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RESEARCH PAPER

Effects of β-1,3-glucan from *Septoria tritici* on structural defence responses in wheat

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Abstract

The accumulation of the pathogenesis-related (PR) proteins β-1,3-glucanase and chitinase and structural defence responses were studied in leaves of wheat either resistant or susceptible to the hemibiotrophic pathogen *Septoria tritici*. Resistance was associated with an early accumulation of β-1,3-glucanase and chitinase transcripts followed by a subsequent reduction in level. Resistance was also associated with high activity of β-1,3-glucanase, especially in the apoplastic fluid, in accordance with the biotrophic/endophytic lifestyle of the pathogen in the apoplastic spaces, thus showing the highly localized accumulation of defence proteins in the vicinity of the pathogen. Isoform analysis of β-1,3-glucanase from the apoplastic fluid revealed that resistance was associated with the accumulation of an endo-β-1,3-glucanase, previously implicated in defence against pathogens, and a protein with identity to ADPG pyrophosphatase (92%) and germin-like proteins (93%), which may be involved in cell wall reinforcement. In accordance with this, glycoproteins like extensin were released into the apoplast and callose accumulated to a greater extent in cell walls, whereas lignin and polyphenolics were not found to correlate with defence. Treatment of a susceptible wheat cultivar with purified β-1,3-glucan fragments from cell walls of *S. tritici* gave complete protection against disease and this was accompanied by increased gene expression of β-1,3-glucanase and the deposition of callose. Collectively, these data indicate that resistance is dependent on a fast, initial recognition of the pathogen, probably due to β-1,3-glucan in the fungal cell walls, and this results in the accumulation of β-1,3-glucanase and structural defence responses, which may directly inhibit the pathogen and protect the host against fungal enzymes and toxins.

Key words: ADPG pyrophosphatase, callose, chitinase, extensin, β-1,3-glucanase, *Mycosphaerella graminicola*, PAMPs/MAMPs, *Septoria tritici*, wheat.

Introduction

The innate ability of plants to detect pathogens is essential for their survival (Nürnberger *et al.*, 2004; Altenbach and Robatzek, 2007). This is made possible by the ability of the plant to recognize non-self structures through receptors (pattern recognition receptors, PRR) and the structures recognized by these receptors are termed pathogen-/microbe-associated molecular patterns or PAMPs/MAMPs (Nürnberger *et al.*, 2004; Altenbach and Robatzek, 2007).

Several different PAMPs have been isolated from bacteria, Oomycetes, and fungi. Examples from fungi include β-1,3-glucan, chitin, and ergosterol (Nürnberger *et al.*, 2004; Altenbach and Robatzek, 2007). Pathogenesis-related (PR)

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proteins have been implicated in disease resistance in several plant–pathogen interactions (Linthorst, 1991; Van Loon and Van Strien, 1999). The roles of some PR-proteins are poorly understood (Kitajima and Sato, 1999; Van Loon and Van Strien, 1999), whereas others are known to have antifungal activity *in vivo*. Thus, the two plant hydrolases PR-2 (β-1,3-glucanase) and PR-3 (chitinase) degrade fungal cell walls and may therefore inhibit pathogen growth directly (Kim and Hwang, 1997; Kini *et al.*, 2000). Oligomers of β-1,3-glucan and chitin released from the hydrolysis of fungal cell walls may, furthermore, act as elicitors of defence reactions (Takeuchi *et al.*, 1990; Wu *et al.*, 1997; Jia and Martin, 1999) and thus serve as PAMPs/MAMPs (Nürnberger *et al.*, 2004; Altenbach and Robatzek, 2007).

As a result of recognition of PAMPs/MAMPs, defence reactions are activated in the plant. These responses include the accumulation of Reactive Oxygen Species (ROS), and PR-proteins as well as the reinforcement of the cell wall by oxidative cross-linking of cell wall components and the deposition of callose and lignin (Nürnberger *et al.*, 2004; Göhre and Robatzek, 2008). The synthesis of callose, a β-1,3-glucan, occurs *de novo* as a response to pathogen attack (Skou *et al.*, 1984; Enkerli *et al.*, 1997; Verma and Hong, 2001) although it is present at a constitutive level in many other regions such as pollen tubes (Meikle *et al.*, 1991) and sieve tubes (Skou *et al.*, 1984). Lignin precursors and other phenolics are themselves directly toxic to pathogens and their polymerization makes cell walls more difficult to penetrate and degrade (Hammerschmidt and Kuć, 1982). Likewise, cross-linking involving hydroxyproline-rich glycoproteins, including extensin may have the same effect (Wei and Shirsat, 2006). In addition to their role in the activation of defence response genes and direct antimicrobial effects, the accumulation of ROS is reported to enhance cross-linking in cell walls (Thordal-Christensen *et al.*, 1997).

Septoria tritici Roberge in Desmaz [teleomorph *Mycosphaerella graminicola* (Fuckel) J. Schröt. in Cohn] is a serious constraint for wheat (*Triticum aestivum* L.) production, causing *Septoria tritici* blotch or speckled leaf blotch. The disease has become more serious worldwide during recent years (Eyal, 1999). The infection biology of *S. tritici* is fairly well understood (Cohen and Eyal, 1993; Kema *et al.*, 1996; Duncan and Howard, 2000; Shetty *et al.*, 2003) whereas detailed studies of host defence responses against *S. tritici* and studies of how the pathogen influences host physiology have only recently begun (Ray *et al.*, 2003; Shetty *et al.*, 2003, 2007; Keon *et al.*, 2007; Rudd *et al.*, 2008). Cohen and Eyal (1993) observed callose to accumulate in a resistant, but not in a susceptible cultivar. However, they stated that there was no conclusive evidence for the involvement of callose in resistance. No evidence has been found that resistance was associated with compartmentalization (Kema *et al.*, 1996), classical hypersensitive responses (HR) (Kema *et al.*, 1996; Shetty *et al.*, 2003; Rudd *et al.*, 2008) or polyphenolic compounds or lignin (Cohen and Eyal, 1993; Kema *et al.*, 1996), although Shetty *et al.* (2003) observed autofluorescence to occur to a significantly higher degree in a resistant than in a susceptible wheat cultivar. Recently, H_2O_2 accumulation was reported as a host response in wheat to infection by *S. tritici* and evidence was presented that H_2O_2 was indeed a factor inhibiting pathogen growth in wheat (Shetty *et al.*, 2003, 2007). However, it was also concluded that H_2O_2 was probably not the only defence response since scavenging of H_2O_2 from a resistant cultivar did not render it fully susceptible to the pathogen (Shetty *et al.*, 2007).

It is shown here that PR-protein accumulation and callose deposition correlate with resistance in wheat against infection by *S. tritici*. Furthermore, it is shown that the application of purified β-1,3-glucan isolated from the cell walls of the pathogen is able to protect a susceptible wheat cultivar from disease development, and that this is accompanied by, among other defences, the accumulation of PR-proteins and callose. Thus our data suggest that β-1,3-glucanase cleaves β-1,3-glucan in the pathogen cell wall, releasing fragments which elicit further structural defence responses, to participate in preventing the colonization of *S. tritici*.

Materials and methods

Plants and inoculation

Two wheat cultivars were used throughout the experiments: cv. Sevin is susceptible to isolate IPO323 of *S. tritici* and cv. Stakado is resistant to this isolate. Plants were grown, inoculum produced, and plants inoculated as described previously by Shetty *et al.* (2003). Control plants were treated with distilled water.

RNA extraction and quantification of gene expression by quantitative real-time RT-PCR

Leaves of *S. tritici*-inoculated or water-treated plants of both cultivars were sampled at 1, 3, 5, 7, 9, 11, 13, and 15 dai, ground in liquid nitrogen, and stored at –80 °C until use. Total RNA was extracted from 100 mg homogenized plant tissue using the RNeasy Plant Mini Kit (Qiagen, Venlo, The Netherlands) following the manufacturer's protocol. Genomic DNA contaminating the samples was removed by treatment with DNase using DNA-free (Ambion, UK) according to the manufacturer's instructions. cDNA synthesis was carried out using iscript (Bio-Rad, USA) according to the manufacturer's protocol. The final concentration of reverse transcribed total RNA was 35 ng μl^{-1}. For each sample, a negative control was made without adding reverse transcriptase to ensure that there was no contamination with genomic DNA.

The primers used are shown in Table 1. The 18S rRNA gene was used as the reference gene (Shimada *et al.*, 2003). The other primers were designed using the primer3 program (http://fokker.wi.mit.edu/primer3/input.htm). Specificities of the genes were tested by blasting analyses of the amplicon. Tests for secondary structure of the amplicon was performed on the website: http://mfold.bioinfo.rpi.edu/. Testing of primers as well as real-time RT-PCR was carried out as described by Bedini *et al.* (2005).

Table 1. Primers used in the quantitative real-time RT-PCR studies

Gene	Accession	Forward primer	Reverse primer
18S ribosomal RNA		5′-CGGCTACCACATCCAAGGAA-3′	5′-GCTGGAATTACCGCGGCT-3′
β-1,3-glucanase	DQ090946.1	5′-AACGACCAGCTCTCCAACAT-3′	5′-GTATGGCCGGACATTGTTCT-3′
Chitinase	AY437443.1	5′-ACGGTGTGATCACCAACATC-3′	5′-CAGTCCAGGTTGTCACCGTA-3′
PAL	AY005474.1	5′-CCAATGTTCTGTCCGTCCTT-3′	5′-CTTCAGCTTGTGGGTCAGGT-3′
Chalcone synthase	AY286097.1	5′-TCACCTTCCACCTCCTCAAG-3′	5′-GGATGCGCTATCCAGAAGAC-3′
Oxalate oxidase	AJ556991	5′-TGCAGTTCAACGTCGGTAAG-3′	5′-ATGGCACGAAGACGATACC-3′

Extraction of apoplastic fluid

Apoplastic fluid was obtained according to the method of Kerby and Somerville (1989). Leaves were sampled at 1, 3, 5, 7, 9, 11, 13, and 15 dai. At each time point, the leaves were immersed in deionized water and then vacuum infiltrated for about 60 min. After infiltration, they were gently dried with tissue paper, wrapped in a plastic film, and centrifuged at 500 *g*, at 4 °C with their cut end down in a centrifuge tube with glass beads at the bottom. The collected apoplastic fluids were immediately frozen at –80 °C until use.

Assays for β-1,3-glucanase and chitinase activity

Activity of the enzymes was assayed in apoplastic fluid and in protein extracts from whole leaves. For both types of extracts, protein was quantified in duplicate in an ELISA reader at 595 nm according to the method of Bradford (1976) using the Bio-Rad protein assay (Bio-Rad Ltd) with bovine albumin (Sigma) as standard.

For whole leaf extracts, leaves were harvested at 1, 3, 5, 7, 9, 11, 13, and 15 dai, ground in liquid nitrogen and stored at –80 °C until use. Protein was extracted in 0.05 M sodium acetate buffer (pH 5.2) at 4 °C and the homogenate centrifuged at 10 000 *g* for 30 min. at 0 °C. The supernatant was used as the source of enzyme and stored on ice until analysis.

β-1,3-glucanase activity was assayed according to Kini *et al.* (2000) with slight modifications. The samples were incubated with 0.1% (w/v) laminarin in 0.05 M sodium acetate buffer (pH 5.2). The mixture was incubated for 15 min. in a shaker at 37 °C. The reaction was stopped by adding DNS reagent [0.5% (w/v) 3,5-dinitrosalicylic acid (Sigma) and 15% (w/v) potassium sodium tartrate tetrahydrate (Sigma)] followed by boiling in a water bath for 10 min. The absorbance was measured in an ELISA-reader at 540 nm. A standard curve relating the amount of glucose equivalents to the absorbance at 540 nm was used to determine the activity.

Chitinase activity was assayed according to Boller and Mauch (1988). The samples were incubated with colloidal chitin (made from chitin, Sigma) for 2 h at 37 °C. The reaction was stopped by centrifugation and the supernatant boiled with 0.8 M potassium borate buffer (pH 9.1) for 3 min. Subsequently, *p*-dimethylaminobenzaldehyde (Sigma) was added and the mixture incubated at 37 °C for 20 min, after which absorbance was measured at 585 nm in an ELISA-reader. A standard curve relating the amount of glucosamine equivalents to the absorbance at 585 nm was used to determine the activity.

All enzyme activities are expressed as specific activity (μmol min^{-1} mg^{-1} protein).

β-1,3-glucanase isoforms

β-1,3-glucanase isoforms were determined by native-PAGE from apoplastic fluid according to the method of Schrauwen (1966). Apoplastic fluid was obtained as before from both Sevin and Stakado, either inoculated with *S. tritici* or treated with water at 1, 3, 5, 7, 9, and 11 dai (data not shown for water controls). Protein was quantified as before. Protein (40 μg) was electrophoresed in 10% polyacrylamide gels. Basic gels (pH 6.8) were used for the detection and separation of acidic isoforms and acidic gels (pH 5.2) for the separation of the basic isoforms. To detect the different isoforms of β-1,3-glucanase, the gel was stained for 30 min after electrophoresis in a solution containing 100 mg Laminarin in 25 ml 0.05 M Na-acetate buffer (pH 5.2) at 40 °C. After three washes, the gel was transferred to a glass tray containing 0.15% 2,3,5 triphenyl tetrazolium chloride (Sigma) in NaOH (150 mg in 20 ml 1 N NaOH) and boiled until red bands appeared. To check for equal loading of protein and for the identification of proteins by mass spectrometry, a gel was run as described above and stained with Coomassie Brilliant Blue.

Mass spectrometry

Gel pieces were excised from the Coomassie Brilliant Blue-stained bands. Protein was digested in-gel with sequencing grade porcine trypsin (Promega) as described by Zhang *et al.* (2007) and applied to a 600 μM AnchorChip™ target (Bruker Daltonics, Bremen, Germany) using a sample matrix wash procedure (Zhang *et al.*, 2007) with α-hydroxycinammic acid as the matrix. MALDI-TOF (matrix assisted laser desorption ionization-time of flight) spectra were acquired using a Bruker Ultraflex II (Bruker Daltonics) in positive ion reflector mode. External calibration was performed using a tryptic digest of β-lactoglobulin. Spectra were processed in the FlexAnalysis software version 3.1 (Bruker Daltonics) and database searching was performed using BioTools version 3.0 (Matrix Science) to search the NCBI sequence database and the DFCI wheat gene index (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=wheat) by applying the following

criteria: taxonomy green plants; monoisotopic mass accuracy <80 ppm; one allowed missed cleavage site; carbamidomethylation of cysteine (complete), and oxidation of methionine (partial). The signal peptide cleavage sites were predicted using SignalP (Nielsen *et al.*, 1997).

Histochemical staining for callose, lignin, polyphenolic substances, and H_2O_2

Leaves were harvested 1, 3, 5, 7, and 9 days after inoculation (dai) and cleared using an ethanol:acetic acid mixture as previously described by Shetty *et al.* (2003).

Callose deposition in cleared leaves was detected after staining the leaves with a solution of 0.005% Aniline Blue in 0.15 M dipotassium hydrogen phosphate (pH 8.2) for 2 h. The samples were observed using epifluorescence microscopy (excitation maximum 330–385 nm, dichroic mirror DM 400, barrier filter >420 nm). Regions with callose deposition emitted a greenish yellow fluorescence. For each time point, four leaves were examined and, on each leaf, 20 microscopic fields were studied (400× magnification, total area in field of vision approximately 0.22 mm^2). The fields were selected randomly across the leaf. In each field, the total number of cells was counted as well as the number of cells with callose. These numbers also comprised cells of which only a part was seen within the field of vision.

To study the distribution of callose inside the tissue, leaf pieces, 4×7 mm were cut from inoculated leaves and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 6.8) under vacuum. After 24 h, the leaf pieces were washed in buffer and dehydrated in a graded series of isopropanol (IPA) and embedded in paraffin. After sectioning and drying of the slides, the paraffin was removed by UltraClear (Mallinckrodt Baker B.V. Devanter, Holland); and the sections transferred to 100% IPA. Finally, the slides were air-dried and mounted with Permount (Fisher Chemical, Fair Lawn, New Jersey, USA).

Lignin accumulation was studied in cleared leaf pieces after staining with 2% phloroglucinol dissolved in 96% ethanol for 1 h. Subsequently, the tissue was placed on a glass slide with 10% HCl, heated slightly over a flame, and examined in the microscope. Pink to reddish staining of the xylem served as a control for the staining procedure.

Polyphenolic substances were detected in cleared leaf pieces after staining with 0.05% Toluidine Blue O (pH 6.8) for 30 min and washing in phosphate buffer (pH 6.8). Turquoise staining of the xylem served as a control for the staining procedure.

In vivo detection of H_2O_2 was carried out using the DAB-staining method as described before (Shetty *et al.*, 2003). These leaves were also stained with Aniline Blue and examined for the presence of callose.

Detection of cell wall polysaccharides in apoplastic fluid and leaves

Leaves of cvs Sevin and Stakado were inoculated with *S. tritici* or treated with water as before and apoplastic fluid collected at 1, 3, 5, 7, 9, 11, 13, and 15 dai. Cell wall-derived polysaccharides in the apoplastic fluid and the leaves from which apoplastic fluid was extracted were analysed by immune-dot-assay and Western blot, respectively, using hybridoma supernatants of rat monoclonal antibodies LM1, LM2, LM5, LM6, LM7, JIM5, JIM7, and XGA2. These antibodies recognize specifically extensin, arabinogalactan, β-1,4-galactan, α-1,5-arabinan, homogalacturonan, sequentially methyl esterified homogalacturonan, non-sequentially methyl esterified homogalacturonan, and xylogalacturonan, respectively. These hybridoma supernatants were a kind gift from Professor P Knox (University of Leeds, UK). Immuno-dot-analysis followed the method described by Willats *et al.* (2002) using 1 µl of apoplastic fluid. Hybridoma supernatants and anti-rat secondary antibody conjugated with horseradish peroxidase were diluted in PBS containing 3% (w/v) skim milk in 1:10 and 1:1000, respectively. The remaining leaf materials, after isolating the apoplastic fluid (three leaves for each treatment; approximately 250 mg), were ground in 0.5 ml of 50 mM Na_2CO_3, 50 mM DTT, 15% (w/v) sucrose, and 2.5% (w/v) SDS using a glass homogenizer. Proteins were separated on a 12% SDS gel and analysed by Western blot using hybridoma supernatants and the secondary antibody diluted 1:40 and 1:1000, respectively.

Preparation of β-glucan fragments from S. tritici *mycelium*

To purify β-glucan from *S. tritici*, the fungus was grown in Fries medium for 3 weeks. After inoculation of the flasks, they were incubated in the dark for 48 h at 27 °C with continuous shaking (80 rpm). Subsequently, the flasks were incubated in darkness and without shaking at room temperature until use. After 3 weeks, the mycelial mat in the flasks was separated from the medium and freeze-dried. Water-soluble and mainly linear β-glucans were extracted from the freeze-dried mycelium (3 g dry matter) essentially as described by Yamaguchi *et al.* (2000).

The dry mycelium was ground in a mortar with liquid N_2. The fine (grey) powder was suspended in 50 ml PBS-buffer [0.1 M phosphate buffer (pH 7.2), 0.5 M NaCl] and the suspension was incubated for 15 min at room temperature. Following centrifugation, the pellet was recovered and re-extracted twice. The insoluble residues were further washed with 50 ml distilled water and then centrifuged followed by delipidation in four steps: (i) 50 ml acetone, (ii) 50 ml 1:1 ratio of chloroform:methanol (v/v), (iii) 50 ml chloroform, and (iv) 50 ml acetone. For each treatment, the insoluble matter was mixed thoroughly, incubated for 15 min at room temperature, and the insoluble residue was recovered by centrifugation. The final pellet was resuspended in 50 ml milliQ water and autoclaved three times for 15 min at 121 °C. The remaining insoluble residues were extracted overnight at room temperature with 50 ml 1 M NaOH containing 0.01% $NaBH_4$. The pellet was re-extracted twice with 1 M NaOH and once with milliQ water. All supernatants were pooled and the mainly linear β-1,3-glucan (Yamaguchi *et al.*, 2000) was precipitated by

neutralization to pH 6.0 with glacial acetic acid and collected by centrifugation at 4 °C according to the protocol of Yamaguchi *et al.* (2000). Preparation of β-glucan fragments was achieved by partial enzymatic digestion using (endo-1,3-β-D-glucanase (*Trichoderma* sp.) EC 3.2.1.39). A total of 100 mg pure β-glucan was dispersed in 3 ml of 20 mM TRIS-HCl buffer (pH 4.5) and digested for 4.5 h at 40 °C with 0.5 U endo-β-1,3-glucanase (Megazyme). The reaction was stopped by boiling and the products recovered freeze-dried. The final product was analysed using High Performance Anion Exchange Chromatography (HPAEC) with pulsed amperometric detection (PAD) using a Dionex BioLC-System (Dionex Corp., Sunnyvale, CA) equipped with a CarboPac PA-200 column operating with a 0.5 ml ml^{-1} flow and an elution profile as described by Blennow *et al.* (1998). Chromatograms were visualized using the Chromeleon V.6.40 software.

Application of purified β-glucan to plants

In order to study the influence of purified β-glucan on the host, a range of experiments were carried out in cv. Sevin. Symptom expression of *S. tritici* was scored in plants either infiltrated (Shetty *et al.*, 2007) or sprayed until run-off with a solution containing 100 μg purified β-glucan ml^{-1} of milliQ water. Plants were inoculated with *S. tritici* 24 h later as before and symptoms recorded 15 dai.

Callose accumulation was studied as before in plants either sprayed with 100 μg purified β-glucan ml^{-1} of milliQ water or water (control), followed by inoculation with the pathogen. Observations were made at 3 dai and 5 dai.

In order to test whether β-glucan application to the plants elicited gene expression, qRT-PCR were performed on plants either sprayed with purified β-glucan (100 μg ml^{-1}) or water followed by inoculation with the pathogen. Samples were taken at 3 dai as before. The primers tested were PAL, chitinase, chalcone synthase, β-1,3-glucanase, and oxalate oxidase (Table 1).

To examine the ability of the wheat plant to produce β-1,3-glucanase, which could degrade fungal cell walls, apoplastic fluid was isolated from cvs Stakado and Sevin (either inoculated with *S. tritici* or treated with water) at 3, 5, and 7 dai. The apoplastic fluid was used as the source of enzyme and purified fungal cell walls as substrate. Protein determination and β-1,3-glucanase assay was performed as described above.

Statistical analysis

Data from enzyme activity assays represent continuous variables and were analysed by analysis of variance assuming a normal distribution. Variances were stabilized by appropriate transformation of data if necessary. Data from studies of callose accumulation represent a discrete variable and were hence analysed by logistic regression, assuming a binomial distribution, as described by Shetty *et al.* (2003). All data were analysed by PC-SAS (release 8.2, SAS Institute, Cary, NC).

For gene expression studies, statistical evaluations of the relative expression level of the target genes was evaluated in *S. tritici*-inoculated material compared with water-treated material and normalized to the 18S rRNA expression level. The analyses were performed using the relative expression software tool REST© as described by Pfaffl *et al.* (2002). The stability of the housekeeping gene was evaluated using the program Bestkeeper as described by Pfaffl *et al.* (2004).

All experiments were repeated at least twice with similar results and representative results are presented. All hypotheses were rejected at $P \leqslant 0.05$. In the following, all differences are significant at $P \leqslant 0.05$ unless specifically mentioned.

Results

Gene expression

Table 2 shows the results on gene expression. β-1,3-glucanase transcript levels in Stakado were elevated in inoculated compared with water-treated control plants at 1–3 dai and again at 9–11 dai, with no significant difference at 5–7 dai and a reduction at 13–15 dai. In Sevin, there was

Table 2. Quantitative real-time RT-PCR experiments of transcript levels of β-1,3-glucanase, chitinase, PAL, and chalcone synthase gene expression in wheat cvs Stakado (resistant) and Sevin (susceptible) after inoculation with *S. tritici*

Values shown represent fold up-regulation in inoculated compared with water-treated plants, after normalization to 18S rRNA (Shimada *et al.*, 2003).

Time	β-1,3-Glucanase (PR-2)[a]		Chitinase (PR-3)[a]		PAL[a]		Chalcone synthase[a]	
	Stakado	Sevin	Stakado	Sevin	Stakado	Sevin	Stakado	Sevin
1 dai	4.5 A	1.4 NS	2.6 NS	0.9 NS	1.3 NS	0.8 NS	2.0 NS	0.6 NS
3 dai	4.1 A	1.1 NS	3.2 A	0.6 A	2.5 A	1.7 NS	0.4 A	1.2 NS
5 dai	2.9 NS	1.5 NS	1.6 A	1.5 NS	1.4 NS	0.8 NS	4.2 NS	0.6 NS
7 dai	1.0 NS	1.1 NS	1.0 NS	0.9 NS	1.1 NS	0.7 NS	0.7 A	0.5 NS
9 dai	3.5 A	52.0 A	5.3 A	54.9 A	2.3 A	0.5 A	0.6 A	0.1 A
11 dai	2.9 A	48.8 A	0.5 A	3.3 A	3.7 A	0.2 A	0.1 NS	0.1 A
13 dai	0.6 A	27.2 A	0.7 NS	317.4 A	1.1 NS	4.5 A	0.6 A	0.3 A
15 dai	0.5 A	322.9 A	0.3 A	2846.6 A	1.8 A	3.4 A	0.4 A	0.0 A,B

[a] A, significant change; NS, non-significant change; B, exact value is 0.017.

no significant difference between inoculated and control plants at 1–7 dai whereas elevated transcript levels were seen in inoculated plants from 9–15 dai. Chitinase transcript levels were elevated in Stakado in inoculated compared with control plants at 3–5 and 9 dai, whereas no significant differences were seen at 1, 7, and 13 dai. Reduced transcript levels were seen at 11 and 15 dai. In Sevin, no significant differences in transcript levels were observed at 1 and 5–7 dai, whereas reduced transcript levels were observed at 3 dai and elevated levels at 9–15 dai. PAL transcript levels were elevated at 3 and again at 9–11 and 15 dai in Stakado, whereas there were no significant differences between inoculated and control plants at the remaining time points. In Sevin, there was no significant difference between inoculated and control plants at 1–7 dai, whereas reduced transcript levels were seen at 9–11 dai followed by elevated levels at 13–15 dai.

Chalcone synthase levels in Stakado were generally reduced (3, 7–9, and 13–15 dai) whereas no significant differences in levels were observed at the remaining time points. In Sevin (Table 2), there were no significant differences between inoculated and control plants 1–7 dai, followed by reduced transcript levels in inoculated plants 9–15 dai.

Activity of β-1,3-glucanase and chitinase in whole leaf extracts and apoplastic fluid

β-1,3-glucanase activity in whole leaf extracts (Fig. 1A) was higher in Stakado than in Sevin at all time points, with an increasing trend, especially from 11 dai. Activity in Stakado was higher in inoculated than in control plants at 5 dai and 13–15 dai, whereas the opposite relationship was observed at 3 dai. In apoplastic fluid (Fig. 1B), activity was also higher in Stakado than in Sevin, with very high activities observed in Stakado at 1 dai with a minor peak at 9 dai. At all time points, activities were higher in inoculated than in control plants. In Sevin, activity was higher in inoculated plants compared with the controls at 5–9 dai.

Chitinase activity in whole leaf extracts (Fig. 1C) fluctuated considerably, often with no major differences between the inoculated and control plants. However,

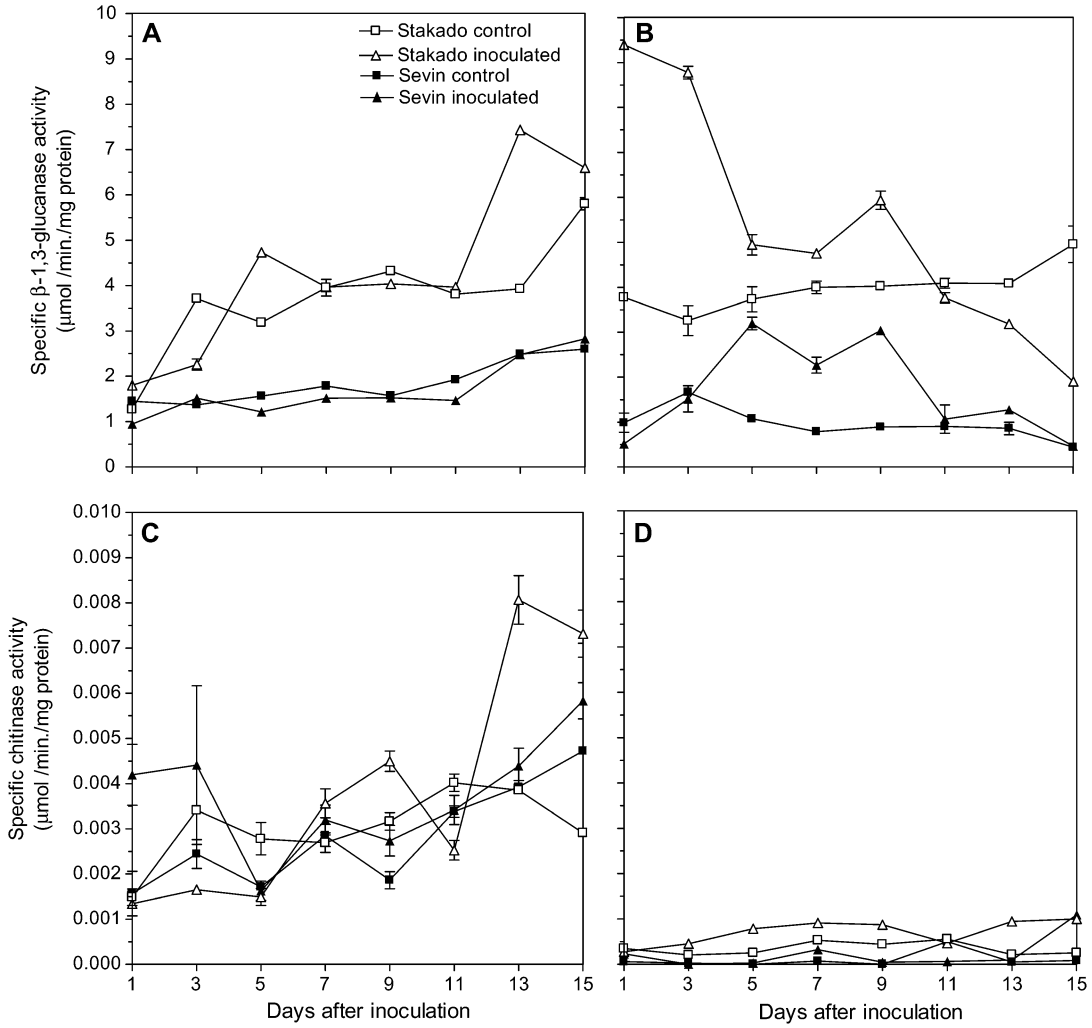

Fig. 1. Time-course of activity of β-1,3-glucanase (A, B) and chitinase (C, D) in Stakado and Sevin with and without inoculation with *Septoria tritici*, isolate IPO 323. (A) and (C) show activity in whole leaf extracts for β-1,3-glucanase and chitinase, respectively, whereas (B) and (D) show activity in the apoplastic fluid. Each value is presented ±standard error.

activity was higher in Stakado than in Sevin at 9 dai and 13–15 dai whereas higher activity was observed in Sevin at 1–3 dai. In apoplastic fluid (Fig. 1D), activity was considerably lower than in whole leaf extracts. Activity was higher in Stakado than in Sevin until 13 dai where activity in Sevin (inoculated with *S. tritici*) increased substantially. In Stakado, activity was higher in inoculated than in control plants at all time points, whereas in Sevin, no difference was seen between treatments until 13 dai after which time activity was higher in the inoculated plants.

β-*1,3-glucanase isoforms and identification by mass spectrometry*

Native-PAGE of apoplastic fluid showed the presence of several bands containing β-1,3-glucanase activity (Fig. 2B). The most intensely β-1,3-glucanase-stained band (Fig. 2, band b) was observed at all time points whereas another band (Fig. 2, band a) accumulated differentially, with high intensity at 3 dai and 7–11 dai. These bands were excised from gels and subjected to tryptic digestion and MALDI-TOF-TOF mass spectrometry. Identifications were performed using samples from Coomassie blue-stained gels, but similar spectra were obtained from the bands excised from activity-stained gels. For band b, database searches revealed a significant match ($P \leqslant 0.05$) to a β-1,3-glucanase sequence from wheat (GenBank accession AAY96422.1), based on nine peptide masses with mass errors <20 ppm and covering 39% of the protein sequence. An additional peptide with [M+H]=2418.2 matched the N-terminus of the sequence after removal of the predicted signal peptide.

The MS data for band a matched a wheat consensus sequence TC232338 which was highly similar to adenosine diphosphate glucose pyrophosphatase from *Hordeum vulgare* ssp. *vulgare* (93% identity to GenBank Accession CAC32847.1) and to germin-like protein 2a (92% identity to GenBank Accession ABG46233.1 also from *Hordeum vulgare* ssp. *vulgare*. Identification was based on three peptides with mass errors <50 ppm and covering 36% of the protein sequence. The identity of all peptides was confirmed by MSMS. The signal peptide cleavage site was confirmed by identification of the peptide LTQDFCVADLACPDTPAGYPCKK.

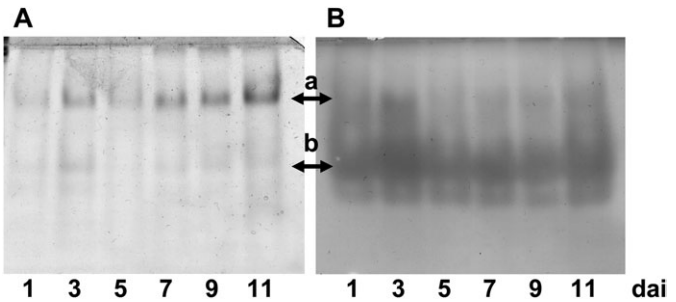

Fig. 2. Native-PAGE of β-1,3-glucanase isoforms accumulating in the apoplastic fluid of cv. Stakado (incompatible interaction) at 1, 3, 5, 7, 9, and 11 d after inoculation with *S. tritici.* (A) Coomassie Brilliant Blue stained gel and (B) activity stained gel. Band a was identified by mass spectrometry as adenosine diphosphate glucose pyrophosphatase and band b as endo-β-1,3-glucanase.

Localization of callose, lignin, and polyphenolics

The deposition of callose as a response to inoculation by *S. tritici* was observed in both resistant and susceptible wheat cultivars but at different rates and amounts (Table 3). Thus, deposition of callose in Stakado started 3 dai and increased in amount and intensity. Initially, callose was observed in and around stomata, (Fig. 3A, B), but at 5 dai, callose started to appear in the mesophyll beneath the stomata (Fig. 3C, D), coinciding with pathogen penetration and its confinement here. At 9 dai, callose was also seen in places far from substomatal cavities (Fig. 3E, F). In Sevin, deposition of callose started only 7 dai (Table 3). Here, deposition was also primarily seen in the stomatal complexes, with only a few reactions in the mesophyll. However, deposition was seen in very few cells and the intensity was very faint when compared to Stakado (data not shown).

Staining of cleared leaf segments with phloroglucinol for the localization of lignin or Toluidine Blue O for the localization of polyphenolic substances did not reveal any accumulation related to pathogen growth in either cultivar at either time point. For both stains, positive reactions in the xylem showed that the staining procedure worked satisfactorily (data not shown).

Detection of various cell wall polysaccharides in apoplastic fluid and leaves

All antibodies tested showed similar patterns in apoplastic fluid. Figure 4 shows an example of LM1, which recognizes glycan components of a hydroxyproline-rich glycoprotein, extensin. In cv. Sevin, the LM1 epitope was relatively low in the apoplastic fluid regardless of the treatment, whereas it was associated well to the cell wall. In Stakado, the LM1 epitope was rather high during the early stages of the interaction (1–9 dai) in the inoculated samples, and particularly high at 11 dai. In the remaining material after collecting apoplastic fluid, the LM1 epitope in Sevin increased by 7–9 dai, whereas it decreased in Stakado in the inoculated samples, thus indicating that it is dissociated

Table 3. Percentage of leaf cells with accumulation of callose in cvs. Stakado and Sevin after inoculation with *Septoria tritici*

	Stakado	Sevin	Odds ratio[a]	
1 dai	0.0	0.0	1.00	NS
3 dai	0.4	0.0	∞	*
5 dai	4.4	0.0	∞	***
7 dai	5.9	0.2	26.07	***
9 dai	11.2	0.3	46.49	***

[a] Odds ratio for comparison of Stakado and Sevin (Sevin used as a reference, odds ratio=1.00). The number of asterisks indicates the degree of significance. NS, non-significant difference; *** significant at $P \leqslant 0.001$; * significant at $P \leqslant 0.05$.

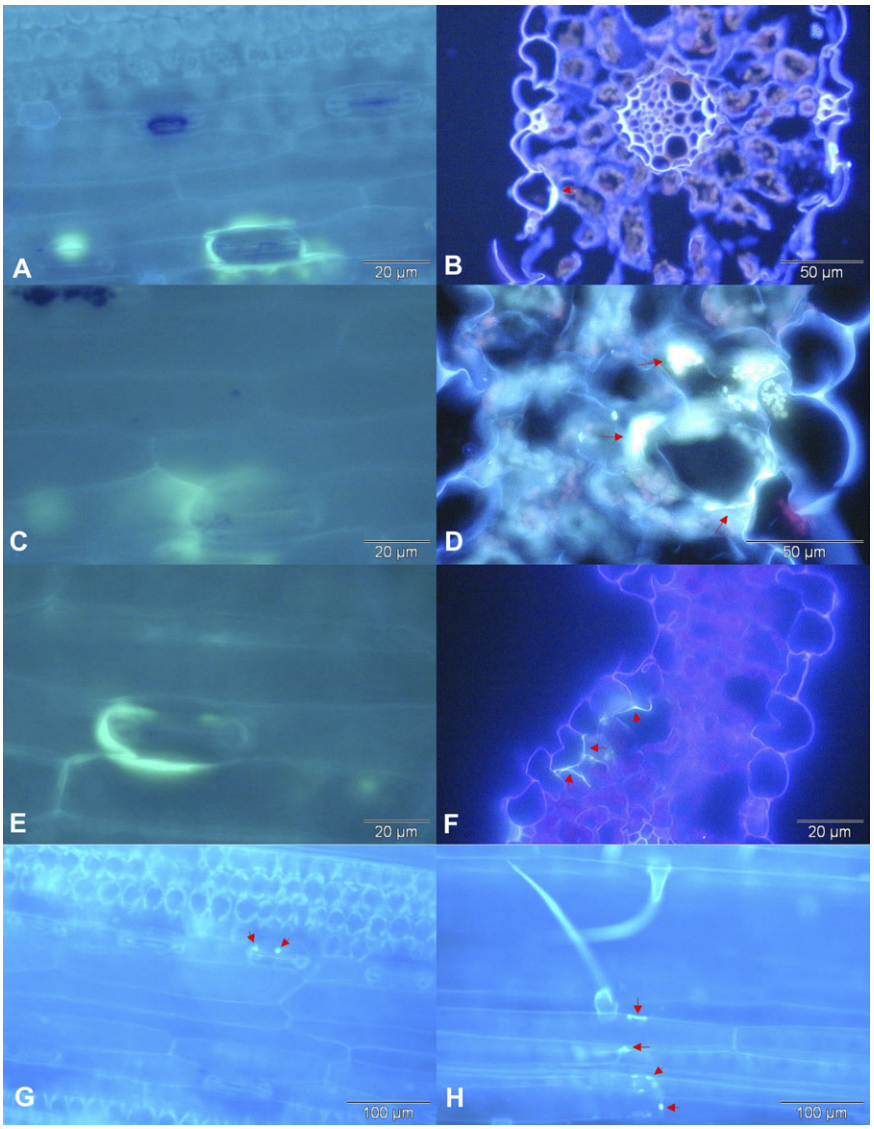

Fig. 3. Deposition of callose in Stakado after inoculation with *S. tritici* isolate IPO323 (incompatible interaction). (A) Callose deposition in stoma in whole leaf mount 5 dai. (B) Deposition of callose in the guard cells in a transverse leaf section 5 dai. (C) Callose deposition in the mesophyll near a penetrated stoma 7 dai. (D) Callose deposition in the mesophyll near a penetrated stoma in a transverse section of a leaf 7 dai. (E, F) Callose deposition at sites remote from penetrated stomata in whole leaf mount (E) and in transverse leaf section (F) 9 dai. (G, H) Callose accumulation in leaves after pretreatment with water (G) or β-1,3-glucan (H) followed by inoculation with *S. tritici* at 5 dai (H). Arrows show deposition of callose.

from the cell wall and flows out to the apoplastic fluid after inoculation.

Application of purified β-glucan to plants

In order to test more specifically whether β-glucans, which are potentially released from *S. tritici* as an effect of induced β-1,3-glucanase activity in wheat, mainly linear β-glucans were prepared from mycelium of the pathogen and fragmented by endo-1,3-β-D-glucanase from *Trichoderma* sp. The chain structure of the generated β-glucan fragments was analysed by High Performance Anion Exchange Chromatography (HPAEC) with pulsed amperometric detection (PAD), using linear malto oligosaccharides (linear α-1,4 glucans) and cellobiose (β-1,4) as standards (Fig. 5). The position of the peaks indicates that the preparation was composed of linear β-1,3-cello oligosaccharides with an apparent degree of polymerization (DP) ranging from 2 to 7. The main product is attributed to β-1,3-linked cellotriose, followed by longer cello oligosaccharides. Only very minor peaks of long fragments eluting at 15–16 min (approximately DP12) were detected (due to scaling, these are not visible in Fig. 5). Both spraying and infiltration of the purified β-glucan on the host resulted in complete inhibition of symptom expression at 15 dai whereas symptoms appeared in control plants treated with water. Figure 6 shows plants sprayed with either water or β-glucan.

Application of a desalted protease and amylase-treated β-glucan preparation to remove possible protein or glycogen contamination (data not shown) showed that less β-glucan elicitor was required to obtain the identical effects on symptom expression. This indicates the presence of some impurities in the β-glucan preparation, but that these impurities had no or only a minor effect on the ability to protect against disease.

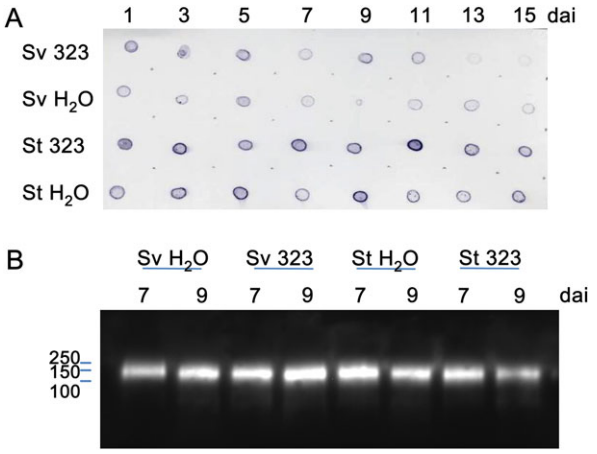

Fig. 4. Detection of cell wall-derived glycoproteins in apoplastic fluid and in the leaves from which apoplastic fluid was isolated. (A) Apoplastic fluid was spotted onto a nitrocellulose membrane and extensin was detected by the LM1 antibody. (B) The remaining leaf material after isolation of apoplastic fluid at 7 and 9 dai was extracted and extensin was analysed by Western blotting using the LM1 antibody. Abbreviations: Sv323, Sevin inoculated with *S. tritici*; SvH_2O, Sevin treated with water; St323, Stakado inoculated with *S. tritici*; StH_2O, Stakado treated with water.

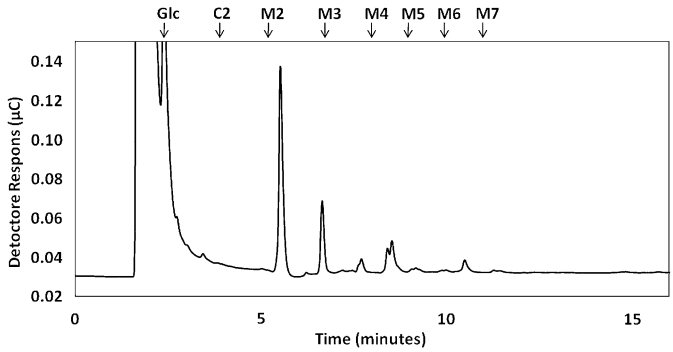

Fig. 5. HPAEC-PAD profile for the linear β-1,3-cello oligosaccharides. Elution time for standard glucose (glc) and larger malto oligosaccharides (linear α-1,4-glucans, M2–M7) and cellobiose (β-1,4, C2) are indicated to show approximate fragment sizes. The large peak at 2 min is eluted salt.

Callose accumulation was studied as before at 3 dai and 5 dai in plants sprayed with β-glucan and it was found here that there was an increased accumulation of callose after β-glucan treatment (Fig. 3G, H).

β-glucan application also affected gene expression. Thus, at 3 dai, chalcone synthase transcript levels were reduced (P <0.001), in β-1,3-glucan-treated compared to water-treated control plants (0.2-fold), whereas chitinase and β-1,3-glucanase levels were elevated (2.4- and 5.9-fold, respectively). There was no significant alteration of the expression of oxalate oxidase and PAL genes (1.2- and 1.3-fold, respectively).

The ability of the wheat plant to produce β-1,3-glucanase, which could degrade fungal cell walls, was studied from apoplastic fluid isolated from cvs Stakado (Fig. 7) and Sevin (data not shown) either inoculated with *S. tritici* or treated with water. Figure 7 shows that apoplastic fluid isolated from Stakado at 5 d and 7 d after inoculation with *S. tritici* was better in degrading β-glucan from the cell walls of *S. tritici* than fluid isolated from plants sprayed with water. On the other hand, fluid from control plants was better in degrading fungal β-glucans than fluid from inoculated plants at 3 dai.

Discussion

After sensing an invading pathogen, plants activate a wide variety of general defence reactions including the oxidative burst, structural cell wall modifications, and the production of defence-related compounds such as PR-proteins (e.g. chitinases and β-1,3-glucanases) (Bolwell, 1999; Kini *et al.*, 2000; Shetty *et al.*, 2008). These proteins can degrade the cell walls of pathogens and inhibit their growth (Kim and Hwang, 1997). Furthermore, these enzymes hydrolyse fungal call walls, releasing β-1,3-glucan and chitin oligomers that act as elicitors of defence reactions (Takeuchi *et al.*, 1990; Wu *et al.*, 1997; Jia and Martin, 1999).

Our data indicate that the PR-protein β-1,3-glucanase (PR-2) in wheat is essential for cleaving the cell walls of the hemibiotrophic pathogen *Septoria tritici* to release elicitors which can act as PAMPs to elicit further defence responses,

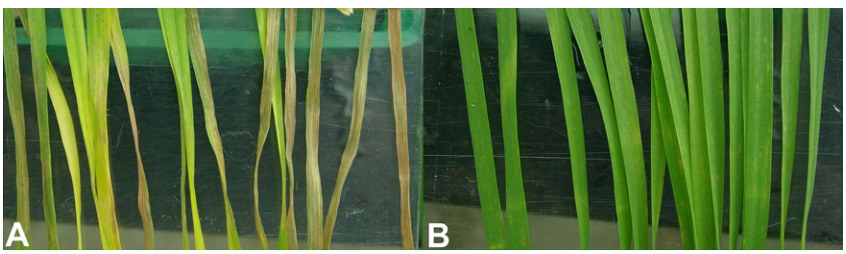

Fig. 6. Spraying of wheat cv. Sevin with (A) water or (B) purified β-glucan from *S. tritici* followed by inoculation with the pathogen 24 h later. Symptoms scored 15 dai.

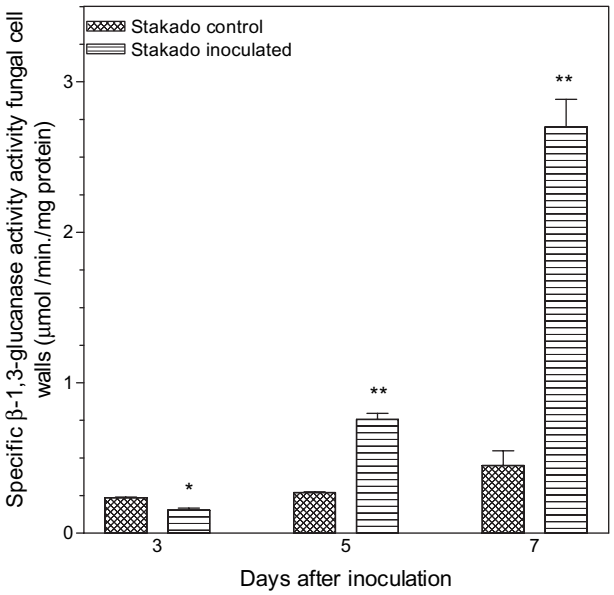

Fig. 7. Time-course study of activity of β-1,3-glucanase using apoplastic fluid from cv. Stakado as the source of enzyme and purified fungal cell walls from isolate IPO323 as the substrate. Apoplastic fluid was isolated from Stakado either inoculated with *S. tritici* or sprayed with water (controls) at different time points after inoculation (3, 5, and 7 dai). Each value is presented ±standard error. Comparisons are possible between inoculated and control plants at each time point. ** Significant at $P \leqslant 0.01$, * Significant at $P \leqslant 0.05$.

which prevent colonization of the pathogen. Therefore, a series of experiments was conducted to elucidate the role of β-1,3-glucanase and β-1,3-glucan in defence. In the resistant cv. Stakado, elevated transcript levels were seen early in the interaction compared with the water-treated control, with a reduction during the late stages. In contrast, in the susceptible cv. Sevin, elevated gene expression was seen only from 9 dai. This change occurred before visible leaf necrosis occurred and probably coincides with the pathogen entering its necrotrophic phase (Shetty *et al.*, 2003, 2007). Activity of β-1,3-glucanase was higher in Stakado than in Sevin, especially in apoplastic fluid, where very high levels were seen at 1 dai with the pathogen, coinciding with initial penetration and colonization of the host, which takes place in the apoplastic space (Shetty *et al.*, 2003). This suggest that β-1,3-glucanase plays a role in the restriction of the pathogen. Since the pathogen lives as a biotroph/endophyte in the apoplastic spaces, this shows the highly localized accumulation of the defence protein close to the pathogen. On the other hand, activity of chitinase did not correlate well with resistance in the host or the infection course of the pathogen. Furthermore, the activity was very low in the apoplastic fluid.

To investigate the potential involvement of β-1,3-glucanase in defence further, the temporal accumulation of different isoforms was studied. Native-PAGE analysis followed by β-1,3-glucanase staining revealed that, in apoplastic fluid from cv. Stakado, two intensely stained bands were present. By mass spectrometry, the band with the highest activity was identified as β-1,3-glucanase and this accession was identical to the accession tested in the transcript accumulation studies (Tables 1, 2). This β-1,3-glucanase has been implicated in defence against *Puccinia striiformis* in wheat (http://www.ncbi.nlm.nih.gov/nuccore/DQ090946.1) and also showed a prominent accumulation in the resistant cv. Stakado (Fig. 2), in compliance with the high β-1,3-glucanase activity (Fig. 1) and the high level of resistance to fungal colonization. The other band showed weaker β-1,3-glucanase activity (Fig. 2, band a) and was matched by MS analysis to adenosine diphosphate glucose pyrophosphatase (AGPPase) and germin-like proteins. One of the main roles of AGPPase is hydrolysis of ADPglucose, which is the universal starch precursor, yielding glucose-1-phosphate and AMP and therefore this enzyme determines the net rate of starch synthesis (Rodrígues-López *et al.*, 2000). Interestingly, it was previously found that sucrose levels were slightly increased in Stakado after inoculation with *S. tritici*, i.e. at 7 dai and 11–15 dai (Shetty *et al.*, 2007). This sugar release essentially correlated with the peaks in AGPPase activity. AGPPases are generally considered to be located in the plastids and has been reported from wheat leaves before, although some have been reported outside the plastids in barley endosperm tissue (Rodrígues-López *et al.*, 2000). However, Rodrígues-López *et al.* (2001) found that two isoforms of AGPPase were oligomers of the germin-like protein HvGLP1. The protein identified here showed 92% identity to the proteins described by Rodrígues-López *et al.* (2001), one of which was found to be soluble. In accordance with this, the protein was found to be present in the apoplast after removal of the signal peptide, indicating its secreted nature. Rodrígues-López *et al.* (2001) furthermore found that neither of the oligomers had oxalate oxidase or superoxide dismutase activity, confirming results by Vallelian-Bindschedler *et al.* (1998). On the other hand, they suggested that AGPPases may regulate the biosynthesis of cell wall polysaccharides, glycoproteins, and glycolipids by controlling the level of nucleotide sugars. In accordance with this, it has previously been shown that the gene *HvGLP1* is constitutively expressed in barley infected by *Blumeria graminis* f.sp. *hordei*, but that expression declined under infection, causing the disappearance of the protein at the same time as cell wall reinforcement occurred (Vallelian-Bindschedler *et al.*, 1998; Schweizer *et al.*, 1999) and Schweizer *et al.* (1999) suggested that HvGLP1 may play a role in stressed leaves, for example, by serving as the substrate for cell-wall reinforcement. Previously, Segarra *et al.* (2003) reported a germin-like protein with SOD-activity and serine protease-inhibiting activity from the apoplast of wheat infected by *S. tritici*. However, the relationship to HvGLP1 could not be confirmed by Zimmermann *et al.* (2006), who did not observe any SOD-activity. Likewise, it has previously been found that even though H_2O_2 accumulated as a defence response against *S. tritici* in cv. Stakado (Shetty *et al.*, 2003), there was no SOD activity (NP Shetty *et al.*, unpublished results).

Increased cell wall reinforcement was observed in the resistant cv. Stakado during the late stages of the interaction with *S. tritici* in the form of deposition of callose and, furthermore, cell wall glycoproteins accumulated in the apoplastic fluid, e.g. extensin (Smallwood *et al.*, 1995) and arabinogalactan proteins as well as different types of pectin fragments from the primary plant cell wall matrix. Vallelian-Bindschedler *et al.* (1998) reported that the germin-like protein HvGLP1 disappeared from the apoplastic fluid of barley infected by the pathogen *B. graminis* f.sp. *hordei*, but not after inoculation with the non-host pathogen *B. graminis* f.sp. *tritici*, thus indicating that the insolubilization reflected infection-related stress rather than resistance. In the wheat–*S. tritici* interaction the reverse situation was observed, with a correlation between resistance to infection and glycoproteins in the apoplastic fluid. Thus, the levels of glycoproteins, including extensins, in the apoplastic fluid was low in the susceptible Sevin and high in the resistant Stakado. Apparently, the glycoproteins were released from the cell walls in the leaves. Extensins are hydroxyproline-rich glycoproteins and two major roles for extensins have been suggested (Wei and Shirsat, 2006). Thus, they may provide strengthening of the cell wall by cross-linking and/or anchorage for lignification in order to form a physical barrier to pathogen ingress. In addition, extensins may directly agglutinate around bacteria to prevent further proliferation. Since *S. tritici* lives in the apoplast and extensins were observed in the apoplastic fluid, the latter role for extensin could be envisaged also to play a role in resistance against this fungal pathogen. Cross-linking of extensins also requires an oxidative burst (Wei and Shirsat, 2006) and this has also been observed in the wheat–*S. tritici* interaction (Shetty *et al.*, 2003).

It was not possible to detect lignin and it was also found that gene expression of two key enzymes in the phenylpropanoid pathway, PAL and CHS, were not elevated after inoculation with *S. tritici*. Lack of lignification is in agreement with previous studies (Cohen and Eyal, 1993; Kema *et al.*, 1996). Only Ride (1975) reported lignification when wounded wheat leaves were inoculated with *S. tritici*. Thus, wounding probably potentiated lignin production which accumulated in the presence of the pathogen.

The accumulation of callose is often used as a marker for PAMP-elicited defence responses (Kim *et al.*, 2005) and it was found that callose accumulated earlier and to a higher degree in Stakado than in Sevin. The accumulation in Stakado increased dramatically by 5 dai, coinciding with penetration of *S. tritici* (Shetty *et al.*, 2003). From 7 dai, accumulation was also seen in the substomatal cavities and the mesophyll following the initial growth of the pathogen. In Sevin, only faint callose accumulation was seen and only from 7 dai. Even though Cohen and Eyal (1993) reported callose accumulation in stomata, they did not consider it important for resistance. However, callose has been shown to be deposited on the inner side of the cell walls in response to invasion by micro-organisms and to restrict their growth (Parker *et al.*, 1993).

Since *S. tritici* penetrates through stomata and then grows between the mesophyll cells without penetrating them (Cohen and Eyal, 1993; Kema *et al.*, 1996; Shetty *et al.*, 2003), the accumulation of callose and cell wall glycoproteins from the primary plant cell wall matrix in the apoplastic fluid in response to pathogen growth in cv. Stakado could be envisaged to prevent nutrient and water transfer to the pathogen. In addition, the cell wall reinforcement could offer protection against cell wall-degrading enzymes or toxins produced by the pathogen (Wei and Shirat, 2006). This would help to inhibit the pathogen from spreading in the mesophyll since it derives its nutrients from the host apoplast (Rohel *et al.*, 2001; Keon *et al.*, 2007) in accordance with its hemibiotrophic nature (Parbery, 1996; Rohel *et al.*, 2001; Shetty *et al.*, 2003, 2007). Cell wall-degrading enzymes have been reported from *in planta* libraries (Kema *et al.*, 2008) and liquid cultures of *S. tritici* (Douaiher *et al.*, 2007), whereas the existence of toxins have only recently been verified (NP Shetty *et al.*, unpublished results) although the possibility of toxins has been suggested previously (Kema *et al.*, 1996; Shetty *et al.*, 2003, 2007).

As a final point, it was tested whether infection by *S. tritici* could release β-glucan fragments from the pathogen cell walls trough the action of β-1,3-glucanase and whether these β-glucan fragments could act as elicitors, signalling the plant to induce further defence responses. For this, β-glucan was isolated from the pathogen and the susceptible cv. Sevin was treated. First, it was necessary to ascertain that the β-1,3-glucanase released from the plants could cleave the fungal cell walls to release elicitor active molecules, which could act as PAMPs. Infiltration and spraying of purified β-glucans from fungal cell walls 1 dai before inoculation resulted in delayed symptom expression in cv. Sevin, thus efficiently protecting this otherwise susceptible cultivar. In order to verify that wheat was able to degrade the fungal cell walls, a β-1,3-glucanase assay was conducted using apoplastic fluid from cv. Stakado as the enzyme source and purified β-glucans from *S. tritici* cell walls as the substrate. In plants inoculated with the pathogen, β-1,3-glucanase activity was high and able to degrade the fungal cell walls. Interestingly, the temporal profile of, β-1,3-glucanase activity in the apoplastic fluid in general (Fig. 1B) was quite different from the activity elicited after treatment of purified β-glucan from cell walls (Fig. 7). This is probably due to the secretion of different isoforms during the infection (targeted specifically against the pathogen after penetration) and differences in substrate specificity for laminarin versus fungal β-glucan.

In cv. Sevin, there was no up-regulation of the PR-proteins or structural defence responses during the early stages of the interaction (Tables 2, 3). Probably, β-1,3-glucanase isoforms required to cleave the fungal cell walls were not elicited, meaning that release of β-glucan fragments did not take place. To test if defence responses could be elicited in cv. Sevin, plants were treated with purified β-glucan fragments one day before inoculation. There was no difference in fungal penetration (data not shown).

However, transcript accumulation of β-1,3-glucanase and chitinase increased and so did callose accumulation, especially in the stomata, indicating that changes in host defence occurred after penetration. Thus, in cv. Sevin, the pathogen is apparently not recognized since PR-protein accumulation is virtually absent, meaning that the fungal cell walls cannot be cleaved and that elicitors and further defence responses are therefore absent. This suggests that when *S. tritici* establishes contact with a leaf surface of a resistant cultivar, H_2O_2 accumulates (Shetty *et al.*, 2003), and this could be the signal for the accumulation of PR-proteins as also shown by Ray *et al.* (2003). When the pathogen penetrates through the stomata, the PR-proteins already present hydrolyse the fungal cell walls, releasing fungal cell wall fragments acting as PAMPs, which induce structural defence responses such as callose, as well as the accumulation of cell wall glycoproteins in the apoplastic fluid (Table 2), to inhibit pathogen growth and reduce the availability of nutrients and water and to protect against fungal cell wall-degrading enzymes and toxins.

Acknowledgements

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