Tissue-specific expression of a defence-related peroxidase in transgenic wheat potentiates cell death in pathogen-attacked leaf epidermis

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ABSTRACT

Gene technology can offer creative solutions to problems of agromonomical relevance, which may not be solved by conventional breeding methods. One of the major problems of wheat cultivation is disease caused by a number of fungal pathogens including the wheat powdery mildew fungus *Blumeria graminis* f.sp. *tritici* (*Bgt*). Transgenic wheat plants that constitutively express the coding sequence of the defence-related wheat peroxidase TaPrx103 (previously TaPERO) in shoot epidermis under the control of the wheat *GstA1* promoter were generated and found to exhibit enhanced resistance to *Bgt* (Altpeter *et al.*, *Plant. Mol. Biol.* 57, 271–283). Here, I report on physiological and molecular analyses of these plants in order to assess the mode of action of the peroxidase encoded by the *TaGstA1:TaPrx103* transgene. Epidermal cells of transgenic lines with enhanced resistance were found to respond to *Bgt* attack more frequently with hypersensitive cell death and the generation of hydrogen peroxide. By contrast, resistance of epidermal cell walls to degradation by fungal enzymes appeared to be similar in transgenic and wild-type plants. Moreover, the analysis of the abundance of approximately 10 000 wheat transcripts revealed no significant effect of the *GstA1:TaPrx103* transgene on host gene expression in non-inoculated leaves and only a marginal effect in *Bgt*-challenged leaves, compared with wild-type plants treated in the same manner. The results indicate that the TaPrx103 protein is involved in generating reactive oxygen species specifically in pathogen-attacked cells, which may lead to localized cell death and resistance. I therefore suggest that the transgenic plants presented here can be regarded as substantially equivalent to non-transgenic wheat.

INTRODUCTION

Wheat plants respond to pathogen attack with the accumulation of a number of transcripts that are speculated to play a role in basal (quantitative) resistance, race-specific resistance mediated by major resistance (R) genes, or non-host resistance (Han *et al.*, 2005; Liu *et al.*, 2005; Schweizer *et al.*, 1989). A role for several differentially expressed host genes in either controlling potentially self-destructive defence responses or in supporting the successful fungus cannot be excluded either (Hückelhoven *et al.*, 2003; Piffanelli *et al.*, 2002). In order to understand gene function in plant pathogen interactions better, a number of genetic and genomic tools and approaches have been developed. In the cereal species of wheat and barley, transient overexpression or silencing of candidate genes in single epidermal cells, which are attacked by powdery mildew fungi, has become instrumental to address gene function (Douchkov *et al.*, 2005; Schweizer *et al.*, 1999b, 2000; Shirasu *et al.*, 1999). This resulted in the identification of a number of host genes in wheat that appear to be required for basal resistance against *Blumeria graminis* f.sp. *tritici* *Bgt*. The gene with the strongest resistance-enhancing effect by transient overexpression encodes a secreted class III peroxidase, TaPERO, with unknown substrate specificity (Christensen *et al.*, 2004; Schweizer *et al.*, 1999b). Plant peroxidases have recently been systematically classified, which resulted in renaming TaPERO as TaPrx103 (Bakalovic *et al.*, 2006; <http://peroxidase.isb-sib.ch>). Thus, I will use the new and more consistent nomenclature for all peroxidases discussed herein. The TaPrx103 protein consists of 312 amino acids, of which the first 22 form a putative signal sequence, and has a calculated mass of 31 kDa and a pI of 5.7 as mature protein, not taking into account possible glycosylation (Rebmann *et al.*, 1991). The barley orthologous protein HvPrx08 has been shown to possess guaiacol-peroxidase activity (Kristensen *et al.*, 1997), which is typical for many class III peroxidases. Interestingly, the *TaPrx103* gene or group of paralogous genes are not expressed in *Bgt*-attacked wheat epidermis, which is the tissue that directly interacts with the pathogen, but are strongly up-regulated in inner leaf tissue (Altpeter *et al.*, 2005).

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This finding was the starting point for the generation of transgenic wheat plants that overexpress *TaPrx103* in the shoot epidermis. For this purpose, the coding sequence of *TaPrx103* was fused to the promoter of the glutathione-S-transferase (*Gst*) *A1* gene, which was found to direct constitutive transgene expression mainly in leaf epidermis. A preliminary characterization of *GstA1i:TaPrx103* transgenic plants revealed that some transgenic lines possessed enhanced resistance of flag leaves to *Bgt*, whereas plant status and yield parameters remained unaffected by the transgene (Altpeter *et al.*, 2005). Together with the fact that peroxidases are potentially harmful proteins that have been reported to cause stunting or wilting of transgenic plants (Lagrimini *et al.*, 1990, 1997), the more specific phenotype of *GstA1i:TaPrx103* transgenic wheat indicated a beneficial effect of using a tissue-specific promoter. The epidermis-specific expression of the *GstA1i:TaPrx103* transgene may also be compatible with the concept of substantial equivalence of transgenic as compared with non-transformed plants. This concept has attracted attention recently in the discussion regarding risks associated with genetically engineered crops including wheat (Baker *et al.*, 2006; Baudo *et al.*, 2006).

Here, I present physiological and molecular characterization of *GstA1i:TaPrx103* transgenic T_2 plants with the aim of addressing the mode of action of *TaPrx103* and substantial equivalence of transgenic lines as compared with wild-type wheat.

RESULTS

Enhanced resistance of transgenic plants

Originally, 19 primary transgenic T_0 plants were obtained from biolistic transformation of immature wheat embryos with plasmid pGstA1i:TaPrx103, as reported earlier (supplementary Fig. S1, Altpeter *et al.*, 2005). A pool of approximately 20 T_1 progeny of these primary transformants were tested for constitutive accumulation of TaPrx103 protein by using an antiserum directed against the orthologous barley HvPrx08 peroxidase (Kerby and Somerville, 1992). As shown in Fig. 1, all transgenic lines expressed

Fig. 1 Analysis by Western blotting of TaPrx103 protein accumulation in T_1 seedlings of transgenic lines carrying *GstA1i:TaPrx103*. Acid-soluble (pH 2.8) proteins in intercellular washing fluid (IWF) were separated by SDS-PAGE and blotted onto nitrocellulose. TaPrx103 protein was detected by an antiserum directed against the homologous protein HvPrx08 of barley. The transgene product has an expected mass of approximately 31 kDa. Coomassie-stained IWF run in parallel served as loading controls (C). Shown is the major protein band corresponding to the germin-like protein HvGER2 (Vallelian-Bindschedler *et al.*, 1998).

TaPrx103 to different extent, in contrast to cv. Bobwhite (BW) wild-type plants in which no peroxidase protein was detected. The TaPrx103 protein was accumulating in the intercellular washing fluid of transgenic plants, indicating correct secretion into the leaf apoplast. Based on this result and on preliminary resistance, tests three lines (nos. 2013, 2062 and 2193) were chosen for further analysis (boxed in Fig. 1).

T_2 progeny of these T_1 plants were tested for resistance to *Bgt* at the adult plant stage (Table 1). All three transgenic lines showed significantly enhanced resistance of flag leaves. Figure 2 shows examples of engineered adult plant resistance against a spontaneous *Bgt* epidemic in the greenhouse that was not controlled by fungicide. Please note that although transgenic lines were not fully resistant the number of sporulating pustules was reduced. The same lines were also found to be partially resistant against *Bgt* at the seedling stage (Table 2). The weaker protection of seedlings compared with adult plants appears not to be due to weak transgene product accumulation in the former because strong signals were obtained in Western blots of first leaves

Table 1 Enhanced resistance of adult transgenic plants expressing the TaPrx103 gene.

Line no.	Bobwhite	Transgenic plants		
	Percentage infection*	Percentage infection*	Relative infection (%)†	Chi-squared test‡
2013	27.3	13.1	48.0	< 0.0001
2062	31.6	11.3	35.7	< 0.0001
2193	33.5	10.9	32.5	< 0.0001

*Infection rates (percentage of mildewed leaf surface) on flag leaves.

†Infection relative to Bobwhite wild-type plants.

‡Test for significant differences in the distribution of disease rating classes between transgenic plants and corresponding Bobwhite plants. Results are based on two to three independent experiments using independent batches of plants sown on different dates.

Fig. 2 Resistance phenotype of adult transgenic T_2 plants compared with wild-type wheat. Pictures were taken approximately 4 weeks after the outbreak of a spontaneous *Bgt* epidemic in the greenhouse. Numbers in lower left corners of panels indicate transgenic lines.

Table 2 Enhanced resistance of transgenic seedlings expressing the *TaPrx103* gene.

Line no.	Bobwhite	Transgenic seedlings		
	Percentage infection*	Percentage infection*	Relative infection (%)†	Chi-squared test‡
2013	42.65	28.21	66.1	0.045
2062	50.5	31.61	62.6	0.0084
2193	65.75	46.78	71.2	0.017

*Average infection rates (percentage of mildewed leaf surface) on second and third leaves of 4-week-old seedlings of the T_2 generation.

†Infection relative to Bobwhite wild-type plants.

‡Test for significant differences in the distribution of disease rating classes between transgenic plants and corresponding Bobwhite plants.

(Fig. 1), although no direct comparison of young versus adult plants has been performed with respect to recombinant *TaPrx103* accumulation. Therefore, substrate availability might be more limiting in seedling leaves compared with the flag leaf of adult plants.

By using a total of ten sister lines from lines 2062 and 2193 I carried out a co-segregation analysis of transgene expression with resistance to *Bgt* (Fig. 3). More precisely, *TaPrx103* mRNA accumulation in the leaf below the flag leaf was quantified by using a spotted RNA array and associated with observed *Bgt* resistance in the flag leaf of the same plant. This should be more conclusive than co-segregation analysis of transgene DNA with protection because transgene expression levels may vary between transgene-carrying plants, e.g. depending on homo- or heterozygosity or silencing of the transgene locus. Line 2013 was not included in this co-segregation analysis because sister lines 2013/3 and 2013/5 were found to be homozygous for *GstA1i:TaPrx103* based on transgene-specific PCR from a total of 96 plants (data not shown). Homozygosity of these sister lines has been previously

suggested, on the basis of segregation of the *NptII* resistance marker (Altpeter *et al.*, 2005). We previously observed that hybridization of Northern blots with a transgene-specific oligonucleotide probe hybridizing to the chimeric *GstA1i:TaPrx103* 5' non-translated leader produced no signal, probably due to limited sensitivity of the assay. Therefore, a near full-length cDNA probe of *TaPrx103* was used to detect transgene expression here. In order to confirm that the *TaPrx103* signal on the RNA array reflected transgene, and not spurious endogenous *TaPrx103* expression, I verified the transgenic nature of the signals on the RNA array by transgene-specific RT-PCR in subline 2193b (supplementary Fig. S2). Clearly, the *TaPrx103* hybridization signals co-segregated with the abundance of an amplification product of the expected size obtained by transgene-specific RT-PCR of the same plants. As shown in Fig. 3A, all of the analysed sister lines showed a segregating pattern of *TaPrx103* mRNA abundance, except for subline 2193d in which no expression was detected. In cv. Bobwhite wild-type plants, *TaPrx103* mRNA was not detectable, unless plants were *Bgt* inoculated (rows 'BW' and 'BW pools').

Fig. 3 Segregation analysis of *GstA1i:TaPrx103* expression in T_2 transgenic lines. (A) A spotted RNA array was used to measure the abundance of *TaPrx103* mRNA in the non-inoculated leaf immediately below the flag leaf of adult individual T_2 plants (transgenic lines indicated to the right side of the array). BW, non-inoculated leaf immediately below the flag leaf of five individual wild-type plants; BW pools, primary leaves of five seedlings (either non-inoculated or *Bgt*-challenged) were pooled for each RNA extraction. The lower panel shows hybridization signals of a 26S rRNA probe that served for signal normalization. (B) Resistance data of two sublines of transgenic line 2193 that either carry the transgene (2193b) or the null-allele (2193d). Susceptibility (%) relative to wild-type plants is shown. Statistical significance of difference from the control was tested by chi-squared analysis, based on two independent experiments with different batches of plants. (C) Bulk analysis of transgene presence in lines 2193b and 2193d by genomic PCR.

Transgene-specific PCR from genomic DNA of five pooled plants confirmed the presence of a null allele in subline 2193d while all other sublines were positive for the *GstA1i:TaPrx103* transgene (Fig. 3C, and data not shown). I conclude from these data that the selected sister sublines were heterozygous for the effector transgene. The presence of approximately 25% null-allele carriers in lines 2062 and 2193 suggests that the observed resistance may be an underestimation of the protective potential of *TaPrx103*. Comparison of *Bgt* resistance of sister lines 2193b and 2193d that were heterozygous and null allelic for the transgene, respectively, resulted in a clear association of resistance with the presence of the transgene (Fig. 3B). The fact that the homozygous line 2013 was not more resistant than the heterozygous lines 2062 and 2193 also indicated that the *GstA1i:TaPrx103* transgene was inherited as a fully dominant trait.

Two groups with highest or lowest *TaPrx103* mRNA abundance were formed, each containing 48 plants across all transgenic (sister) lines. These two groups of plants were analysed for association of *TaPrx103* mRNA abundance with resistance of the flag leaves against *Bgt* (Fig. 4). There was a significant, positive association between *TaPrx103* mRNA abundance and resistance against *Bgt* ($\chi^2 = 0.03$), indicating that plant protection was due to *GstA1i:TaPrx103* transgene expression.

Fig. 4 Co-segregation analysis of transgene expression with resistance to *Bgt*. Expression of *TaPrx103* in non-inoculated leaves immediately below the flag leaf of adult transgenic plants was related to *Bgt* resistance of flag leaves from the same individuals. Two groups with highest or lowest *TaPrx103* mRNA abundance were formed, each consisting of 48 plants. For this analysis, plants from all transgenic lines shown in Fig. 3 were pooled. CI 0 to CI 3, disease scoring classes 0 (resistant) to 3 (fully susceptible).

Hypersensitive cell death, H_2O_2 generation and cell-wall degradation of transgenic plants

Epidermal cell-wall reinforcement (papilla formation) and hypersensitive cell death (HR) are typical defence reactions of wheat

Table 3 Increased hypersensitive cell death of attacked epidermal cells of transgenic seedlings expressing the *GstA1i:TaPrx103* transgene.

Leaf	Line*	Autofluorescing cells (%)†	P (t-test)	Leaves	Sites
1st	Bobwhite	9.2 ± 2.7		20	353
1st	2013	22.6 ± 3.1	0.002	19	371
1st	2062	20.8 ± 1.9	0.001	20	384
1st	2193	17.0 ± 2.4	0.037	19	326
2nd	Bobwhite	12.3 ± 2.4		20	401
2nd	2013	25.1 ± 3.8	0.006	19	359
2nd	2062	19.6 ± 2.6	0.041	19	375
2nd	2193	25.8 ± 3.9	0.005	19	380

*Four-week-old seedlings of the T₂ generation were used (one subline per line).

†Mean values ± SE of pooled data from two independent inoculation experiments with different batches of plants.

against *Bgt* or *B. graminis* f.sp. *hordei* whereas no clear association of either reaction with race specificity of an interaction can be found. For example, basal, race-non-specific host resistance has been described to be characterized by papilla formation, whereas race-non-specific non-host resistance was found to be associated with HR (Kmecl *et al.*, 1995; Wei *et al.*, 1994). These two types of response can be easily distinguished by a local versus a whole-cell autofluorescence that builds up in attacked cells. In order to address the question of whether the observed protection by epidermal *TaPrx103* overexpression was the result of (1) a direct antifungal effect of the recombinant peroxidase, (2) enhanced papilla formation at sites of attempted penetration or (3) enhanced HR, I performed microscopic analysis of attacked epidermal cells in leaves of 4-week-old seedlings that were found to show enhanced resistance against *Bgt*, although somewhat weaker than flag leaves (Tables 1 and 2). As shown in Table 3 all three analysed transgenic lines exhibited a significantly increased rate of whole-cell autofluorescence, indicating increased HR (see Fig. 5A for an example of an autofluorescing cell). Virtually no localized autofluorescence was detected on Bobwhite wild-type or transgenic lines (data not shown). I therefore conclude that the papilla response is not important in the observed wheat–*Bgt* interaction. Moreover, no enhanced whole-cell autofluorescence was found in non-attacked cells of transgenic plants, indicating the absence of spontaneous cell death due to transgene expression (data not shown).

The next question was whether the observed HR was associated with enhanced formation of H₂O₂ as has been observed previously in barley interacting with powdery mildew fungi (Hückelhoven *et al.*, 2001; Piffanelli *et al.*, 2002; Thordal-Christensen *et al.*, 1997). Indeed, the results presented in Table 4 suggest that attacked epidermal cells of transgenic 4-week-old seedlings generated more H₂O₂ that was reflected by enhanced 3,3-diaminobenzidine (DAB) staining, although statistical significance was lower compared with the autofluorescence data. In line with the autofluorescence data, DAB staining also encompassed entire cells, supporting the conclusion that these underwent an HR (Fig. 5B).

Fig. 5 Whole-cell autofluorescence and H₂O₂ burst revealed by DAB staining of *Bgt*-attacked epidermal cells of transgenic plants. Whole-cell autofluorescence (A) and DAB staining (B) was recorded at 36 and 20 h post-inoculation, respectively. Arrows point at fungal appressoria. Scale bars = 25 μm.

Class III peroxidases have long been implicated in cell-wall lignification although only a limited number of reports are available suggesting a direct effect of altered peroxidase expression on cell-wall properties of plants (Elfstrand *et al.*, 2002; Li *et al.*, 2003; Ostergaard *et al.*, 2000). In the present study, the enhanced cell death of transgenic plants could have been the consequence of impaired fungal cell-wall penetration due to constitutive, Prx103-catalysed cell-wall modification, thereby preventing the timely release of suppressors of defence (Lyngkjaer and Carver, 1999). Since *Bgh*, which is closely related to *Bgt*, has been shown to penetrate barley cell walls with the aid of cell-wall-degrading enzymes it appears possible that enhanced penetration resistance of *TaPrx103*-overexpressing wheat was due to reduced digestibility of epidermal cell walls by these fungal enzymes (Pryce-Jones *et al.*, 1999). This possibility was addressed by using a newly developed assay that is based on the quantification of remaining epidermal cross-cell walls after incubation with cell-wall-degrading enzymes (Fig. 6A,B; see Experimental procedures for details). In order to

Line*	DAB-stained cells (%)†	P (Mann–Whitney)‡	Leaves	Sites
Bobwhite	6.4 ± 1.1		51	1090
2013	10.9 ± 2.0	0.056	39	813
2062	15.6 ± 2.2	0.0001	34	772
2193	10.6 ± 2.2	0.14	40	852

*Secondary and third leaves from 4-week-old seedlings of the T₂ generation were analysed (one subline per line).

†Mean values ± SE of pooled data from two independent inoculation experiments using different batches of plants.

‡A non-parametric test was used because data were non-normally distributed.

Table 4 Increased generation of H₂O₂ of attacked epidermal cells of transgenic seedlings expressing the *GstA1i:TaPrx103* transgene.

Fig. 6 Epidermal cell walls of transgenic plants do not exhibit enhanced resistance to fungal cell-wall-degrading enzymes. (A) The cell-wall degradation of immobilized epidermis was measured after incubation beneath a drop of a cell-wall-degrading enzyme cocktail. (B) Microscopic image (100× magnification) of partially degraded epidermal cross-cell walls after 120 min of incubation. Black arrows, still intact walls; red arrows, digested walls. Scale bar = 50 μm. (C) Different rates of cross-cell walls from old versus young, elongating leaves. Data are shown as mean ± SE from four parallel plants. (D) Comparison of cell-wall digestibility of wild-type plants versus homozygous transgenic line #2013. Data are shown as mean ± SE from five parallel plants. A repeated independent experiment using plants from a different sowing date produced a similar result.

test whether the assay was capable of reporting altered cell-wall digestibility, I compared the rate of cross-cell-wall degradation in older versus young, elongating leaves from wild-type plants. The result shows a clearly enhanced resistance of cell walls from older leaves to enzymatic degradation, according to previous observations and knowledge regarding secondary cell-wall formation (Fig. 6C; Scobbie *et al.*, 1993). In contrast to this result, no significant difference in epidermal cell-wall digestibility was observed between wild-type and transgenic, 4-week-old seedlings, suggesting that preformed cell-wall reinforcement was not a major mechanism of Prx103-mediated resistance to *Bgt* (Fig. 6D).

Host-gene expression in *GstA1i:TaPrx103* transgenic plants

The ectopic expression of potentially harmful transgenes like peroxidases may lead to pleiotropic effects in the recipient organism. This is usually not a desired trait and might be avoided by

expressing such transgenes under the control of a tissue-specific and/or inducible promoter. In order to test whether constitutive *TaPrx103* expression in wheat epidermis caused pleiotropic effects, I monitored transcript abundance of approximately 10 000 unigenes by using the 'barleyPGRC1' cDNA array of the IPK. This array was found to be useful for transcript profiling not only in barley but also in wheat based on the following observations. First, powdery mildew attack did not result in lower numbers of differentially regulated genes based on statistical criteria mentioned below (558 in wheat at 15 h post-inoculation versus 187 in barley at 12 h post-inoculation), indicating that gene-regulation events were picked up in wheat in a sensitive manner. Secondly, regulation trends of the 558 powdery mildew-responsive transcripts in wheat were similar in barley (correlation coefficient = 0.56; supplementary Table S1). Moreover, the technical reproducibility of the barleyPGRC1 array was found to be high, based on repeated hybridization of a single ³³P-labelled cDNA probe to different membranes (supplementary Fig. S3).

Fig. 7 *GstA1:TaPrx103* expression in wheat epidermis does not affect host gene expression in leaves. Hierarchical clustering of 558 pathogen-regulated host genes in wild-type and transgenic plants. Signal intensities were log-transformed and median centred per gene, prior to hierarchical clustering by using Euclidian distance and average linkage settings.

In resistant interactions of barley with *Bgt*, which are probably physiologically similar to resistant wheat–*Bgt* interactions, whole-cell DAB staining (reflecting HR) was observed from 18 h post-inoculation onwards (Hückelhoven *et al.*, 2001). Therefore, I decided to analyse the wheat transcriptome at 15 h post-inoculation, which is 3 h prior to the first reported manifestation of HR. This should maximize the chance to see cell death-related changes in the transcriptome of *Bgt*-attacked, transgenic lines. The data of two independent inoculation experiments are shown in Fig. 7 and supplementary Table S1. In a split-plot design, half of the Bobwhite wild-type or *TaPrx103*-transgenic seedlings were left non-inoculated as controls whereas the other half were inoculated with *Bgt*. Statistical analysis of the normalized signal intensities resulted in the identification of 558 genes that were

significantly regulated by *Bgt* attack in cv. Bobwhite [$> two-fold$; $P < 0.05$ and false-discovery rate (FDR) < 0.05]. The most dramatically up-regulated transcripts coded for several peroxidases, a thaumatin-like protein, ethylene-forming enzyme, phenylalanine-ammonia lyase, protein phosphatase, chitinase and glutathione-S-transferase (supplementary Table S1). Many of the most dramatically down-regulated transcripts coded for components and enzymes of photosynthesis. These results are in good agreement with previous findings of *Blumeria*-attacked wheat and barley and confirm the sensitivity of the barleyPGRC1 array for the detection of transcriptional changes in the closely related species wheat (Bruggmann *et al.*, 2005; Zierold *et al.*, 2005). By contrast, hierarchical clustering of transcript abundance of these pathogen-regulated genes did not reveal any clear difference between wild-type plants and transgenic lines in non-inoculated or in *Bgt*-challenged leaves although the effect of *Bgt* attack was evident irrespective of plant genotype (Fig. 7). In general, expression patterns among samples from non-inoculated tissue were more similar, indicating that the external stimulus (*Bgt*) increased some scattering of the data. By interrogating the entire unigene set of the 10k cDNA array I could not identify any differentially regulated gene between transgenic and wild-type plants by applying the above-mentioned statistical criteria, irrespective of whether non-inoculated or *Bgt*-inoculated plants were used for the analysis (data not shown). In inoculated leaves, this lack of differential gene expression might reflect dilution of those epidermal cells that were in direct contact with the fungus and that showed enhanced HR. Therefore, single-cell sampling of such attacked cells might reveal transgene-dependent gene regulation events. One transcript that would be expected to show differential abundance between *GstA1:TaPrx103* transgenic lines and Bobwhite would be *TaPrx103* itself. However, the array design does not include *HvPrx08*, which is the proposed barley gene orthologue to *TaPrx103*. Blast analysis suggested that none of the peroxidase-encoding cDNAs on the array produced strong enough cross-hybridizing signals to *TaPrx103* (data not shown).

DISCUSSION

Transgenic approaches to durable disease resistance not based on transfer of NBS-LRR type R-genes in wheat have produced the first encouraging data regarding the interaction with *Bgt*, *Puccinia* sp., *Tilletia* and *Fusarium* sp., although in only one case was resistance also tested under field conditions (Altpeter *et al.*, 2005; Bieri *et al.*, 2003; Makandar *et al.*, 2006; Schlaich *et al.*, 2006). The results presented here represent a mode of action study to one of these beneficial transgene products, the *TaPrx103* peroxidase of wheat.

Plant class III peroxidases have been proposed as key regulators of the level of extracellular H_2O_2 and as producers of the extremely reactive oxygen intermediates (ROI) $OH^\bullet$ and $OOH^\bullet$,

depending on whether the peroxidative (catalytic) or the hydroxylic cycles of the enzyme are operating (Kawano, 2003; Passardi *et al.*, 2004). During one peroxidative cycle, one consumed molecule of H_2O_2 can maximally lead to two produced H_2O_2 molecules if the two oxidized peroxidase products (X^*) react with two O_2 molecules to produce superoxide radicals, which are converted spontaneously or catalysed by superoxide dismutase to H_2O_2 . Apoplastic class III peroxidases have long been proposed as producers of ROI including enzymes from tobacco and french bean (Bindschedler *et al.*, 2006; Bolwell *et al.*, 2002; Mader and Ambergfisher, 1982; Peng and Kuc, 1992). Current models of plant defence responses include peroxidase-generated radicals and ROI as catalysts of covalent cell-wall modifications (Barcelo, 1995; Harkin and Obst, 1973; Ostergaard *et al.*, 2000) as well as signals for cell-death reactions (Bestwick *et al.*, 1998; Martinez *et al.*, 1998; Passardi *et al.*, 2004). NADPH-oxidase encoded by $RBOH_{A-F}$ genes is considered to represent the second, main source of apoplastic ROI, and many conclusions about the importance of this enzyme in the generation of stress-induced oxidative burst have been drawn from pharmacological studies using the inhibitor diphenyleneiodonium. However, this compound also inhibits class III peroxidases, which might have led to an underestimation of their role in ROI generation (Frahry and Schopfer, 1998). The enhanced DAB staining in epidermal cells of *GstA11:TaPrx103* transgenic lines that are in contact with an appressorium of *Bgt* supports a role of the TaPrx103 peroxidase in the generation of H_2O_2 in pathogen-attacked wheat. The co-incidence of enhanced DAB staining and enhanced autofluorescence of attacked epidermal cells of transgenic plants suggests that the proposed H_2O_2 -generating activity of TaPrx103 provides a signal for cell death. However, the substrate(s) for the underlying peroxidative reaction are currently unknown. Interestingly, one strongly accumulating extracellular marker protein of powdery mildew-attacked barley epidermis, HvGER4, possesses superoxide-dismutase activity (Christensen *et al.*, 2004). Powdery mildew attack of wheat causes the accumulation of mRNA encoding an orthologous protein named TaGLP4, which most probably is a functional superoxide dismutase, too, because both HvGER4 and TaGLP4 enhanced resistance upon transient overexpression in wheat and barley (Christensen *et al.*, 2004; Schweizer *et al.*, 1999a). Therefore, it may be that TaGLP4 is supporting TaPrx103 in generating the observed H_2O_2 burst upon pathogen attack by efficiently converting the secondary peroxidase product superoxide radical into H_2O_2 .

The question arises as to the role of endogenous *TaPrx103*, which is expressed in inner leaf tissues, in the pathogen defence of wheat. Why has co-evolution of wheat with *Bgt* not selected for enhanced *TaPrx103* expression in wheat epidermis (Altpeter *et al.*, 2005)? One possibility would be that expression of the *TaPrx103* gene in the epidermis was lost during wheat domestication. Recently, the existence and expression of *TmPrx01*, the

orthologous gene to *TaPrx103*, has been shown in powdery-mildew-attacked *Triticum monococcum* (Liu *et al.*, 2005), one of the wild relatives of hexaploid wheat. Interestingly, *T. monococcum* possesses a high level of basal pathogen resistance reflected by scoring data of many accessions in *ex-situ* collections (Borner *et al.*, 2006). I tested the hypothesis of domestication-related loss of ancient gene function by analysing gene expression of three genetically divergent *T. monococcum* plus three *T. aestivum* accessions (Hammer *et al.*, 2000). However, no enhanced expression of the orthologous gene to *TaPrx103* was found in *Bgt*-attacked, stripped epidermis of *T. monococcum* (supplementary Fig. S4). A second pathogen-induced peroxidase gene encoding *TaPrx02-1* (orthologous gene in *T. monococcum* named *TmPrx03*) was also a marker of inner leaf tissues of *Bgt*-attacked leaves (supplementary Fig. S4). These findings are in agreement with the results of Liu *et al.* (2005) who also considered *TmPrx01* and *TmPrx03* proteins as markers of inner leaf tissues. Finally, a recently identified, novel peroxidase with specific expression in powdery mildew-attacked barley epidermis was found to be weakly but constitutively expressed in wheat epidermis as well as inner leaf tissues (Zierold *et al.*, 2005; supplementary Fig. S4). Taken together, these results do not support a natural selection of any of the tested peroxidases for enhanced expression in powdery mildew-attacked wheat epidermis. On the other hand, a possible role of TaPrx103 might be the triggering of epidermal cell death in incompatible *Bgt* interactions by mesophyll cells directly underlying the epidermis. This speculation is based on early, conspicuous patterns of DAB staining of powdery mildew-attacked, resistant barley leaves that start at contact sites between mesophyll and epidermis (Thordal-Christensen *et al.*, 1997). The re-location of *TaPrx103* from inner leaf tissues to the epidermis in the transgenic lines described here may have enhanced its natural cell-death triggering function. It also appears possible that wheat does possess defence-related peroxidase(s) that are specifically expressed in the epidermal layer, comparable with barley peroxidase HvPrx40, which has been shown to enhance resistance upon transient overexpression comparable with *TaPrx103* overexpression (Zierold *et al.*, 2005; A. Jhrde and P. Schweizer, unpublished data). Several peroxidase transcripts have recently been described to accumulate in wheat epidermis after inoculation with *Bgt* (Liu *et al.*, 2005). However, all of the encoded proteins are predicted to accumulate in the vacuoles due to the presence of C-terminal retention signals. Therefore, a functional homologue to HvPrx40 would still have to be identified in wheat. As summarized in Fig. 8, an epidermal defence-inducing or defence-enhancing shunt of ROI might consist of an inducible substrate, TaPrx103 or related class III peroxidases plus extracellular SOD encoded by *TaGLP4*, which would result in the extracellular burst of H_2O_2 that has been observed in many pathosystems including powdery mildew-attacked wheat and barley.

The analysis of the transcriptome in transgenic versus wild-type wheat revealed no clear transgene-induced changes in host gene

Fig. 8 Model of the pathogen-specific effect of the *GstA1:TaPrx103* transgene. Together with the pathogen-induced TaGLP4 proteins (orthologous to extracellular SODs of barley), the recombinant *TaPrx103* protein might constitute a shunt of reactive oxygen species in pathogen-attacked epidermal cells, resulting in the accumulation of H_2O_2 that leads to cell death.

expression prior or after *Bgt* challenge. In particular, no marker genes for induced resistance of wheat (Görlach *et al.*, 1996; Schweizer *et al.*, 1989) were up-regulated in non-attacked transgenic plants. This argues against a pleiotropic effect of the *TaPrx103* transgene under the control of the *GstA1* promoter, in contrast to the known biological or chemical resistance inducers of wheat. Therefore, the transgene-encoded protein might have caused conditioning of wheat epidermis to respond more strongly to pathogen attack, as outlined in Fig. 8. Such a scenario appears likely if the substrate for the transgene-encoded peroxidase accumulates only locally upon pathogen attack. As a consequence, the transgenic plants behave like wild-type except for those cells in direct contact with a pathogen, where the beneficial transgenic phenotype becomes evident. Thus, the *GstA1:TaPrx103* transgenic plants described here represent an example of substantial equivalence to non-transformed wheat resulting from the combination of a wheat antifungal coding sequence with an epidermis-specific wheat promoter.

EXPERIMENTAL PROCEDURES

Plant and fungal material

Transgenic wheat plants have been obtained by biolistic transformation of immature embryos with plasmid pGstA1:TaPrx103 (supplementary Fig. S1), as previously described. Selected transgenic lines and corresponding wild-type plants of cv. Bobwhite were grown in compost soil in an air-conditioned greenhouse with automatic shading and supplementary light (SonAgro 400 high-pressure sodium bulbs), resulting in a light period of 16 h and a minimum light intensity of 30 000 lux at plant height.

Temperature ranged from 18 °C (night) to 21 °C (day). For experiments at the seedling stage (DAB staining, autofluorescence, transcript profiling), 4-week-old plants grown in a climate chamber were used (16 h light from metal halogen lamps; 8 h dark, 60–70% relative humidity, 20 °C constant temperature). All plants were grown in pots of compost soil from IPK nursery.

Blumeria graminis DC Speer f.sp. *tritici* Em Marchal (*Bgt*) (Swiss field isolate FAL92315) was maintained at 22 °C constant temperature and 16 h light by weekly transfer to fresh wheat cv. Kanzler.

Infection assays

Resistance of freshly emerged flag leaves of adult transgenic plants was scored in a detached leaf assay, as described (Altpeter *et al.*, 2005). The same detached leaf assay was also applied to 4-week-old seedlings for scoring resistance of the second and third leaves. For natural infection experiments in the greenhouse, local *Bgt* isolates were allowed to spread (Gatersleben, spring/summer 2005) by omitting fungicide treatments. Disease scoring was performed approximately 4 weeks after first recording of mildewed leaves in the greenhouse.

Cell death and H_2O_2 accumulation

Whole-cell autofluorescence of wheat epidermis was monitored by using a Zeiss Axioplan 2 epifluorescence microscope (excitation filter 450–490 nm BP, emission filter 515–565 nm BP) at 200× magnification. Autofluorescence of epidermal cells attacked by exactly one *Bgt* appressorium was recorded at 36 h post inoculation. Prior to microscopy, inoculated leaves were fixed and epiphytic fungal structures were stained with Coomassie blue, as described (Schweizer *et al.*, 1993).

Staining of the H_2O_2 burst by DAB was performed by leaf infiltration of a solution of 1% (w/v) DAB in H_2O (pH adjusted to 4.0 with HCl) using a needle-less syringe. Detached leaves on water agar containing 20 µg/mL benzimidazole were inoculated with *Bgt* at a low density (1–10 conidia/mm²) and incubated at 20 °C for 20 h, prior to DAB infiltration. Six hours after DAB infiltration, leaves were destained in 15% (w/v) TCA in ethanol/chloroform (4 : 1 v/v) overnight at room temperature, followed by light microscopy of those cells attacked by exactly one fungal appressorium.

Cell-wall digestibility

An assay for the direct quantification of *in-situ* cell-wall digestion was developed because the release of epidermal protoplasts as an indirect measure of cell-wall degradation could not be measured, probably due to rapid bursting of these very large and delicate protoplasts. The *in-situ* assay is based on the statistical analysis of remaining cross-cell walls after the incubation of an

immobilized epidermal mono-cell layer with fungal cell-wall-degrading enzymes. Leaf segments were fixed with their abaxial surface onto adhesive tape, followed by one cross-cut by a scalpel. The upper epidermis as well as the mesophyll were carefully scratched off starting at the cut, resulting in an immobilized epidermal mono-cell layer. After thoroughly rinsing the treated leaf area with distilled water in order to remove cell debris, the number of epidermal cross-cell walls of type A, B and C cells not bordering stomata or hair cells was counted per microscopic field. One hundred and fifty microlitres of a cell-wall-degrading enzyme cocktail consisting of 0.5% (w/v) Cellulysin (Calbiochem/Merck, Darmstadt, Germany) and 0.05% (w/v) Macerace (Duchefa, Haarlem, the Netherlands) in 5 mM potassium phosphate buffer, pH 5.7, 10 mM L-arginine and 0.6 M mannitol was added as a drop onto the free epidermis. After 60–180 min incubation at 32 °C, the treated leaf area was gently rinsed with distilled water, followed by quantification of the number of remaining cross-cell walls.

Co-segregation analysis

For segregation analysis at the RNA level, total RNA was extracted from the leaf below the flag leaf of adult plants used for resistance scoring of detached flag leaves. For this purpose I used a previously described procedure, except that leaves were squeezed in RNA extraction buffer inside sealed plastic bags by using a rolling device (Stein *et al.*, 2001; Vallelan-Bindschedler *et al.*, 1998). RNA concentration was adjusted to 1 µg/µL, mixed 1 : 1 (v/v) with bromo-phenol blue solution in DEPC-treated H₂O and used for spotting onto nylon membranes (Hybond N) by using a spotting robot equipped with a 384-pin tool with 0.4-mm tip diameter (MicroGrid II, BioRobotics, Zinsser Analytic GmbH, Frankfurt, Germany). Four spot replicas per RNA sample in a 2 × 2 spotting pattern with four needle strokes per spot were applied. Spotted RNA was crosslinked to the membranes under UV light (Stratalinker, Stratagene Co, La Jolla, CA; 1200 energy units) and used for hybridization with random-prime ³²P-labelled probes (either *TaPrx103* near full-length cDNA or bean cDNA corresponding to the 26S rRNA gene) as described (Zierold *et al.*, 2005). Hybridization signals were detected by using a Fuji-BAS 3000 phosphorimager (Raytest, Straubenhardt, Germany). Spot detection and signal-intensity quantification were performed by using the AIDA Image Analyser 4.08 software package (Raytest). Per subarray (four replicate spots) mean signal intensity was normalized to the mean signal intensity from hybridization with the 26S rRNA probe.

Segregation analysis at the (c)DNA level was performed by PCR by using the PCR MasterMix (Qiagen, Hilden, Germany) and transgene-specific primers 5'-GAAACCTCGCTCGAAACGCAC (sense) and 5'-GCCGGCTTAGCTACCTG (reverse) that give rise to 304- and 190-bp fragments from unspliced and spliced templates, respectively. Thirty and 42 cycles (94 °C/58 °C/72 °C) were applied for amplification from cDNA and genomic DNA templates, respectively.

Semi-quantitative RT-PCR

Total RNA was extracted from epidermal peels (abaxial) or the remainder of peeled leaves of *T. aestivum* accessions (accession nos. TRI 7405, TRI 8989 and TRI 2206) or *T. monococcum* accessions (TRI 4319, TRI 2381 and TRI 2006) of the *ex-situ* collection of the Leibniz Institute of Plant Genetics and Crop Plant Research (http://gbis.ipk-gatersleben.de/gbis_i/). One microgram of RNA was reverse transcribed by using the iScript kit (Bio-Rad Co., München, Germany). Three microlitres of the 50 µL of resulting cDNA solution was used for semi-quantitative PCR by using the PCR MasterMix (Qiagen Co.), followed by gel electrophoresis on 1% w/v agarose gels in TAE containing 0.5 µg/mL ethidium bromide. The following PCR primers were used: *TaPrx103* and *TmPrx01*, 5'-AAGGACAGGATCTACAATGAG (sense) plus 5'-TGTCTTSG-GCGCGATGTTG (reverse); *TaPrx02-1* and *TmPrx03*, 5'-ACTCG-GATCTACGGCGGTG (sense) plus 5'-GGTGAGCGGCGCATGTTT (reverse); *TaPrx47*, 5'-GACTGCTTCGTTCGGGGG (sense) plus 5'-CTGAGGAAGTAGGACGCG (reverse); *UBC*, 5'-AAGCAGCCAGAATG-TACAGCGAGAAC (sense) and 5'-GGTACAGACCAGCAAAGCCA-GAAATG (reverse); *GAPDH*, 5'-CAACGCTAGCTGCACCACTA (sense) 5'-TTCCACCTCTCCAGTCCTTG (reverse). *UBC* and *GAPDH* primers were based on cDNA sequences encoding a ubiquitin-conjugating enzyme (TC234903 of TIGR wheat gene index) and cytosolic glyceraldehyde dehydrogenase (accession no. AF251217). Primers (except for *GAPDH*) were designed such as to match perfectly to orthologous sequences of *T. aestivum* and *T. monococcum*.

The barleyPGRC1 cDNA array and transcript profiling

A total of 41 600 vector-trimmed expressed sequence tags (ESTs) of more than 100 bp length were used for clustering by using the StackPack™ software (Electric Genetics PTY Ltd, Reston). In the case of sequence clusters, one representative clone associated with the longest EST sequence was manually selected per primary consensus. Singleton sequences were subjected to ID-based clone-linking, and corresponding EST clones were only selected for the unigene set if neither their 5'- nor their 3'-orientated EST was found in a cluster. Diluted plasmid DNA used for sequencing (approximately 1 : 20) of the thus defined unigene set of barley was re-arrayed by using a Genesis Workstation 100 (TECAN Co., Männedorf, Switzerland). Each re-arrayed cDNA clone was PCR amplified (30 cycles 95 °C/65 °C/72 °C) in four 50-µL reactions by using in-house produced Taq polymerase and primers M13-21PE (5'-ACGACGTTGTAAACGACGCGCCAG) and MVR26 (5'-CTCACTAAAGGGAACAAAAGCTGG). The pooled, amplified PCR fragments were controlled by agarose gel electrophoresis for multiple bands and for the estimated DNA concentration. PCR reactions with more than two DNA fragments or with no visible product were eliminated from the set by re-arraying. A minority of PCR reactions containing two DNA fragments were left in the

unigene set if the abundance of the second fragment was clearly below that of the major one. The accuracy of re-arraying and clone handling was verified by sequencing (four clones per 384-well destination plate). No inconsistencies were identified in the set of re-arrayed PCR fragments. After purification and two-fold concentration by ultrafiltration (MinElute, Qiagen, Hilden, Germany) resulting in an estimated DNA concentration of 50 ng/ μ L, samples were mixed 1 : 1 with spotting solution (1 M NaOH, 5 M NaCl, bromophenol blue). DNA was spotted in duplicate onto nylon membranes (Biodyne B, PALL Co., Dreieich, Germany) by using a BioGrid II Robot (Genomics Solutions, Cambridgeshire, UK) equipped with a 0.2-mm pin tool. Five strokes of the pin tool were applied to each spot, resulting in the transfer of approximately 50 nL DNA, and each cDNA fragment was spotted twice. Membranes were treated as described (Sreenivasulu *et al.*, 2002).

RNA isolation, labelling and hybridization of wheat cDNA probes by 32 P-dCTP was performed as described (Zierold *et al.*, 2005) except for reduced washing stringency (0.5 $\times$ SSC instead of 0.1 $\times$ SSC at 65 °C).

Spots were detected and signals were quantified by using the AIDA Image Analyzer 4.08 software package (Raytest Co.). For background subtraction, dynamic definition of background spots was used by selecting four spots producing the lowest signals per subarray and by subtracting the mean intensity of those four spots from all spots of the corresponding subarray. The rationale behind this background subtraction was the presence of four empty spots per subarray that, however, did not always produce the lowest signals due to sporadic overshining from neighbouring spots with extremely strong hybridization signals. Spot-intensity values were normalized by median centring of the signal distribution per array hybridization (Sreenivasulu *et al.*, 2002). Spotted unigenes producing no signals above 2.5 $\times$ local background at two hybridizations were excluded from the analysis. Pathogen-regulated genes in wild-type plants were identified by using the EDGE software developed by Storey *et al.* (<http://faculty.washington.edu/jstorey/edge/>) with significance thresholds set to $P < 0.05$ and false-discovery rate (FDR) < 0.05 (Storey and Tibshirani, 2003). Pathogen-regulated genes were subjected to hierarchical clustering by using the Cluster3 software of Eisen and de Hoon (<http://rana.lbl.gov/EisenSoftware.htm>). Settings were as follows: $\log_2$ transformation of signal intensities, median centring of genes, Euclidian distance, average linkage.

MAGE-ML export of the complete set of array data to ArrayExpress (<http://www.ebi.ac.uk/arrayexpress/>) has been performed.

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SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article:

Fig. S1 Schematic drawing of expression plasmid pGstA1i:TaPrx103 that carries the TaPrx103 cDNA (previously named TaPERO) under the control of the epidermis-specific wheat promoter GstA1. The 2.3 kb GstA1 promoter is followed by part of exon 1 and the single 118 bp intron of the pathogen-induced wheat WIR1a gene. T, CamV 35S terminator.

Fig. S2 Co-segregation of *TaPrx103* mRNA abundance and *GstA1i:TaPrx103* transgene expression in subline 2193b. RNA was extracted from non-inoculated plants and used for spotting

onto an RNA array hybridized with a ³²P-labelled *TaPrx103* full-CDS probe (top panel) or for semi-quantitative RT-PCR using a transgene-specific primer pair (middle panel). The arrow points at the expected amplified fragment from spliced mRNA. The weak upper band either corresponds to genomic DNA contamination or non-spliced transcript. As control for cDNA synthesis, primers for the constitutively-expressed reference gene encoding an ubiquitin-conjugating enzyme (UBC) were used (lower panel).

Fig. S3 Technical reproducibility of the barleyPGRC1 cDNA array. Two membranes of type 'A' were hybridized with an identical cDNA probe derived from barley leaves, as described in Materials and Methods. Similar results were obtained by hybridizing membranes of type 'B' (not shown).

Fig. S4 Semi-quantitative RT-PCR of peroxidase mRNA in peeled epidermis and remaining leaf tissues of cultivated (Acc. 1–3, *T.aestivum*) and wild (Acc. 4–6, *T.monococcum*) wheat in response to *Bgt* attack. Names of peroxidase-encoding genes are indicated at the right hand side of the figure, and both putative gene orthologues from *T.aestivum* and *T.monococcum* (*TaPrx103* and *TmPrx01*; *TaPrx02-1* and *TmPrx03*) are indicated. No gene orthologue to *TaPrx47* has been described in *T.monococcum* so far. UBC, ubiquitin-conjugating enzyme used as constitutively-expressed reference gene. Peroxidase mRNAs were detected by 24 PCR cycles unless otherwise specified; UBC mRNA was detected by 22 PCR cycles.

Table S1 Regulation of identified, defense-related genes in wheat and barley.

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