

A class III peroxidase specifically expressed in pathogen-attacked barley epidermis contributes to basal resistance

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SUMMARY

Higher plants possess large multigene families encoding secreted class III peroxidase (Prx) proteins. In barley, two *Prx* cDNAs encoding HvPrx07 and HvPrx08 have been isolated and characterized to some extent with respect to a resistance-mediating function upon attack by the powdery-mildew fungus *Blumeria graminis* f.sp. *hordei* (*Bgh*). Here we present evidence for the tissue-specific accumulation of a new Prx mRNA, *HvPrx40*, in *Bgh*-attacked epidermis of barley (*Hordeum vulgare*). The encoded protein is predicted to be secreted into the apoplastic space of epidermal cells due to the absence of a C-terminal extension, which distinguishes it from other Prx proteins reported to accumulate in leaf epidermis. Transient overexpression of *HvPrx40* enhanced the resistance of wheat (*Triticum aestivum*) and barley against *Blumeria graminis* f.sp. *tritici* (wheat powdery mildew) and *Bgh*, respectively. These findings were complemented by transient-induced gene silencing showing hypersusceptibility of barley leaf epidermal cells to *Bgh*. The local accumulation of oxidized 3,3-diaminobenzidine that reflects H₂O₂ production at sites of attempted fungal penetration was not reduced in *HvPrx40*-silenced cells, suggesting a role of this peroxidase other than the production of reactive oxygen species.

INTRODUCTION

Higher plants possess large multigene families encoding class III peroxidase (Prx) proteins (Passardi *et al.*, 2004a). This subgroup of peroxidases is secreted into the apoplastic space or the plant vacuole, depending on the presence of a C-terminal extension serving as a vacuolar retention signal, and is characterized by a number of conserved residues or domains that include two heme-binding histidines of the catalytic centre, eight cysteine residues forming disulfide bridges, plus a highly conserved domain rather close to the N-terminus of the protein. Plant class III peroxidases

have been proposed as key regulators of the level of extracellular H₂O₂ and as producers of the extremely reactive oxygen intermediates (ROI) OH* and OOH*, depending on whether the peroxidative (catalytic) or the hydroxylic cycles of the enzyme are operating (Kawano, 2003; Passardi *et al.*, 2004b). During one peroxidative cycle, one consumed molecule of H₂O₂ can maximally lead to two produced H₂O₂ molecules if the two oxidized peroxidase products (X*) react with two O₂ molecules to produce superoxide radicals, which are converted spontaneously or catalysed by superoxide dismutase to H₂O₂. There has been evidence from tobacco and french bean that Prx proteins do indeed produce H₂O₂ *in vivo* (Bindschedler *et al.*, 2006; Bolwell *et al.*, 2002; Mader and Ambergfisher, 1982; Peng and Kuc, 1992). According to these results Prx proteins produce 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.*, 2004a).

In *Arabidopsis thaliana* and rice (*Oryza sativa*), a total of 73 and 138 *Prx* genes have been annotated, respectively, but a physiological function for most of the encoded proteins is still missing. This may be largely due to the complexity of *Prx* gene families and to suspected functional redundancy between individual, duplicated family members. One way to obtain further information about the function of individual *Prx* genes is to employ methods of genomics such as transcript profiling or the generation of transgenic plants resulting in gain or loss of gene function by overexpression or RNAi-mediated gene silencing. A number of transcript profiling approaches to multiple *Prx* genes per genome has been undertaken in rice, wheat and *Arabidopsis* (Liu *et al.*, 2005; Sasaki *et al.*, 2004; Welinder *et al.*, 2002). These studies revealed that *Prx* mRNAs often accumulate upon biotic stress, indicating a role in warding off pathogens. Recently, plant class III peroxidases have been catalogued by using a consistent nomenclature (Bakalovic *et al.*, 2006). This nomenclature has been adopted for the peroxidases addressed in the study presented here.

In the cereal crop barley, a similar level of complexity of class III peroxidases appears to exist at the genomic level, with a total of 105 unigenes presently known (Bakalovic *et al.*, 2006)

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(Supporting Information Fig. S1). However, only a very limited number of *Prx* genes have been studied in more detail until now. HvPrx07 has been described as a protein accumulating in the central vacuole of *Bgh*-attacked barley leaf epidermis, and a role in the accumulation of antifungal compounds such as hordatines has been proposed (Kristensen *et al.*, 1999). However, transient overexpression in barley epidermal cells enhanced susceptibility, which might reflect a more complex function of this peroxidase or a pleiotropic effect of overexpression (Kristensen *et al.*, 2001). Another peroxidase of barley, HvPrx08, has also been described as pathogen-induced at the mRNA as well as protein level (Scott-Craig *et al.*, 1995; Thordal-Christensen *et al.*, 1992). HvPrx08 is likely to be orthologous to the wheat gene *TaPrx103*, which has also been described to be induced by powdery mildew and for which functional data are available in a transient expression system as well as in transgenic plants (Altpeter *et al.*, 2005; Schweizer *et al.*, 1999; Schweizer, 2008).

Attack of barley by powdery mildew fungi resulted in either local or whole-cell accumulation of H₂O₂, reflected by the oxidation of 3,3-diaminobenzidine (DAB) (Thordal-Christensen *et al.*, 1997). Sources for this type of reactive oxygen species (ROI) might be a shunt consisting of NADPH oxidase plus superoxide dismutase. In accordance with this model, germin-like proteins including HvGER4 and HvGER5 from barley have recently been described as apoplastic SOD enzymes (Zimmermann *et al.*, 2006). In addition, the shunt might also involve peroxidases as signal amplifiers, as discussed above. Here, we functionally address a new peroxidase protein that specifically accumulates in *Blumeria graminis* f.sp. *hordei* (*Bgh*)-attacked barley epidermis and tested its association with disease resistance and H₂O₂ formation.

RESULTS

Sequence analysis

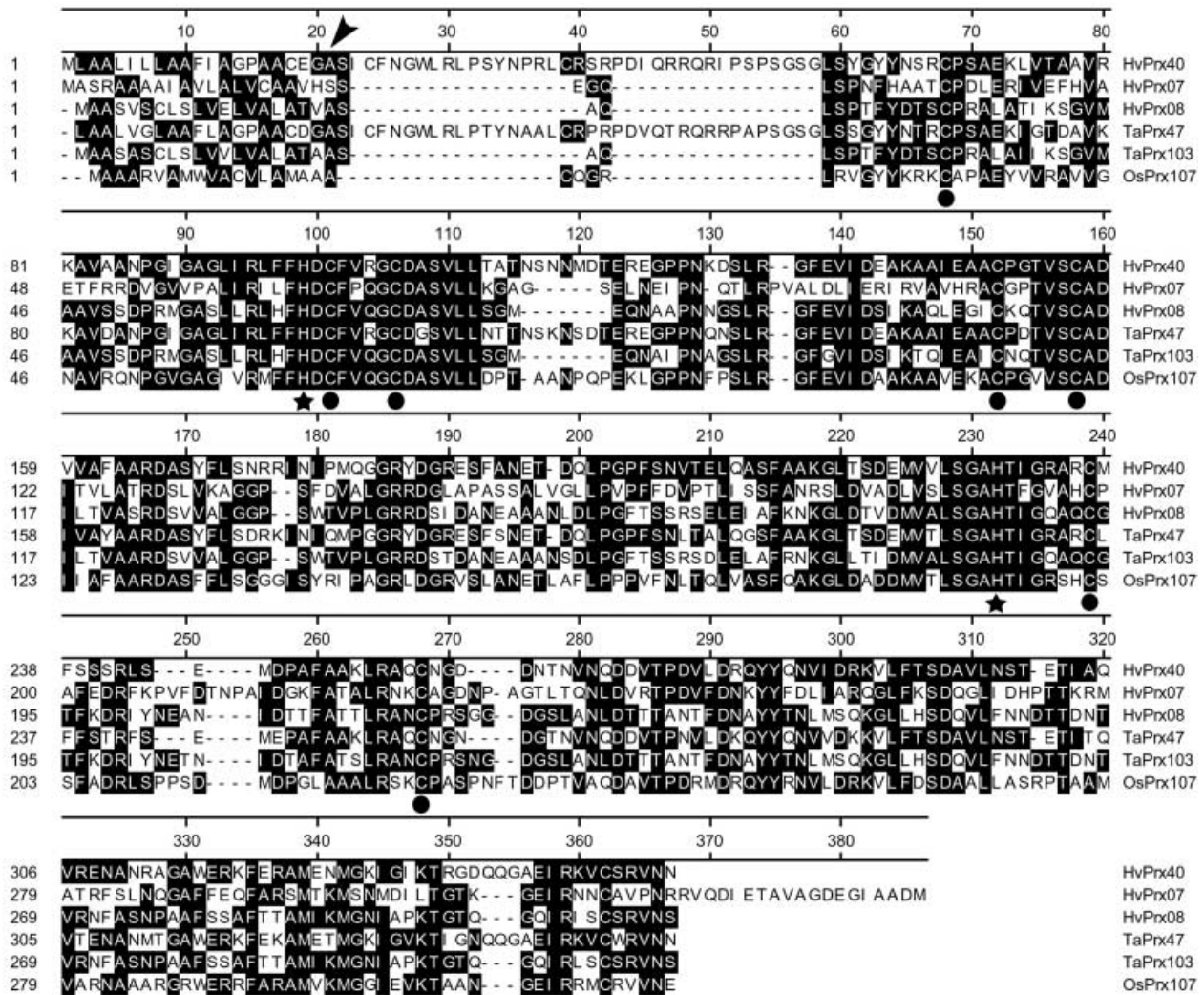
Gene-expression profiling revealed a transcript that was up-regulated in *Bgh*-attacked barley epidermis (Zierold *et al.*, 2005). Moreover, this transcript hybridizing to cDNA clone HO13D15 (accession no. CD056766) appeared to accumulate more strongly in resistant barley epidermis carrying the *mlo5* resistance gene, compared with the near-isogenic susceptible barley cultivar Ingrid. Therefore, the encoded protein might contribute to race-nonspecific basal or *mlo*-mediated resistance. The sequence of EST clone HO13D15 was analysed by BLASTX and found to be similar to class III peroxidases of rice and other plant species. A full-coding-sequence (CDS) cDNA clone was obtained (HO26F22; accession no. DN180661) and its sequence compared with 5470 manually curated peroxidase sequences (<http://peroxidase.isb-sib.ch/index.php>). This analysis revealed identity of the protein encoded by cDNA HO26F22 to a barley peroxidase of unknown function annotated as HvPrx40 with a predicted mass of 38.3 kDa and a

pI of 7.95. The entire cDNA sequence of *HvPrx40* is available as unigene consensus no. 14156 of assembly 35 in the HarvEST database (<http://harvest-web.org/>). Figure 1 shows the alignment of HvPrx40 to a number of peroxidases of barley, wheat and rice, as well as their phylogenetic relationship. In contrast to HvPrx07 and a number of peroxidases described in *Triticum monococcum* to accumulate in leaf epidermis, HvPrx40 is predicted to be secreted into the apoplast due to the absence of a C-terminal vacuolar retention signal (Fig. 1A). All analysed peroxidases contain the two highly conserved, catalytic histidine as well as eight conserved cysteine residues that build four intramolecular disulfide bonds (Hiraga *et al.*, 2001). Interestingly, HvPrx40 and TaPrx47 contain an N-terminal extension of 36 amino acids starting just after the predicted signal peptide-cleavage site, which is highly basic (pI = 11.7) due to seven arginine residues. Two cysteine residues in this basic peptide might form a fifth disulfide bond of the protein. The role of this N-terminal extension is unknown and possesses no similarity to any functional domain. However, similar sequences were identified in two additional barley and two wheat peroxidases (HvPrx61, HvPrx75, TaPrx47, TaPrx51) whereas HvPrx61 and HvPrx75 are not the most closely related peroxidases to HvPrx40 (Supporting Information Fig. S1). Clearly, HvPrx40 is distinct from the previously described peroxidases HvPrx07 and HvPrx08 of barley but corresponds to a putative protein orthologue in wheat, TaPrx47 (Fig. 1B).

Transcript accumulation

In order to test pathogen-induced *HvPrx40* transcript accumulation in susceptible barley and in near-isogenic lines carrying two different alleles of *Mlo* (*mlo3* and *mlo5*), we carried out quantitative real-time reverse-transcriptase (RT) PCR in leaves and stripped epidermis at 12 h post-inoculation (hpi) (Fig. 2). For comparison, mRNA accumulation of *HvPrx08* was also examined. Transcripts of both peroxidases accumulated upon attack by *Bgh*. However, the tissue distribution of mRNA accumulation was clearly different between the two genes: whereas *HvPrx08* transcripts mainly accumulated in inner leaf tissue, as reflected by higher signals in entire-leaf samples where epidermal RNA is diluted, transcripts of *HvPrx40* mainly accumulated in attacked epidermis. The weak *HvPrx08*-specific signal in stripped epidermis probably reflects contamination by adhering mesophyll cells (data not shown). The mesophyll-specific accumulation of *HvPrx08* mRNA was also in agreement with a previous report (Gregersen *et al.*, 1997). No difference of *HvPrx40* transcript abundance between attacked susceptible and near-isogenic, *mlo*-resistant lines was found, although a stronger accumulation was reported based on cDNA array data (Zierold *et al.*, 2005). However, as confirmed in a second independent series of array hybridizations, the differential accumulation between susceptible and resistant plants was only observed 24 hpi and may therefore have escaped detection by

A



B

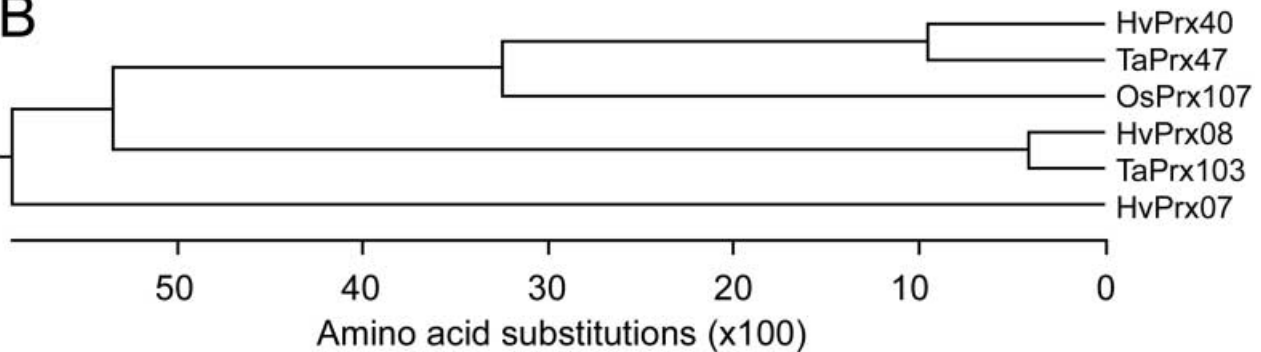


Fig. 1 Sequence comparison of HvPrx40 protein with selected peroxidases from cereal species. (A) Amino-acid alignment of HvPrx40 with other peroxidases by Clustal W. Conserved residues are shaded in black. Arrow, putative cleavage site of N-terminal signal peptide; dots, conserved cysteine residues forming four disulfide bridges; stars, conserved histidine residues of the catalytic centre. (B) Phylogenetic tree of HvPrx40 and selected peroxidases, based on Clustal W.

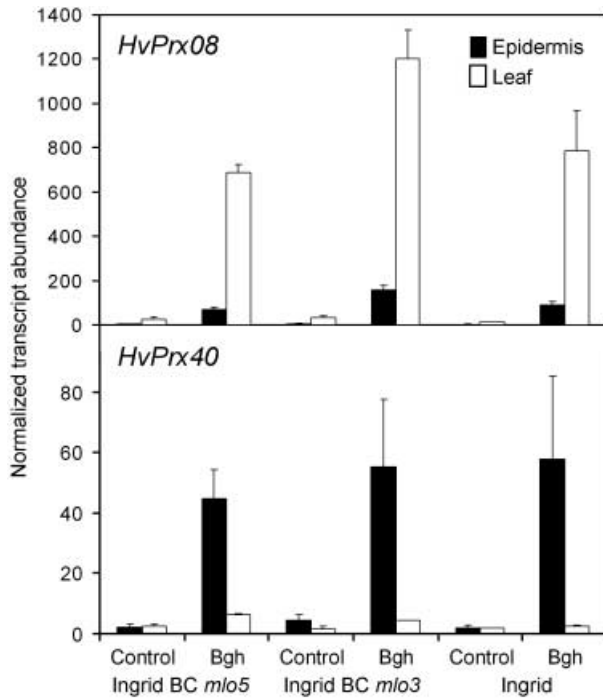


Fig. 2 mRNA Abundance of two selected peroxidases following inoculation with *B.graminis* f.sp. *hordei* (*Bgh*). RNA was isolated from barley leaves or stripped leaf epidermis at 12 h post inoculation with *Bgh*, followed by reverse transcription and quantitative real-time PCR. Data are normalized to constitutively expressed control gene *UBC* encoding a ubiquitin-conjugating enzyme. Values are means \pm SE from two independent inoculation experiments.

the qPCR reported here, which was carried out 12 hpi (Supporting Information Fig. S2). It should also be noted that transcript abundance of *HvPrx40* was considerably lower, compared with *HvPrx08*, arguing against *HvPrx40* protein to be a mass protein in epidermal tissue (Fig. 2). *HvPrx40* appears to be a member of a *Prx40*-like subfamily with at least three paralogous genes that are all characterized by the presence of the N-terminal basic peptide and that are represented by unigenes no. 14156 (*HvPrx40*), 14158 and 31622 of the HarvEST assembly 35 of barley (<http://www.harvest-web.org/>). EST abundance data revealed unigene 14156 to be the highest expressed in *Bgh*-challenged epidermis, followed by unigene 14158. Unigene 31622 was represented by one EST only from etiolated leaves.

HvPrx40 and disease resistance

We have previously shown that transient or stable overexpression of *TaPrx103*, which is the proposed wheat orthologue of *HvPrx08*, enhanced resistance to wheat powdery mildew (Altpeter *et al.*, 2005). However, because *TaPrx103* was found to be mainly expressed in inner leaf tissues, the overexpressing epidermal cells represent an artificial system that is not easy to relate to naturally occurring defence reactions. By contrast, *HvPrx40* was found to be naturally expressed in the relevant tissue for *Bgh* interactions. This prompted us to test a possible role of this peroxidase in basal resistance of barley by transiently over-expressing or silencing *HvPrx40* in single epidermal cells, followed by *Bgh* inoculation. The plasmid constructs used for these experiments are shown in Fig. 3. Transient over-expression significantly enhanced penetration

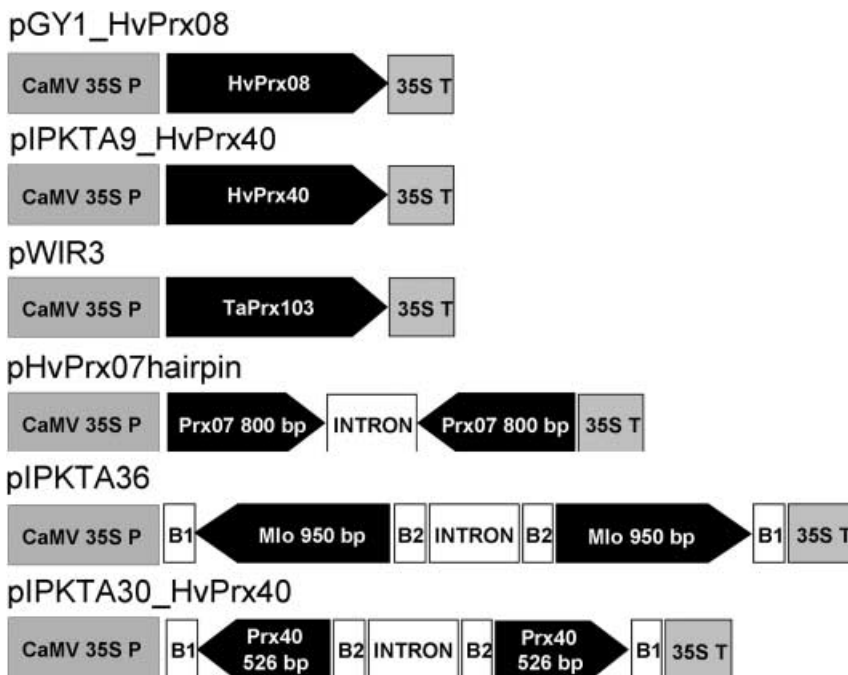


Fig. 3 Plasmid constructs used for transient overexpression and transient-induced gene silencing (not drawn to scale). The intron in the RNAi hairpin constructs was derived from the wheat *RGA2* gene (AF326781). P, promoter; T, terminator; B1 and B2, attachment sites B1 and B2 for LR clonase, respectively.

Table 1 Effect of transient overexpression and TIGS of *Prx* genes on basal resistance of barley and wheat.

Construct*	Relative SI (% of empty vector control)†			
	Overexpression		TIGS	
	Barley G.Pr.‡	Wheat	Barley G.Pr.	Barley Ingrid
pGY1_HvPrx08 (OEx HvPrx08)	70 ± 11 (<i>P</i> = 0.0391)	n.a.	—	—
pIPKTA9_HvPrx40 (OEx HvPrx40)	83 ± 4 (<i>P</i> = 0.0008)	45 ± 3 (<i>P</i> < 0.0001)	—	—
pWIR3§ (OEx TaPrx103)	54 ± 4 (<i>P</i> < 0.0001)	27 ± 2 (<i>P</i> < 0.0001)	—	—
pHvPrx07hairpin¶ (TIGS HvPrx07)	—	—	116 ± 56 (<i>P</i> = 0.7872)	124 ± 45 (<i>P</i> = 0.5527)
pIPKTA30N_HvPrx40 (TIGS HvPrx40)	—	—	184 ± 28 (<i>P</i> = 0.0105)	112 ± 10 (<i>P</i> = 0.2547)
pIPKTA34** (TIGS HvMlo)	—	—	14 ± 4 (<i>P</i> = 0.0001)	11 ± 2 (<i>P</i> < 0.0001)

*All constructs contain the CamV 35S promoter and terminator. See Fig. 3.

†SI, susceptibility index = number of GUS-positive cells containing at least one haustorium/total number of GUS-positive cells.

‡G.Pr, barley cv. Golden Promise.

§Described by Schweizer *et al.* (1999).

¶Described by Christensen *et al.* (2004).

**Described by Douchkov *et al.* (2005).

Table 2 Specific silencing of a *HvPrx40:GFP* fusion gene by the co-bombarded RNAi construct that targets *HvPrx40*.

Co-bombardment	GFP cells*	GFP/GUS†
pUbiGUS+pGFP+pIPKTA30N	951.5 ± 89.5	2.30 ± 0.18 (100)
pUbiGUS+pGFP+pIPKTA30N_HvPrx40	812.0 ± 144.0	2.04 ± 0.25 (89)
pUbiGUS+pGFP+pIPKTA30N_HO14J07‡	682.5 ± 30.5	1.89 ± 0.19 (82)
pUbiGUS+pIPKTA40_HvPrx40 _{deltaSP} +pIPKTA30N	37.0 ± 2.0	0.10 ± 0.01 (100)
pUbiGUS+pIPKTA40_HvPrx40 _{deltaSP} +pIPKTA30N_HvPrx40	16.3 ± 6.3	0.03 ± 0.01 (30)
pUbiGUS+pIPKTA40_HvPrx40 _{deltaSP} +pIPKTA30N_HO14J07	138.0 ± 10.5	0.35 ± 0.07 (359)

*Number of GFP-expressing cells per bombardment. Means ± range of two independent experiments.

†Ratio of GFP-expressing to GUS-expressing cells. Mean ± range of two independent experiments. Values in parentheses indicate relative values in percentage of the corresponding empty RNAi-vector control.

‡Control RNAi construct targeting a transaldolase gene (represented by EST clone HO14J07).

resistance of transformed cells against *Bgh*, as reflected by a reduced susceptibility index that corresponds to the relative rate of haustorium-containing, GUS-expressing cells (Table 1). A similar, enhanced resistant single-cell phenotype was observed by overexpressing the orthologous genes encoding either *TaPrx103* or *HvPrx08*, too, whereby *TaPrx103* served as a positive control (Schweizer *et al.*, 1999). Complementary to the overexpression data, transient-induced gene silencing (TIGS) of *HvPrx40* by RNAi caused hypersusceptibility of transformed cells of barley cv. Golden Promise. The efficiency of the TIGS construct targeting *HvPrx40* was tested in a co-bombardment experiment using pIPKTA30N_HvPrx40 (RNAi construct) plus pIPKTA40_HvPrx40_{deltaSP} (*HvPrx40:GFP* target construct). Table 2 demonstrates that the

number of fluorescent cells expressing *HvPrx40:GFP* was largely reduced in the presence of the corresponding co-bombarded RNAi construct, whereas the number of fluorescing cells expressing wild-type GFP was not affected by the same RNAi construct. The RNAi effect on cells expressing pIPKTA40_HvPrx40_{deltaSP} was sequence-specific because the control RNAi construct pIPKTA30N_HO14J07 targeting a non-related transaldolase gene of barley increased the number of GFP-fluorescing cells compared with the empty RNAi vector (Table 2). The overall decreased number of fluorescent cells expressing the pIPKTA40_HvPrx40_{deltaSP} construct probably reflects negative interference of the Prx fusion partner with correct GFP folding, or reduced *HvPrx40:GFP* mRNA or fusion-protein stability. In conclusion, the two opposing effects

of over-expression *versus* gene silencing of *HvPrx40* argues against a non-specific effect caused by either construct and suggests the *HvPrx40* peroxidase as a relevant factor of basal resistance in barley. Unlike *HvPrx40*, a second peroxidase specifically accumulating in attacked epidermis, *HvPrx07*, did not significantly affect the susceptible interaction with *Bgh* upon transient overexpression or TIGS (Table 1). The absence of a resistance-enhancing effect by *HvPrx07* overexpression is in accordance with previous data, where enhanced susceptibility was observed. Finally, it should be noted that the transient overexpression of *HvPrx40* in wheat epidermal cells also caused powdery-mildew resistance, indicating that the mode of action of the encoded protein is similar in wheat and barley.

HvPrx40 and localized H₂O₂ production

Class III peroxidases have been implicated in the production of reactive oxygen species, especially H₂O₂ (Passardi *et al.*, 2004b). The localized accumulation of H₂O₂ at cell-wall appositions (papillae) that are produced by barley epidermal cells beneath primary germ tubes and appressoria of *Bgh* has been shown and associated with enhanced basal resistance mediated by the *mlo* gene (Piffanelli *et al.*, 2002). Because we have demonstrated an effect of *HvPrx40* over-expression or silencing on basal resistance, we tested whether TIGS of *HvPrx40* affects the local accumulation of H₂O₂ at sites of attempted penetration by *Bgh*. For this purpose, we infiltrated bombarded leaf segments with DAB, which is oxidized to an insoluble reddish brown product at sites of H₂O₂ or OH^{*} production. As shown in Fig. 4, localized DAB oxidation

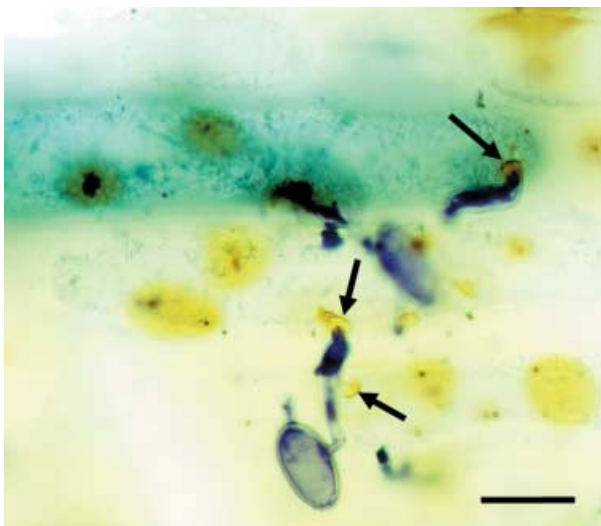


Fig. 4 Detection of localized DAB oxidation reflecting H₂O₂ accumulation in transformed, GUS-stained cells as well as in surrounding cells. Arrows point at sites of attempted penetration associated with DAB staining. Epiphytic fungal structures were stained with Coomassie blue. Scale bar, 25 µm.

Table 3 Hydrogen peroxide accumulation in cell-wall appositions upon transient silencing of *HvPrx40*.

	DAB-stained CWA (% of surrounding cells)*	
	pIPKTA30N	pIPKTA30N_ <i>HvPrx40</i>
Bombardment 1	81.9	99.1
Bombardment 2	97.0	87.0
Bombardment 3	109.9	76.8
Bombardment 4	110.3	86.7
Bombardment 5	97.1	117.1
Mean	99.2	93.3
SEM	5.2	6.9
p (one sample <i>t</i> -test)	0.891	0.39

*Local accumulation of hydrogen peroxide in cell-wall appositions (CWA) beneath fungal penetration attempts was monitored by DAB staining. The percentage of DAB-stained CWA of GUS-positive, transformed cells was compared with four surrounding, attacked cells (north, east, south and west) that were not transformed. The analysis is based on a total of 119–124 GUS-positive, attacked cells per construct.

was efficiently recorded in transformed, GUS-stained cells as well as in surrounding, non-transformed cells. Next, we compared the rate of detectable DAB oxidation in GUS-expressing cells, relative to four indirect neighbouring cells (north, east, south and west) of the transformed cell, separated by one cell in order to reduce possible neighbouring effects; Carver *et al.*, 1999). TIGS of *HvPrx40* caused no significant decrease of relative DAB oxidation (Table 3), suggesting that *HvPrx40* is not a major catalyst of localized production of H₂O₂ at sites of attempted fungal penetration.

DISCUSSION

Defence of barley epidermal cells against *Bgh* or other, inappropriate powdery mildew fungi is likely to include peroxidase isoforms for apoplastic cross-linking events, lignification or the production of H₂O₂. The peroxidase-encoding gene *HvPrx40* attracted our attention because its transcripts accumulated specifically in *Bgh*-attacked leaf epidermis, with stronger accumulation in *mlo*-resistant versus susceptible plants. A closer look at the complexity of peroxidase-encoding gene expression in barley using a cDNA array of 10 297 spotted unigenes revealed 69 unigenes producing detectable signals in stripped leaf epidermis. However, transcripts of only six unigenes were both enriched by a factor of at least two in epidermis and up-regulated at least two-fold by pathogen attack (A. Himmelbach and P. Schweizer, unpublished data). Four unigenes belonged to subfamilies that include *HvPrx07* and *HvPrx40*, leaving only two as yet completely unknown peroxidase genes (*HvPrx52* and *HvPrx97*) for future functional analysis.

Transient overexpression and TIGS of *HvPrx40* reduced and enhanced haustorium formation of a compatible *Bgh* isolate in

cv. Golden Promise, respectively. However, an assessment of off-targets for the *Prx40*-RNAi construct used here revealed a total of 16 tentative unigene consensi of the barley Gene Index of TIGR as potential off-targets besides *HvPrx40*, most of them annotated as peroxidases (<http://bioinfo2.noble.org/RNAiScan.htm>). Therefore, the phenotypic effect observed upon TIGS of *HvPrx40* might be actually due to silencing of multiple *Prx* genes. Nevertheless, the transient overexpression experiments, which revealed an interaction phenotype in barley (and wheat) opposite to the TIGS effect, suggest that *HvPrx40* is indeed a factor of basal resistance in barley, similar to *HvGER4*, another secreted protein with superoxide-dismutase activity that accumulates in *Bgh*-attacked epidermis (Christensen *et al.*, 2004; Zimmermann *et al.*, 2006). As we found in the case of *HvGER4*, silencing of *HvPrx40* did not affect basal resistance in cv. Ingrid, indicating that the importance of the encoded protein is dependent on the host genotype, a common phenomenon in the analysis of quantitative-trait loci (QTL) for basal resistance. The product of *HvGER4* is H_2O_2 , which can either be consumed by *Prx* proteins such as those involved in lignification or be further amplified by *Prx* proteins such as NAD(P)H oxidizing isoforms. However, the unaffected DAB oxidation of cells with silenced *HvPrx40* does not support the appealing hypothesis of an ROI shunt in leaf epidermis consisting of *HvGER4* and *HvPrx40* or *HvPrx40*-like proteins. This of course does not exclude the existence of such an ROI shunt involving other *Prx* genes. Data from *TaPrx103*-overexpressing transgenic wheat that exhibits enhanced DAB oxidation upon wheat powdery-mildew attack support the participation of this *Prx* protein in H_2O_2 formation (Schweizer, 2008). However, because *TaPrx103/HvPrx08* and *TaGER4/HvGER4* proteins are predicted to accumulate in mesophyll and epidermis, respectively, they are not likely candidates for the above-mentioned ROI shunt, except perhaps at the interface of the two tissues. Recently, an involvement of chelatable Fe^{3+} in the localized DAB oxidation of barley epidermal cells was shown, and free Fe^{3+} plus H_2O_2 was found to be sufficient to oxidize DAB *in vitro* in a non-enzymatic reaction (Liu *et al.*, 2007). Despite these findings, the question as to the mechanism of localized H_2O_2 formation remains open.

The mature *HvPrx40* protein possesses a highly basic N-terminal extension of 36 amino acids containing seven arginine residues that is not found in any other *Prx* besides two and one close homologues in barley and wheat, respectively. This basic peptide is predicted to be positively charged in the acidic apoplast environment and therefore possesses affinity to negatively charged structures such as pectate. Indeed, (Ca^{2+}) -pectate binding of peroxidases from zucchini and *Arabidopsis* has been demonstrated, and binding of the zucchini protein was found to be dependent on a cluster of four arginine residues whereas the two pectate-binding peroxidases from *Arabidopsis* were found to be required for root-hair elongation (Carpin *et al.*, 2001; Passardi *et al.*, 2006). Based on these data it appears possible that *HvPrx40* is

guided to negatively charged epidermal cell-wall components such as partially acetylated arabinoxylans. However, its defence-related function in the cell wall remains to be examined.

EXPERIMENTAL PROCEDURES

Plants and pathogens

Barley (*Hordeum vulgare*) plants cv. Golden Promise, Ingrid, the backcross lines Ingrid BC *mlo3* and Ingrid BC *mlo5* as well as the wheat (*Triticum aestivum*) cv. Kanzler were grown in pots of compost soil (from the IPK nursery) in a growth chamber at 20 °C with 70% relative humidity and 16 h light from metal halogen lamps and 8 h of dark. *Blumeria graminis* f.sp. *hordei* (*Bgh*) (Swiss field isolate CH4.8) was maintained at 22 °C and 16 h of light by weekly transfer to fresh barley cv. Golden Promise. *Blumeria graminis* f.sp. *tritici* (*Bgt*) (Swiss field isolate FAL92315) was maintained at 20 °C and 16 h of light by weekly transfer to fresh wheat cv. Kanzler.

For quantitative RT-PCR experiments, 7-day-old barley plants (cv. Ingrid) or Ingrid backcross lines carrying *mlo3* or *mlo5* alleles were inoculated with *Bgh*. Twelve hours after inoculation the abaxial epidermis of control and inoculated plants was stripped and the epidermis plus remaining leaf material were immediately frozen in liquid N_2 .

Real-time RT-PCR

Total RNA was extracted from epidermal peels (abaxial) and the remainder from peeled leaves of barley plants. By using the iScript cDNA synthesis kit (Bio-Rad, München, Germany) 1 µg RNA was reverse transcribed, following the manufacturer's instructions. One µL of 100 µL of the resulting cDNA solution was used for real-time RT-PCR by using the POWER SYBR® GREEN PCR MASTER MIX kit (Applied Biosystems) in an ABI 7900HT Fast Real-Time PCR system (Applied Biosystems). The PCR conditions were: 50 °C for 2 min, followed by a denaturation step at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 58 °C for 1 min and extension at 72 °C for 30 s, followed by a dissociation step at 95 °C for 15 s, 60 °C for 15 s and finally 95 °C for 15 s. The mRNA abundance of *HvPrx08* and *HvPrx40* was normalized to *HvGAPDH* and *HvUBC*.

The following PCR primers were used: *HvPrx08* (TC139154 DFCI *Hordeum vulgare* Gene Index), 5'-CTCGGACCAGTGCTCTCAACA-3' (sense) plus 5'-TGGCCTGCTCCCCGT-3' (reverse); *HvPrx40* (TC123108, DFCI *Hordeum vulgare* Gene Index), 5'-GAGCAATGGAGAATATGGGAAAATC-3' (sense) plus 5'-GGAGCCAGCCAGCAAACAC-3' (reverse); *HvGAPDH* (TC131363, DFCI *Hordeum vulgare* Gene Index), 5'-CAATGCTAGCTGCACCACCAACTG-3' (sense) plus 5'-CTAGCAGCCCTCCACCTCTCCA-3' (reverse); *HvUBC* (TC139319, DFCI *Hordeum vulgare* Gene

Index), 5'-AAGCAGCCAGAATGTACAGCGAGAAC-3' (sense) plus 5'-GGTACAGACCAGCAAAGCCAGAAATG-3' (reverse).

Reverse Northern blot

Reverse Northern blot experiments were performed by using a barley cDNA array of 10 297 spotted unigenes, as described (Zimmermann *et al.*, 2006).

Plasmid constructs for transient overexpression

For *HvPrx08* the full CDS-cDNA was cloned into the *SphI* site of the transient expression vector pGY1 (Shinshi *et al.*, 1990), which contains the CaMV 35S promoter, a multiple cloning site and the CaMV 35S terminator.

The full CDS-cDNA for *HvPrx40* was cloned into the *PstI* and *XhoI* sites of the transient expression vector pPKTA9, which is identical to pGY1, except for a more complex multiple cloning site.

Plasmid construct for TIGS

The amplification of DNA fragments for the RNAi construct pPKTA30N_HvPrx40 was performed using cDNA clone HO13D15, the non-specific sense primer MVR-26 (5'-CTCACTAAAG-GGAACAAAAGCTGGAG-3') plus the gene-specific reverse primer pPKTA30_HO13D15 (5'-GATTGTGTGTGCACCGGAGA-3'). The preparation of the RNAi construct was performed as described by Douchkov *et al.* (2005) by using the Gateway™ recombination system, except that pPKTA33 was used as entry vector (cloning site: *EcoRV*). The construct pHvPrx07hairpin has been generated by conventional ligation, as described (Christensen *et al.*, 2004). However, no effects of the B1 and B2 attachment sites for Gateway™ recombination on stability of constructs or silencing efficiency have been observed (Douchkov *et al.*, 2005).

GFP-fusion construct

An *HvPrx40* cDNA fragment was amplified by using the primer pair 5'-AAAAGGCGCGCCGGCATTGGCCAGCATTTGCTTCAACGGC-3' (sense) and 5'-AAAAACCTGCAGGGTTGTTGACTCTGGAGCAT-ACCTTCC-3' (reverse). The design of the sense primer resulted in a deleted N-terminal signal peptide of *HvPrx40*. This should enhance GFP fluorescence of the fusion protein because GFP is known to fluoresce weakly in the acidic apoplast. The amplified fragment was ligated into the *SwaI* site of the vector pPKTA43 to create intermediate plasmid pPKTA43_HvPrx40, as described by Douchkov *et al.* (2005). The translational *HvPrx40*:GFP fusion construct pPKTA40_HvPrx40_{deltaSP} was obtained by directional subcloning of an *AscI*-*SbfI* fragment of *HvPrx40* from pPKTA43_HvPrx40 into pPKTA40, upstream of GFP.

Transient single-cell expression, TIGS and H₂O₂ detection

Different peroxidases were transiently overexpressed in bombarded barley or wheat epidermal cells by using a PDS-1000 System (Bio-Rad, Munich, Germany) as described (Christensen *et al.*, 2004). For each experiment, seven primary leaves from 7-day-old plants were used. Leaf segments were challenge-inoculated with *Bgh* 4 h post bombardment at a density of 150–200 conidia/mm² and stained for GUS reporter-protein activity 48 hpi. TIGS of *HvPrx40* was performed in barley cv. Golden Promise as described (Douchkov *et al.*, 2005). Bombarded barley leaf segments were challenge-inoculated with *Bgh* 72 h post bombardment and stained for GUS reporter-protein activity 48 hpi.

The number of GFP-expressing cells was counted 24 h post bombardment using a Zeiss Axioplan 2 microscope with filterset 10 (excitation: BP450–490; beam splitter: FT510; emission: BP505–530) and normalized to GUS-expression resulting from co-bombarded plasmid pUbiGUS (Schweizer *et al.*, 1999). Sporadically occurring spherical, GFP-expressing mesophyll cells were excluded from the analysis.

H₂O₂ *in situ* production in bombarded barley leaf segments was detected using the 3,3-diaminobenzidine (DAB) method (Thordal-Christensen *et al.*, 1997). A solution of 1% (w/v) DAB in H₂O (pH adjusted to 4.0 with HCl) was injected into the leaves by using a needle-less syringe 18 h after *Bgh* inoculation. Twenty-four hours after inoculation the leaf material was stained for GUS reporter-protein activity and for epiphytic fungal structures by X-Gluc and Coomassie-blue staining solutions, respectively (Schweizer *et al.*, 1993). Detectable DAB staining at appressorium contact sites on the transformed cells and on four surrounding, non-transformed cells was determined. The relative DAB staining of appressorial contact sites on GUS-positive cells was calculated, relative to the average DAB staining of such sites on GUS-negative, surrounding cells.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Fig. S1 Phylogenetic tree of translated cDNA sequences of the barley class III Pxr multigene family, based on clustallW. The three Prx proteins used for transient expression and TIGS are highlighted by red frames.

Fig. S2 Reverse RNA blot experiments of *HvPrx40* by using a barley cDNA macroarray. RNA was isolated from epidermal peels at the times indicated post inoculation with *Bgh* and used for array hybridization as described (Zimmermann *et al.*, 2006). Mean values \pm range from two independent biological replicates.

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