Respiratory Burst Oxidase Homologue A of barley contributes to penetration by the powdery mildew fungus *Blumeria graminis* f. sp. *hordei*

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Abstract

Reactive oxygen intermediates (ROI) are closely related to defence reactions of plants against pathogens. A prominent role in the production of ROI has been attributed to the plant respiratory burst oxidase homologues (RBOH) of the human phagocyte GP91(phox). A barley RBOH, which encodes a putative superoxide (O$_2^-$/C1/C0$_2$) producing NADPH oxidase, is described here. Histochemical analysis of the barley-Blumeria graminis f. sp. hordei (Bgh) interaction showed that O$_2^-$ is produced locally at the site of penetration. In contrast, hydrogen peroxide (H$_2$O$_2$) is produced in non-penetrated cell wall appositions. A barley RBOHA cDNA was isolated and a minor induction of expression of RBOHA was observed during the interactions of barley with Bgh. Transient RNA interference-mediated gene silencing of HvRBOHA during the penetration process of Bgh led to an increase of basal penetration resistance. The results support a potential role of HvRBOHA in cellular accessibility to *Blumeria graminis*

Key words: *Blumeria graminis*, cell wall softening, hydrogen peroxide, HvRBOH, penetration, resistance, superoxide (O$_2^-$), susceptibility.

Introduction

Since Doke (1983) demonstrated that superoxide (O$_2^-$) was generated during an incompatible plant–microbe interaction, a plethora of roles have been attributed to reactive oxygen intermediates (ROI). The NADPH oxidizing phagocyte respiratory burst oxidase homologues (RBOHs) were proposed to be a source of O$_2^-$ and indirectly of proximate ROI such as hydrogen peroxide (H$_2$O$_2$). In recent years, several studies have provided ample genetic evidence supporting RBOHs as a source for ROI production (Torres et al., 2002, 2005; Foreman et al., 2003; Kwak et al., 2003; Sagi et al., 2004).

Several RBOH genes have been isolated from different plant species (Groom et al., 1996; Torres et al., 1998; Amicucci et al., 1999; Simon-Plas et al., 2002; Yoshioka et al., 2003). RBOHs are partially similar to the GP91(phox) subunit of the mammalian respiratory burst NADPH oxidase found in phagocytes. GP91(phox) is responsible for the production of O$_2^-$, which acts as a signal leading to protease activation (Reeves et al., 2002).

Mutations in the catalytic subunit lead to chronic granulomatous disease in humans, a disorder of the immune system. By contrast with the mammalian respiratory burst oxidase, plant RBOHs possess an N-terminal extension of approximately 300 amino acids. This additional domain includes in most RBOHs two EF-hand motifs for potential Ca$^{2+}$ regulation (Keller et al., 1998).

Both superoxide and hydrogen peroxide are produced during pathogenesis. Superoxide can be converted into H$_2$O$_2$, either spontaneously or by the superoxide dismutase. Although both oxygen species are moderately reactive, in the presence of transition metal ions as Fe$^{2+}$ and Cu$^{+}$, H$_2$O$_2$ is converted into the extremely reactive free hydroxyl radical (OH-) via the Fenton reaction.

O$_2^-$ and H$_2$O$_2$ have been proposed to act as signals. Kwak et al. (2003) showed that *Arabidopsis thaliana* AtRBOHD and AtRBOHF are necessary for H$_2$O$_2$ production, which is...
a second messenger in the abscisic acid signal transduction pathway in stomatal closure. AtRBOHD is also responsible for ROI production to maintain elevated levels of Ca\(^{2+}\) at the tip of root hairs during root hair growth (Foreman et al., 2003; Carol et al., 2004). Concomitantly, RBOH knock-downs in tomato display growth anomalies accompanied by loss of tissue-specific regulation of genes related to development (Sagi et al., 2004). Liszkay and colleagues (Liszky et al., 2004) have proposed a more direct role of the hydroxyl radical in cell wall softening during cell expansion. Downstream responses induced by ROI were also shown for activation of OXI1, an Arabidopsis serine/threonine kinase (Rentel et al., 2004) and a MAPK-induced cell death in Nicotiana benthamiana (Yoshioka et al., 2003).

In barley (Hordeum vulgare), O\(^{2-}\) and H\(_{2}\)O\(_{2}\) are produced in different spatiotemporal patterns during host as well as non-host interaction with the grass powdery mildew fungus (Blumeria graminis ff. spp.). O\(^{2-}\) is generated at sites of fungal penetration, during establishment of the fungal haustorium, and in cells neighbouring hypersensitive cell death reactions (HR). By contrast, H\(_{2}\)O\(_{2}\) is produced in cell wall appositions during penetration resistance and throughout the cell during HR (Hückelhoven and Kogel, 2003; Trujillo et al., 2004).

In Arabidopsis, atrbohF mutants show increased HR during the interaction with Hyaloperonospora parasitica, while mutation of atrbohF eliminates the majority of H\(_{2}\)O\(_{2}\) (Torres et al., 2003). Interestingly, no difference was observed in the resistance of atrbohF or atrbohD to Pseudomonas syringa pv. tomato. Knock-down of two NADPH oxidase genes known in potato during the incompatible interaction with Phytophthora infestans led to partially induced susceptibility (Yoshioka et al., 2003).

Regulation of plant RBOHs seem to involve both pre- and post-transcriptional elements. RBOH activity is stimulated by Ca\(^{2+}\) (Sagi and Fluhr, 2001) and also seems to be triggered by fungal elicitors like cryptogein in Nicotiana tabacum (Allan and Fluhr, 1997), hyphal wall components, arachidonic acid, and salicylic acid in potato tubers (Yoshioka et al., 2003). The catalytic subunit GP91(phox) of the multicomplex enzyme in mammals is regulated in part by the small GTP-binding protein RAC (Babior, 1999). In plants, there is evidence which suggests that a regulatory function of RAC is conserved (Kawasaki et al., 1999; Morel et al., 2004). Plant homologues of the regulatory subunits of mammalian oxidase p22, p47 or p67 have not been found and oxidase activity can be independent of activating proteins (Sagi and Fluhr, 2001).

The aim of this work was to investigate the role of a RBOH in grasses by analysing the barley–Blumeria graminis f. sp. hordei (Bgh) interaction. A barley NADPH oxidase homologue, designated HvRBOHA (Hordeum vulgare respiratory burst oxidase homologue A) was isolated. Transient knock-down via RNA interference using dsRNA was performed to assess its role in plant resistance. In addition, the expression profile of HvRBOHA was analysed during the interaction with Bgh and O\(^{2-}\) production using histochemical detection.

**Materials and methods**

**Plants, pathogens and inoculation**

Barley line Ingrid was grown in a growth chamber at 18 °C with 60% humidity and a photoperiod of 16 h (60 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) photon flux density). Inoculation was performed by air current dispersion in an inoculation tower. Primary leaves were inoculated 7 d after germination with Blumeria graminis f. sp. hordei isolate A6 (BghA6). Inoculation density for RNA extraction was 50 conidia mm\(^{-2}\) and for the RNA interference experiments 100 conidia mm\(^{-2}\). Fungi were produced under the same conditions. BghA6 was grown on barley line Golden Promise. Primary leaves were harvested at 15, 24, and 48 h after inoculation (hai) and shock frozen with liquid nitrogen. Mock inoculation and harvesting was carried out in parallel for each time point.

**Cytological analysis**

The solution used for in situ detection of O\(^{2-}\) contained 1 mg NBT ml\(^{-1}\) in 10 mM NaNO\(_{2}\) and 10 mM potassium phosphate buffer pH 7.8. For O\(_{2}\) production analysis, 1 ml of the solution was injected into primary leaves 25 min before fixation as described by Hückelhoven and Kogel (1998). Leaves were bleached and fungal structures were subsequently stained by acidic ink (Hückelhoven and Kogel, 1998). Only short epidermal cells were utilized for analysis (type A and B: Koga et al., 1990).

Leaves used for H\(_{2}\)O\(_{2}\) detection were transiently transformed with DsRed using a protocol from Schweizer et al. (2000) and modified according to Schultheiss et al. (2002). The detection of H\(_{2}\)O\(_{2}\) was carried out 20 hai by immersing leaf segments in 10 ml of a loading buffer (KCl 30 mM, MES-KOH 10 mM, pH 6.15) with 4 \(\mu\)l of a 100 mM 2,7'-dichlorofluorescin diacetate stock in DMSO (H\(_{2}\)-DCF-DA; Invitrogen, Karlsruhe, Germany). After 15 min of staining, leaves were washed with fresh loading buffer and fluorescence intensity was evaluated in a time frame of 30 min by confocal laser scanning microscopy (Leica TCS SP2, Leica Microsystems, Bensheim Germany). Values represent the average relative fluorescence on an arbitrary scale of a minimum of 30 interactions in four independent experiments. H\(_{2}\)-DCF-DA was excited at 488 nm laser line and detected at 500–535 nm; DsRed was excited at 543 nm laser line and detected at 580–650 nm.

**RNA extraction and reverse transcription**

Tissue was ground into a fine powder in liquid nitrogen. Total RNA was then extracted and integrity was assessed by examining rRNA bands after electrophoresis on a standard gel. DNA contamination was removed using RNase-free (Fermentas, St Leon-Rot, Germany) with 1 U DNase I \(\mu\)g\(^{-1}\) sample RNA, incubated at 37 °C for 30 min and inactivated by adding 1 \(\mu\)l EDTA (25 mM) and incubating 10 min at 70 °C.

One \(\mu\)g of RNA was reverse transcribed using the BioRad iScript cDNA synthesis kit (BioRad Laboratories Inc., California, USA) according to the manufacturer's instructions. The cDNA was diluted 5-fold and used as the template for PCR amplifications.

**Isolation of HvRBOHA partial clones and fragments used for dsRNA synthesis**

cDNA was used to obtain partial clones of 5' and 3'-fragments of HvRBOHA and for dsRNA synthesis of a 5'-fragment (nucleotide...
position 49–251, GenBank accession number AJ871131), a predicted transmembrane regions 3 to 4 fragment (nucleotide position 1452–1819, GenBank accession number AJ871131) and a 3′-fragment (nucleotide position 1135–1434, GenBank accession number AJ251717).

Primers flanking the HvRBOHA gene were designed using sequence information obtained by sequencing bacterial artificial chromosomes (BAC)-clones with the following primers: fwd 5′-GAATTCATAGGAGCATTCT-3′, rev1 5′-TGAGCCCTGTGGACAACT-3′ and rev2 5′-GACAATGGTGTCGCTCTCT-3′. Primers were designed from the target gene sequence using Primer3 software (O'Connell et al., 2004). The following primers were generated for amplification of the 5′ and 3′ overlapping fragments containing the complete coding sequence of HvRBOHA. Primers for 5′ fragment: fwd 5′-AAATCTGTCACCCAGTTGG-3′, rev 5′-CAGAAAGCCA-TTGAAAGCCGCT-3′. Primers for 3′ fragment: fwd 5′-AACCCTCCTTGGTGCG-3′ and rev 5′-ACATAAGGCGCAATCTCCTG-3′. PCR products were isolated after electrophoresis, cloned into pCR 4-TOPO (Invitrogen, Karlsruhe, Germany) and sequenced (GenBank accession numbers AJ871131, AJ251717).

Fragments used for dsRNA synthesis were obtained using the following primers. 5′-fragment: fwd 5′-ACTGCTCTCCCGGA-CAAGG-3′, rev 5′-GACAATGGTGTCGCTCTCT-3′. TMR 3–4: fwd 5′-AACCCTCCTTGGTGCG-3′, rev 5′-CAGAAAGGCATGTCGCTCT-3′. 3′-fragment: fwd 5′-AGCAATGGTGTCGCTCTCT-3′, rev 5′-GAATGTGGTGGAGTATGCTCCA-3′. PCR products were isolated after electrophoresis, cloned into pGEM-T (Promega, Mannheim, Germany) and sequenced.

Screening of BAC-libraries

PCR screening was performed with the primers, fwd 5′-CGAT-CAGATGGATGCTGCTCA-3′ and rev 5′-CAGAAAGCCGGAATGCTCT-3′ to isolate a BAC containing genomic HvRBOHA from the Barley Hv_MBa DNA pools (Clemson University, http://www.genome.clemson.edu/orders/HV_MBa_Cloned.html).

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed using the Platinum SYBR Green qPCR SuperMix UDG Kit (Invitrogen, Karlsruhe, Germany) in a MX3000P QPCR System from Stratagene (Stratagene, California, USA). Reactions contained 10 μl of reaction mixture, 2 μl of cDNA, and 0.5 pmol of each primer in a total volume of 20 μl. All reactions were carried out in triplicate for each sample. Primers were designed from the target gene sequence using Primer3 software (Rozen and Skaltsky, 2000) and optimized to anneal at an equal temperature of 60 °C. The following primers were generated for gene expression analysis (MGW Biotech, Ebersberg, Germany). Target gene 1, NADPH oxidase (GenBank accession number: AJ251717), forward 5′-CGATCAGATGGATGCTGCTCA-3′ and reverse 5′-CAGAAAGCCGGAATGCTCT-3′; target gene 2, PR-1b (GenBank accession number: X74940) forward 5′-GGAC-TACGACTACGGCTCACA-3′, reverse 5′-GGCTGCTGTAGGTCGCT-3′; reference gene Ubiquitin (GenBank accession number: M60175), forward 5′-ACCTGGGCGACTACACAT-3′, reverse 5′-CATAGTGGCGCCTGAAGCT-3′; reference gene 18s (GenBank accession number: AY552749), forward 5′-CGGGATGTTCTATAACGC-3′, reverse 5′-GGGACCGACC-GACCTACT-3′.

PCR for quantification started with 2 min incubation at 50 °C and 3 min denaturation at 95 °C followed by 50 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 20 s (1. fluorescence acquiring) and 82 °C for 15 s (2. fluorescence acquiring). Subsequently, a melting curve was performed in which temperature was incremented from 50 °C to 90 °C in one degree steps each held for 10 s.

Identity of qRT-PCR products was confirmed by the observation of a single melting peak. In addition, products were separated by agarose-gel electrophoresis to verify product size, and sequenced. Standard curves and threshold values were calculated with a 5-fold dilution series (total of five dilutions) of template pools using the Stratagene software (Stratagene, California, USA). Relative quantification of fold expression changes were calculated according to Pfaffl (2001).

Transient transformation assay and RNAi

Transient transformation protocol to assess gene function in the barley–powdery mildew interaction via RNA interference (RNAi) through a biolistic approach was taken from Schweizer et al. (2000) and modified according to Schultheiss et al. (2002). Plants were grown under the same conditions as previously described (Schultheiss et al., 2002). For RNAi, dsRNA of the three different HvRBOHA fragments were synthesized and leaf segments bombarded with the coated particles 4 h before inoculation with Bgh race A6. Inoculation density was 100 conidia mm⁻². Interaction outcome (i.e. penetration or effective cell wall apposition) was analysed 40 h after inoculation by light microscopy. Transformed GFP expressing cells were identified under blue light excitation. Surface structures of Bgh were detected by light microscopy or by fluorescence staining of the fungus with 0.3% calcofluor (w/v) in 50 mM TRIS pH 9.0.

Relative penetration efficiency (PE), referring to average control

Results

Cytological analysis of ROI production

A microscopic analysis of the interaction between the host barley (cv. Ingrid) and virulent Bgh (race A6) was performed.

Superoxide production was detected employing the in situ nitroblue tetrazolium (NBT) reduction assay, considered as specific for O²⁻ (Doke, 1983; Fryer et al., 2002). The first ROI to be detected at sites of attempted penetration was O²⁻. At first, O²⁻ was detected locally at the plasma membrane adjacent to successful penetration attempts in the early stage of the interaction, approximately 16 h after inoculation (hai; Fig. 1A). Importantly, no O²⁻ was detected in non-penetrated cell wall appositions. By contrast, H₂O₂ accumulation detected with 2′,7′-dichlorofluorescein diacetate (H₂DCF-DA), was primarily produced in non-penetrated cell wall appositions, starting at approximately 18 hai (Fig. 1B). Staining patterns observed with H₂DCF-DA were very similar to those observed with the H₂O₂-specific dye 3,3-diaminobenzidine (Thordal-Christensen et al., 1997; Hückelhoven et al., 1999; Trujillo et al., 2004).
Isolation of HvRBOHA

To isolate HvRBOHA, a reverse transcription (RT) step was performed using total RNA of barley (*Hordeum vulgare*) cv. Ingrid as the template followed by PCR. Degenerate primers were designed based on the conserved C-terminal region of the human GP91(phox) subunit (Royer-Pokora et al., 1986) and of rice OsRBOHA (Groom et al., 1996; Hückelhoven et al., 2001). Additional primers for upstream annealing were designed based on the rice OsRBOHA genomic sequence (GenBank accession number AP003560).

This allowed the isolation of overlapping cDNA fragments covering a total of 2215 base pairs of the 3′ part of a barley putative NADPH oxidase. However, the 5′ end of the cDNA could not be isolated via RT-PCR with degenerate primers. Therefore, the barley bacterial artificial chromosome (BAC)-library HV_MBa of the Clemson University (http://www.genome.clemson.edu/orders/HV_MBa_Clon.html) was screened. PCR-based screening of DNA pools and sub-pools was performed using a primer combination that generated an amplicon of 190 bp of a conserved region for barley (GenBank accession number AM265370) is provided here. The nucleotide sequence showed an estimated 87% identity to a rice cDNA clone, which will be referred to as OsRBOH, and 84% identity to OsRBOHA using BLASTN (GenBank accession numbers AK120905, AF015302 partial mRNA; Altschul et al., 1997; Keller et al., 1998). The nearest Arabidopsis orthologue is AtRBOHF with a similarity of 81% (GenBank accession number AB008111; Torres et al., 1998).

The deduced amino acid sequence of the RBOH ORF is predicted to encode 963 amino acids and shows an extended N-terminal region not present in the human GP91(phox) sequence (GenBank accession number NM_000397). The C-terminal region of the deduced amino acid sequence shares a 47% similarity to the GP91(phox) of human phagocytes, but domains relevant for the activity of the catalytic subunit are conserved (Fig. 2). Binding sites for NADPH- and FAD of the human NADPH oxidase (Yoshida et al., 1998; Vignais, 2002) are conserved in the barley homologue. Although plants lack clear P47 phox homologues, residues involved in the binding of P47phox (De Leo et al., 1996) are partially conserved as shown for the barley and the Arabidopsis sequences (Fig. 2). Six hydrophobic stretches in a similar arrangement as in transmembrane regions of GP91(phox) were predicted using the dense alignment surface program (Cserzo et al., 1997), implying analogous topological arrangement (data not shown). In addition, haem-ligating histidines (Bibers-tine-Kinkade et al., 2001) of the human NADPH oxidase are conserved in barley.

Two putative Ca2+-binding EF-hand motifs preceding the first residue aligned with GP91(phox), are present in the N-terminus in barley (Fig. 2). This is a typical feature
of plant RBOHs and is also described for rice and Arabidopsis (Keller et al., 1998; Torres et al., 1998).

The deduced amino acid sequence of the isolated RBOH gene was compared with divergent RBOHs to construct a phylogenetic tree using ClustalW series program (Chenna et al., 2003). The deduced amino acid sequence of a rice RBOH (GenBank accession number AK120905) is predicted to be related closest to HvRBOHA and displays 91% similarity (Fig. 3). OsRBOHA is the second closest homologue in the same subgroup. The barley NADPH oxidase will be referred to as HvRBOHA. AtRBOHf is the closest Arabidopsis homologue with 79% similarity.

Expression analysis of HvRBOHA

A check was made for epidermal expression of HvRBOHA, because this is the only tissue with direct contact to Bgh. Similar to that described previously (Schultheiss et al., 2003), expression was compared in epidermal peels of barley inoculated with Bgh and the rest of the leaves at 24 h after inoculation (hai). Expression of HvRBOHA was compared with that of actin, epidermis-expressed oxalate oxidase-like protein (HvGLP4), and ribulose bisphosphate carboxylase for mesophyll contamination by semi-quantitative RT-PCR. When compared to strong signals of germin-like protein 4 and ribulose bisphosphate carboxylase in the epidermis and the rest of the leaves, respectively, HvRBOHA appeared equally expressed in both epidermis and the rest of leaves. It was further examined if HvRBOHA expression was induced by the interaction of barley with Bgh. For this purpose, total RNA was isolated from epidermal peels at 0, 15, and 24 hai and after mock inoculation and subjected to first strand cDNA synthesis. No HvRBOHA signal could be detected by northern-blot analysis. Therefore, relative gene expression was determined by quantitative real-time PCR (qRT-PCR). Low abundance of transcripts was confirmed by the threshold values obtained by qRT-PCR. On average, nine additional cycles of amplification were necessary to obtain the threshold value of HvRBOHA (target) compared to Ubiquitin used as a constitutively expressed reference (Schultheiss et al., 2002). HvRBOHA expression increased by a maximum of 2.7-fold during the interaction of barley with Bgh at 15 hai. To verify the induction of defence responses, expression of the pathogenesis-related-1b (PR-1b) gene was also analysed. A strong increase of 36-fold of transcript abundance was detected after inoculation of barley with Bgh at 15 hai (Table 1). In independent experiments, a similar expression pattern of HvRBOHA was detected. In addition, expression level of HvRBOHA decreased at 48 hai to the same levels as the control. Semi-quantitative reverse transcription-PCR resulted in similar expression patterns as those obtained by qRT-PCR for HvRBOHA and PR-1b. Expression patterns during the non-host interaction of barley with Blumeria graminis f. sp. tritici were similar. Epidermal expression of HvRBOHA is thus slightly and transiently induced by Bgh.

Functional analysis of HvRBOHA

To analyse the function of HvRBOHA during the interaction of barley with Bgh, a transiently induced gene-silencing assay was performed via RNAi in the barley cultivar Ingrid (Schweizer et al., 2000; Schultheiss et al., 2002). Subsequently, dsRNA of the target gene together with GFP was introduced in an expression vector as reporter gene into epidermal cells via a biolistic approach. Interaction outcome was evaluated 40 h after inoculation and compared with control cells containing heterologous human thyroid receptor (TR) dsRNA. To determine the effect of HvRBOHA on penetration efficiency, dsRNA of three different fragments including a 5’-fragment (nucleotide position 49–251, GenBank accession number AJ871131), a fragment containing the predicted transmembrane stretches 3 and 4 (nucleotide position 1452–1819, GenBank accession number AJ251717) was used. The average penetration efficiency (PE) of the fungus in the controls (cells transformed with TR-dsRNA and GFP) was 48% over all experiments. Fragments corresponding to the highly conserved sequences of the TMR 3–4 and 3’–region induced a similar PE decrease of 36% of Bgh on a susceptible barley cultivar (Fig. 4). The 5’-region is the most variable between RBOHs, thus the most specific of all fragments used for RNAi. Cells transformed with dsRNA from the 5’-stretch, showed 28% penetration on average, while 59% of interaction sites of control cells were penetrated. This translates in a highly significant (Student’s t-test, P <0.001) decrease of 52% of the relative penetration efficiency of the fungus (Fig. 4). Similar results were obtained when instead of Ingrid, the also susceptible barley cultivar Pallas was transformed with dsRNA from the 3’-stretch. Together, knock-down of HvRBOHA reduced susceptibility to penetration by Bgh.

H₂O₂ accumulation was quantified after transient knock-down of HvRBOHA by measuring H₂DCF-DA fluorescence intensities of papillae at 20 hai by confocal laser scanning microscopy. However, H₂DCF-DA fluorescence was unchanged in Bgh-attacked cells in which HvRBOHA was knocked down (Fig. 5). Hence, the abundance of HvRBOHA does not seem to limit H₂O₂ in cell wall appositions produced in response to Bgh.

Discussion

Plant RBOHs have been shown to be a source for ROI production and play a pivotal role in manifold processes. Our purpose was to investigate a possible role of a barley RBOH in the interaction of barley with the powdery mildew fungus.

An RNAi method was employed for transient silencing of the barley NADPH oxidase homologue HvRBOHA. Knock-down of HvRBOHA led to a decrease of fungal
involved in p47phox binding in mammals. Sites identified for FAD- and NADPH-binding in GP91(phox) FAD-binding I and II, NADPH units III
GP91(phox) homologues. Conserved amino acid residues are shaded in grey. EF-hand motifs are encased and highlighted by bars. (A–C) Residues
Alignment of deduced amino acid sequences of the barley (HvRBOHA),
Fig. 2.
3–4 and the 3
of resistance achieved varied dependent on the dsRNA used. Nevertheless, the degree
penetration, which was independent of the cDNA fragment
used for the generation of dsRNA. Nevertheless, the degree of resistance achieved varied dependent on the dsRNA used. Fragments spanning the predicted transmembrane regions 3–4 and the 3’ stretch are highly conserved in other RBOHs and induced an increase in penetration resistance of 36%. Sequence conservation implies homology to any other RBOH possibly present in barley and their concomitant down-regulation. Maximum resistance induction was achieved with the 5’ stretch (52%). This region shows the highest sequence variability between RBOH in different species. Thus, the 5’ stretch might be the most specific for silencing of HvRBOHA. Accordingly, specific silencing of highly similar RAC-gene family members in rice was achieved by using gene-specific inverted repeat constructs (Miki et al., 2005). On the other hand, it is also possible that different dsRNA fragments had different suitability as substrate for RNAi machinery (Yiu et al., 2004). The latter explanation is supported by the fact that no barley EST could be detected out of more than 300 000 public sequences, showing more than 64% sequence identity to HvRBOHA (tentative consensus sequence: TC147482,
www.tigr.org/tdb/tgi/nucleotide). Together, this suggests that RNAi was specific for HvRBOHA.
In the barley–Bgh interaction, H2O2 has been related to resistance (Christensen et al., 1997; Hückelhoven et al., 1999). Nevertheless, O2– production occurs concomitant to penetration during the early interaction stages (Fig. 1A) and around growing fungal haustoria (Hückelhoven and Kogel, 1998; Trujillo et al., 2004). This hints at the possibility that O2– either contributes to penetration or acts as a signal supporting biotrophy. In addition, distinct spatiotemporal production patterns of H2O2 and O2–, respectively, suggest different roles as well as different sources of ROI (Allan and Fluhr, 1997; Hückelhoven and Kogel, 2003; Trujillo et al., 2004). Superoxide, produced by RBOH and the hydroxyl radicals that can be generated by the Fenton reaction, have been shown to be involved in cell wall polymer breakdown during cell growth (Foreman et al., 2003; Liszkay et al., 2004). The same process leading to polymer breakdown may provoke local cell wall softening. This could be induced by the invading fungus to promote penetration by triggering RBOH activity. Hence, knock-down of HvRBOHA might lead to decreased levels of hydroxyl radicals and enhance penetration resistance by preventing host cell wall softening. In this context it is interesting that an extracellular germin-like protein GLP4 with superoxide dismutase activity is involved in background penetration resistance of barley to Bgh (Christensen et al., 2004). Simultaneous histochemical detection of O2– and H2O2 during the interaction of barley with Blumeria graminis f. sp. tritici showed that non-penetrated papillae contained H2O2 whereas O2– could almost never be detected (Trujillo

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“ Total RNA was extracted from 8-d-old barley plants of cv. Ingrid after inoculation with Bgh and mock inoculation. RNA was subsequently subjected to reverse-transcription, followed by real-time PCR analysis. Expression of the target genes Hv/RBOHA and PR1-b is displayed relative to the expression of the reference gene Ubiquitin. Relative change of expression was calculated according to Pfaffl (2001). Both technical and biological repetitions of the experiment led to similar results.

Fig. 2. Alignment of deduced amino acid sequences of the barley (HvRBOHA), Arabidopsis (AtRBOH), and human NADPH oxidase subunit GP91(phox) homologues. Conserved amino acid residues are shaded in grey. EF-hand motifs are encased and highlighted by bars. (A–C) Residues involved in p47phox binding in mammals. Sites identified for FAD- and NADPH-binding in GP91(phox) FAD-binding I and II, NADPH units III pyrophosphate, IV ribose, V adenine, and VI nicotinamide. GP91(phox) haem-ligating histidines, asparagines involved in N-glycosylation as well as proline and aspartate, involved in chronic granulomatous disease are denoted by white letters with black background. Dashes represent gaps introduced to optimize alignment.

Fig. 3. Tentative phylogenetic tree of plant respiratory burst oxidase homologues (RBOH). Tree was constructed by alignment of deduced amino acid sequences using CLUSTALW (http://www.ebi.ac.uk/clustalw/, Chenna et al., 2003). Estimated branch lengths given after abbreviation are proportional to the amount of inferred evolutionary change. Sequences were obtained from GenBank. Abbreviations: barley (Hv), rice (Os), Arabidopsis (At) GenBank accession numbers: AtRBOHA (At5g07390), AtRBOHB (At1g09090), AtRBOHC (At5g10600), AtRBOHD (At5g476910), AtRBOHE (At5g12300), AtRBOHF (At1g64060), AtRBOHG (At4g25090), AtRBOHH (At5g51060), AtRBOHI (At4g11230), AtRBOHJ (At3g45810), AtRBOHK (At3g45810) as reported by Torres and Dangl (2005). For rice accession numbers, see figure.

Table 1. Relative expression levels of barley RBOHA and PR1-b in response to inoculation with virulent Bgh

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In the same study, O$_2^-$ production at sites of fungal attack by $Bgh$ on wheat was associated with successful penetration and subsequent HR. It was also observed that the flavin-enzyme inhibitor diphenylene iodonium at a high concentration of 200 $\mu$M inhibited penetration by $Bgh$ and maturation of haustoria by 90% in susceptible barley (Hückelhoven, 1999). The same concentration also inhibited HR in an incompatible interaction without allowing fungal development (Hückelhoven and Kogel, 1998). This further supports a role of flavin-containing enzymes such as RBOH in accessibility to $Bgh$. By contrast, HvRBOHA is not the major source of papilla-associated H$_2$O$_2$ production, because H$_2$O$_2$-sensitive fluorescence of 2',7'-dichlorofluorescein diacetate was unchanged by silencing of HvRBOHA (Figs 5, 1B). This supports the view, that NADPH oxidase-dependent O$_2^-$ production is not the source of H$_2$O$_2$ in papillae (Hückelhoven and Kogel, 1998). Similarly, Arabidopsis RBOHF is not the major source of H$_2$O$_2$ during defence against avirulent $P$. syringae or the oomycete $H$. parasitica (Torres et al., 2002). Taking these results together, there is accumulating evidence for different sources of ROI in the interaction of barley and $Bgh$ (Hückelhoven and Kogel, 2003). There are data indicating that papillae and HR associated H$_2$O$_2$ may be alternatively produced in grasses by peroxidases, oxalate oxidases, or amine oxidases (Zhang et al., 1995; Walters, 2003; Altpeter et al., 2005; Liu et al., 2005; Passardi et al., 2005). However, HvRBOHA activity might be compensated by other RBOHs.

Dicotyledonous plants appear to have evolved several RBOH genes, which are expressed in a tissue-specific manner and display specialized functions as well as regulation (Foreman et al., 2003; Kwak et al., 2003; Yoshioka et al., 2003; Torres et al., 2002; Sagi et al., 2004). In the barley unigene set, which is the basis for a 22K commercially available barley microarray (Close et al., 2004), only one cDNA fragment was found identical to HvRBOHA, but no further clear homologues (Contig12420_at, www.barleybase.org). However, intensive searches for barley ESTs revealed at least six RBOH-like fragments in barley EST databases (tentative consensus sequences at www.tigr.org/tdb/tgi/nucleotide). Sequences were related to HvRBOHA, but except TC149618, which represents a fragment of the HvRBOHA cDNA, RBOH-like sequences had a maximal similarity of only 64%. The corresponding predicted open reading frames, however, represent potential orthologues of rice RBOHs from all phylogenetic clades (Fig. 3 and data not shown). In rice, a close relative of barley, the deduced amino acid sequence of a RBOH (accession AAU43962) is predicted to be related...
Therefore, suppression of RBOH-dependent O$_2^-$ production may result in distinct modulations of resistance, probably depending on fungus-specific infection strategy and/or signalling pathways. In this investigation effects on HR could not be detected, because transiently induced gene silencing was performed on a single cell level with GFP as a marker to detect transformed cells. Nevertheless, the effect on reduced penetration may well rely on a missing signal necessary for the establishment of biotrophy, which would impede fungal growth in the initial stage. Genetic evidence indicates that RBOH is involved in restriction of pathogen-induced cell death in Arabidopsis (Torres et al., 2005), which is in accordance with the assumption that HvRBOHA may have a function in the survival of barley cells in a compatible interaction with Bgh. Alternatively, lack of HvRBOHA and its potential role in defence might be over-compensated by alternative host defence strategies.

There are also data supporting a function of RBOH in the induction of cell death. Barley plants expressing a constitutively activated RAC1 GTPase (Schultheiss et al., 2003), the presumptive rice orthologue of which was shown to induce RBOH dependent cell death (Kawasaki et al., 1999), are more susceptible to penetration by Bgh and, at the same time, show more hypersensitive cell death reactions than wild type (I Priyadarshini, G Hensel, J Kumlehn, and R Hückelhoven, unpublished results). During barley race-specific response to penetration by Bgh a long-lasting O$_2^-$ production is detected that may facilitate cell death (Hückelhoven and Kogel, 1998). Therefore, O$_2^-$ may also reduce the threshold of cell death by sensitizing penetrated cells. It is plausible that O$_2^-$ can support cell death initially, whereas after cell death has been triggered, it is required for the restriction of lesions (Fig. 6). This suggests the involvement of additional specific components to determine the outcome of O$_2^-$ production during defence responses.

Because expression of HvRBOHA is only weakly induced by Bgh (this study; Hückelhoven et al., 2001; Jain et al., 2004), local O$_2^-$ generation may be regulated rather at the activity level, potentially after Ca$^{2+}$ influx sensed by the EF-hand motifs of RBOH (Sagi and Fluhr, 2001). There is indirect evidence for Ca$^{2+}$ influx into susceptible barley cells attacked by Bgh because apoplastic [Ca$^{2+}$] decreases when Bgh attempts to penetrate (Felle et al., 2004). Moreover, Ca$^{2+}$ is linked to susceptibility because the major susceptibility factor MLO binds calmodulin in a Ca$^{2+}$-dependent manner to fulfil its role in accessibility to Bgh (Kim et al., 2002). Astonishingly, little O$_2^-$ is observed in resistant mlo5-mutant lines at the time when O$_2^-$ is produced in susceptible near-isogenic MLO genotypes during fungal penetration (Hückelhoven and Kogel, 1998). Together, HvRBOHA may support fungal penetration and/or be indispensable for the establishment of haustoria.

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**References**


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