Downregulation of a barley (*Hordeum vulgare*) leucine-rich repeat, non-arginine-aspartate receptor-like protein kinase reduces expression of numerous genes involved in plant pathogen defense

David L. Parrott^{a,1}, Li Huang^a and Andreas M. Fischer^{a,*}

^a*Department of Plant Sciences and Plant Pathology, Montana State University, Bozeman, MT 59717-3150 (USA)*

¹*Present address: Department of Biology, University of Utah, Salt Lake City, UT 84112 (USA)*

*Corresponding author.

E-mail address: fischer@montana.edu

ABSTRACT

Pattern recognition receptors represent a first line of plant defense against pathogens. Comparing the flag leaf transcriptomes of barley (*Hordeum vulgare* L.) near-isogenic lines varying in the allelic state of a locus controlling senescence, we have previously identified a leucine-rich repeat receptor-like protein kinase gene (LRR-RLK; GenBank accession: AK249842), which was strongly upregulated in leaves of early- as compared to late-senescent germplasm. Bioinformatic analysis indicated that this gene codes for a subfamily XII, non-arginine-aspartate (non-RD) LRR-RLK. Virus-induced gene silencing resulted in a two-fold reduction of transcript levels as compared to controls. Transcriptomic comparison of leaves from untreated plants, from plants treated with virus only without any plant sequences (referred to as 'empty virus' control), and from plants in which *AK249842* expression was knocked down identified numerous genes involved in pathogen defense. These genes were strongly induced in 'empty virus' as compared to untreated controls, but their expression was significantly reduced (again compared to 'empty virus' controls) when *AK249842* was knocked down, indicating that their expression partially depends on the LRR-RLK investigated here. Expression analysis, using datasets from BarleyBase/PLEXdb, demonstrated that *AK249842* transcript levels are heavily influenced by the allelic state of the well-characterized mildew resistance *a* (*Mla*) locus, and that the gene is induced after powdery mildew and stem rust infection. Together, our data suggest that *AK249842* is a barley pattern recognition receptor with a tentative role in defense against fungal pathogens, setting the stage for its full functional characterization.

Keywords:

Barley (*Hordeum vulgare* L.)

Leucine-rich repeat (LRR)

Mildew resistance locus *a* (*Mla*)

Pathogen defense

Pattern recognition receptor (PRR)

Receptor-like protein kinase (RLK)

1. Introduction

Plants, like other organisms, are constantly challenged by interactions with pathogens from all kingdoms of life. Sophisticated defense mechanisms have evolved in response to this situation. A first layer of defense is provided by plants' ability to detect conserved molecular signatures (microbe/pathogen-associated molecular patterns; MAMPs/PAMPs) which are present in a wide range of microbes including pathogens (Boller and Felix 2009). Such signatures/patterns are detected by 'pattern recognition receptors' (PRRs), some of which are present at the cell surface, while others are intracellular (Dardick et al. 2012). Plant responses to the detection of PAMPs are known as PAMP-triggered immunity or PTI. A particular category of cell surface receptors is represented by receptor(-like) kinases (RKs, RLKs) consisting of extracellular receptor, transmembrane and intracellular protein kinase domains. Various types of receptor domains including the lysine-motif (LysM) and leucine-rich-repeat (LRR) domains have been identified; PRRs can be classified based on the identity of the extracellular domains and phylogenetic relationships between the kinase domains (Dardick et al. 2012; Shiu and Bleecker 2001). While numerous RLKs appear to be involved in plant pathogen defense, a second functional category of RLKs is important for cell growth (e.g. BRASSINOSTEROID INSENSITIVE1, BRI1) and developmental processes (e.g. CLAVATA1) (Afzal et al. 2008).

Plant RLKs belong to a large monophyletic gene family with >600 members in *Arabidopsis*. In contrast, animal genomes contain very few (between one and six in those investigated) such genes (Shiu and Bleecker 2003). **Additionally, some RLK subfamilies are considerably larger in rice than in *Arabidopsis*. Subfamilies differ widely in the degree of expansion after the *Arabidopsis*-rice split, with tandem duplication as the major factor contributing to family expansion in rice (Shiu et al. 2004).** One RLK sub-family that very conspicuously expanded is LRR XII, which has >150 members in rice, but only six members in *Arabidopsis* (Shiu et al. 2004). Several of the best-investigated PRRs including *Arabidopsis* FLS2 (detecting the presence of a conserved 22-amino acid peptide from flagellin, flg22) and EF-Tu receptor/EFr (detecting a bacterial elongation factor) as well as rice Xa21 belong to this subfamily (Antolin-Llovera et al. 2012; Shiu et al. 2004), but many monocot LRR XIIs (and other PRRs) remain uncharacterized. Drawing attention to this fact, such putative PRRs have been called 'orphan PRRs' by Boller

Abbreviations: BSMV, Barley Stripe Mosaic Virus; LRR, leucine-rich repeat; MAMP, microbe-associated molecular pattern; *Mla*, mildew resistance locus *a*; PAMP, pathogen-associated molecular pattern; *pds*, phytoene desaturase gene; PRR, pattern recognition receptor; PTI, PAMP-triggered immunity; qRT-PCR, quantitative real-time RT-PCR (for gene expression analysis); R(L)K, receptor (-like) protein kinase; RT-PCR, reverse transcription PCR (for gene cloning); VIGS, virus-induced gene silencing.

and Felix (2009). To extend our basic knowledge of plant-pathogen interactions, it is imperative to characterize a larger number of these receptors, at least in the predominant model species and in major crops, and to identify the microbial elicitors they recognize.

A majority of investigated plant and animal kinases are so-called ‘RD’ kinases, due to the presence of a conserved arginine (R) residue immediately preceding an invariant aspartate (D) in kinase subdomain VI, which is required for catalytic activity (Johnson et al. 1996). However, a smaller number of kinases have been grouped in the ‘non-arginine-aspartate’ (‘non-RD’) class which, as indicated by the name, lacks the conserved arginine in subdomain VI. In its place members of this group carry uncharged cysteine, glycine, phenylalanine or leucine residues. Non-RD kinases do generally not autophosphorylate their activation loop and are either always active, or they are regulated through other mechanisms (Dardick et al. 2012; Johnson et al. 1996). A systematic survey of RLKs in plants and other organisms suggests that the presence of the non-RD motif is diagnostic for a role in pathogen defense. Specifically, all characterized members of the LRR XII family are non-RD, and are involved in the recognition of bacterial or fungal pathogens (Dardick et al. 2012). In contrast, RLKs involved in development (such as CLAVATA1) belong to the RD class of protein kinases (Afzal et al. 2008).

A putative LRR-RLK (Affymetrix Barley1 GeneChip designation: rbaal9i05_at; GenBank accession: AK249842; see materials and methods for details) has been identified in our laboratory through a transcriptomic comparison of flag leaves of early- vs. late-senescing near-isogenic barley germplasm. Analyzed plant material differs in the allelic state of a chromosome 6H locus (delineated by molecular markers HVM74 and ABG458) controlling both leaf senescence and grain protein concentration (Jukanti et al. 2008). Based on the transcriptomic screen and additional data obtained by quantitative real-time RT-PCR (qRT-PCR), *AK249842* was upregulated >100-fold in flag leaves of early- vs. late-senescing germplasm at 7, 14, 21 and 28 past anthesis, and also in lower leaves during vegetative plant growth (Jukanti et al. 2008; Lacerenza et al. 2010). Conspicuously, this gene is part of a small cluster of genes with similar expression patterns, which contains four additional differentially regulated RLKs (Jukanti et al. 2008) (see online supplementary data of that publication).

Using a map-based cloning approach, Uauy et al. (2006) established that a NAC transcription factor (*TtNAM-B1*) is responsible for the influence of a wheat chromosome 6B locus on senescence control and grain protein concentration. A potential orthologue, *HvNAM-1*, was found in a co-linear region of barley chromosome 6H (Distelfeld et al. 2008); allelic variation in the regulation or function of this gene is most likely responsible for differences in the senescence behavior of the barley germplasm mentioned in the previous paragraph. The functional

importance of *NAC* genes in senescence regulation is now well established (Balazadeh et al. 2010; Fischer 2012; Guo and Gan 2006; Zhang and Gan 2012), but NAC transcription factors also have roles in plant responses to abiotic stress and pathogen attack (Al-Daoud and Cameron 2011; Carviel et al. 2009; Jensen et al. 2007; Mao et al. 2012; You et al. 2013). Furthermore, cross-talk between processes involved in senescence regulation and plant pathogen defense has repeatedly been demonstrated (Fischer 2012). Based on this background, the goal of the present study was to initiate the functional characterization of *rbaal9i05_at/AK249842*, and to dissect its roles in plant senescence control, plant pathogen defense, and crosstalk between these processes.

2. Materials and methods

2.1 Bioinformatic analyses

Previous transcriptomic analysis has identified a putative *LRR-RLK* (Affymetrix Barley1 GeneChip designation: *rbaal9i05_at*) as highly expressed in line ‘10_11’ (Jukanti et al. 2008) (see introduction). The *rbaali05_at* sequence was obtained from HarvEST barley (<http://harvest.ucr.edu/>) (Close et al. 2007), and the corresponding full-length cDNA sequence (GenBank accession: AK249842) was identified through a BLAST search at <http://blast.ncbi.nlm.nih.gov/Blast.cgi>. The translated AK249842 sequence was then analyzed using the following bioinformatic tools: (1) MyDomains (<http://prosite.expasy.org/mydomains/>) (Sigrist et al. 2013) and SignalP (<http://www.cbs.dtu.dk/services/SignalP/>) (Petersen et al. 2011) were used to characterize the protein’s domain structure; (2) BLAST searches against the Rice Kinase Phylogenomics Database (<http://ricephylogenomics.ucdavis.edu/kinase/>) (Dardick et al. 2007; Jung et al. 2010) were used to determine to which LRR-RLK subfamily AK249842 belongs and to identify homologs from rice; (3) multiple full-length sequence alignments with homologs from *Arabidopsis* and rice were performed using CLUSTAL W (Thompson et al. 1994); and (4) evolutionary relationships between AK249842, closely related rice sequences and previously characterized rice and *Arabidopsis* PRRs were inferred using the neighbor-joining method (Saitou and Mei 1987). Specifically, the optimal phylogenetic tree with a total branch length of 3.94 was calculated. The percentage of replicate trees in which the associated sequences clustered together in the bootstrap test (1000 replicates) were also determined (Felsenstein, 1985). Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007).

Data deposited in BarleyBase/PLEXdb (<http://www.plexdb.org/plex.php?database=Barley>) (Dash et al. 2012; Wise et al. 2007) were used for expression analysis, specifically to determine

the dependence of *AK249842* transcript levels on the allelic state of the mildew resistance *a* (*Mla*) locus (see results). Gene co-expression analysis was performed using both BarleyBase/ PLEXdb and the ‘co-expressed gene network in barley’ (Mochida et al. 2011) (<http://coexpression.psc.riken.jp/barley/index.pl>).

2.2 Plant material and growth conditions

Barley plants (*Hordeum vulgare* L.; early-senescing line ‘10_11’) (described by Jukanti et al. 2008) were grown two plants per four-liter pot in potting soil in a growth room (Convion, Winnipeg, Manitoba, Canada) with a 25/20 °C day/night temperature cycle, a 16 h photoperiod and a light intensity of $\sim 250 \mu\text{mol}^{-2} \text{s}^{-1}$ at leaf level. Plants were fertilized with 250 ml of Peter’s Professional General Purpose fertilizer (4 g l⁻¹; Scotts-Sierra Horticultural Products Company, Marysville, OH, USA) per pot at 2, 4, and 6 weeks after planting. Leaves were sequentially tagged along the main shoot from leaf 1 (the first, oldest leaf) through leaf 8.

2.3 Virus-induced gene silencing (VIGS) experimental design

Experiments consisted of four groups, namely (1) untreated control plants (‘control’), (2) plants inoculated with *Barley Stripe Mosaic Virus* (BSMV) without any included plant sequences (‘empty virus’ or ‘EV’), (3) plants inoculated with BSMV containing a silencing construct for *rbaal9i05_at / AK249842* (‘rbaal’), and (4) plants inoculated with two silencing constructs, for both *rbaal9i05_at / AK249842* and for *phytoene desaturase* (‘co-silencing’) (see below for details). Sixteen (16) plants (2 plants each in 8 pots) were used per treatment, and the entire experiment was independently replicated three times.

All plants in groups 2, 3 and 4 were evaluated visually for viral infection symptoms and for photobleaching (group 4 only); plants which did not pass these tests (indicating that inoculation was not successful) were excluded from further analyses. Additionally, target gene (*rbaal9i05_at / AK249842*) knockdown was tested individually for plants in groups 3 and 4, using gene-specific qRT-PCR assays (described in section 2.7). Based on these tests, slightly lower numbers of successful inoculation and gene knockdown were observed in the ‘co-silencing’ than in the ‘rbaal’ samples. Therefore, for each of the three independent experiments, leaves from five plants (‘co-silencing’) or seven plants (‘control’, ‘EV’, ‘rbaal’) were utilized for further analysis. For microarray hybridization, validation of microarray experiments by qRT-PCR and for peroxidase activity assays, leaves from each treatment within an experiment were pooled to obtain sufficient material, leading to three independent ‘control’, ‘EV’, ‘rbaal’ and

‘co-silencing’ samples from the three experimental replications, with each sample consisting of five or seven leaves.

2.4 AK249842 cloning and VIGS silencing construct

Primers for RT-PCR were designed from the full-length cDNA sequence (see appendix A, Table A.1 for new primer sequences used in this study). RNA extractions were performed as described below for gene expression analysis. cDNA was synthesized using the RetroScript First Strand Synthesis Kit (Ambion, Austin, TX, USA). PCR was performed using Phusion High Fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA) followed by an overhang A addition reaction (Qiagen A-Addition Kit, Qiagen, Valencia, CA, USA) to facilitate cloning into the pDrive vector using the Qiagen PCR Cloning Plus kit following the manufacturer’s instructions. Inserts from the isolated plasmids were sequenced for confirmation.

Virus-induced gene silencing (VIGS) constructs were designed following the guidelines in Scofield et al. (2005). **PCR primers for the VIGS-*rbaal* silencing construct (γ -*rbaal*) were designed to amplify a 188 bp product coding for a part of the protein’s extracellular LRR domain (and matching the length of the 185 bp γ -*PDS4* insert to facilitate co-silencing, see below).** The 188 bp product was ligated into the γ -PCR vector, a modified version of the γ vector containing PCR-ready cloning sites (Campbell and Huang 2010). To ensure specificity of the utilized sequence for *AK249842*, the sequence was BLASTed against the whole-genome shotgun assembly of the barley genome (Mayer et al. 2012) available at <http://webblast.ipk-gatersleben.de/barley/viroblast.php>. Results identified one barley gene (*AK361316*) with a single 25 bp perfect match with the sequence used for *AK249842* silencing. It has previously been reported that VIGS requires a minimum stretch of 23 identical nucleotides, suggesting that our silencing construct could also influence expression of *AK361316* (Lacomme et al. 2003; Thomas et al. 2001). We therefore identified the most closely related probe on the Barley1 GeneChip, using the ‘find your gene’ function of the BarleyBase/PLEXdb database. Results indicated that nine of the eleven probes (25-mers) used for contig26180_at detection are perfect matches for the *AK361316* sequence. Re-analyzing raw microarray expression values (see below) for all perfect-match probes across all experimental conditions indicated that expression of this gene, unlike *AK249842*, is not influenced by the VIGS treatment. Therefore, effects observed in the present study can be assumed to be due to specific silencing of the investigated gene, *AK249842*.

2.5 VIGS inoculation

The *Barley Stripe Mosaic Virus* (BSMV) VIGS vectors α , β , γ and γ -*PDS4* (Scofield et al. 2005) were obtained from Large Scale Biology Corporation (Vacaville, CA, USA). The vector γ -*PDS4* contains part of the phytoene desaturase (*pds*) gene used as a silencing reporter. RNA transcripts of the VIGS vectors were synthesized by *in vitro* transcription using RNA cap structure analog, rNTP mixture and T7 RNA polymerase (New England Biolabs, Ipswich, MA) using the linearized α , β , γ , γ -*rbaal* and γ -*pds4* plasmids and following the protocols outlined in Scofield et al. (2005).

For leaf inoculation, BSMV VIGS RNA was combined in equal ratios of α , β , and γ transcripts in an inoculation buffer containing a bentonite/celite wounding agent (Scofield et al. 2005). There were four VIGS treatments: Untreated control ('control'); empty virus control ('EV') consisting of α : β : γ RNA in a 1:1:1 ratio; *rbaal9i05_at* ('rbaal') consisting of α : β : γ -*rbaal* RNA in a 1:1:1 ratio; *rbaal9i05/pds4* ('co-silencing') consisting of α : β : γ -*rbaal*: γ -*pds4* RNA in a 1:1:0.5:0.5 ratio. Each transcript combination was mixed with excess inoculation buffer and leaf 4 was inoculated with 22 μ l of the appropriate transcript mix as described by Scofield et al. (2005). As described under 'experimental design', 16 plants (2 plants each in 8 pots) were used per treatment ('control', 'EV', 'rbaal', 'co-silencing') and the entire experiment was independently replicated three times.

The plants were evaluated visually for viral infection and photobleaching patterns on all subsequent emerging leaves, as described by Holzberg et al. (2002). Based on this evaluation, 7th leaves were harvested individually from each inoculated plant one week after full expansion, immediately frozen in liquid nitrogen and stored at -80 °C until analysis. Photobleaching effects indicated that strong gene knockdown was also present in 8th leaves, but those were not analyzed for the present study.

2.6 RNA extraction

RNA was extracted from individual leaves as described in Parrott et al. (2007). RNA samples were incubated with recombinant RNAsin (Promega, Madison, WI, USA), and RNA concentration and integrity were determined using an RNA 6000 nano assay (Bioanalyzer 2100, Agilent Technologies, Palo Alto, CA, USA). For qRT-PCR screening of gene silencing, RNA samples from each harvested leaf were kept separate and analyzed individually. As mentioned under 'experimental design', RNA samples from five ('co-silencing') or seven (all other treat-

ments) leaves were pooled and used for microarray analysis, qRT-PCR validation of microarray data, and for peroxidase activity assays.

2.7 Gene expression analysis

Quantitative real-time RT-PCR (qRT-PCR) was used to both screen individual leaf 7 RNA for determination of successful *rbaal9i05_at* gene silencing and to validate microarray data. qRT-PCR was performed as previously described (Parrott et al. 2007). Expression levels of target genes were normalized to the levels of the two genes chosen as standards (high, contig97_s_at; low, contig13298_at) utilizing a standard curve (Rotor-Gene Q 2.0.24, Qiagen, Valencia, CA, USA) (Parrott et al. 2007; Parrott et al. 2010).

Primers for the detection of VIGS-*rbaal* silencing (appendix A, Table A.1) were designed approximately 2 kb downstream of the silencing region in order to avoid false positive detection of the silencing construct. VIGS EV, *rbaal* and co-silencing treated leaves were individually screened for *rbaal* gene expression. RNA samples (from *rbaal*- and co-silencing treated plants) with expression levels that were at least 50 percent lower than empty virus control samples were considered silenced and used for further experiments (section 2.8). Screening was necessary to avoid using samples in which gene knockdown was not successful, which would have confounded microarray analysis.

Primers for microarray validation were designed using the sequence information in HarvEST Barley (<http://harvest.ucr.edu/>) (Close et al. 2007) for contig2768_s_at (germin), contig7007_s_at (thionin), contig21617_at (peroxidase), contig12191_s_at (peroxidase), contig3214_at (defensin), and contig4336_at (disease resistance gene/protein) using the OligoPerfect primer design software (Invitrogen) as previously described (Parrott et al. 2007; Parrott et al. 2010) (sequences are shown in appendix A, Table A.1).

2.8 Microarray analysis

Pooled leaf RNA samples were used for microarray analysis. Three biological replications (from three independent experiments; see ‘experimental design’) were tested for each of the treatments (‘control’, ‘EV’, ‘*rbaal*’ and ‘co-silencing’) resulting in 12 samples (three replications of 4 treatments), each hybridized to a corresponding chip (Barley1 22k genome array, Affymetrix, Santa Clara, CA, USA) (described in Close et al. 2004). Chip preparation, hybridization and analysis were performed at the Montana State University Functional Genomics Core Facility (Bozeman, MT, USA). Procedures for reverse transcription, cRNA synthesis and label-

ing, chip hybridization, washing, staining and scanning were performed according to the manufacturer's instructions (Affymetrix). For the Barley1 GeneChip, 10 µg cRNA was hybridized to each chip.

For microarray data analysis, the 12 CEL files were imported into FlexArray v1.5 (Blazejczyk et al. 2007), and were normalized using gcRMA (display is log2). Data was filtered for: (1) present calls in at least one replicate set, (2) scaled signal intensity >100 in at least one replicate set, and (3) a fold change of at least 2 in one or more comparisons. An analysis of variance (ANOVA) with Benjamini and Hochberg (1995) false discovery rate correction was also performed, and a final list containing 352 genes was generated. The log2 values of the 352 genes were imported into Genesis (Sturn et al. 2002), were median centered (centers expression on 0), and hierarchically clustered.

Microarray data were deposited in both BarleyBase/PLEXdb (Experiment BB108) and Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>; Accession GSE34102) (Barrett et al. 2013).

2.9 Peroxidase assays

Peroxidases were extracted from three replicates of 500 mg each of 'control', 'EV', 'rbaal' and 'co-silenced' leaf 7 tissue. Samples were ground on ice in 500 µl of cold extraction buffer (2 mM MgCl₂, 20 mM NaCl, 0.01% NP-40, 10 mM Tris-HCl, pH 8.0), in a 1.5 ml centrifuge tube using a motorized pestle, then centrifuged at 15,000 x g for 10 min to pellet cellular debris. Supernatants were transferred to fresh tubes and placed on ice.

Peroxidase assays were conducted using the peroxidase guaiacol assay (Faizyme Laboratories, Philippi, South Africa; <http://faizyme.com/assaperg.htm>), based on Bergmeyer (1974). Briefly, 2.8 ml buffer (0.1 M potassium phosphate, pH 7.0), 0.05 ml guaiacol (0.018 M), 0.05 ml substrate (0.1 ml 30% H₂O₂ diluted to 120 ml in ddH₂O) were mixed and equilibrated at 25 °C in a 1 cm cuvette. Leaf extract (0.10 ml) was added and mixed and the absorbance was read kinetically at 436 nm for 3 minutes. Peroxidase activity was calculated over a 1 min period using the linear portion of the curve (Bergmeyer 1974).

2.10 Statistical analysis

Analysis of microarray raw data is described above. Microarray-based, normalized expression values of selected genes, qRT-PCR-based gene expression and peroxidase activity data

were evaluated by One-Way Analysis of Variance (ANOVA) and post-hoc testing, using the corresponding functions in SigmaPlot v.12 (Systat Software, Inc., San Jose, CA, USA).

3. Results

3.1 *AK249842 is a member of subfamily XII, non-RD LRR-RLKs*

Bioinformatic analysis of the protein sequence translated from the *AK249842* full-length cDNA identifies all domains commonly associated with LRR-RLKs, including a signal peptide (presumably absent in the mature protein), at least 14 leucine-rich repeats, a membrane-spanning domain, and a canonical (intracellular) protein kinase domain (Fig. 1A, B). BLAST-

Fig. 1. Predicted protein domains and functional sites for rbaal9i05_at / *AK249842*. (A) Domain image showing signal peptide (SP), leucine-rich repeats (LRR), transmembrane domain (TM) and protein kinase domain. The diamond indicates the kinase active site. (B) Protein sequence locations for N-terminal signal peptide and transmembrane domain (black highlighted text), leucine-rich repeats (light gray highlighted text) and protein kinase domain (dark gray highlighted text). Underlined text indicates the location of the VIGS silencing construct, and underlined/italicized text shows the sequence used for qRT-PCR analysis of gene expression. The cysteine and aspartate (CD) residues identifying this protein as a non-RD protein kinase are boxed.

ing of the *AK249842* protein sequence against the Rice Kinase Phylogenomics Database (Dardick et al. 2007; Jung et al. 2010) (<http://ricephylogenomics.ucdavis.edu/kinase/>) indicates that the 10 closest matches all belong to LRR-RLK subfamily XII, with the five closest matches grouped in a conspicuous cluster within this subfamily (appendix A, Fig. A.1). The relationship

between these sequences was further explored through full-length sequence alignments of AK249842, four rice LRR XIIs from the BLAST search, and the best-characterized plant LRR XIIs from *Arabidopsis* and rice (EFR, FLS2 and Xa21), with human protein kinase B (UniProt P31751), a typical RD kinase, for comparison (appendix A, Fig. A.2). This analysis identified important catalytic (conserved) residues in all compared kinase domains, and also demonstrated that AK249842, like EFR, FLS2 and Xa21, belongs to the non-RD kinases (see introduction). Data shown in Fig. 2 (phylogenetic analysis) and in Fig. A.2 (appendix A) indicate that the

Fig. 2. Evolutionary relationships between rbaal9i05_at / AK249842, closely related rice sequences (see appendix, Fig. A.1), and previously characterized rice and *Arabidopsis* LRR XII-RKs. See materials and methods for details.

compared sequences are substantially different from each other (mostly due to divergence in the LRR domains important for ligand binding), and that Os02g40240 is most closely related to AK249842 in this comparison.

3.2 Knockdown of AK249842 expression by virus-induced gene silencing reduces transcript levels of numerous genes involved in pathogen defense

Virus-induced gene silencing (VIGS) has been successfully used to analyze the functions of diverse genes in cereals, including genes involved in pathogen resistance (Hein et al. 2005; Hu et al. 2009; Meng et al. 2009; Meng and Wise 2012; Panwar et al. 2013; Scofield et al. 2005). To gain direct functional information on barley AK249842, we used a VIGS system derived from *Barley Stripe Mosaic Virus* (BSMV) (Bruun-Rasmussen et al. 2007; Campbell and Huang 2010; Holzberg et al. 2002; Scofield et al. 2005). We included a sequence stretch coding for

part of the LRR domain (Fig. 1B; appendix A, Table A.1) in the silencing vector to target *AK249842* transcripts. During the VIGS assay, we noted that target gene expression was reduced to some extent by virus inoculation *per se* (no included plant gene sequences, labeled ‘empty virus’ or EV in figures and tables), but was further reduced by at least 50% (as compared to the EV control) when attempting to silence *AK249842* (treatment labeled ‘rbaal’; Fig. 3). Furthermore, when plants were co-inoculated with constructs silencing both *AK249842* and

Fig. 3. VIGS knockdown of *rbaal9i05_at* / *AK249842* in barley leaf 7. Gene expression levels were compared in untreated control (‘control’) leaves, in leaves obtained from plants treated empty virus, in leaves obtained from plants treated with BSMV containing the *rbaal9i05_at* silencing construct (‘rbaal’), and in leaves obtained from plants co-inoculated with *rbaal9i05_at* and *pds* silencing constructs (‘co-silencing’). Means and standard deviations are shown for three independent experiments.

phytoene desaturase (*pds*, used as a visual marker for successful gene silencing) (Holzberg et al. 2002; Scofield et al. 2005), the same level of knockdown was achieved (‘co-silencing’, Fig. 3). This result confirms observations by Campbell and Huang (2010), indicating that the utilized method can be used to silence two genes simultaneously by mixing the corresponding RNA products immediately prior to inoculation. Seventh and 8th leaves of plants co-inoculated with *AK249842* and *pds* silencing constructs exhibited typical photobleaching symptoms (data not shown), as previously described by other authors utilizing the BSMV system for *pds* knockdown in wheat and barley (Holzberg et al. 2002; Scofield et al. 2005).

To characterize leaves/plants, in which successful *AK249842* knockdown was achieved, 7th leaves were analyzed through transcriptomics, using the Affymetrix Barley1 GeneChip (described in Close et al. 2004), previously utilized in our laboratory to analyze barley leaf senescence (Jukanti et al. 2008; Parrott et al. 2007). This approach identified 352 genes, which were (significantly) differentially regulated between two or more of the applied controls and treatments (appendix A; Fig. A.3 and Table A.2). Based on expression patterns, these genes fell into 8 clusters (appendix A, Fig. A.3). Numerous genes were more or less strongly induced by virus treatment *per se* (‘empty virus’), but their expression was reduced with respect to the ‘empty virus’ treatment when the mentioned *AK249842* sequence segment was included in the

virus γ -genome. Genes in clusters 1, 4, 5, 6, 7 and 8 in Fig. A.3 (appendix A) fell into that category. In contrast, expression of a smaller number of genes in clusters 2 and 3 was reduced, to a similar extent, by all virus treatments (including ‘empty virus’ treatment, *rbaal9i05_at* / *AK249842* silencing, and co-silencing with *pds*). Importantly, behavior of these genes indicates that virus virulence (speed of spreading in the plant) was similar for all three treatments, leading to comparable defense reactions. This fact allows the conclusion that differences in gene behavior between ‘empty virus’ and *rbaal9i05_at* / *AK249842* silencing seen in the other clusters (1, 4, 5, 6, 7 and 8) are specific for knockdown of the gene analyzed here, and can be used to analyze *rbaal9i05_at* / *AK249842* function.

Many of the genes upregulated by BSMV treatment but downregulated (with respect to that control) when *AK249842* was knocked down have well-established or tentative functions in plant pathogen defense. These genes include several proteases, protease inhibitors, cell-wall-modifying proteins (glucanases, extensins, expansin), three disease resistance response proteins, three peroxidases, a germin, a thionin, and a defensin (Table 1). A majority of these genes were strongly induced by virus inoculation (‘empty virus’ treatment, EV). Furthermore, their transcript levels, while still higher than in untreated controls, were substantially lower in leaves with knocked-down *rbaal9i05_at*/*AK249842* expression, indicating that their expression partially depends on transcript (and presumably protein) levels of the LRR-RLK investigated here (Table 1).

To confirm and expand microarray data, a subset of six defense-related genes from Table 1 were further analyzed by qRT-PCR (Table 2). This experiment confirmed gene expression trends shown in Table 1. Virus treatment strongly enhanced expression of these genes, while downregulating *rbaal9i05_at* / *AK249842* reduced expression of analyzed genes by 28-76% with respect to ‘empty virus’ controls (Table 2). Regulation of the expression of genes listed in Tables 1 and 2 by *AK249842* knockdown indicates a role for *AK249842* in plant pathogen defense, possibly (based on bioinformatic analysis) as a novel pattern recognition receptor (PRR).

3.3 AK249842 expression is controlled by allelic variation at the Mla locus, and is induced by Blumeria graminis and by Puccinia graminis infection

With respect to a role for *AK249842* in plant pathogen defense, it appears intriguing that its expression levels are strongly influenced by allelic variation at the mildew resistance locus *a* (*Mla*), which is located on the short arm of chromosome 5 (1H) (Wei et al. 1999). Based on an experiment performed with near-isogenic germplasm varying in the allelic state of that locus

Table 1

Expression values of plant defense-related genes, as determined by microarray analysis.

Expression values are shown in untreated control leaves, in leaves obtained from plants treated with BSMV without any inserted plant sequences ('empty virus'), in leaves obtained from plants treated with BSMV containing the *rbaal9i05_at* silencing construct ('rbaal'), and in leaves obtained from plants co-inoculated with *rbaal9i05_at* and *phytoene desaturase* silencing constructs ('co-silencing'). Contig numbers are based on Affymetrix Barley1 GeneChip designations (Close *et al.*, 2004).

Contig number	Putative function	Normalized gene expression			
		Control	Empty virus	rbaal	Co-silencing
Contig5807_s_at	Protease inhibitor/seed storage/LTP family protein precursor	13.7 ± 9.5 ^a	2767 ± 569 ^b	1409 ± 108 ^{a,b}	1790 ± 490 ^{a,b}
Contig12421_at	Extensin family protein	2.9 ± 1.1 ^a	290 ± 126 ^b	188 ± 62.4 ^{a,b}	151 ± 28.7 ^{a,b}
HVSMEh0020G09f_s_at	Protease inhibitor/seed storage/LTP family protein precursor	32.0 ± 18.9 ^a	2922 ± 596 ^b	1673 ± 354 ^c	1764 ± 674 ^c
Contig5760_at	Stress-responsive α/β barrel domain-containing protein	3.7 ± 0.9 ^a	679 ± 241 ^b	273 ± 59.1 ^{a,c}	305 ± 152 ^{a,b}
Contig2768_s_at	Germin-like protein 1	8.0 ± 7.4 ^a	1210 ± 324 ^b	402 ± 53.0 ^{a,c}	454 ± 198 ^{a,c}
HS05F12u_s_at	<i>A. thaliana</i> expansin A1	20.8 ± 9.2 ^a	919 ± 319 ^b	701 ± 28.6 ^b	741 ± 246 ^b
Contig553_s_at	Hydroxyproline-rich glycoprotein	3.9 ± 0.8 ^a	364 ± 54.7 ^b	168 ± 7.1 ^c	204 ± 72.6 ^c
HVSMEh0099O01f_s_at	Proteinase inhibitor	1.7 ± 0.2 ^a	199 ± 127 ^b	66.6 ± 42.6 ^{a,b}	83.1 ± 14.1 ^{a,b}
Contig4321_at	Extensin-like protein	8.5 ± 4.1 ^a	538 ± 143 ^b	224 ± 41.5 ^c	242 ± 119 ^c
Contig61_s_at	Extensin family protein precursor	3.6 ± 0.6 ^a	251 ± 56.3 ^b	88.4 ± 23.5 ^{a,c}	107 ± 45.5 ^c
Contig5808_at	Extensin-like protein	14.7 ± 9.1 ^a	856 ± 251 ^b	257 ± 92.4 ^{a,c}	335 ± 178 ^{a,c}
Contig4320_at	Extensin-like protein	5.7 ± 2.6 ^a	620 ± 199 ^b	161 ± 28.4 ^{a,c}	203 ± 112 ^c
Contig10558_at	Putative aspartic proteinase nepenthesin precursor	9.6 ± 3.3 ^a	287 ± 117 ^b	133 ± 19.2 ^{a,b}	114 ± 62.4 ^{a,b}
Contig2671_at	Xyloglucan endo-1,4- β -D-glucanase	4.3 ± 0.9 ^a	223 ± 96.0 ^b	65.3 ± 19.3 ^{a,c}	77.6 ± 36.9 ^{a,c}
HF25F14r_s_at	Leucine-rich repeat family protein / extensin family protein	4.4 ± 1.0 ^a	203 ± 41.0 ^b	67.1 ± 30.1 ^{a,c}	88.7 ± 48.8 ^{a,c}
Contig7007_s_at	Thionin Osth1	3.6 ± 1.0 ^a	210 ± 83.4 ^b	50.9 ± 30.9 ^{a,c}	66.2 ± 43.3 ^{a,c}
HVSMEb0012G11r2_s_at	Extensin-like protein	7.3 ± 6.2 ^a	596 ± 227 ^b	118 ± 9.7 ^{a,c}	141 ± 95.0 ^{a,c}
Contig5759_at	Cysteine endopeptidase EP-A	3.1 ± 0.2 ^a	7.2 ± 1.1 ^b	5.6 ± 0.2 ^{b,c}	5.1 ± 1.1 ^c
Contig5272_s_at	Putative carboxypeptidase	3.8 ± 0.2 ^a	13.1 ± 1.1 ^b	7.7 ± 0.1 ^c	9.5 ± 2.5 ^c
Contig14613_at	Leucine-rich repeat family protein / extensin family protein	3.3 ± 0.6 ^a	12.4 ± 3.0 ^b	8.1 ± 1.6 ^{a,b}	8.1 ± 2.1 ^{a,b}
Contig18702_at	Endo-1,4- β -glucanase precursor	5.6 ± 0.3 ^a	22.8 ± 5.4 ^b	12.1 ± 1.0 ^{a,c}	13.4 ± 5.4 ^{a,b}
Contig10481_at	Protease inhibitor/seed storage/LTP family protein precursor	3.2 ± 0.9 ^a	13.5 ± 3.0 ^b	5.2 ± 0.3 ^{a,c}	6.6 ± 1.8 ^{a,c}
HE01I24u_s_at	Xyloglucan endo-1,4- β -D-glucanase	82.8 ± 25.9 ^a	369 ± 109 ^b	233 ± 26.1 ^{a,b}	262 ± 39.2 ^b
Contig18723_at	Putative subtilisin-like protease	5.0 ± 2.5 ^a	77.6 ± 38.3 ^a	39.6 ± 7.4 ^a	38.1 ± 6.2 ^a
Contig21617_at	Putative peroxidase	1.8 ± 0.3 ^a	30.3 ± 10.5 ^b	13.4 ± 3.0 ^{a,c}	13.1 ± 5.0 ^{a,c}
Contig13657_at	Putative peroxidase	3.8 ± 0.8 ^a	44.9 ± 30.0 ^b	23.0 ± 1.5 ^{a,b}	18.4 ± 3.7 ^{a,b}
HVSMEg0005E17r2_s_at	Disease resistance-responsive family protein	10.0 ± 3.0 ^a	84.3 ± 17.6 ^b	45.5 ± 4.9 ^c	58.0 ± 13.0 ^c
Contig20222_at	Putative carboxypeptidase D	2.9 ± 0.4 ^a	25.0 ± 14.7 ^a	12.8 ± 0.2 ^a	15.6 ± 5.2 ^a
Contig11980_at	Putative subtilisin homolog	21.3 ± 8.5 ^a	124 ± 16.1 ^b	83.7 ± 24.8 ^c	83.3 ± 12.8 ^{b,c}
Contig20713_at	Stress-responsive α/β barrel domain-containing protein	14.0 ± 5.2 ^a	124 ± 21.1 ^b	49.1 ± 8.9 ^c	67.6 ± 14.3 ^c
Contig13906_at	β -1,3-glucanase-like protein	4.6 ± 0.2 ^a	56.1 ± 15.0 ^b	21.5 ± 8.0 ^{a,c}	23.6 ± 3.0 ^{a,c}
Contig12191_s_at	Peroxidase	4.4 ± 0.5 ^a	134 ± 79.7 ^b	35.1 ± 5.6 ^{a,b}	36.0 ± 18.2 ^{a,b}
Contig16732_at	Extensin-like protein	3.4 ± 1.1 ^a	99.3 ± 43.9 ^b	20.9 ± 2.6 ^{a,c}	29.5 ± 23.1 ^{a,c}
Contig3214_at	Defensin	3.5 ± 0.4 ^a	76.9 ± 15.9 ^b	18.6 ± 8.2 ^{a,c}	20.0 ± 13.0 ^{a,c}
Contig11988_at	CND41-like protease	17.0 ± 2.4 ^a	93.0 ± 17.2 ^b	54.7 ± 10.7 ^c	55.5 ± 6.0 ^c
Contig4336_at	Disease resistance response protein-related / dirigent protein-related	5.4 ± 0.9 ^a	45.1 ± 3.2 ^b	17.6 ± 5.7 ^c	17.9 ± 5.7 ^c
Contig4483_s_at	Protease inhibitor / seed storage / LTP family protein precursor	9.7 ± 4.7 ^a	41.7 ± 9.1 ^b	22.4 ± 0.9 ^{a,c}	21.0 ± 2.3 ^{a,c}
HVSMEb0012G11r2_x_at	Extensin-like protein	4.0 ± 0.7 ^a	19.8 ± 2.4 ^b	8.8 ± 0.5 ^{a,b}	9.3 ± 2.9 ^{a,b}
Contig7916_at	Disease resistance response-like protein	7.3 ± 0.3 ^a	40.9 ± 11.0 ^b	17.8 ± 4.7 ^{a,c}	21.3 ± 3.3 ^{a,c}
Contig2673_at	Xyloglucan endo-1,4- β -D-glucanase	4.3 ± 0.7 ^a	73.6 ± 13.4 ^b	13.7 ± 3.7 ^{a,c}	20.0 ± 2.7 ^{a,c}

Table 2

Relative expression values of six plant defense-related genes from Table 1, as determined by qRT-PCR.

Gene expression levels were compared in untreated control leaves, in leaves obtained from plants treated with BSMV without any inserted plant sequences ('empty virus'), in leaves obtained from plants treated with BSMV containing the *rbaal9i05_at* silencing construct ('rbaal'), and in leaves obtained from plants co-inoculated with *rbaal9i05_at* and *phytoene desaturase* silencing constructs ('co-silencing'). Contig numbers are based on Affymetrix Barley1 GeneChip designations (Close *et al.*, 2004).

Contig number	Putative function	Control	Empty virus	rbaal	Co-silencing
Contig2768_s_at	Germin	0.18 ± 0.20 ^a	17.3 ± 7.13 ^b	5.61 ± 0.90 ^{a,b}	6.32 ± 2.37 ^{a,b}
Contig7007_s_at	Thionin	0.019 ± 0.016 ^a	1.40 ± 0.38 ^b	0.66 ± 0.29 ^c	0.68 ± 0.17 ^c
Contig21617_at	Peroxidase	<0.01 ^a	1.06 ± 0.36 ^b	0.25 ± 0.11 ^{a,b}	0.30 ± 0.19 ^{a,b}
Contig12191_s_at	Peroxidase	<0.01 ^a	0.84 ± 0.13 ^b	0.31 ± 0.11 ^c	0.34 ± 0.09 ^c
Contig3214_at	Defensin	<0.01 ^a	0.71 ± 0.37 ^b	0.33 ± 0.12 ^{a,b}	0.51 ± 0.25 ^{a,b}
Contig4336_at	Disease res.	<0.01 ^a	0.12 ± 0.01 ^b	0.058 ± 0.028 ^{a,c}	0.068 ± 0.029 ^c

(Moseman 1972), *AK249842* expression levels are constitutively high in lines containing the *Mla1* and *Mla13* alleles, and powdery mildew infection (*Blumeria graminis* f. sp. *hordei*; *Bgh*) further increased *AK249842* expression (Fig. 4A; data obtained from BarleyBase/PLEXdb experiment BB4 at <http://www.plexdb.org/plex.php?database=Barley>; Caldo et al. 2004). Data shown in Fig. 4A are based on inoculation with isolate *Bgh* 5874 (*AvrMla1* and *AvrMla6*), but identical results were obtained by Caldo et al. (2004) with a different isolate (*Bgh* K1; *AvrMla1* and *AvrMla13*) (data not shown; available in BarleyBase). In contrast, *AK249842* expression was low in a line containing the *Mla6* allele, and it was not induced by infection with either *Bgh* 5874 (Fig. 4A) or *Bgh* K1 (not shown). Experiment BB10 (Caldo et al. 2006; Moscou et al. 2011a) indicates that *AK249842* expression levels in an *Mla1* deletion mutant (*m1a1-m508*) were identical to results obtained with the *Mla1* line, including gene induction after *Bgh* 5874

Fig. 4. Influence of mildew resistance locus *a* (*Mla*) allelic variation and *Blumeria graminis* f. sp. *hordei* inoculation on *rbaal9i05_at* / *AK249842* expression. Expression data were obtained from BarleyBase/PLEXdb, and are based on deposited experiments BB4 (Caldo et al., 2004) and BB10 (Caldo et al., 2006; Moscou et al., 2011a). (A) Black columns, germplasm containing the *Mla1* allele; white columns, germplasm containing the *Mla6* allele; gray columns, germplasm containing the *Mla13* allele. (B) Black columns, germplasm containing the *Mla1* allele; white columns, germplasm containing the *Mla6* allele; dark gray columns, germplasm containing the *Mla1* deletion mutant *m1a1-m508*; light gray columns, germplasm containing the *Mla6* deletion mutant *m1a6-m9472*. Means and standard deviations of three replications are shown. *Mla6* is significantly different from *Mla1/13* and from deletion mutants for all time points.

inoculation (Fig. 4B). Interestingly, gene expression levels in an *Mla6* deletion mutant (*m1a6-m9472*) were almost as high as in plants containing *Mla1* or *m1a1-m508*, suggesting that presence of a functional *Mla6* allele is responsible for low *AK249842* expression (Fig. 4B). Experiment BB49 (Zhang et al. 2008) shows strong induction of *AK249842* expression after stem rust (*Puccinia graminis* f. sp. *tritici*; *Pgt*) infection of barley variety ‘Golden Promise’, and this induction was independent of the presence of the *Resistance to Puccinia graminis 1* [*Rpg1*] gene (Fig. 5). Experiments were performed with *Pgt* pathotypes ‘MCC’ (avirulent for *Rpg1*) and

Fig. 5. Influence of *Puccinia graminis* f. sp. *tritici* inoculation on *rbaal9i05_at* / *AK249842* expression. Expression data were obtained from BarleyBase/PLEXdb, and are based on deposited experiment BB49 (Zhang *et al.*, 2008). Black columns, inoculation of barley variety ‘Golden Promise’; white columns, inoculation of variety ‘Golden Promise’ transformed with the *Rpg1* gene (see text). Means and standard deviations of three replications are shown.

‘QCC’ (virulent for *Rpg1*); while data for experiments performed with MCC are shown in Fig. 5, the pathotype had no influence on *AK249842* expression (data not shown; available in BarleyBase). In an independent experiment (BB64) (Moscou et al. 2011b) using *Pgt* race TTKSK on barley lines Q21861, SM89010 and a doubled-haploid mapping population generated from a single Q21861 x SM89010 F1 plant (Steffenson et al. 1995), *AK249842* expression was primarily controlled by plant genetic background, and not induced by *Pgt* inoculation (data not shown; available in BarleyBase).

Considering organ-specific gene expression, BarleyBase experiment BB3 (Druka et al. 2006) indicates that *AK249842* expression is strongly upregulated in seedling leaves as compared to all other analyzed organs and tissues (data not shown), suggesting that this LRR-RLK is primarily associated with leaf defense responses.

3.4 *AK249842* expression is co-regulated with several additional receptor-like protein kinases

Analysis of co-expressed genes within experiment BB4 identifies several additional (LRR)-RLKs which, like *AK249842*, show low expression in lines containing the *Mla6* allele, but high or higher expression in presence of *Mla1* and *Mla13* (appendix A, Fig. A.4). Interestingly, three additional (putative) LRR-RLKs were identified which exhibit an inverse pattern in response to the allelic state of the *Mla* locus (low in presence of *Mla1* and *Mla13*, but high or higher in presence of *Mla6*) (appendix A, Fig. A.5), while yet other putative PRRs show low expression in presence of *Mla13*, but high or higher expression in presence of both *Mla1* and *Mla6* (appendix A, Fig. A.6). Differential regulation of RLKs in response to the *Mla* allelic status, including some of those shown in Figs. A.4 and A.5, has previously been noted by Hu and Wise (2008). Together, these data suggest that the *Mla* locus exerts its influence on plant pathogen resistance at least partially through the control of a series of RLKs.

Broader co-expression analysis of *AK249842* across 45 publicly available experiments, using the dataset published by Mochida et al. (2011) (<http://coexpression.psc.riken.jp/barley/index.pl>), identified nine (putative) RLKs among the 10 most highly co-expressed genes (the exception is a β -glucanase), indicating co-regulation of *AK249842* with multiple related genes across a series of genetic backgrounds and experimental conditions (appendix A, Fig. A.7).

3.5 Knockdown of *AK249842* reduces peroxidase activities

To obtain protein/activity-level information on one functional category of genes listed in Tables 1 and 2, peroxidase activity was assayed using the guaiacol assay. Similarly to transcript levels of genes influenced by treatments used in this study, peroxidase activity was strongly (~6-fold) induced by ‘empty virus’ treatments, but reduced (with respect to that control) when

Fig. 6. Peroxidase activity in barley leaf 7. Activities were compared in untreated control (‘control’) leaves, in leaves obtained from plants treated with BSMV without any inserted plant sequences (‘empty virus’), in leaves obtained from plants treated with BSMV containing the *rbaal9i05_at* silencing construct (‘rbaal’), and in leaves obtained from plants co-inoculated with *rbaal9i05_at* and *phytoene desaturase* silencing constructs (‘co-silencing’). Means and standard deviations of three independent experiments are shown.

AK249842 expression was knocked-down, and in *AK249842/pds* co-silencing treatments (Fig. 6). These data indicate that, at least for some of the genes controlled by *AK249842*, reductions in gene expression are correlated with reductions in protein level and activity.

4. Discussion

Analysis of the *Arabidopsis* and rice genomes has indicated the presence of numerous genes with high degrees of homology to the few functionally characterized plant microbial pattern recognition receptors (PRRs). Also, as discovered by Shiu et al. (2004), certain PRR families are considerably larger in rice than in *Arabidopsis*. Functional characterization of additional PRRs, in model species as well as in major crops, has the potential to expand our fundamental understanding of plant-microbe (including plant-pathogen) interactions, and may enlarge the arsenal of genes that can be deployed to protect crop yields from excessive losses.

Based on a transcriptomic screen comparing near-isogenic barley germplasm varying in its leaf and whole-plant senescence behavior, the authors' lab has previously identified a small cluster of putative LRR-RLKs which are highly upregulated in early vs. late-senescing lines (Jukanti et al. 2008; Lacerenza et al. 2010) (see introduction). These lines differ in the allelic state of a NAC transcription factor (*HvNAM-1*) (Distelfeld et al. 2008). The importance of NAC proteins in senescence regulation, plant pathogen defense and abiotic stress resistance is now well-established, and extensive cross-talk exists between signaling pathways controlling senescence and stress responses (see introduction; also Gepstein and Glick 2013). To contribute to the dissection of that regulatory network, it appeared promising to initiate the functional characterization of the LRR-RLK which is most highly upregulated in the early-senescing lines of our original transcriptomic analysis, *rbaal9i05_at* /*AK249842* (Jukanti et al. 2008).

Bioinformatic analysis clearly established that *AK249842* is a subfamily XII, non-RD LRR-RLK, and therefore related to some of the best-investigated plant PRRs (*Arabidopsis* EFR and FLS2, and rice Xa21) (Figs. 1 and 2; appendix A, Figs. A.1 and A.2). Virus-induced silencing of the gene allowed a consistent two-fold reduction in *AK249842* transcript levels in the investigated leaves, as compared to controls inoculated with BSMV without any included plant gene sequences ('empty virus' control) (Fig. 3). While that reduction may appear modest, it is within the range of previous VIGS studies, which have led to successful gene functional analysis (Campbell and Huang 2010; Scofield et al. 2005; Van der Linde et al. 2010). Furthermore, co-silencing of *AK249842* and *pds* (coding for phytoene desaturase, a gene needed for carotenoid biosynthesis) led to the previously described photobleaching phenotype, confirming compatibil-

ity of the used barley germplasm with BSMV-based VIGS (Holzberg et al. 2002; Scofield et al. 2005).

Visual observation of *AK249842* knockdown leaves did not indicate differences in plant senescence behavior, and non-destructive leaf chlorophyll analysis using a Minolta SPAD-502 meter was not practicable, as the instrument transferred virus particles from virus-treated to untreated control plants, most likely through small lesions (unpublished observations). Leaves in which successful *AK249842* knockdown was achieved (Fig. 3) were therefore subjected to transcriptomic analysis, to identify genes whose expression is directly or indirectly dependent on *AK249842* (Table 1; appendix A, Fig. A.3 and Table A.2).

Numerous genes with confirmed or putative roles in pathogen defense were strongly induced in response to plant inoculation with BSMV ('empty virus' vs. untreated controls; Table 1). These genes are likely involved in anti-viral defense responses triggered after BSMV inoculation. Induction of different functional classes of 'pathogenesis-related proteins' after virus infection has previously been demonstrated (Bol et al. 1990; Sudisha et al. 2012); this finding is therefore not surprising. Importantly, these genes were downregulated (with respect to the 'empty virus' control) when *AK249842* expression was reduced through gene-specific VIGS. This result clearly indicates that expression of these genes, while induced by BSMV infection, is also dependent on the LRR-RLK investigated here (Fig. 7). Our data therefore strongly sug-

Fig. 7. Control of pathogenesis-related genes/proteins by *AK249842* (solid arrows) and by host defense against BSMV (dashed arrows). Pathogenesis-related genes, including those listed in Tables 1 and 2, are induced both in response to viral (BSMV) infection and in response to the detection of MAMPs by *AK249842*. VIGS-induced knockdown of *AK249842* interferes with signaling dependent on this protein, leading to reduced *PR* gene expression (as compared to the 'empty virus' control; indicated by the solid ⊥ symbol). In addition, this model postulates that plant defense against BSMV leads to lowered *AK249842* expression (Fig. 3), as indicated by the dashed ⊥ symbol.

gest a role for *AK249842* in plant pathogen defense, possibly (based on bioinformatic analysis) as a novel PRR. This interpretation assumes that some level of *AK249842*-dependent signaling

is occurring in our experimental plants, which appears feasible, as they were not grown under aseptic conditions.

To expand microarray data, a subset of six defense-related genes from Table 1 were further analyzed by qRT-PCR (Table 2). Barley germins have been demonstrated to be induced by pathogen attack and exogenous hydrogen peroxide application, and a role for some of these proteins in pathogen defense was demonstrated by transient silencing and overexpression assays (Zimmermann et al. 2006). Contig7077_s_at shows homology to thionin precursors. Mature thionins are members of a family of basic peptides with low molecular weight (~5 kDa), which present toxicity against bacterial, fungal, animal and even plant cells (Castro and Fontes 2005). Barley leaf thionin expression is induced after inoculation with *Blumeria graminis* f. sp. *hordei* (Bohlmann et al. 1988), and transgenic expression of a barley thionin in tobacco increased resistance against *Pseudomonas syringae* (Carmona et al. 1993). Peroxidases have long been known as ‘pathogenesis-related’ (PR) proteins, and are upregulated during plant-pathogen interactions (Geddes et al. 2008). Many plant defensins, sometimes also called γ -thionins (despite low similarity to the previously discovered α/β -thionins), demonstrate potent fungicide action (Castro and Fontes 2005). The sequence represented by contig4336_at encodes a dirigent(-like)/disease resistance responsive protein, which may be involved in enhanced cell wall lignification and pathogen resistance (Shi et al. 2012). Confirming microarray data, expression of all these genes was low in untreated controls (Table 2). As previously shown for different classes of PR genes/proteins (Bol et al. 1990; Sudisha et al. 2012), these genes were strongly induced after viral inoculation (‘empty virus’ control). Importantly, again as indicated by microarray analysis, their expression was reduced by AK249842-specific VIGS, with statistically significant reductions observed for the analyzed thionin, one peroxidase and the dirigent-like genes (Table 2). These data strengthen the hypothesis that AK249842 functions in plant pathogen defense.

Intriguingly, despite the fact that AK249842 was identified in a screen comparing near-isogenic germplasm varying in senescence behavior, experiments reported here did not identify differential regulation of known or putative ‘Senescence-Associated Genes’ (SAGs) in response to AK249842 knockdown. It can, however, not be excluded that analyzed leaves were harvested too early in their development (they were fully expanded, but clearly not senescing) to identify effects on senescence regulation. Alternatively, achieved knockdown, while sufficient to identify downstream genes important for pathogen defense, may have been insufficient to probe a pleiotropic influence on senescence regulation. Our data could also be explained by the possibility that, while HvNAM-1, like its homolog in wheat (*TtNAM-B1*) (Uauy et al. 2006) is re-

sponsible for observed differences in senescence behavior of our near-isogenic germplasm (Heidlebaugh et al. 2008; Jukanti et al. 2008), a different, linked gene controls expression of *AK249842* and additional *LRR-RLKs*. This question could only be resolved through additional experiments aimed at fine-mapping the barley chromosome 6H locus.

Roles in both bacterial and fungal perception have been demonstrated for subfamily XII LRR-RLKs (Dardick et al. 2012). At this point, it is unclear what type of microbe or pathogen might be perceived by *AK249842*, but dependence of *AK249842* expression on the allelic state of the *Mla* locus (Mildew Resistance Locus *a*) is intriguing. It has previously been pointed out that *Mla1*, 6, 7, 10, 12 and 13 could be alleles of one of several intracellular *Nucleotide Binding Site-Leucine-Rich Repeat* (*NBS-LRR*) genes present at that complex locus (Halterman and Wise 2004; Seeholzer et al. 2010). *Mla 1*, 6 and 13 are all known to confer resistance against *Blumeria graminis* f. sp. *hordei* infection (Caldo et al. 2006); yet *AK249842* expression was constitutively high when the *Mla1* or *Mla13* alleles were present, but low in presence of the *Mla6* allele. Furthermore, some gene induction was observed after *Bgh* inoculation in germplasm with the *Mla1* or 13, but not *Mla6* alleles (Fig. 4A). Data shown in Fig. 5 indicate that *AK249842* expression is also induced after inoculation of barley variety ‘Golden Promise’ with the causing agent of stem rust, *Puccinia graminis* f. sp. *tritici* (*Pgt*), suggesting that *AK249842* is involved in defense reactions against fungal pathogens. Intriguingly, expression of several additional *RLK* genes is regulated in parallel to *AK249842* in response to the *Mla* allelic status (appendix A, Fig. A.4), while other *RLKs* are regulated differently in the various barley lines containing *Mla1*, *Mla6* or *Mla 13* (appendix A, Figs. A.5 and A.6). Differential regulation of putative barley *RLK* genes in response to the *Mla* allelic state has previously been noted by Hu and Wise (2008). In this context, it has been demonstrated that co-silencing of three wheat *RLK* genes (*TaRLK-R1*, -R2 and -R2) led to a more susceptible phenotype than specific silencing of single genes (Zhou et al. 2007); control of several *RLKs* by specific *Mla* alleles may therefore lead to more robust disease resistance than upregulation of a single gene. Hu and Wise (2008) speculated that *RLKs* may have coevolved with different *Mla* alleles, which would lead to their cultivar-dependent expression in different *Mla* backgrounds.

In summary, the combination of experimental and bioinformatic approaches utilized here suggests that *AK249842* is an LRR-RLK, possibly involved in the detection of fungal pathogens, and co-regulated with several similar genes/proteins in response to the allelic state of the *Mla* locus. In contrast, senescence regulation, downstream of *HvNAM-1*, may not be an (important) function of this kinase. Our results will facilitate further functional studies, including identification/confirmation of the detected pathogens. Informed by previous studies charac-

terizing plant PRRs such as FLS2, EFR and CERK1 (Felix et al. 1999; Kunze et al. 2004; Petutschnig et al. 2010), it will be necessary to identify the molecular pattern recognized by AK249842 to establish its function as a PRR and fully evaluate its importance in barley defense responses.

Author contributions

David L. Parrott performed the experiments, with technical expertise provided by Li Huang. David L. Parrott, Li Huang and Andreas M. Fischer designed the experiments. David L. Parrott and Andreas M. Fischer wrote the manuscript, with critical comments and help from Li Huang.

Acknowledgements

Help with microarray experiments (chip hybridization, washing; data analysis) was provided by Kate McInnerney at the Montana State University Genomics Core Facility. The *Barley Stripe Mosaic Virus* (BSMV) VIGS vectors α , β , γ and γ -*PDS4* (Scofield et al. 2005) were obtained from Large Scale Biology Corporation (Vacaville, CA, USA). This work was supported by grant IOS-0918037 from the National Science Foundation to AMF, by the USDA National Institute of Food and Agriculture (NIFA; Hatch project 0192396), and by the Montana Agricultural Experiment Station (MAES). Funding sources had no role in study design.

Appendix A: Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/xxx>.

Fig. A.1. Identification of closely related rice receptor(-like) protein kinases. The *rbal9i05_at* / AK249842 sequence was BLASTed against the Rice Kinase Phylogenomics Database (Dardick *et al.*, 2007; Jung *et al.*, 2010; <http://ricephylogenomics.ucdavis.edu/kinase/>). Twenty-seven (27) sequences producing significant alignments are listed in the right-side panel. The left-side panel shows the LRR XII (sub)family from the cited web resource, with the 10 sequences most closely related to AK249842 highlighted by red numbers. The 5 closest matches form a distinct sub-cluster (boxed in red).

Fig. A.2. Multiple sequence alignment of *rbal9i05_at* / AK249842, closely related rice sequences (see Fig. A.1), previously characterized rice and *Arabidopsis* LRR XII-RKs, and human protein kinase B (for comparison). Sequence alignments were obtained using CLUSTAL W (Thompson *et al.*, 1994). Catalytic residues are repeated below the alignment segments in black and bold; contact residues in green, and other residues interacting with contact residues and specifically conserved in serine/threonine or tyrosine kinases are highlighted in red (see Krupa *et al.*, 2004 for detailed information on kinase structure and function). The location of the cysteine-aspartate residues (CD) identifying AK249842 and related proteins as non-RD kinases is boxed in blue (note that protein kinase B, used for comparison, is a ‘classical’ RD kinase).

Fig. A.3. Hierarchical cluster analysis of all differentially regulated genes. Gene expression levels (based on

microarray analysis of three independent experiments) are compared in untreated control ('control') leaves, in leaves obtained from plants treated with BSMV without any inserted plant sequences ('EV'), in leaves obtained from plants treated with BSMV containing the *rbaal9i05_at* silencing construct ('rbaal'), and in leaves obtained from plants co-inoculated with *rbaal9i05_at* and phytoene desaturase silencing constructs ('co-inoc'). Red and blue correspond to up- and downregulation, respectively.

Fig. A.4. Heat map of 30 genes with similar (to *rbaal9i05_at* / *AK249842*) expression profiles. BarleyBase/PLEXdb experiment BB4 (Caldo *et al.*, 2004) was used to obtain a heat map of 30 genes, which demonstrate similar expression profiles (to *rbaal9i05_at* / *AK249842*) in response to the *Mla* allelic status. The row representing *rbaal9i05_at* / *AK249842* is boxed in red to facilitate orientation. Putative functions of the listed contigs are shown on the second page. hai, hours after inoculation; Bgh, *Blumeria graminis* f. sp. *hordei*; CI, Moseman Cereal Introduction lines (Moseman, 1972).

Fig. A.5. Heat map of 30 genes with opposite (to *rbaal9i05_at* / *AK249842*) expression profiles. BarleyBase/PLEXdb experiment BB4 (Caldo *et al.*, 2004) was used to obtain a heat map of 30 genes, which demonstrate low expression in lines containing the *Mla1* and *Mla13* alleles, but higher expression in the line containing the *Mla6* allele (compare with Fig. A.4). Putative receptor-like protein kinases are boxed in red. Rice sequences most closely related to contig4666_at and contig6205_at code for LRR XII, non-RD kinases, while the rice sequence most closely related to contig4998_s_at codes for an LRK10L-2, non-RD kinase. hai, hours after inoculation; Bgh, *Blumeria graminis* f. sp. *hordei*; CI, Moseman Cereal Introduction lines (Moseman, 1972).

Fig. A.6. Heat map of 15 genes with different (from *rbaal9i05_at* / *AK249842*) expression profiles. BarleyBase/PLEXdb experiment BB4 (Caldo *et al.*, 2004) was used to obtain a heat map of 15 genes, which demonstrate moderate to high expression in lines containing the *Mla1* and *Mla6* alleles, but low expression in the line containing the *Mla13* allele (compare with Figs. A.4 and A.5). Putative receptor-like protein kinases are boxed in red. Rice sequences most closely related to contig19845_at and contig22980_at code for LRR XII, non-RD kinases, while no hits were found in the rice kinase database for HV_CEB0001H04r2_at. hai, hours after inoculation; Bgh, *Blumeria graminis* f. sp. *hordei*; CI, Moseman Cereal Introduction lines (Moseman, 1972).

Fig. A.7. *AK249842* co-expression analysis, using the 'co-expressed gene network in barley' database (Mochida *et al.*, 2011; <http://coexpression.psc.riken.jp/barley/index.pl>).

Table A.1. Primer sequences used for cloning, VIGS and qRT-PCR.

Table A.2. All (significantly) differentially regulated genes detected by microarray analysis.

References

- Afzal AJ, Wood AJ, Lightfoot DA (2008) Plant receptor-like serine threonine kinases: roles in signaling and plant defense. *Mol. Plant Microbe In.* 21: 507–517
- Al-Daoud F, Cameron RK (2011) ANAC055 and ANAC092 contribute non-redundantly in an EIN2-dependent manner to Age-Related Resistance in *Arabidopsis*. *Physiol. Mol. Plant Pathol.* 76: 212–222
- Antolin-Llovera M, Ried MK, Binder A, Parniske M (2012) Receptor kinase signaling pathways in plant-microbe interactions. *Annu. Rev. Phytopathol.* 50: 451–473
- Balazadeh S, Siddiqui H, Allu AD, Matallana-Ramirez LP, Caldana C, Mehrnia M, Zanol M-I, Köhler B, Mueller-Roeber B (2010) A gene regulatory network controlled by the NAC transcription factor ANAC092/AtNAC2/ORE1 during salt-promoted senescence. *Plant J.* 62: 250–264

Barrett T, Wilhite SE, Ledoux P, Evangelista C, Kim IF, Tomashevsky M, Marshall KA, Phillippy KH, Sherman PM, Holko M, Yefanov A, Lee H, Zhang N, Robertson CL, Serova N, Davis S, Soboleva A (2013) NCBI GEO: archive for functional genomics data sets--update. *Nucleic Acids Res.* 41: D991-D995

Benjamini Y, Hochberg Y (1995) Controlling the False Discovery Rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society Series B - Methodological OR Series B - Statistical Methodology* 57: 289-300

Bergmeyer HU (1974) *Methods of enzymatic analysis* 1. Academic Press, New York

Blazejczyk M, Miron M, Nadon R (2007) FlexArray: A statistical data analysis software for gene expression microarrays. Genome Quebec, Montreal, Canada

Bohlmann H, Clausen S, Behnke S, Giese H, Hiller C, Reimann-Philipp U, Schrader G, Barkholt V, Apel K (1988) Leaf-specific thionins of barley - a novel class of cell wall proteins toxic to plant-pathogenic fungi and possibly involved in the defence mechanism of plants. *EMBO J.* 7: 1559-1565

Bol JF, Linthorst HJM, Cornelissen BJC (1990) Plant pathogenesis-related proteins induced by virus infection. *Annu. Rev. Phytopathol.* 28: 113-138

Boller T, Felix G (2009) A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annu. Rev. Plant Biol.* 60: 379-406

Bruun-Rasmussen M, Madsen CT, Jessing S, Albrechtsen M (2007) Stability of *barley stripe mosaic virus*-induced gene silencing in barley. *Mol. Plant Microbe In.* 20: 1323-1331

Caldo RA, Nettleton D, Peng J, Wise RP (2006) Stage-specific suppression of basal defense discriminates barley plants containing fast- and delayed-acting *Mla* powdery mildew resistance alleles. *Mol. Plant Microbe In.* 19: 939-947

Caldo RA, Nettleton D, Wise RP (2004) Interaction-dependent gene expression in *Mla*-specified response to barley powdery mildew. *Plant Cell* 16: 2514-2528

Campbell J, Huang L (2010) Silencing of multiple genes in wheat using barley stripe mosaic virus. *J. Biotech Res.* 2: 12-20

Carmona MJ, Molina A, Fernández JA, López-Fando JJ, García-Olmedo F (1993) Expression of the α -thionin gene from barley in tobacco confers enhanced resistance to bacterial pathogens. *Plant J.* 3: 457-462

Carviel JL, Al-Daoud F, Neumann M, Mohammad A, Provart NJ, Moeder W, Yoshioka K, Cameron RK (2009) Forward and reverse genetics to identify genes involved in the age-related resistance response in *Arabidopsis thaliana*. *Mol. Plant Pathol.* 10: 621-634

Castro MS, Fontes W (2005) Plant defense and antimicrobial peptides. *Protein and Peptide Letters* 12: 11-16

Close TJ, Wanamaker S, Roose ML, Lyon M (2007) HarvEST. An EST database and viewing software. *Meth. Mol. Biol.* 406: 161-177

Close TJ, Wanamaker SI, Caldo RA, Turner SM, Ashlock DA, Dickerson JA, Wing RA, Muehlbauer GJ, Kleinhofs A, Wise RP (2004) A new resource for cereal genomics: 22K barley GeneChip comes of age. *Plant Physiol.* 134: 960-968

Dardick C, Chen J, Richter T, Ouyang S, Ronald P (2007) The rice kinase database. A phylogenomic database for the rice kinome. *Plant Physiol.* 143: 579-586

Dardick C, Schwessinger B, Ronald P (2012) Non-arginine-aspartate (non-RD) kinases are associated with innate immune receptors that recognize conserved microbial signatures. *Curr. Opin. Plant Biol.* 15: 358-366

- Dash S, Van Hemert J, Hong L, Wise RP, Dickerson JA (2012) PLEXdb: gene expression resources for plants and plant pathogens. *Nucleic Acids Res.* 40: D1194-D1201
- Distelfeld A, Korol A, Dubcovsky J, Uauy C, Blake T, Fahima T (2008) Colinearity between the barley grain protein content (GPC) QTL on chromosome arm 6HS and the wheat *Gpc-B1* region. *Mol. Breeding* 22: 25-38
- Druka A, Muehlbauer G, Druka I, Caldo R, Baumann U, Rostoks N, Schreiber A, Wise R, Close T, Kleinhofs A, Graner A, Schulman A, Langridge P, Sato K, Hayes P, McNicol J, Marshall D, Waugh R (2006) An atlas of gene expression from seed to seed through barley development. *Funct. Integr. Genomics* 6: 202-211
- Felix G, Duran JD, Volko S, Boller T (1999) Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *Plant J.* 18: 265-276
- Felsenstein J (1985) Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39: 783-791
- Fischer AM (2012) The complex regulation of senescence. *Crit. Rev. Plant Sci.* 31: 124-147
- Geddes J, Eudes F, Laroche A, Selinger LB (2008) Differential expression of proteins in response to the interaction between the pathogen *Fusarium graminearum* and its host, *Hordeum vulgare*. *Proteomics* 8: 545-554
- Gepstein S, Glick BR (2013) Strategies to ameliorate abiotic stress-induced plant senescence. *Plant Mol. Biol.* 82: 623-633
- Guo Y, Gan S (2006) AtNAP, a NAC family transcription factor, has an important role in leaf senescence. *Plant J.* 46: 601-612
- Halterman DA, Wise RP (2004) A single-amino acid substitution in the sixth leucine-rich repeat of barley MLA6 and MLA13 alleviates dependence on RAR1 for disease resistance signaling. *Plant J.* 38: 215-226
- Heidlebaugh NM, Trethewey BR, Jukanti AK, Parrott DL, Martin JM, Fischer AM (2008) Effects of a barley (*Hordeum vulgare*) chromosome 6 grain protein content locus on whole-plant nitrogen reallocation under two different fertilisation regimes. *Funct. Plant Biol.* 35: 619-632
- Hein I, Barciszewska-Pacak M, Hrubikova K, Williamson S, Dinesen M, Soenderby IE, Sundar S, Jarmolowski A, Shirasu K, Lacomme C (2005) Virus-induced gene silencing-based functional characterization of genes associated with powdery mildew resistance in barley. *Plant Physiol.* 138: 2155-2164
- Holzberg S, Brosio P, Gross C, Pogue GP (2002) Barley stripe mosaic virus-induced gene silencing in a monocot plant. *Plant J.* 30: 315-327
- Hu P, Meng Y, Wise RP (2009) Functional contribution of chorismate synthase, anthranilate synthase, and chorismate mutase to penetration resistance in barley-powdery mildew interactions. *Mol. Plant Microbe In.* 22: 311-320
- Hu P, Wise RP (2008) Diversification of *Lrk/Tak* kinase gene clusters is associated with subfunctionalization and cultivar-specific transcript accumulation in barley. *Funct. Integr. Genomics* 8: 199-209
- Jensen MK, Rung JH, Gregersen PL, Gjetting T, Fuglsang AT, Hansen M, Joehnk N, Lyngkjaer MF, Collinge DB (2007) The *HvNAC6* transcription factor: a positive regulator of penetration resistance in barley and *Arabidopsis*. *Plant Mol. Biol.* 65: 137-150
- Johnson LN, Noble MEM, Owen DJ (1996) Active and inactive protein kinases: structural basis for regulation. *Cell* 85: 149-158
- Jukanti AK, Heidelbaugh NM, Parrott DL, Fischer IA, McInnerney K, Fischer AM (2008) Comparative transcriptome profiling of near-isogenic barley (*Hordeum vulgare*) lines differing in the allelic state of a major grain protein content locus identifies genes with possible roles in leaf senescence and nitrogen reallocation. *New Phytol.* 177: 333-349

Jung K-H, Cao P, Seo Y-S, Dardick C, Ronald PC (2010) The Rice Kinase Phylogenomics Database: a guide for systematic analysis of the rice kinase super-family. *Trends Plant Sci.* 15: 595-599

Krupa A, Preethi G, Srinivasan N (2004) Structural modes of stabilization of permissive phosphorylation sites in protein kinases: distinct strategies in Ser/Thr and Tyr kinases. *J. Mol. Biol.* 339: 1025-1039

Kunze G, Zipfel C, Robatzek S, Niehaus K, Boller T, Felix G (2004) The N terminus of bacterial elongation factor Tu elicits innate immunity in *Arabidopsis* plants. *Plant Cell* 16: 3496-3507

Lacerenza JA, Parrott DL, Fischer AM (2010) A major grain protein content locus on barley (*Hordeum vulgare* L.) chromosome 6 influences flowering time and sequential leaf senescence. *J. Exp. Bot.* 61: 3137-3149

Lacomme C, Hrubikova K, Hein I (2003) Enhancement of virus-induced gene silencing through viral-based production of inverted-repeats. *Plant J.* 34: 543-553

Mao X, Zhang H, Qian X, Li A, Zhao G, Jing R (2012) *TaNA C2*, a NAC-type wheat transcription factor conferring enhanced multiple abiotic stress tolerances in *Arabidopsis*. *J. Exp. Bot.* 63: 2933-2946

Mayer KF, Waugh R, Langridge P, Close TJ, Wise RP, Graner A, Matsumoto T, Sato K, Schulman A, Muehlbauer GJ, Stein N, Ariyadasa R, Schulte D, Poursarebani N, Zhou R, Steuernagel B, Mascher M, Scholz U, Shi B, Madishetty K, Svensson JT, Bhat P, Moscou M, Resnik J, Hedley P, Liu H, Morris J, Frenkel Z, Korol A, Berges H, Taudien S, Felder M, Groth M, Platzer M, Himmelbach A, Lonardi S, Duma D, Alpert M, Cordero F, Beccuti M, Ciardo G, Ma Y, Wanamaker S, Cattonaro F, Vendramin V, Scalabrin S, Radovic S, Wing R, Morgante M, Nussbaumer T, Gundlach H, Martis M, Poland J, Spannagl M, Pfeifer M, Moisy C, Tanskanen J, Zuccolo A, Russell J, Druka A, Marshall D, Bayer M, Swarbreck D, Sampath D, Ayling S, Febrer M, Caccamo M, Tanaka T, Wanamaker S, Schmutzer T, Brown JW, Fincher GB (2012) A physical, genetic and functional sequence assembly of the barley genome. *Nature* 491: 711-716

Meng Y, Moscou MJ, Wise RP (2009) *Blufensin1* negatively impacts basal defense in response to barley powdery mildew. *Plant Physiol.* 149: 271-285

Meng Y, Wise RP (2012) HvWRKY10, HvWRKY19, and HvWRKY28 regulate *Mla*-triggered immunity and basal defense to barley powdery mildew. *Mol. Plant Microbe In.* 25: 1492-1505

Mochida K, Uehara-Yamaguchi Y, Yoshida T, Sakurai T, Shinozaki K (2011) Global landscape of a co-expressed gene network in barley and its application to gene discovery in Triticeae crops. *Plant Cell Physiol.* 52: 785-803

Moscou MJ, Lauter N, Caldo RA, Nettleton D, Wise RP (2011a) Quantitative and temporal definition of the *Mla* transcriptional regulon during barley-powdery mildew interactions. *Mol. Plant Microbe In.* 24: 694-705

Moscou MJ, Lauter N, Steffenson B, Wise RP (2011b) Quantitative and qualitative stem rust resistance factors in barley are associated with transcriptional suppression of defense regulons. *PLoS Genet.* 7: e1002208

Moseman JG (1972) Isogenic barley lines for reaction to *Erysiphe graminis* f. sp. *hordei*. *Crop Sci.* 12: 681-682

Panwar V, McCallum B, Bakkeren G (2013) Host-induced gene silencing of wheat leaf rust fungus *Puccinia tritici-na* pathogenicity genes mediated by the Barley Stripe Mosaic Virus. *Plant Mol. Biol.* 81: 595-608

Parrott DL, Martin JM, Fischer AM (2010) Analysis of barley (*Hordeum vulgare*) leaf senescence and protease gene expression: a family C1A cysteine protease is specifically induced under conditions characterized by high carbohydrate, but low to moderate nitrogen levels. *New Phytol.* 187: 313-331

Parrott DL, McInnerney K, Feller U, Fischer AM (2007) Steam-girdling of barley (*Hordeum vulgare*) leaves leads to carbohydrate accumulation and accelerated leaf senescence, facilitating transcriptomic analysis of senescence-associated genes. *New Phytol.* 176: 56-69

- Petersen TN, Brunak S, von Heijne G, Nielsen H (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nature methods* 8: 785-786
- Petutschnig EK, Jones AM, Serazetdinova L, Lipka U, Lipka V (2010) The lysin motif receptor-like kinase (LysM-RLK) CERK1 is a major chitin-binding protein in *Arabidopsis thaliana* and subject to chitin-induced phosphorylation. *J. Biol. Chem.* 285: 28902-28911
- Saitou N, Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4: 406-425
- Scofield SR, Huang L, Brandt AS, Gill BS (2005) Development of a virus-induced gene-silencing system for hexaploid wheat and its use in functional analysis of the *Lr21*-mediated leaf rust resistance pathway. *Plant Physiol.* 138: 2165–2173
- Seeholzer S, Tsuchimatsu T, Jordan T, Bieri S, Pajonk S, Yang W, Jahoor A, Shimizu KK, Keller B, Schulze-Lefert P (2010) Diversity at the *Mla* powdery mildew resistance locus from cultivated barley reveals sites of positive selection. *Mol. Plant Microbe In.* 23: 497-509
- Shi H, Liu Z, Zhu L, Zhang C, Chen Y, Zhou Y, Li F, Li X (2012) Overexpression of cotton (*Gossypium hirsutum*) *dirigent1* gene enhances lignification that blocks the spread of *Verticillium dahliae*. *Acta Biochim. Biophys. Sin.* 44: 555-564
- Shiu S-H, Bleecker AB (2001) Receptor-like kinases from Arabidopsis form a monophyletic gene family related to animal receptor kinases. *Proc. Natl. Acad. Sci. USA* 98: 10763-10768
- Shiu S-H, Bleecker AB (2003) Expansion of the receptor-like kinase/Pelle gene family and receptor-like proteins in Arabidopsis. *Plant Physiol.* 132: 530–543
- Shiu S-H, Karlowski WM, Pan R, Tzeng Y-H, Mayer KFX, Li W-H (2004) Comparative analysis of the receptor-like kinase family in Arabidopsis and rice. *Plant Cell* 16: 1220–1234
- Sigrist CJ, de Castro E, Cerutti L, Cuche BA, Hulo N, Bridge A, Bougueleret L, Xenarios I (2013) New and continuing developments at PROSITE. *Nucleic Acids Res.* 41: D344-347
- Steffenson BJ, Jin Y, Rossmann BG, Rasmussen JB, Kao K (1995) Genetics of multiple disease resistance in a doubled-haploid population of barley. *Plant Breeding* 114: 50-54
- Sturn A, Quackenbush J, Trajanoski Z (2002) Genesis: cluster analysis of microarray data. *Bioinformatics* 18: 207-208
- Sudisha J, Sharathchandra RG, Amruthesh KN, Kumar A, Shetty HS (2012) Pathogenesis related proteins in plant defense response. In: Mérillon JM, Ramawat KG (eds) *Plant defence: Biological control*. Springer, New York, pp 379-403
- Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24: 1596-1599
- Thomas CL, Jones L, Baulcombe DC, Maule AJ (2001) Size constraints for targeting post-transcriptional gene silencing and for RNA-directed methylation in *Nicotiana benthamiana* using a potato virus X vector. *Plant J.* 25: 417-425
- Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22: 4673-4680

- Uauy C, Distelfeld A, Fahima T, Blechl A, Dubcovsky J (2006) A NAC gene regulating senescence improves grain protein, zinc, and iron content in wheat. *Science* 314: 1298-1301
- Van der Linde K, Kastner C, Kumlehn J, Kahmann R, Doeblemann G (2010) Systemic virus-induced gene silencing allows functional characterization of maize genes during biotrophic interaction with *Ustilago maydis*. *New Phytol.* 189: 471-483
- Wei F, Gobelmann-Werner K, Morroll SM, Kurth J, Mao L, Wing R, Leister D, Schulze-Lefert P, Wise RP (1999) The *Mla* (powdery mildew) resistance cluster is associated with three NBS-LRR gene families and suppressed recombination within a 240-kb interval on chromosome 5S (1HS) of barley. *Genetics* 153: 1929-1948
- Wise RP, Caldo RA, Hong L, Shen L, Cannon E, Dickerson JA (2007) BarleyBase/PLEXdb. A unified expression profiling database for plants and plant pathogens. *Meth. Mol. Biol.* 406: 347-363
- You J, Zong W, Li X, Ning J, Hu H, Xiao J, Xiong L (2013) The SNAC1-targeted gene *OsSRO1c* modulates stomatal closure and oxidative stress tolerance by regulating hydrogen peroxide in rice. *J. Exp. Bot.* 64: 569-583
- Zhang K, Gan SS (2012) An abscisic acid-AtNAP transcription factor-SAG113 protein phosphatase 2C regulatory chain for controlling dehydration in senescing *Arabidopsis* leaves. *Plant Physiol.* 158: 961-969
- Zhang L, Castell-Miller C, Dahl S, Steffenson B, Kleinhofs A (2008) Parallel expression profiling of barley-stem rust interactions. *Funct. Integr. Genomics* 8: 187-198
- Zhou H, Li S, Deng Z, Wang X, Chen T, Zhang J, Schen S, Ling H, Zhang A, Wang D, Zhang X (2007) Molecular analysis of three new receptor-like kinase genes from hexaploid wheat and evidence for their participation in the wheat hypersensitive response to stripe rust fungus infection. *Plant J.* 52: 420-434
- Zimmermann G, Baumlein H, Mock HP, Himmelbach A, Schweizer P (2006) The multigene family encoding germin-like proteins of barley. Regulation and function in basal host resistance. *Plant Physiol.* 142: 181-192

Author contributions

David L. Parrott performed the experiments, with technical expertise provided by Li Huang. David L. Parrott, Li Huang and Andreas M. Fischer designed the experiments. David L. Parrott and Andreas M. Fischer wrote the manuscript, with critical comments and help from Li Huang.