Expression of β-1,3-glucanase and chitinase in healthy, stem-rust-affected and elicitor-treated near-isogenic wheat lines showing *Sr5*-or *Sr24*-specified race-specific rust resistance

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Abstract. Pathogenesis-related expression of the two antifungal hydrolases β-1,3-glucanase (EC 3.2.1.39) and chitinase (EC 3.2.1.14) was studied in wheat (*Triticum aestivum* L.) as part of the defence response to stem rust (*Puccinia graminis* f.sp. *tritici*; *Pgt*), mediated by the semi-dominantly acting resistance genes *Sr5* and *Sr24*. Complete resistance (infection type 0), mediated by the *Sr5* gene in cultivar Pre-*Sr5*, closely correlates with the hypersensitive response of penetrated cells at early stage of the interaction, when the first haustorium is formed. In contrast, cultivar Pre-*Sr24* shows intermediate resistance (infection type 2–3) which is not directly linked to cell death. In both cases, the plant response included a rapid increase in β-1,3-glucanase activity between 24 and 48 h after inoculation. One main extracellular 30-kDa isoform of β-1,3-glucanase was present in both lines, as shown by polyacrylamide-gel electrophoresis. Two additional minor isoforms (32 and 23 kDa) were detected only in Pre-*Sr24*, and only at later time points. Increased enzyme activity and the appearance of new isoforms in the resistant lines was preceded by accumulation of mRNAs encoding β-1,3-glucanases and chitinases. However, there were no changes in chitinase activity or isoforms. A high constitutive level of chitinase activity was observed in all wheat genotypes. Serological studies indicated the presence of a class II chitinase of 26 kDa. Accumulation of β-1,3-glucanase and chitinase transcripts was detected before the pathogen penetrated the leaves through stomata and approximately 16 h before the typical hypersensitive response was observed, indicating that signal(s) for defense gene activation were recognised by the host plant long before a tight contact between the pathogen and a host cell is established. A glycoprotein (*Pgt* elicitor) derived from hyphal walls, strongly induced β-1,3-glucanase. We discuss the possible role of the elicitor in the early signalling mediating *Sr5*- and *Sr24*-specified resistance in wheat.

Key words: Chitinase – Elicitor – Glucanase – Pathogenesis-related proteins – *Puccinia* – *Triticum*

Introduction

Stem rust is a severe fungal disease of wheat (*Triticum aestivum* L.) caused by *Puccinia graminis* f.sp. *tritici*. Many wild wheat varieties are naturally resistant to the fungus (Anikster 1984). The resistance traits have been transferred to susceptible wheat cultivars by crossing to obtain several pairs of near-isogenic lines which differ in their resistance to the fungus (McIntosh 1988). These lines serve as well-defined genetic materials for experiments that are used to study the nature of resistance to stem rust.

In this study, we have chosen *Sr5* and *Sr24* as representatives of wheat resistance genes for an investigation of the role of the two pathogenesis-related (PR) proteins β-1,3-glucanase and chitinase in the defence of wheat plants against the stem rust fungus. The genes *Sr5* and *Sr24* govern resistance in a typical race-specific and dominant manner and meet the criteria of the gene-for-gene model (Flor 1971). Resistant cultivars bearing these genes show dramatic changes at the cellular and molecular level at different stages of stem rust development. Several investigations have indicated that, based on the presence of the *Sr5* gene, fungal development is arrested by a hypersensitive response (HR), i.e. by the rapid death of cells penetrated by the avirulent fungus (Rohringer at al. 1979; Tiburzy et al. 1990). The appearance of an HR is correlated with enhanced synthesis of lignin-like material (Moerschbacher et al. 1988). In plants bearing the *Sr24* gene, no direct correlation between lignin formation and fungal growth inhibition has been observed. The type of resistance governed by *Sr24* is characterised by a retarded growth

of the invading pathogen during the entire vegetative developmental cycle (Tiburzy 1984), resulting in significantly reduced sporulation (infection type 2–3) compared to the fully compatible interaction.

Induction of extracellular or intracellular PR proteins may be a defence trait additional or alternative to lignification. β-1,3-Glucanase and chitinase, two often-studied PR proteins, have been shown to have antifungal properties (Schlumbaum et al. 1986; Mauch et al. 1988; Sela-Buurlage et al. 1993). Beside this function, the two enzymes may generate glucan and chitin fragments that act as signal molecules in plants (Boller 1989, 1995).

There are numerous reports on β-1,3-glucanase and chitinase in dicotyledonous plants, but rather limited numbers of studies in cereals (reviews: Meins et al. 1992; Sahai and Manocha 1993). Pathogen-induced β-1,3-glucanase activity has been detected in wheat (Sock et al. 1990), barley, rice, sorghum (Jutidamrongphan et al. 1991; Ignatius et al. 1994), oat (Fink et al. 1988) and maize (Nasser et al. 1990), indicating that the activation of this enzyme may be a general response of cereals to infection. Infection-related expression of chitinase has been found in barley in response to inoculation with the powdery mildew fungus, and specific defence-related isoforms have been isolated (Kragh et al. 1993; Ignatius et al. 1994). In wheat, the presence of chitinase has been demonstrated by several authors (Molano et al. 1979; Boller et al. 1983; Ride and Barber 1990), but its regulation during infection or stress has not yet been studied.

Here, we examine expression of different β-1,3-glucanase and chitinase genes in response to stem rust infection at the transcript, isoenzyme and enzyme activity level. To gain more insight into the signalling of defence gene activation, we also analyzed induction of both PR proteins by a biotic elicitor previously shown to induce part of the defence reactions in wheat plants.

Materials and methods

Plants, fungi, and cultivation conditions. Near-isogenic backcross lines of the wheat cultivar *Triticum aestivum* L. cv. Prelude carrying functional alleles of stem rust resistance genes *Sr5* or *Sr24* were supplied by R. Rohringer (Agriculture Research Station, Winnipeg, Canada). Seven-day-old seedlings, grown under controlled conditions (Kogel et al. 1985), were inoculated with uredospores of *Puccinia graminis* f.sp. *tritici* (*Pgt*) Erik. & Henn, race 32, bearing the *avrP5* and the *avrP24* avirulence genes. Infectious stock cultures of *Pgt* were obtained from infected susceptible wheat cv. Little Club.

Preparation of intercellular washing fluid (IWF), intracellular extract (ICE) and assays for malate dehydrogenase (MDH) and glucose-6-phosphate dehydrogenase (G6PDH). Intercellular washing fluid was obtained from leaves using 10 mM sodium acetate buffer (pH 5.0) for infiltration (Deverall and Deakin 1985). Subsequent to IWF preparation, primary leaves (1 g) were homogenised with 4 ml of 10 mM sodium acetate buffer (pH 5.0) and sea sand using mortar and pestle. Homogenates were centrifuged for 20 min at 15 000 · *g*. The supernatant (ICE) was stored at –20 °C. The MDH was determined according to Deverall and Deakin (1985) and G6PDH was measured according to Kogel and Beißmann (1992).

β-1,3-Glucanase and chitinase assays. To remove low-molecular-mass carbohydrates, extracts were purified by gel filtration on PD10-columns (Pharmacia, Freiburg, Germany). Chitinase activity was determined as described by Boller and Mauch (1989). The β-1,3-glucanase assay was carried out according to Fink et al. (1988) with modifications. The enzyme assay contained 10 μl of 1 M sodium acetate buffer (pH 5.0) and 400 μl of extracts (0.1–0.4 μg protein). The reaction was started with 100 μl laminarin (2 mg · ml^{-1}). The assay was carried out at 37 °C for 1 h. The amount of reducing sugars released by enzymatic cleavage of laminarin was determined according to Somogyi (1952).

Elicitor assays. The *Pgt* elicitor, a glycoprotein of 67 kDa, was prepared from germ tube walls of uredosporelings of *Pgt* (Kogel and Beißmann 1992). Elicitor activity was tested by injection of 50 μl of test solution into the intercellular spaces of 7-d-old wheat primary leaves (Kogel et al. 1985). After appropriate time points, treated leaves were harvested and used for β-1,3-glucanase and chitinase assays.

Gel electrophoresis and Western blot analysis. The SDS-PAGE (8–25% polyacrylamide) was performed on a commercial system according to the producer's recommendation (PhastSystem; Pharmacia). Proteins were transferred onto nitrocellulose filters (Schleicher & Schüll, Dassel, Germany) by electro-blotting (Kogel and Beißmann 1992). Immunostaining of the blots was carried out using antisera produced against β-1,3-glucanase from barley, class II chitinase from barley (both provided by T. Bryngelsson, Department of Plant Breeding Research, Svalöv, Sweden), or class I chitinase from potato (E. Kombrink, MPI für Züchtungsforschung, Köln, Germany). For calculating the size of the bands, pre-stained SDS-PAGE standards (low range; BioRad, München, Germany) were used.

Cloning of chitinase and glucanase sequences. Specific RNA probes for chitinase and β-1,3-glucanase transcripts were amplified by polymerase chain reaction (PCR) from total plant DNA, extracted from leaves of the parent wheat Prelude by the CTAB method (Rogers and Bendich 1988), using oligonucleotide primers based on highly conserved sequences of plant β-1,3-glucanase and chitinase. Oligonucleotides 5′-GGG AAT TCG GAT CCA TCG GCG TBT GCT ACG G (forward primer) and 5′-CCG GAT CCG AAT TCA RGT ANG GGT AYA YGT TNG C (backward primer) amplified a fragment of approx. 230 basepairs (bp) representing the 5′ part of a β-1,3-glucanase gene. It was cloned into plasmid pTZ18 U as clone pTaGL-1 and partially sequenced with Sequenase (USB, Braunschweig, Germany). Oligonucleotides 5′-GGC ATG CCT GCA GAC NTC NCA YGA RAC NAC (forward primer) and 5′-GGG AAT TCG GAT CCK WNK DCA TCC ARW ACC A (backward primer) amplified a 284-bp fragment internal to the catalytic domain typical of class I and II chitinases. It was cloned as clone pTaCH-1 and sequenced as above. In addition, a class III cDNA chitinase clone from maize (pPRm3; gift from G. Burkhard, Institute de Biologie Moléculaire des Plantes, Straßbourg, France) was also used to detect the respective transcripts.

Extraction of RNA, blotting and hybridization techniques. Total RNA was extracted from 5-cm-long primary leaf segments at the indicated time points after inoculation with *Pgt* uredospores as described by Martin et al. (1993). Ten micrograms of total RNA per lane was loaded on 1% denaturing formaldehyde-agarose gels and transferred, after electrophoretic size separation, to Hybond-N+ membranes (Amersham, Braunschweig, Germany) by alkaline downward transfer, as described by Chomezynski (1992). Hybridizations with digoxigenin-labeled RNA probes were performed as described (Kogel et al. 1994). The size of the bands were calculated using RNA-molecular weight marker II (Boehringer, Mannheim, Germany). The constitutively expressed chitinase III was used as an internal standard to check loading of the gels with equal amounts of RNA.

Table 1. β-1,3-Glucanase and chitinase activities (nkat · (g FW)$^{-1}$) in homogenates (ICE) and extracts of intercellular fluids (IWF) of 7-d-old primary leaves from different genotypes of the wheat cv. Prelude bearing distinct genes for stem rust resistance

Genotype	Infection type[b] with P. graminis	β-1,3-Glucanase		Chitinase	
		ICE[a]	IWF	ICE	IWF
Prelude (Sr5)	(0) resistant	1.07 ± 0.227	0.32 ± 0.07	3.2 ± 0.6	0.24 ± 0.05
Prelude (Sr24)	(1–2) resistant	0.36 ± 0.08	0.18 ± 0.04	2.9 ± 0.3	0.25 ± 0.04
Prelude (sr5/sr24)	(4) susceptible	0.81 ± 0.07	0.31 ± 0.06	3.7 ± 0.40	0.27 ± 0.03

[a]ICE was extracted subsequent to IWF extraction from the same leaves
[b]Designation according to Roelfs and Groth (1987)

Results

β-1,3-Glucanase and chitinase activities in healthy and stem-rust-affected wheat leaves. All the genotypes of the wheat cv. Prelude investigated showed activities of β-1,3-glucanase and chitinase before infection (Table 1). High intracellular (ICE) chitinase activities compared to extracellular (IWF) chitinase and β-1,3-glucanase activities were found. The β-1,3-glucanase activity of Pre-Sr24 was only 30–50% of that of Pre-Sr5. Following inoculation with Pgt, a strong increase in β-1,3-glucanase activity was detected in resistant lines Pre-Sr5 and Pre-Sr24, but not in the susceptible, recurrent parent line Prelude (Pre-sr5/sr24) carrying the non-functional alleles sr5 and sr24 (Fig. 1). Enhanced enzyme activity was found in the resistant lines from 2 days post inoculation (dpi) onwards, and could be attributed to an increase in intracellular activity,

measured in the ICE, as well as to an increase in extracellular activity, measured in the IWF (Fig. 1).

A slight increase in total chitinase activity was found in some experiments with increasing leaf age. However, as shown in Fig. 2, no significant pathogenesis-related increase of chitinase activity was detected in either incompatible or compatible combinations of Pgt with the different Prelude genotypes.

Defence-related isoforms of β-1,3-glucanase in wheat primary leaves. To study specific defence-related isoforms of β-1,3-glucanase, a gel blot analysis was performed after separation by SDS-PAGE, using antiserum raised against β-1,3-glucanase from barley (Fig. 3). In Pre-Sr5, accumulation of an extracellular 30-kDa isoform was detected in IWF from 2 dpi onwards. In Pre-Sr24, the 30-kDa isoform accumulated even more strongly. In addition, a 32-kDa isoform was detected
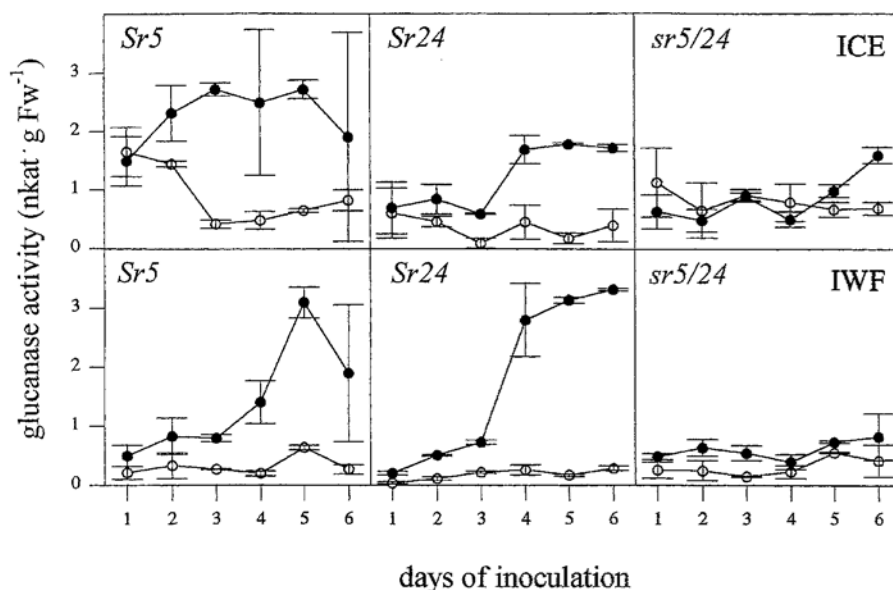

Fig. 1. Changes in the intra- and extracellular activities of β-1,3-glucanase in leaves of *Sr5*- and *Sr24*-resistant backcross lines as well as in the susceptible recurrent parent Prelude after inoculation with *Pgt* (*closed circles*) or mock-inoculation (*open circles*). Intracellular enzyme activities were measured in homogenates (ICE) from those leaves which had been used previously for extraction of extracellular enzymes. Extracellular enzyme activities were measured in the intercellular washing fluids (IWF). Data shown are representatives of four replicate experiments. (± SD)
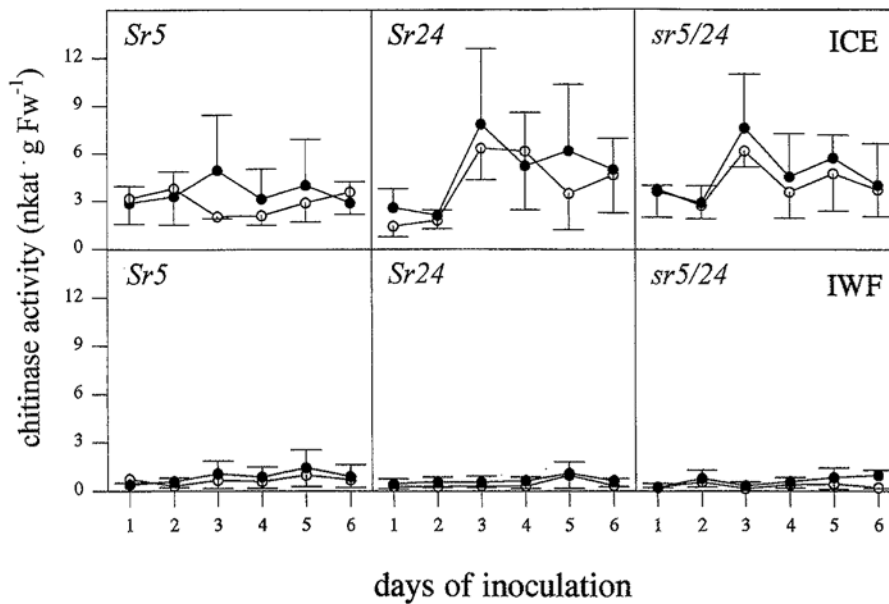
Fig. 2. Changes in the intra- and extracellular activities of chitinase in leaves of *Sr5*- and *Sr24*-resistant backcross lines as well as in the susceptible recurrent parent Prelude after inoculation with *Pgt* (*closed circles*) or mock-inoculation (*open circles*). Intracellular enzyme activities were measured in homogenates (*ICE*) from those leaves which had been used previously for extraction of extracellular enzymes. Extracellular enzyme activities were measured in the intercellular washing fluids (IWF). Data shown are representatives of four replicate experiments (± SD)

from 3 dpi, and a 23-kDa isoform from 5 dpi onwards. In the susceptible Pre-*sr5/sr24*, comparatively low amounts of the 30-kDa isoform accumulated at later time points of inoculation (3–6 dpi). Only traces of the

30-kDa isoform were found in ICE at late time points after infection (data not shown).

Chitinase isoforms in wheat primary leaves. Gel blot analysis of chitinase isoforms from total leaf extracts separated by SDS-PAGE and stained with antiserum raised against barley class II chitinase showed a single constitutively present isoform with a molecular mass of 26 kDa in all Prelude genotypes (Fig. 4A, shown for Pre-*Sr5*). Using an antiserum raised against a potato class I chitinase, several bands were seen corresponding to molecular masses in the range of approx. 17 through 47 kDa (Fig. 4B, shown for Pre-*Sr5*). This indicates the presence of class I-type chitinases in all the wheat
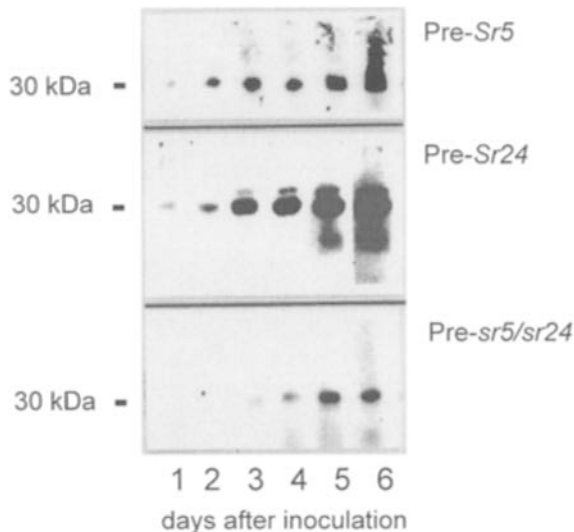

Fig. 3. Characterization of extracellular β-1,3-glucanase isoenzymes in leaves of *Sr5*- and *Sr24*-resistant backcross lines as well as in the susceptible recurrent parent Prelude (Pre-*sr5/sr24*) after inoculation with *Pgt*. Immunostaining of β-1,3-glucanases separated by SDS-PAGE (1 µg of total IWF protein per well) using an antiserum against a β-1,3-glucanase from barley. A 30-kDa isoform was found in Pre-*Sr24* and two additional isoforms of 32 and 23 kDa appeared from 3 dpi onwards. No qualitative differences were observed when an antiserum against a potato β-1,3-glucanase was used instead. No intracellular β-1,3-glucanase isoforms were detected
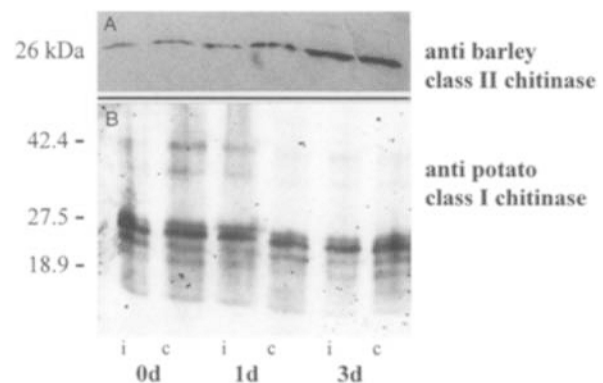

Fig. 4A,B. Characterisation of chitinase isoenzymes after inoculation with *Pgt*. Immunostaining of chitinases separated by SDS-PAGE (1 µg of total leaf protein per well) using an antiserum against a class II chitinase from barley (**A**) or a class I chitinase from potato (**B**). All the isoforms could be found in ICE and IWF of all genotypes. As an example, the result for Pre-*Sr5* is shown

genotypes. However, we did not make an attempt to test whether all of these bands actually represent chitinases. The putative chitinase isoforms were constitutively expressed or only slightly enhanced with leaf age. A pathogenesis-related accumulation of a distinct isoform or a genotype-specific variation of the band profile was not detected.

Both antisera showed the same bands in ICE and IWF (data not shown), indicating that all the putative isoforms were located at least partially in the extracellular space.

Defence-related β-1,3-glucanase and chitinase gene expression. To check in an exemplary study whether β-1,3-glucanase and chitinase transcripts were induced in wheat leaves after fungal attack, clones pTaGL-1 and pTaCH-1 were used as hybridization probes. The sequence of pTaGL-1 (EMBL Nucleotide Sequence Database, Cambridge, UK; accession number, X95647) was obtained by PCR with oligonucleotide primers corresponding to two conserved motifs within the N-terminal region of β-1,3-glucanases. The nucleotide sequence is 70% homologous to β-1,3-glucanases from maize and barley but only 63% homologous to a β-1,3–1,4-glucanase from wheat (Fig. 5). The level of β-1,3-glucanase transcript (1.4 kb in size) represented by pTaGL-1 was markedly elevated in response to inoculation with *Pgt* in lines Pre-*Sr5* and Pre-*Sr24*, but not in Pre-*sr5/sr24* (Fig. 6). Enhanced amounts of β-1,3-glucanase transcripts were already detected at the earliest time point measured in this experiment (1 dpi). In Pre-*Sr5*, the β-1,3-glucanase transcript accumulated over the whole time range measured, whereas in Pre-*Sr24*, accumulation showed a pronounced biphasic profile with maxima at 1 and 5 dpi.

Likewise, a pathogenesis-related accumulation of chitinase mRNA (1.6 kb in size) was detected in lines Pre-*Sr5* and Pre-*Sr24* with clone pTaCH-1 (EMBL Nucleotide Sequence Database, accession number X95000) as hybridization probe. The sequence was obtained by PCR with oligonucleotide primers corresponding to two conserved motifs within the catalytic domain of chitinases class I and II. The nucleotide
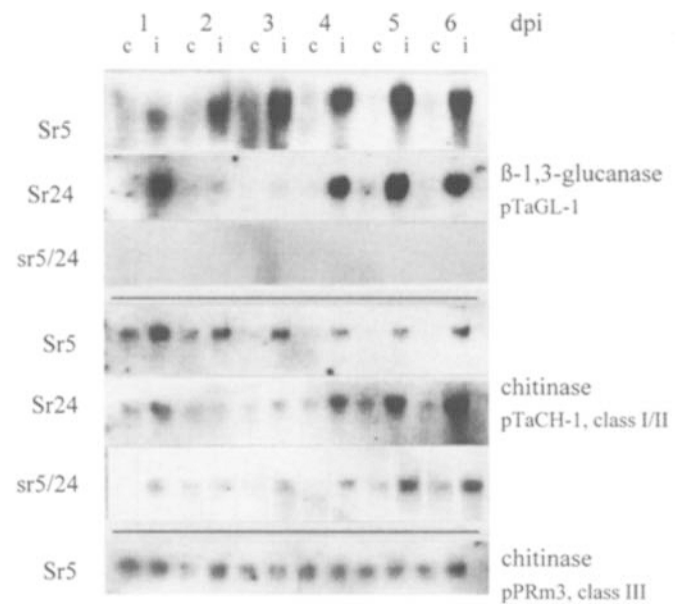

Fig. 6. Time courses of β-1,3-glucanase and chitinase transcript accumulation in leaves of *Sr5*- and *Sr24*-resistant backcross lines as well as in the susceptible recurrent parent Prelude (Pre-*sr5/sr24*) after inoculation with *Pgt*. Each lane contained 10 μg of total RNA extracted from 15 individuals per time point and per genotype. Filters were hybridized with digoxigenin-labeled RNA probes representing the 5′ part of a β-1,3-glucanase (*pTaGL-1*; Fig 5), the 5′ part of the catalytic domain typical for class I and II-chitinases (*pTaCH-1*; Fig. 7), and a class III chitinase from maize (*pPRm3*). The sizes of mRNAs detected by pTaGL-1, pTaCH-1 and pPRm3 were 1.4 kb, 1.6 kb and 1.4 kb, respectively. Wash stringency: 0.5 × SSC at 68 °C. *i* inoculated plants; *c* non-inoculated controls. The blot is a representative of three independent experiments. The size of the pTaGL-1-specific transcript found in the *Sr5* cultivar does not reproducibly vary between days 1 and 6

sequence shares 76–78% homology with wheat and barley class I chitinases and with a barley seed chitinase, but only 63% with a barley class II chitinase (Fig. 7). When compared to class I chitinases, the latter shares with tobacco pathogenesis-related class II chitinases a

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Wheat pTaCH-1   HETTGGWPTAPNGPYTWGYCYKHERTTSS-YCTPSEQWPCAPRRRYY
Wheat ClassI    **********D***S****FNQ**GAA*D**S*NS*****GKK*F
Barley ClassI   *******AT**D*AFA****F*Q**GAT*N*****A*****GKS**
Barley ClassIIa *******AT**D*AFA****F*Q**GA**D*****A*****GK***
Barley ClassIIb ******TRG*AD-QFQ****F*E*ISKAT------------SPP**

Wheat pTaCH-1   GRGPIQLTHNYNYGQAGRAIGADLLGNPNLVVVNPTVSFETAMWFWM
Wheat ClassI    ******ISY*****P******T***N**D**ATDA****K******
Barley ClassI   *******S******P******V***R**D**ATDP****K**M***
Barley ClassIIa *******S******P******V***A**D**ATDA*G*K*I****
Barley ClassIIb *******GRS**DL******K**VS**D**STDAV***R*******
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Fig. 7. Comparison of the deduced amino acid sequence of clone pTaCH-1 with sequences of chitinases from several cereals. The nucleotide sequence was 76–78% homologous to a wheat class I chitinase (Liao et al. 1994), a barley class I (Swegle et al. 1989), or a class II chitinase[a] (Leah et al. 1991), but only 63% homologous to another barley class II chitinase[b] (T. Bryngelsson, Department of Plant Breeding Research, Svalöv, Sweden, personal communication; AC X78671). *Underlined* sequences corresponding to the primers used for PCR amplification; [*], amino acids identical to pTaCH-1; -, deletions

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Wheat pTaGL-1   GSISLCYGMQGDSQSLPSRADVVQFYGTKGISAMRIYE
Barley          H**GV*N*VL*NN--**A*S***TL*RS*R*D******
Maize           Q**GV***VN**N--**PAS****L*QSN**NLL***F
Wheat 1,3-1,4   E**GV****SANN--**AAST**SMFKSN**NS**L*A

Wheat pTaGL-1   PEPETLAALDGTEIDLIMDVGGSFAAIASDPAAAAGWI
Barley          **SKV*T**S**G*AV*****PALPSL**S*S****A*V
Maize           *DANP*N**S**S*G*****PNTDL*S****S***A*V
Wheat 1,3-1,4   *DQAA*Q*VG**GVNVVVGAPNDVLSA*AS*****S*V
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Fig. 5. Comparison of the partial amino acid sequence deduced from the 5′ end of clone pTaGL-1 with sequences of β-1,3- and β-1,3–1,4-glucanases from several cereals. The nucleotide sequence was 70% homologous to maize and barley β-1,3-glucanases (Wang et al. 1992; Wu and Kriz 1992), and 63% homologous to a wheat β-1,3-1,4-glucanase (Lai et al. 1993). *Underlined* sequences corresponding to the 5′ primer used for PCR amplification; [*], amino acids identical to pTaGL-1; -, deletions

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  -30      0     +30
  c  i   c  i   c  i
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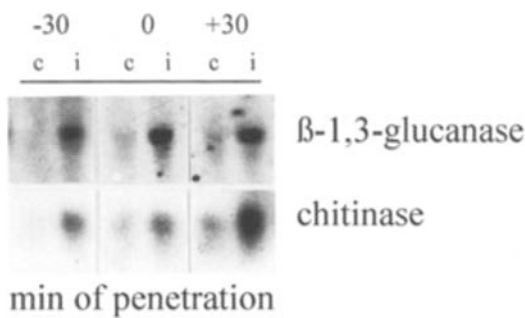ß-1,3-glucanase

chitinase

min of penetration

Fig. 8. Early time course of β-1,3-glucanase and chitinase transcript accumulation in leaves of Pre-*Sr5* after inoculation with *Pgt*. Each lane contained 10 μg of total RNA extracted from 15 individuals shortly before (−30 min), at (0 min), and shortly after (+30 min) penetration of the pathogen. Filters were hybridized with digoxigenin-labeled RNA clone (pTaGL-1; Fig 5) and (pTaCH-1; Fig. 7). For details see Fig 6. Data shown are representative of four replicate experiments

deletion within the amplified region; this sequence represents the chitinase detected by the anti-class II-chitinase antibodies used in this study. In Pre-*Sr5*, a maximum of transcript accumulation was found at 1 dpi. In Pre-*Sr24*, transcript accumulation showed a biphasic profile (Fig. 6; identical to β-1,3-glucanase), suggesting a coordinated regulation of the genes represented by these transcripts. A slight increase in the chitinase transcript is also detected in Pre-sr5/sr24, but only at late time points of the interaction (4–6 dpi). A high constitutive, pathogenesis-unrelated level of a chitinase transcript of approx. 1.4 kb was detected in all genotypes with pPRm3, which represents a class III chitinase (Fig. 6; shown for Pre-*Sr5*).

As shown in Fig. 6, enhanced amounts of mRNAs specific for β-1,3-glucanase and chitinase were already present at very early stages of fungal development. Therefore, we addressed the question whether fungal penetration into the host plant is the signal for PR gene expression. Because rust fungi penetrate leaves through stomata, fungal penetration can be synchronized by illumination, provided that the pathogen has had time to form a mature appressorium above the closed stoma in the dark. Hence, transcript accumulation was measured before, at, and after the onset of illumination of the inoculated plants. Figure 8 shows β-1,3-glucanase and chitinase transcript both being accumulated in Pre-*Sr5* before the onset of illumination, suggesting that fungal penetration is not the signal for defence gene expression. Instead, the data suggest the recognition by the plant of compounds with defence-gene-eliciting activity before host leaves are penetrated by the pathogen, and long before the tight contact between the infection structures of the pathogen and the first-attacked host cell.

β-1,3-Glucanase and chitinase induction in response to treatment with fungal elicitors. The *Pgt* elicitor, a glycoprotein of 67 kDa isolated from fungal germ tubes, has previously been shown to induce an HR in wheat leaves (Kogel et al. 1988). To assess whether this elicitor

concomitantly induced accumulation of PR proteins, β-1,3-glucanase and chitinase activities were measured after elicitor injection into wheat leaves. Enhanced β-1,3-glucanase activities were detected in all the Prelude genotypes. The elicitor concentration needed for half-maximal enzyme activity (EC_{50}) was 0.1 $\mu g \cdot ml^{-1}$ (Fig. 9A, shown for Pre-*Sr5*). β-1,3-Glucanase activity increased between 12 and 24 h and reached a plateau at approx. 50 h after injection (Fig. 9B). Accumulation of glucanase transcripts was found using pTaGL-1 as hybridization probe (Fig. 9B, inset), and SDS-PAGE showed the 30 kDa isoform of β-1,3-glucanase to be induced by the elicitor in all genotypes (data not shown).

No increase in chitinase activity was observed after *Pgt* elicitor treatment in any genotype and, unlike the avirulent fungus, the elicitor also did not induce an increase in chitinase transcript. This implies the existence of further fungal or endogenous elicitors responsible for the induction of chitinase transcript accumulation during *Sr5*- and *Sr24*-specified resistance responses.

Discussion

Pathogenesis-related accumulation of β-1,3-glucanase and chitinase. Our work shows that defence reactions governed by the functional alleles of the stem rust resistance loci *Sr5* and *Sr24* are associated with the accumulation of mRNAs encoding β-1,3-glucanase and chitinase.

The pathogenesis-related induction of a 30-kDa β-1,3-glucanase was clearly established at the transcript, protein, and activity levels. It also occurred after *Pgt* elicitor treatment. A similar β-1,3-glucanase induction has been shown to occur in barley, when a 33-kDa isoform accumulated after inoculation with *Erysiphe graminis*, especially in plants with *Mla*- and *Ml-[La]*-specified powdery mildew resistance (Jutidamrongphan et al. 1991).

A chitinase gene expressed upon inoculation encodes a class I or class II chitinase because the transcript hybridised to pTaCH-1, a 284-bp PCR product, which was selected in an exemplary study to examine accumulation of chitinase transcripts. The fragment represents the 5′-part of the catalytic domain typical of class I and II chitinases and shows ca. 75% homology at the amino acid level to a endochitinase from barley (Leah et al. 1991) or rice (Nishizawa and Hibi 1991). A gene encoding a class III chitinase is constitutively expressed, because the amount of transcript detected with cDNA clone pPRm3, encoding a class III chitinase from maize (Nasser et al. 1990), did not change during the infection process. Cross-hybridization of pTach-1 and pPRm3 could be excluded because the sizes of the RNAs detected were different and because a different band pattern appeared on one and the same blot when the blot was probed successively with pTaCH-1 and pPRm3.

The antiserum raised against a barley class II chitinase detected a single 26-kDa isoform which slightly accumulated with leaf age but not after pathogen attack (Fig. 4). Interestingly, the heterologous antiserum raised
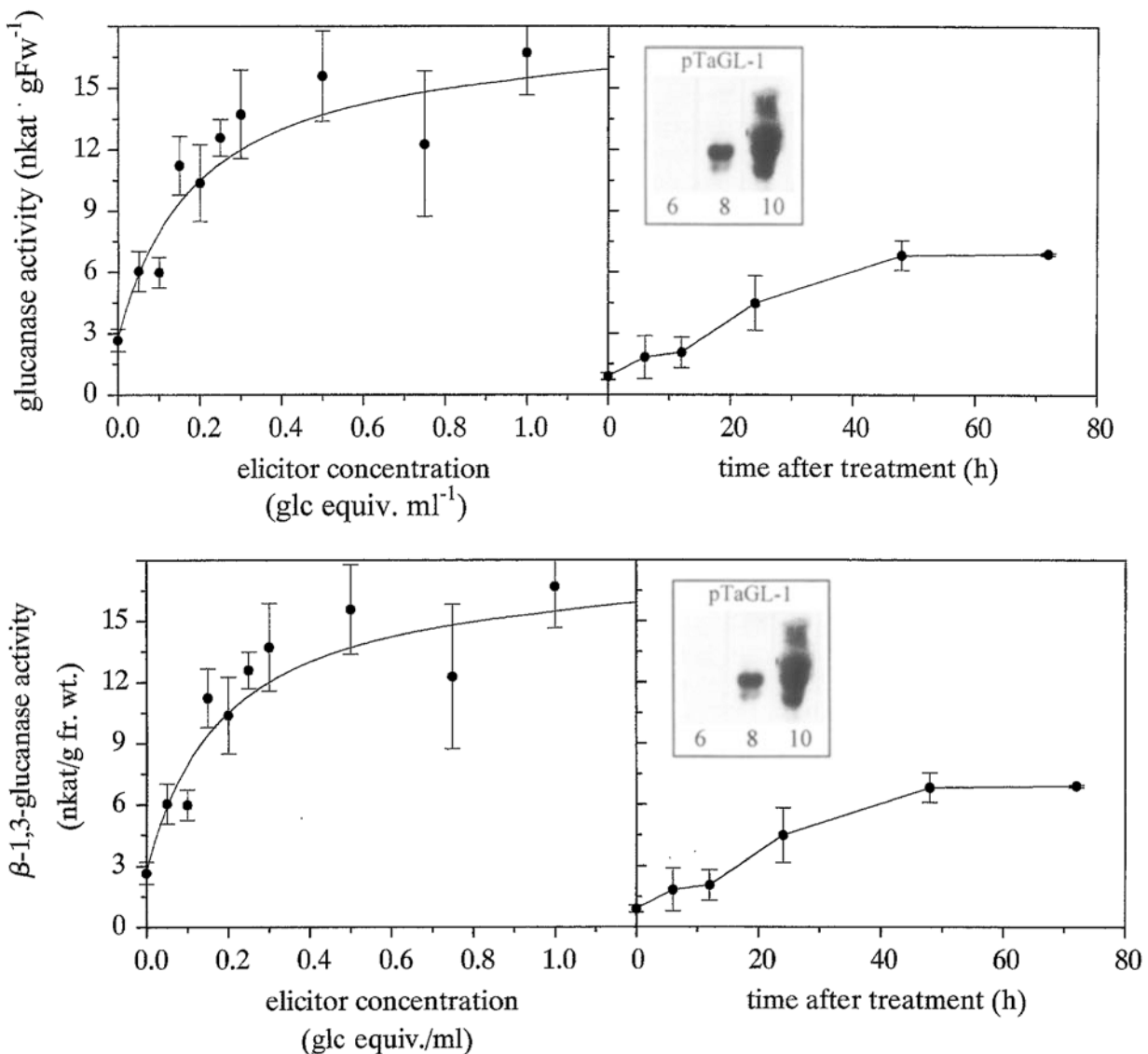
Fig. 9. A. Dose-dependent increase in β-1,3-glucanase activity in response to treatment with *Pgt* elicitor in leaves of Pre-*Sr5*. Half-maximum activation of enzyme activity (EC_{50}) was accomplished by an elicitor concentration of 0.1 μg · ml^{-1}, based on glucose equivalents. Similar EC_{50} values were calculated for Pre-*Sr24* and Pre-*sr5/sr24* genotypes (data not shown). **B.** Changes in β-1,3-glucanase activity in response to treatment with *Pgt* elicitor (1 μg · ml^{-1}) in the same wheat line. *Inset:* Time course (h) of transcript accumulation. Filters were hybridized with digoxigenin-labeled RNA clone (*pTaGL-1*; Fig. 5). For details, see Fig. 6. Data shown are representative of four replicate experiments

against a potato class I chitinase gave strong signals with several bands indicating that wheat contains class I chitinases. However, no further information is available regarding whether all of these bands correspond to distinct chitinase isoenzymes. Thus, the results show that the chitinase activities measured (Fig. 2) reflect a complex situation in which sereral isoenzymes belonging to different chitinase classes contribute to total activity. In this connection, it is important to realize that different classes of chitinases may have significantly different activities with a given, artificial substrate (Melchers et al. 1994).

In barley, similar chitinase isoenzymes have been found. In addition to a 26-kDa isoform, two constitutively expressed 30-kDa polypeptides have been identified and characterised as class I and class III chitinases,

respectively (Collinge et al. 1993). However, in contrast to what is found in wheat, Kragh et al. (1993) described an additional extracellular 27-kDa isoform in barley that was induced by infection with the powdery mildew fungus. This enzyme showed high homology to a class II chitinase from bean and barley grain.

Interpretation of the role of β-1,3-glucanase and chitinase on the basis of the cytological analysis of the Sr5- and Sr24-mediated resistance responses. In the highly resistant line Pre-*Sr5*, an HR occurred in the majority of invaded epidermal cells 32–34 h after inoculation with *Pgt*. This corresponds to a time range of 16–18 h after penetration of the leaves (Tiburzy et al. 1990). Because the HR of invaded cells and growth inhibition of the pathogen correlates well with lignin formation, lignifica-

tion has been suggested to be a decisive defence mechanism governed by the *Sr5* gene. Resistance correlates with enhanced activities of enzymes of the phenylpropanoid pathway such as phenylalanine ammonia lyase, 4-coumarate: CoA ligase, and coumaryl alcohol dehydrogenase. Inhibition of these enzymes with appropriate inhibitors results in reduced lignification and in intensified fungal growth (Moerschbacher et al. 1990). However, complete compatibility between plant and fungus was not accomplished by any of the enzyme inhibitors, pointing to additional distinct traits of the *Sr5*-specified defence mechanism.

Compared with the fully compatible interaction, the infection phenotype governed by *Sr24* is characterised by a retardation of fungal development over the whole vegetative growth cycle. The gene affects sporulation quantitatively but does not completely prevent it. Fungal growth retardation is accompanied by some HR in mesophyll cells invaded by the pathogen or in cells that are in close vicinity to fungal hyphae. However, no cytological or biochemical evidence has been furnished for the assumption that the cell death is directly related to growth inhibition. Our data show a strong accumulation of β-1,3-glucanase, especially in the *Sr24*-mediated resistance response. The extracellular localisation of β-1,3-glucanases subsequent to fungal inoculation suggests a defensive role for these enzymes, because they are in close contact to the invading pathogen. These enzymes may attack the rust fungus by direct lytic action or may release elicitor signals. Moreover, constitutive extracellular chitinases may attack fungal hypha directly, while the constitutive intracellular chitinases may become active after plant cell collapse, which occurs in Pr-*Sr5* when the pathogen attacks epidermal cells and in Pre-*Sr24*, to some extent, when the pathogen invades the mesophyll (for a general discussion see Mauch and Staehelin 1989). Since fungi may adapt to these enzymes, a sudden release of high concentrations of these hydrolases may be a very effective mechanism with which to challenge fungal pathogens (Boller 1993). Furthermore, a synergistic action between both types of hydrolase may effectively optimize the defence (Mauch et al. 1988, Roberts and Selitrennikoff 1988). However, although it has been demonstrated in thin sections that *Pgt* contains chitin in the cell walls of intercellular infection structures (infection hyphae, haustorium mother cells) and intracellular infection structures (haustorial body, Chong et al. 1986), arguments cannot be disregarded that the surface composition of fungal structures formed in the intercellular space is altered (Mendgen and Deising 1993). A model presented by Freytag and Mendgen (1991) suggests that the chitin of rust infection structures formed inside the mesophyll is covered by apposition of glycoproteins, proteins and α- and β–1,3-glucans, and/or may, additionally, be converted to chitosan by action of chitin deacetylase. Studies using thigmoinductive membranes have shown that chitin deacetylase is secreted at the onset of penetration-hypha formation (H. Deising, Institut für Phytopathologie, Universität Konstanz, Germany, personal communication). Thus,

the defensive role of chitinase and β-1,3-glucanase in plants against rust fungi is far from being fully understood.

Molecules involved in host-pathogen recognition. In all inoculation experiments, uredospores germinated in the dark and fungal penetration through stomata started synchronously at the onset of illumination (16 h post inoculation). The observation that transcripts for β-1,3-glucanase and chitinase had already accumulated before leaves had been illuminated and, consequently, before stomatal opening and fungal penetration, strongly suggests that the pathogen is recognized in the ectophytic phase of its development. More importantly, the differential early expression of β-1,3-glucanase and chitinase in resistant and susceptible genotypes shows race-cultivar specificity to be expressed before a direct contact of fungal hyphae with leaf cells (biotrophic phase). One possible signal molecule in the interaction of stem rust and wheat is the *Pgt* elicitor which is released from hyphal walls during the interaction (Beißmann et al. 1992). Synthesis of β-1,3-glucanase was induced very strongly after injection of this glycoprotein elicitor (Fig. 8). The EC_{50} value of $0.1 \ \mu g \cdot ml^{-1}$ is low compared with those of enzymes of the phenylpropanoid pathway like phenylalanine ammonia-lyase (PAL; EC50 = 5 μg ml^{-1}; Kogel et al. 1988). However, in accordance with previous observations, the elicitor does not show race-cultivar specificity because β-1,3-glucanase is equally induced in all wheat genotypes. Unlike the pathogen, the elicitor also does not induce chitinase transcript, if pTaCH-1 is used as probe, demonstrating that this elicitor is not capable of inducing the complete set of defence genes. Although the data presented here might be explained by the action of suppressor molecules (Beißmann and Kogel 1992), we suggest that an elicitor with properties different from *Pgt* elicitor must be present in the interaction. Previously, the ability of chitin hydrolysate and chitosan to act as elicitors was demonstrated in wheat (Moerschbacher et al. 1986; Barber et al. 1989). In accordance with these reports, we found accumulation of both, β-1,3-glucanase and chitinase transcripts, homologous to pTaGL-1 and pTaCH-1, when wheat leaves were treated with chitin oligomers (data not shown). Hence, further work must show whether chitin derivatives are involved in the signalling during the wheat-stem rust interaction and whether they could be responsible for the induction of the race-cultivar-specific resistance responses.

Albeit the significance for a decisive role of the PR proteins β-1,3-glucanase and chitinase in the resistance mechanism of cereals against biotrophic fungi is far from being elucidated, our data show that the PR proteins are excellent tools with which to study early signalling in these interactions.

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