Fincher GB (1992) Evolution and differential expression of two (1→3)-β-glucan endohydrolase-encoding gene families in barley, *Hordeum vulgare*. Gene 120: 157–165