

Regulation of basal resistance by a powdery mildew-induced cysteine-rich receptor-like protein kinase in barley

CBGOWDA RAYAPURAM¹, MICHAEL K. JENSEN^{1,2}, FABIAN MAISER¹, JOHANNA VIKGREN SHANIR¹, HENRIK HORNSHØJ^{1,†}, JESPER H. RUNG^{1,‡}, PER L. GREGERSEN^{1,3}, PATRICK SCHWEIZER⁴, DAVID B. COLLINGE¹ AND MICHAEL F. LYNKJÆR^{1,*}

¹Department of Plant Biology and Biotechnology, Faculty of Life Science, University of Copenhagen, 1871 Frederiksberg, Denmark

²Department of Biology, University of Copenhagen, 2200 Copenhagen, Denmark

³Department of Genetics and Biotechnology, Research Centre Flakkebjerg, University of Aarhus, 4200 Slagelse, Denmark

⁴Leibniz-Institute of Plant Genetics and Crop Plant Research, 06466 Gatersleben, Germany

SUMMARY

The receptor-like protein kinases (RLKs) constitute a large and diverse group of proteins controlling numerous plant physiological processes, including development, hormone perception and stress responses. The cysteine-rich RLKs (CRKs) represent a prominent subfamily of transmembrane-anchored RLKs. We have identified a putative barley (*Hordeum vulgare*) CRK gene family member, designated *HvCRK1*. The mature putative protein comprises 645 amino acids, and includes a putative receptor domain containing two characteristic 'domain 26 of unknown function' (duf26) domains in the N-terminal region, followed by a rather short 17-amino-acid transmembrane domain, which includes an AAA motif, two features characteristic of endoplasmic reticulum (ER)-targeted proteins and, finally, a characteristic putative protein kinase domain in the C-terminus. The *HvCRK1* transcript was isolated from leaves inoculated with the biotrophic fungal pathogen *Blumeria graminis* f.sp. *hordei* (*Bgh*). *HvCRK1* transcripts were observed to accumulate transiently following *Bgh* inoculation of susceptible barley. Transient silencing of *HvCRK1* expression in bombarded epidermal cells led to enhanced resistance to *Bgh*, but did not affect *R*-gene-mediated resistance. Silencing of *HvCRK1* phenocopied the effective penetration resistance found in *mlo*-resistant barley plants, and the possible link between *HvCRK1* and MLO was substantiated by the fact that *HvCRK1* induction on *Bgh* inoculation was dependent on *Mlo*. Finally, using both experimental and *in silico* approaches, we demonstrated that *HvCRK1* localizes to the ER of barley cells. The negative effect on basal resistance against *Bgh* and the functional aspects of MLO- and ER-localized *HvCRK1* signalling on *Bgh* inoculation are discussed.

INTRODUCTION

Receptor-like protein kinases (RLKs) constitute a diverse group of proteins involved in many plant signal transduction pathways regulating numerous plant physiological processes, including growth and development, hormone signalling, transpiration, symbiosis and pathogen defence (Dievart and Clark, 2004; Tichtinsky *et al.*, 2003). A prominent subfamily of RLKs, known as cysteine-rich RLKs (CRKs), are predicted to be transmembrane receptor proteins and are localized in the plasma membrane (Chen, 2001; Shiu and Bleecker, 2001). CRKs are characterized by the presence of one to four copies of domain 26 of unknown function (duf26), a C-X8-C-X2-C motif in the extracellular receptor region in the N-terminus and a serine/threonine kinase domain in the C-terminus (Chen, 2001; Shiu and Bleecker, 2001). The duf26 motifs are conserved domains spread throughout various protein families. According to EMBL-EBI (<http://www.ebi.ac.uk>), there are 520 proteins in Viridiplantae (green plants) that possess one or more duf26 domains. Of these, 263 are found in rice (*Oryza sativa*) and 97 in Arabidopsis (*Arabidopsis thaliana*), including 46 CRKs (Wrzaczek *et al.*, 2010). It has been suggested that the conserved cysteine residues in the duf26 motif direct the formation of three-dimensional structures through disulphide bonds (Chen, 2001) and that C-X2-C could be a binding site for transition metal ions (Dykema *et al.*, 1999). Both features may mediate protein-protein interactions important for the activation of CRKs. The expression of CRKs is induced by oxidative stress, pathogen attack and the application of salicylic acid (SA) (Du and Chen, 2000; Wrzaczek *et al.*, 2010), and it has been shown that CRK4, CRK5, CRK13, CRK19 and CRK20 are involved in the regulation of cell death in Arabidopsis (Acharya *et al.*, 2007; Chen *et al.*, 2003, 2004). Furthermore, overexpression of *CRK5* and *CRK13* in Arabidopsis increases resistance against different pathogens of the bacterial pathogen *Pseudomonas syringae* (Acharya *et al.*, 2007; Chen *et al.*, 2003).

Plant defence responses are primarily controlled by the transcriptional activation of specific stress genes (Glazebrook, 2001;

*Correspondence: Email: mlyn@life.ku.dk

†Present address: Department of Genetics and Biotechnology, Research Centre Foulum, University of Aarhus, Blichers Allé 20, 8830, Tjele, Denmark.

‡Present address: Novozymes, Krogshøjvej 36, Bagsværd 2880, Denmark.

Singh *et al.*, 2002). Several of these are induced in barley during attack of the biotrophic phytopathogenic fungus *Blumeria graminis* f.sp. *hordei* (*Bgh*), which causes powdery mildew (Collinge *et al.*, 2002; Gregersen *et al.*, 1997). Among these, a CRK gene transcript has also been reported to be induced after *Bgh* attack (Gregersen and Collinge, 2001). Interestingly, *Bgh* inoculated onto nonhost *Arabidopsis* induces the same expression profile of CRKs as both ozone and the bacterial microbe-associated molecular pattern (MAMP) molecules flagellin (flg22) and hairpin Z (HrpZ) (Wrzaczek *et al.*, 2010), indicating that CRKs in both *Arabidopsis* and barley play a central role in the mediation of the stress responses driven by apoplastic reactive oxygen species (ROS).

For successful infection, *Bgh* must overcome at least two levels of defence in barley: first, an early race-nonspecific basal resistance (i.e. MAMP-triggered immunity) and, second, a later race-specific (*R*-gene) resistance (i.e. effector-triggered immunity) involving hypersensitive host cell death, which prevents the establishment of a biotrophic interaction. Both types of resistance involve the accumulation of ROS (Thordal-Christensen *et al.*, 1997). Basal resistance is exhibited in susceptible as well as *R*-gene-resistant barley cultivars and is associated with cell wall modifications, including the formation of papillae that form in the apoplast in order to prevent *Bgh* germlings from penetrating the epidermal cell walls (Zeyen *et al.*, 2002). Papilla formation is complex and involves many cellular and biochemical processes, including the early generation of nitric oxide (Prats *et al.*, 2005), hydrogen peroxide (H₂O₂) (Thordal-Christensen *et al.*, 1997) and phenolics (Carver *et al.*, 1994), and localized deposition of callose below the attempted site of penetration (Zeyen *et al.*, 2002). If the host plant possesses *R* genes recognizing the attacking avirulent *Bgh* isolate, a hypersensitive cell death response is initiated in successfully penetrated epidermal cells and the *Bgh* biotrophic life cycle is thereby interrupted. However, if the *Bgh* isolate is virulent, penetrated host epidermal cells survive and *Bgh* forms an intracellular haustorium that mediates nutrient uptake and supports ectophytic hyphal development.

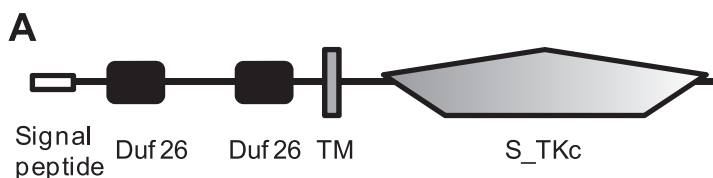
In barley, recessive loss-of-function alleles of the *Mlo* locus are known to mediate durable and broad-spectrum resistance towards *Bgh* as a result of highly effective penetration resistance (Jørgensen, 1994). Papilla formation is reported to occur earlier and is associated with higher levels of ROS and more rapid cross-linking of phenolics in *mlo*-resistant barley compared with susceptible wild-type *Mlo* barley (Lyngkjaer *et al.*, 2000). The functional *Mlo* gene encodes a seven-transmembrane-domain G-coupled receptor protein (Buschges *et al.*, 1997; Devoto *et al.*, 1999). MLO appears to mediate susceptibility to *Bgh* via a Ca²⁺-dependent interaction with calmodulin (Bhat *et al.*, 2005; Kim *et al.*, 2002), and it is considered that MLO is a target used by *Bgh* to gain entry to the host epidermal cell (Panstruga and Schulze-Lefert, 2003).

In this article, we present results concerning the functional characterization of *HvCRK1*, the first CRK isolated from barley. We examined *HvCRK1* expression in relation to *Bgh* attack on barley with or without *mlo* resistance and after treatment with H₂O₂ and SA. H₂O₂ treatment induced the accumulation of *HvCRK1* transcripts and we demonstrated that transcript accumulation after *Bgh* inoculation was dependent on MLO. Furthermore, reduction of *HvCRK1* expression by transient-induced gene silencing (TIGS) resulted in enhanced resistance to *Bgh*, whereas transient overexpression had no effect on basal and *R*-gene resistance. The cellular localization of *HvCRK1* was examined and the protein was found to accumulate in the endoplasmic reticulum (ER), which was also supported by an *in silico* approach. Taken together, these data suggest that *HvCRK1* is involved in the negative regulation of basal resistance.

RESULTS

Isolation of the *HvCRK1* cDNA and phylogenetic relationship

Using the differential display of mRNA, we identified a transcript encoding a putative barley member of the CRK gene family which accumulated following *Bgh* inoculation of barley (Gregersen and Collinge, 2001). The full-length cDNA of 2198 bp was obtained from a cDNA library of *Bgh*-inoculated barley by rapid amplification of cDNA ends (RACE). The cDNA included a single 1935-bp-long open reading frame, representing a coding region of 645 amino acids (Fig. 1) (EMBL-EBI Accession: FR717136). SMART analysis of the domain architecture of the deduced primary sequence identified a predicted extracellular region with a 22-amino-acid signal peptide and two cysteine-rich duf26 (C-X8-C-X2-C and C-X10-C-X2-C) domains, a transmembrane region and an acid serine/threonine kinase domain (Fig. 1). The gene product was designated *HvCRK1* as it represents the first report of a duf26-related CRK from barley. The closest homologue in rice was the AK120072 gene which encodes a protein product with 58.3% identity and 71.6% similarity to *HvCRK1* (EMBL-EBI, <http://www.ebi.ac.uk>). To delineate the evolutionary relationship of *HvCRK1* with other annotated plant CRKs, we performed a phylogenetic analysis using the conserved kinase region. RLK families can often be subdivided into clades based on the phylogenetic relationships between members, and genes encoding RLKs with related kinase sequences tend to have similar extracellular domains (Shiu and Bleecker, 2001; Shiu *et al.*, 2004). For this purpose, we used SMART (Schultz *et al.*, 2000) to define the kinase domain sequences of the entire genomic complement of duf26 subfamily RLKs from rice, poplar (*Populus trichocarpa*) and *Arabidopsis*. From the analysis, it is clear that the kinase domains cluster in distinct groups according to species, as only one small group contained members from rice, poplar and *Arabidopsis*. Accordingly,



B

```

1   gaacctcactctggcaaaagagaacaagaacctgtttagttagctactcttctgactcgc
61  tgtagcttgccatggcgaccatcgcgctcgtctctctctctctctaccacaccc
      M A T I G V V L L L L L L P T P
121 acacccttctggggcccaatttctgcaaacgctcaaggtagcgcgtgcccgcctt
      T P F L V A A N F C D N V K V A A A A L
181 tccaagaacgtaacctctcccggaaaacttcgccaccgaccttggccagcccccc
      S K N V T S S P E N F A T A T F G Q P P
241 gacgtcgtctacgcgctcgcgctctgcgcgcccggtagcgtcgtcgacgctccacctg
      D V V Y A L A L C R A G D V V D G S T C
301 agtgactgcctcactgggttcgctgcagtaaacagacggagtgccacaaggctccgc
      S D C I A N W F A A V N O T E C H K V R
361 tccaactacagcactctattctcgtctacacgcccagcagcaaacctaccgccacc
      S S Y G D C I V V Y S A G D D N L P P P
421 tcaaatgctatgggaggatcctccggtgagaacacgctgcctctcgagcagtgggacatc
      S N V M G G S S G E N T L P F E Q W D I
481 acgaacgtcaccaccggcgaactcactcgtcggcctaccgcgagttgctggtc
      T N V T T G D V Q L I V G L T R E L L V
541 gagacagcagacggcggcagcagcagcagcagcagcagcagcagcagcagcagcagc
      E T A E T A A S T T P S R Y A T G V I D
601 atggacagagtgacacactaccgaaggtgtactcccagcgcattgcatccgggacctg
      M D R V T T Y P K V Y S Q A H C M P D L
661 tccgagataactcctggcagcctcggcgtctcctcgggatggtcaactccaccatg
      S A D N C L A C L R R L L G M V N S T M
721 tccctccgcacgggtggcaaatgggcatcacgcaatgctatttcagtgcaagcttat
      S L R T G G Q M G I T Q C Y F R Y E A Y
781 cggttctatggcggcaaccatgctgattgtgccattgccgaacggcgtgcaacc
      R F Y G A Q P M L I V P L P T P A L T
841 aacacagggagcagcagcagcagcagcagcagcagcagcagcagcagcagcagcagc
      K H R S M L W V I L A V V P L S A A A
901 ttgtctctcgtctgttactctcgtcggctcaggagcacaagaaaaggatcaagact
      F V F F V C Y S R R R L R S Q R K G S R R
961 gattggaatttaaggagatctagttggcaagggaaaaattcggagttctctttgttt
      D W N L K G D L V W Q G K N S E F S L G F
1021 gacttccaccagctagtgaggccacagacagttttcagaagaaaacaaactggacag
      D F H Q L V E A T D S F S E E N K L G Q
1081 ggtgcttggcgtctctacaagggcaactcccagggattggaggtagcagcagaag
      G G F G A V Y K G E L P E G L E V A V K
1141 agactgtctcgcattctggcaaggtttcagtgagttcaaaaatgaagtcacaactaata
      R L S S H S G Q G F M E F K N E V Q L I
1201 gcaaacctcagcacaacaaattgggttaggctcttgggatgtgtcctcaagatgagggag
      A K L Q H T N L V R L L G C C S Q D E E
1261 aacatactggttatgaatacttgccaacagaagtctggatttctctcatctctgacgtg
      N I L V Y E Y L P N R S L D F F I S D V
1321 aataaaagagccttaattgattgggtcaacacatgtagcaataattggaaggtagcacat
      N K R A L M D W S T H V A I I E G V A E
1381 ggactcttactacataagcactctcgtctgctgtctacacacagcagccttaaacca
      G L L Y L H K H S R L L V I H R D L K P
1441 agcaatattctcgtgactacgaactgaatccaaaaattcagatttgggctagcaaaa
      S N I L L D Y E L N P K I S D F G L A A K
1501 atattgagctcaaatgacactgaaggaataccacaagacagtagtggcagatctggc
      I L S N D T E G N T T R R V V G T S G
1561 tacatggcaccagatgctcctcaagggcgtattctcaattaaatccgatgcttccagt
      Y M A P E Y A S K G V F S I K S D V F S
1621 tttgggtgtgctcattttgagatccttagcggaaaacagaattctggtaacgaacaat
      F G V V I F E I L S G K Q N S G N E Q Y
1681 ggaggttctcctcaatcgttggacatgcatggcaattatgggaagaaggaagtgggct
      G G F L N L L G H A W Q L W E E G K W A
1741 gatctcattgctcaccattgctaccgggaagtcactcagcaagatgatgagatattt
      D L I A A P L L P G S H S A K M M R Y F
1801 aatatagcattatgtgtgtaacagaagaatgcaactgatagaccaaccatggcgatatt
      N I A L L C V Q E N A T D R P T M G D I
1861 gttgcaatgtaagcaatgatgctatgatattggctgagcctaagcagcagcagatatt
      V A M L S N D A M I L A E P K Q P A Y I
1921 aatgtaagggtgggaatgaagaagcgtccacagctcttgaggcgtcaaatattaaagat
      N V R V G N E E A S T A L E A C N I K D
1981 atgaccatctcgcgcaaatctatggatagcttattccctggcagatgttttaaaattt
      M T I S A T I H G -
2041 ccagtcgtaattaccacaacaaatggtgataatgcgtaccgtaaatgggtgttcaaat
2101 gtgaggttagtgccctaagagatcaaaagaagcctcccggttagctggagtaaac
2161 atca
    
```

Fig. 1 Domain architecture, deduced amino acid sequence and phylogenetic analysis of *Hordeum vulgare* cysteine-rich receptor-like protein kinase (*HvCRK1*). (A) Schematic diagram of *HvCRK1* protein domain architecture showing the signal peptide and two characteristic domain 26 of unknown function (*duf26*) domains at the N-terminus, followed by a transmembrane TM domain and a well-conserved kinase domain. (B) Deduced amino acid sequence of *HvCRK1*, showing two *duf26* domains (highlighted in black), TM domain (highlighted in grey) and the kinase domain (following the TMD). Conserved amino acids in the predicted serine–threonine kinase domain are highlighted in grey boxes (National Center for Biotechnology Information conserved domain search; <http://www.ncbi.nlm.nih.gov>).

Fig. 2 Phylogenetic tree of the predicted amino acid sequence of the kinase domain of *Hordeum vulgare* cysteine-rich receptor-like protein kinase (*HvCRK1*) and 101 other kinase domains of annotated receptor-like protein kinase (RLK) genes from *Arabidopsis thaliana* (At), *Hordeum vulgare* (Hv), *Oryza sativa* (Os) and *Populus trichocarpa* (XP). The kinase domains cluster in species-distinct groups, except for *HvCRK1*. These groups are highlighted to the right of the tree. Bootstrap values from 1000 replicates are indicated at each node. Scale bar indicates amino acid substitutions.

the protein kinase domain sequence in *HvCRK1* also seems to be species distinct (Fig. 2).

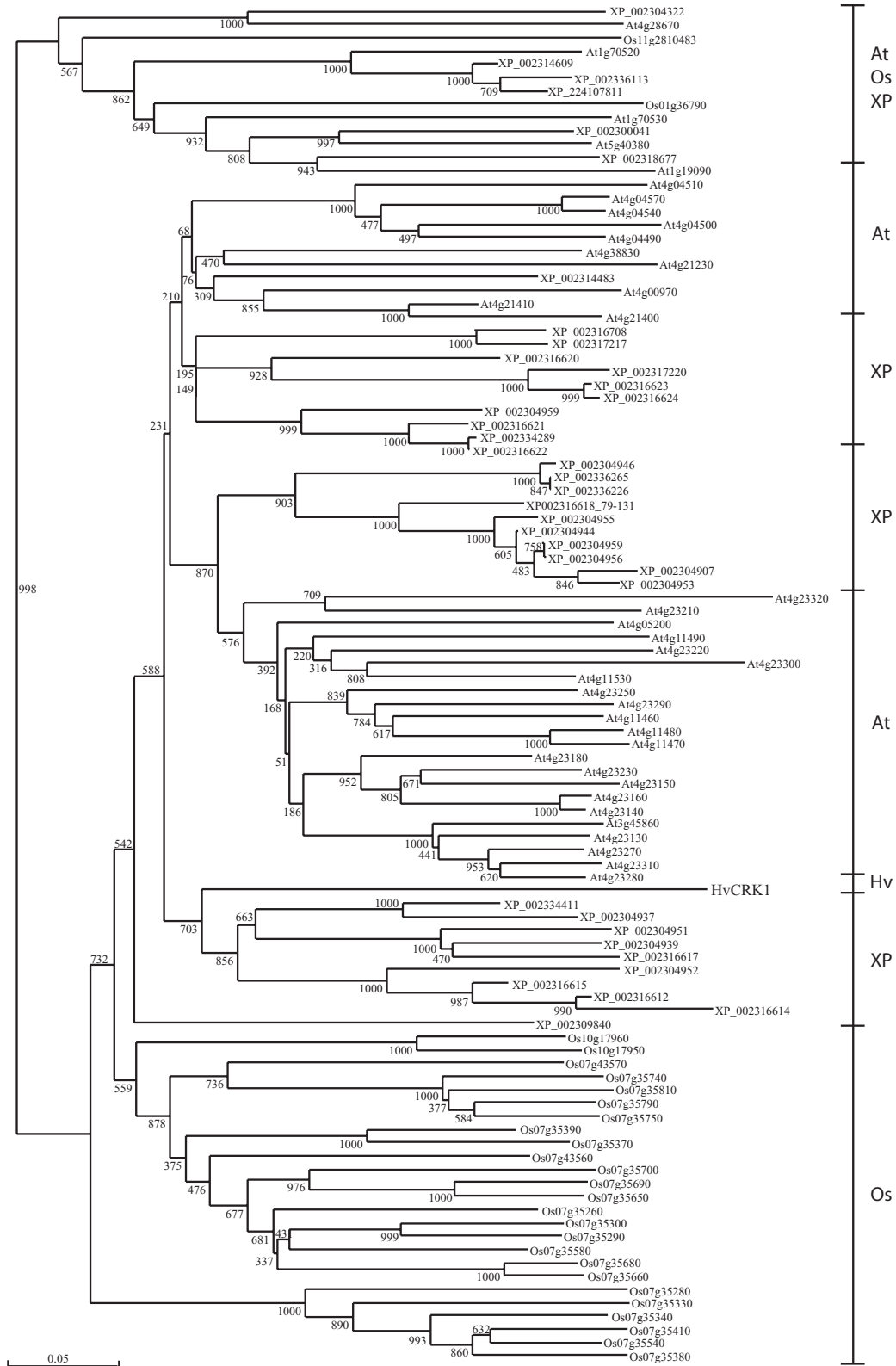
Silencing *HvCRK1* increases basal resistance in barley towards *Bgh*

As signal perception in barley mediating the response to *Bgh* attack involves both early and late recognition, leading to either penetration-based basal resistance or *R*-gene-mediated race-specific resistance, involving programmed cell death (PCD), we examined whether the manipulation of *HvCRK1* expression influenced basal resistance in compatible interactions and/or PCD in incompatible interactions mediated by *Mla9* *R*-gene resistance. This was achieved by RNAi gene silencing (TIGS) and overexpression. TIGS was performed using a 536-bp fragment encoding the extracellular domain of *HvCRK1*. The *HvCRK1* RNAi construct pIPKTA30_CRK1 was generated to produce spliced hairpin double-stranded RNAs *in vivo*, and transformed by particle bombardment into barley epidermal cells together with a pUbiGUS reporter plasmid encoding β -glucuronidase (GUS) (Douchkov *et al.*, 2005). Three days after bombardment, leaf segments were inoculated with either a virulent or avirulent *Bgh* isolate, and transformed cells were screened for possible effects on the barley–*Bgh* interaction. In the compatible interaction, epidermal cells transformed with pIPKTA30_CRK1 exhibited a significant decrease in the infection index (Fig. 3A, $P < 0.05$), i.e. the cells showed increased basal penetration resistance compared with cells carrying the empty vector (pIPKTA30N). In contrast, no effect of the silencing of *HvCRK1* was observed in the incompatible interaction, and all *Bgh* attack was either blocked at the penetration stage or terminated by PCD in the same way as in the empty vector control (Fig. 3A). As a positive control for effects on basal resistance, we included an RNAi construct for *Mlo* (pIPKTA36), as mutation of this gene provides host resistance to *Bgh*. In compatible interactions, pIPKTA36-transformed cells showed a significant reduction (approximately 75%) (Fig. 3A, $P < 0.05$) in the infection index compared with cells transformed with the empty vector control, as reported previously (Douchkov *et al.*, 2005). No effect of *Mlo* silencing was observed in the incompatible interaction as a result of *mlo* resistance or PCD. As a positive control for effects on PCD, we included an RNAi construct expressing the inverted repeat sequence of *Mla6*, which effectively silences the expression of both the *Mla6* and *Mla9* *R* genes because of their high sequence homology (Douchkov *et al.*, 2005; Seeholzer *et al.*, 2010). In the compatible interaction, there was no effect of *Mla6* silencing (Fig. 3A), whereas, in the incompatible interaction, we

found an increase in the infection of cells transformed with this RNAi construct, indicating successful suppression of *R*-gene recognition and subsequent PCD. In an attempt to complement the RNAi studies, we performed overexpression of *HvCRK1*. The pIPKTA9_CRK1 construct for *HvCRK1* overexpression was transformed into barley epidermal cells, together with the reporter plasmid encoding GUS (Douchkov *et al.*, 2005). Four hours after bombardment, leaf segments were inoculated with either a virulent or avirulent *Bgh* isolate. However, cells transformed with the plasmid overexpressing *HvCRK1* did not show any significant changes in infection index compared with leaves transformed with the empty vector (Fig. 3B), irrespective of whether the interaction was compatible or incompatible, indicating that the overexpression of *HvCRK1* does not affect basal resistance and PCD. As a positive control for the effects of overexpression, we included the overexpression of a peroxidase gene (*TaPrx103*) that reduced *Bgh* infection after overexpression in a compatible interaction, as shown previously (Johrde and Schweizer, 2008). No effect of *TaPrx203* overexpression was observed in the incompatible interaction mediated by *Mla9* (Fig. 3B). In summary, the silencing of *HvCRK1* increased basal resistance, but did not affect PCD, in barley after *Bgh* attack.

HvCRK1 transcript accumulation in barley leaves after *Bgh* attack is transient and dependent on MLO

To investigate the spatiotemporal transcript accumulation of *HvCRK1* in response to *Bgh*, we performed a detailed time-course study using both epidermal cell layer and total leaf extracts of the susceptible barley line P01 following virulent *Bgh* inoculation. Transcript profiles of *Bgh*-inoculated epidermal samples showed that *HvCRK1* was strongly expressed, with approximately 50-fold induction at 6 h after inoculation (hai) and 30-fold induction at 12 hai, compared with uninoculated plants (Fig. 4A). In our studies, this time-dependent induction of *HvCRK1* expression coincides with the formation of the primary germ tubes by *Bgh* conidia (6 hai) and the time of *Bgh* penetration of attacked epidermal cells (12 hai) (data not shown). In total leaf samples (i.e. predominantly mesophyll cells which do not come into direct contact with this fungus), *HvCRK1* expression also increased strongly at 6 hai, exhibiting approximately 25-fold induction, followed by a later induction between 12 and 30 hai, with up to 16-fold induction (Fig. 4B), compared with uninoculated control plants. The early and transient induction of *HvCRK1* transcripts suggests a possible function for *HvCRK1* in barley cells during the early recognition of and responses to *Bgh*.



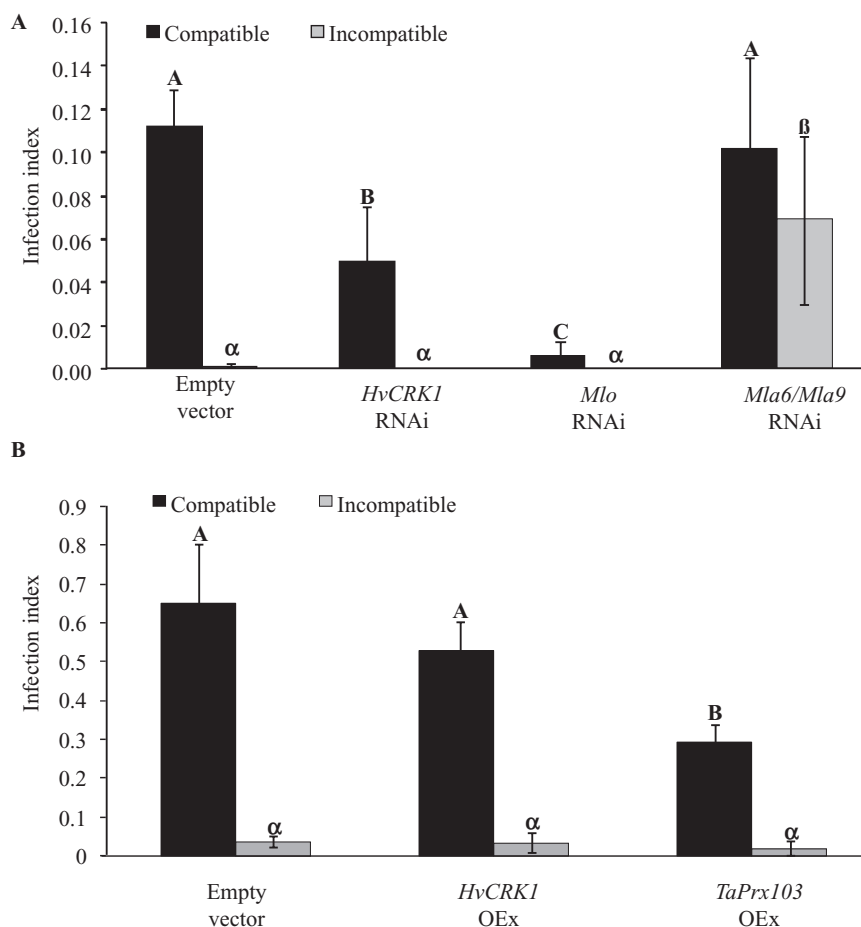


Fig. 3 Effects of silencing (RNAi) or overexpression (OEx) of *Hordeum vulgare* cysteine-rich receptor-like protein kinase (*HvCRK1*) on barley responses to *Blumeria graminis* f.sp. *hordei* (*Bgh*) attack. *Bgh* infection is given as the infection index, which is the number of haustoria divided by the total number of transformed cells. (A) *Bgh* infection of barley cells transiently expressing empty vector as control (pIPKTA30N), the RNAi construct for *HvCRK1* (pIPKTA30_CRK1), the RNAi construct for *Mlo* (pIPKTA36) or the RNAi construct for *Mla6* and *Mla9* (pIPKTA30_Mla6). In the compatible interaction, silencing of *HvCRK1* resulted in reduced infection, when compared with empty vector or RNAi against *Mla6/Mla9*, which have no function in the compatible interaction. RNAi against *Mlo* gave the expected strong reduction in *Bgh* infection. In the incompatible interaction, there was no infection as a result of *Mla9* *R*-gene-mediated programmed cell death (PCD) of *Bgh*-infected cells, except for RNAi against *Mla6/Mla9* which silenced the *R* gene and allowed *Bgh* infection. (B) *Bgh* infection of barley cells transiently overexpressing the empty vector as control (pIPKTA9), overexpression of *HvCRK1* (pIPKTA9_CRK1) or overexpression of the peroxidase gene *TaPrx103* (pWIR3). In the compatible interaction, overexpression of *HvCRK1* had no effect on *Bgh* infection. Overexpression of *TaPrx103* gave the expected reduction in *Bgh* infection. In the incompatible interaction, there was no infection as a result of *Mla9* *R*-gene-mediated PCD of *Bgh*-infected cells. The infection index is given as the mean \pm standard error, and different letters or symbols within each interaction indicate significant differences ($n = 5$, $P = 0.05$).

Like the barley *Mlo* gene, *HvCRK1* is responsive to *Bgh* and compromises penetration resistance. To investigate whether *HvCRK1* transcript levels are dependent on MLO, we analysed *HvCRK1* expression in total leaves in the *mlo*-resistant barley line P22 containing the mutant *mlo5* allele which is near-isogenic to P01 (Kølster *et al.*, 1986). Interestingly, the accumulation of the *HvCRK1* transcript observed in wild-type barley cultivar P01 after *Bgh* attack was completely absent in *mlo5*-resistant P22 plants from 6 h onwards. From this time point, we did not observe any induction of *HvCRK1* expression (Fig. 4B) in *mlo* plants. *HvCRK1* expression levels in uninoculated P01 and P22 leaves showed no

difference (data not shown), suggesting that basal levels of *HvCRK1* were unaltered in uninoculated *mlo5*-resistant P22. The low expression of *HvCRK1* in P22 is unlikely to be caused by a *cis*-allelic effect on the introgressed donor genome fragment (linkage drag) in this backcross line because, according to information from flow-sorted barley chromosome arms (<http://webblast.ipk-gatersleben.de/barley/index.php>), it mapped to chromosome 2HL, whereas the introgressed *mlo* mutation maps to chromosome 4HL.

To ascertain whether there was normal and expected accumulation of defence signalling components in our *mlo5*-resistant

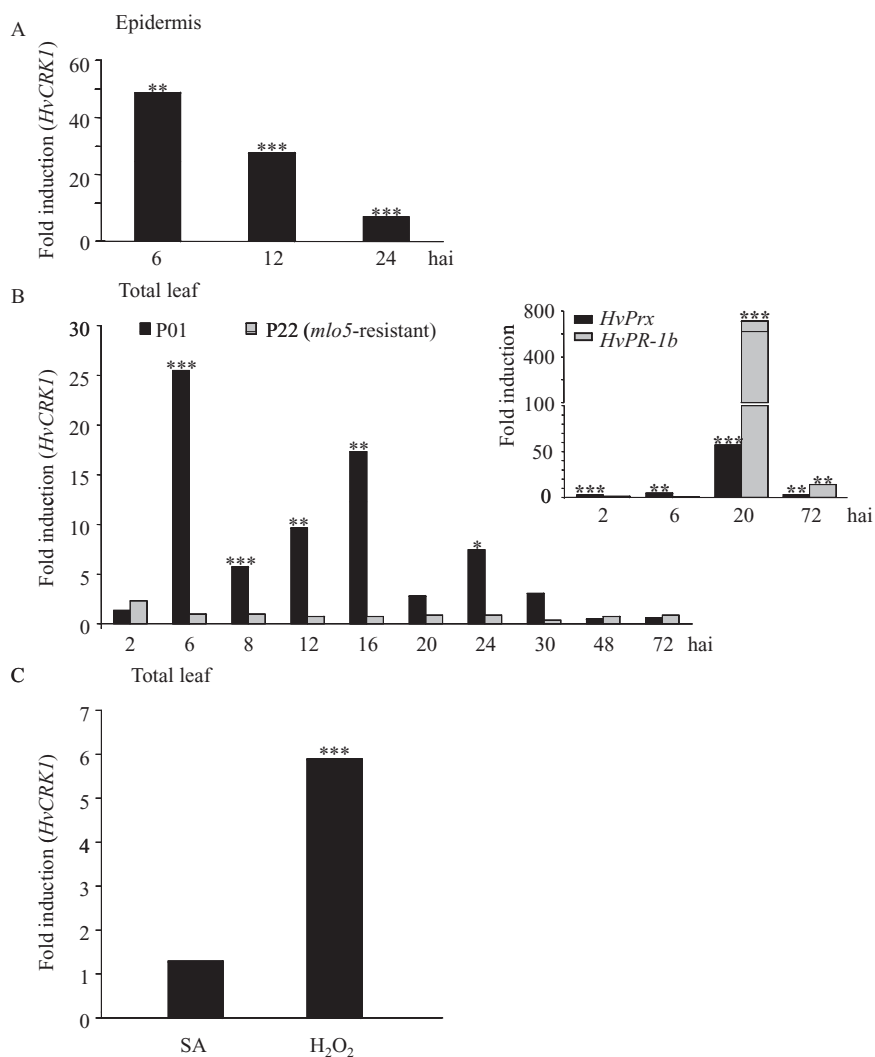


Fig. 4 *Hordeum vulgare* cysteine-rich receptor-like protein kinase (*HvCRK1*) transcript accumulation in the epidermis and total leaves of barley in response to virulent *Blumeria graminis* f.sp. *hordei* (*Bgh*). (A) *HvCRK1* transcript accumulation in abaxial epidermal tissue of the barley line P01 at 6, 12 and 24 h after inoculation (hai) of *Bgh*. (B) *HvCRK1* transcript accumulation in total leaves of barley line P01 and the near-isogenic *mlo5*-resistant P22 barley line between 2 and 72 hai. Inset: transcript accumulation of peroxidase (*HvPrx*) and the pathogenesis-related protein (*HvPR-1b*) gene in the same P22 cDNA. (C) *HvCRK1* transcript accumulation in response to 250 μ M salicylic acid (SA) or 5 mM hydrogen peroxide (H_2O_2) 24 h after infiltration into leaves of P01. Values represent the fold induction in *Bgh*-inoculated or SA/ H_2O_2 -treated plants. Asterisks indicate significant differences in *Bgh*-infected samples compared with uninfected epidermis or leaf samples at the respective time points ($n = 5$, *** $P < 0.001$, ** $P = 0.01$ – 0.001 , * $P = 0.05$ – 0.01).

P22/*Bgh* inoculation assay, we analysed the early *Bgh*-responsive gene (*peroxidase*) and late *Bgh*-induced gene (*PR-1b*) (Gregersen *et al.*, 1997) in the same P22 cDNA as above at 2, 6, 20 and 72 hai. The gene encoding *peroxidase* was induced significantly at all tested time points and *PR-1b* was strongly induced at the 20 and 72 hai time points (Fig. 4B), confirming that there was the expected induction of defence signalling components. The differential accumulation patterns of *HvCRK1* transcripts in *Bgh*-attacked P01 and *mlo5*-resistant P22 suggest that the induction of *HvCRK1* expression after *Bgh* attack is dependent on MLO activity in barley.

Effects of SA and H_2O_2 on *HvCRK1* transcript accumulation

As Arabidopsis CRKs are transcriptionally induced by oxidative stress and the application of SA (Wrzaczek *et al.*, 2010), we tested how *HvCRK1* transcripts accumulated in whole barley leaves 24 h after infiltration of 5 mM H_2O_2 or 250 μ M SA. Transcripts of *HvCRK1* accumulated strongly after H_2O_2 treatment, but not after SA treatment, suggesting that *HvCRK1* is part of a signalling pathway responsive to oxidative stress, but not SA (Fig. 4C).

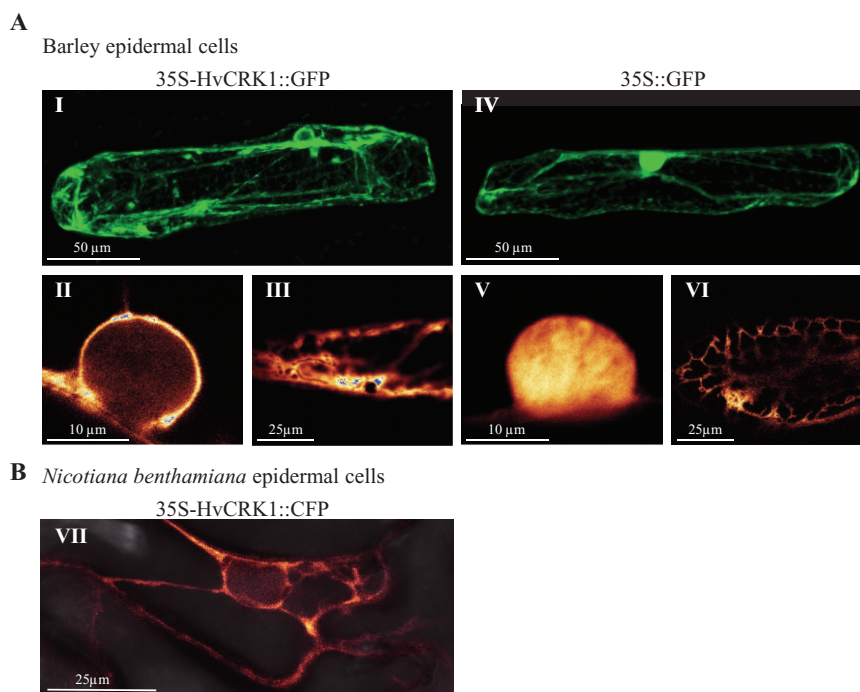


Fig. 5 Cellular localization of *Hordeum vulgare* cysteine-rich receptor-like protein kinase (*HvCRK1*) is mainly associated with the endoplasmic reticulum (ER). (A) *HvCRK1*::GFP (left column) and green fluorescent protein (GFP) alone (right column), expressed under the control of the cauliflower mosaic virus (CaMV) 35S promoter, after biolistic delivery of vector DNA to barley epidermal cells and analysis for GFP fluorescence by confocal microscopy. Panels I and IV show fluorescent (green colour) overall morphology of cells; panels II and V show fluorescent (intensity colour) close-up images of the nuclei; panels III and VI show fluorescent (intensity colour) images of the central part of transformed cells. *HvCRK1*::GFP is localized in a finely polygonal structured network traversing the cytoplasm, similar to the ER (I and III), and a local concentration of the network can be observed aligning around the nucleus, like the ER (II). GFP alone exhibited a characteristic diffuse cytoplasmic fluorescent staining (IV and VI) and a dense fluorescent staining in the nucleus (V). (B) Fluorescence (intensity colour) of *HvCRK1*::CFP *Agrobacterium*-transformed *Nicotiana benthamiana* leaves. *HvCRK1*::CFP is also localized in a polygonal structured ER-like network traversing the cytoplasm in *N. benthamiana* cells (VII). Photographs are maximum intensity images of z-stack projections.

Subcellular localization of *HvCRK1*

To study the subcellular localization of *HvCRK1* *in vivo*, we fused the open reading frame of *HvCRK1* to the green fluorescent protein (GFP) gene (*HvCRK1*::GFP) and expressed it under the control of the cauliflower mosaic virus (CaMV) 35S promoter. Using SMART (<http://smart.embl-heidelberg.de/>) (Schultz *et al.*, 2000) and PSORT (<http://wolfpsort.org/>) (Horton *et al.*, 2007), we found that *HvCRK1* encoded a potential transmembrane domain (Fig. 1) and had predicted plasma membrane localization. As a result of this prediction, we expected *HvCRK1*::GFP to accumulate around the plasma membrane. Initially, the fusion protein was transiently expressed in barley epidermal cells after the biolistic delivery of vector DNA, and was analysed by confocal microscopy. *HvCRK1*::GFP was found to localize in a finely polygonal structured network traversing the cytoplasm, similar to the ER (Fig. 5A; panels I and III). A local concentration of the ER network can be seen to align around the nucleus (Fig. 5A; panel II), in agreement with the actual orientation of this organelle in plant cells. GFP alone exhibited a characteristic diffused cytoplasmic fluorescent staining (Fig. 5A; panels IV and VI)

and a dense fluorescent staining in the nucleus (Fig. 5A; panel V). We also included the co-expression of *HvCRK1*::GFP and the ER marker *YFP::HDEL* (Runions *et al.*, 2006), which supported the localization of ER (Fig. S1, see Supporting Information). Although *HvCRK1* has a predicted plasma membrane localization, we did not observe any clear indication of the plasma membrane localization of *HvCRK1*::GFP. Because wounding and a limited number of transformed epidermal cells are major limitations when using biolistic delivery of plasmid DNA for localization studies, we also examined the subcellular localization of *HvCRK1* using a more robust *Agrobacterium*-mediated transient expression system in *Nicotiana benthamiana*. Consistent with our observations from barley, a clear cyan fluorescent protein (CFP) fluorescence from the *HvCRK1*::CFP construct used was observed in the finely polygonal structured ER (Fig. 5B; panel VII), and no clear plasma membrane localization was observed. The gene *AHA2* (Liu *et al.*, 2009), a plasma membrane-associated transporter, was included as a plasma membrane localization control (Fig. S1). These results suggest that our *HvCRK1* fusion proteins accumulate mainly in the ER.

Table 1 Length and hydrophobicity of the transmembrane domain (TMD) in *Hordeum vulgare* cysteine-rich receptor-like protein kinase (*HvCRK1*) and the Arabidopsis homologue *AtCRK6* (At4g23140).

CRK proteins	TMD alignment	TMD length†	Hydrophobicity score‡	Subcellular localization in <i>Nicotiana benthamiana</i>
<i>AtCRK6</i>	VLVVAVVVVLAVLLFIALVGYCFL	23	3.148	Plasma membrane
<i>HvCRK1</i>	-----VILAVVVPLSAAA FVFF * : * * * : : : : * :	17	2.662	Endoplasmic reticulum

* = identical amino acid, : = similar amino acid.

†<http://expasy.org/tools/protscale.html> (Kyte and Doolittle, 1982).

‡<http://msa.sbc.su.se/cgi-bin/msa.cgi> (Lassmann *et al.*, 2009).

Arabidopsis *AtCRK6* (At4g23140), showing a similar domain structure and sequence homology to *HvCRK1* (39% identity and 54% similarity, EMBL-EBI, <http://www.ebi.ac.uk>), was shown to be a plasma membrane-localized protein when transiently expressed under a 35S promoter in *N. benthamiana* using *Agrobacterium* transformation (Thomas *et al.*, 2008). However, these authors also showed that single amino acid changes in the transmembrane domain can move the localization from the plasma membrane to the ER (Thomas *et al.*, 2008). Therefore, we compared the transmembrane domains of *HvCRK1* and *AtCRK6* to predict possible sequence variation that might explain the unexpected subcellular localization of *HvCRK1* in *N. benthamiana* (Table 1). The transmembrane domain of *HvCRK1* is predicted to consist of 17 amino acids, whereas *AtCRK6* is predicted to comprise 23 amino acids. Five amino acids are identical and five belong to similar hydrophobic groups. The most distinct difference is at positions 17–19, with amino acids leucine–valine–glycine (LVG) in *AtCRK6*, accounting for more hydrophobicity (3.148), and alanine–alanine–alanine (AAA) in *HvCRK1*, accounting for less hydrophobicity (2.662). The length and low hydrophobicity of the *HvCRK1* transmembrane domain support the observed ER localization (Brandizzi *et al.*, 2002; Szczesna-Skorupa and Kemper, 2000). ER localization is also supported by the presence of an ER retention signal in the *HvCRK1* amino acid sequence. This –R–R–L–R– motif is placed adjacent to the transmembrane region (Fig. 1B).

DISCUSSION

Barley *HvCRK1* is a putative CRK. The closest homologue in rice is the gene AK120072 encoding a CRK protein with 58.3% identity and 71.6% similarity to *HvCRK1* (EMBL-EBI, <http://www.ebi.ac.uk>). In Arabidopsis, the closest homologues have about 30–35% identity and 50–60% similarity to *HvCRK1* and, in general, the Arabidopsis CRKs are much more closely related to each other than to *HvCRK1*. In both rice and Arabidopsis, duf26-containing CRKs constitute large families of genes, some of which are known to be induced in response to pathogens and defence-inducing signals, such as SA and ROS (Dievart and Clark, 2004; Du and Chen, 2000; Shiu and Bleecker, 2001; Wrzaczek *et al.*, 2010). Several of the Arabidopsis CRKs (*CRK4*, *CRK5*, *CRK13*, *CRK19* and *CRK20*) have also been shown to be involved in the regulation of

defence reactions and PCD against *Pseudomonas syringae*, and the overexpression of CRKs leads to increased PCD (Acharya *et al.*, 2007; Chen *et al.*, 2003, 2004).

HvCRK1 regulates basal resistance, but not R-gene-mediated PCD

Barley possesses two major types of defence against *Bgh*. Firstly, an early race-nonspecific basal resistance which may block *Bgh* penetration and thereby prevent further fungal infection. However, if this fails and the barley line contains an effective race-specific *R* gene, a hypersensitive response leading to PCD will be induced in the next layer of defence in the infected cells preventing the establishment of biotrophy and further fungal growth. The silencing and overexpression studies of *HvCRK1* showed that this CRK negatively influenced the level of basal resistance, but did not influence *R*-gene-mediated PCD responses against *Bgh*, suggesting a role in defence different from that observed for the Arabidopsis CRKs 4, 5, 13, 19 and 20.

Basal resistance in barley against *Bgh* is characterized by the formation of cell wall appositions, known as papillae, that involve the deposition of callose (a β -1,3-glucan polymer) and phenolic compounds on the inner surface of the cell wall at the site of penetration (Zeyen *et al.*, 2002). These physiological changes are mediated by a strong local H_2O_2 burst which is induced on fungal challenge (Thordal-Christensen *et al.*, 1997; Zeyen *et al.*, 2002). In Arabidopsis, transcript accumulation of several CRKs is induced by ROS and as a nonhost response to *Bgh* (Wrzaczek *et al.*, 2010), and, indeed, we observed a strong induction of *HvCRK1* following both *Bgh* attack and the application of H_2O_2 (Fig. 4), suggesting that the induction of *HvCRK1* in response to *Bgh* in barley could be dependent on H_2O_2 . This is supported by a meta-analysis of Arabidopsis CRK expression in relation to biotic and abiotic stresses (Wrzaczek *et al.*, 2010), which showed that *CRK3*, *CRK10*, *CRK11* and *CRK19* are responsive to H_2O_2 and that *CRK11*, *CRK12*, *CRK13*, *CRK20*, *CRK28*, *CRK29* and *CRK36* are responsive to *Bgh*, indicating that at least the induction of *CRK11* in Arabidopsis could be dependent on both *Bgh* and H_2O_2 .

ROS and SA seem to be connected in a positive feedback loop that amplifies signals leading to defence responses and cell death in Arabidopsis (Overmyer *et al.*, 2003). However, the accumulation of SA could not be detected in barley seedlings after *Bgh*

inoculation in either compatible or incompatible interactions, indicating that defence responses against *Bgh* do not rely on or induce SA accumulation in barley (Huckelhoven *et al.*, 1999). We also found that treatment with SA did not result in any noticeable changes in *HvCRK1* transcript accumulation (Fig. 4).

Finally, we observed no enhanced *HvCRK1* transcript accumulation in the *mlo5* mutant on *Bgh* inoculation. The penetration resistance caused by the recessive barley *mlo5* allele is physiologically similar to the nonhost penetration resistance in grasses (Zeyen *et al.*, 2002), and it has been shown that one effect of MLO is to negatively regulate the cell wall-restricted H₂O₂ burst that is induced by fungal penetration attempts, whereas, in the subtending mesophyll cells, it suppresses a second oxidative burst and cell death (Piffanelli *et al.*, 2002). Although H₂O₂ can upregulate *HvCRK1*, the increased H₂O₂ observed in the fully resistant *mlo5* genotype (Piffanelli *et al.*, 2002) could not compensate for the lack of *HvCRK1* transcript accumulation, suggesting that an MLO-dependent factor(s) in addition to H₂O₂ is important for the upregulation of *HvCRK1* transcript levels. More information about the *HvCRK1*–MLO signalling hierarchy may come from the investigation of *HvCRK1* expression in barley lines mutated in the *Ror1* and/or *Ror2* genes that compromise *mlo* resistance and make these lines partially susceptible to *Bgh*. Interestingly, these mutations are also known to cause changes in the accumulation of H₂O₂ during *Bgh* attack in *mlo*-resistant barley (Huckelhoven *et al.*, 1999).

A reduction in the length and hydrophobicity of the transmembrane domain determines the localization of *HvCRK1* to the ER

Results from the subcellular localization of *HvCRK1* *in vivo* indicate that the *HvCRK1*::GFP fusion protein is targeted primarily to the ER (Fig. 5). However, to rule out any possible effects of wounding responses caused by the biolistic bombardment method in barley, we also investigated the localization of *HvCRK1*::CFP in tobacco (*N. benthamiana*) using an *Agrobacterium*-mediated transformation system with the same result. Few *duf26* proteins have been studied experimentally with respect to their subcellular localization. However, two *duf26*-related CRKs, *OsRMC* (Jiang *et al.*, 2007) and the *HvCRK1* homologue, *AtCRK6*, have been reported to be localized in the plasma membrane (Thomas *et al.*, 2008). Comparing the transmembrane domains of *AtCRK6* and *HvCRK1*, we noticed that the domains differed in length and hydrophobicity (Table 1). It has been postulated that the main characteristic feature determining protein localization to the plasma membrane is the length of the hydrophobic transmembrane domain. As a result, it has been noted that longer and more hydrophobic transmembrane domains tend to be localized to the plasma membrane (Bretscher and Munro, 1993; Szczesna-Skorupa and Kemper, 2000). Some studies have also indicated specific

amino acid sequences in the transmembrane domain as key determinants of extracellular localization (Thomas *et al.*, 2008). In agreement with these observations, it has been shown that the shortening of the transmembrane domain of *ATCRK6* from 23 to 18 amino acids results in the retention of the protein in the ER as opposed to its original localization, i.e. plasmodesmata (Thomas *et al.*, 2008). The same study reported that the substitution of the hydrophobic C-terminal amino acids (LVL) in the domain of PDLP1a (a *duf26*-related transmembrane protein) with less hydrophobic amino acids (AAA) resulted in the ER retention of PDLP1a. Based on these previous results, it is plausible that the less hydrophobic distal amino acids (AAA) in the putative transmembrane domain of *HvCRK1* might represent specific signals that determine ER retention. In summary, a short transmembrane domain (17 amino acids) and the presence of less hydrophobic C-terminal amino acids (AAA) may represent key characteristics of *HvCRK1* responsible for its apparent ER localization. In addition to the amino acid sequence in the transmembrane domain, both luminal and membrane-localized ER proteins have been predicted traditionally on the basis of classical C-terminus-located retention signals ([KH]DEL) (Teasdale and Jackson, 1996). However, other noncanonical arginine-based signals directing proteins to the ER have been identified (Michelsen *et al.*, 2007). These trafficking signals conform to the consensus –R–X–R, where –X– denotes a large neutral or positively charged amino acid, and have been found in both ion channels and G-coupled receptors (Ren *et al.*, 2005). Interestingly, analysis of the deduced amino acid sequence of *HvCRK1* identified an –R–R–L–R– motif adjacent to the transmembrane region (Fig. 1B). Hence, the predicted subcellular localization using the transmembrane domain as a query is in agreement with our *in vivo* studies showing *HvCRK1* to be localized in the ER. Future studies should focus on the negative defence regulation exerted by ER-localized *HvCRK1*.

In summary, *HvCRK1* is a putative CRK, which is probably ER-associated and involved in the negative regulation of basal resistance in barley against *Bgh*. We argue that this regulation could be dependent on H₂O₂. However, this regulation occurs in the signalling pathway downstream from MLO function, as *HvCRK1* transcripts do not accumulate after *Bgh* inoculation in *mlo*-resistant barley. Overall, this suggests that *HvCRK1* could be part of the basal defence regulation controlled by MLO and, through this, a possible direct or indirect target for *Bgh*-derived effector proteins.

EXPERIMENTAL PROCEDURES

Plant material and fungal inoculations

The near-isogenic barley (*Hordeum vulgare*) lines P01 (susceptible) and P22 (*mlo5* resistant) (Kölster *et al.*, 1986) and the cultivars Golden Promise (susceptible) and Roland (carrying *Mla9*-mediated resistance) were grown

in pots of compost soil in growth chambers (16 h light and approximately 20 °C) for 7 days before the experiments. The fungus *Blumeria graminis* f.sp. *hordei* (*Bgh*) race A6, virulent on P01, was used for gene expression and localization studies. The *Bgh* isolates 4.8 (avirulent on *Mla9*) and K1 (virulent on *Mla9*) were used for RNAi and overexpression experiments. All *Bgh* isolates were maintained on compatible barley lines by weekly transfer to fresh plants.

Differential display, RACE and cloning of *HvCRK1*

Differential display was performed as described previously (Jensen *et al.*, 2007). RNA was isolated from abaxial epidermal tissue of barley leaves, 20 h after *Bgh* inoculation. The reverse transcriptase-polymerase chain reaction (RT-PCR) was performed with oligoT12CG and random hexamer primers. Amplification of 5' and 3' ends was carried out according to the SMART™ RACE kit (Clontech, Mountain View, CA, USA). Two 5' cDNA clones were obtained using gene-specific primers in combination with SMART II oligo primers, and one 3' cDNA clone was obtained using 3' CDS primers (SMART™ RACE kit) in combination with the gene-specific primer. Differential display fragments and RACE products were all cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) and sequenced. The overlapping sequences were combined to obtain the 2198-bp sequence of *HvCRK1*, which included a single 1935-bp-long open reading frame, representing a coding region of 645 amino acids.

RNA extraction and cDNA synthesis

For transcript analyses, 20 abaxial epidermal peels, or the 7-cm central part of five fully expanded seedling leaves, were prepared for each time point of both control and inoculated plants for each biological replicate. For the analysis of the effects of SA and H₂O₂, five fully matured seedling leaves of the barley line P01 were infiltrated with 250 µM SA or 5 mM H₂O₂ and sampled 24 h later. Total RNA was isolated using the RNA mini kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. One microgram of purified DNaseI-treated (Ambion, Austin, TX, USA) quality-checked RNA was used for cDNA synthesis employing the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

Quantitative real-time PCR analysis

Quantitative real-time PCR was performed for studies of *HvCRK1* expression using the primers RLK729 (5'-CGCACGGTGGACAAATG-3') and RLK825 (5'-TCGGCAATGGCACAATCAGC-3'). *Ubiquitin conjugating enzyme 2* (*UBC2*, AY220735) was used for the normalization of variations in cDNA quantity employing the primers UBC423 (5'-TCTCGTCCCTGACATTGCCAACAT-3') and UBC552 (5'-TTTCTCGGACAGCAACAATCTTCT-3'). The expression of *peroxidase* (X58396) and *PR-1b* (X74940) was tested in P22 (*mlo* resistant) using the primers Prx299 (5'-GGCATGGAACAAAACGCTAT-3') and Prx419 (5'-TGAGTATGTCGGCACAGGAG-3'), and PR1b158 (5'-CCGCAGGACTACGTATCACC-3') and PR1b282 (5'-TTGCAGTCGTTGATCCTCTG-3'), respectively. Relative gene expression differences (*R*) and statistical significance levels for *Bgh*-inoculated samples compared with uninoculated control samples were quantified

using REST software (Pfaffl *et al.*, 2002) and expressed as fold induction. *C_T* values included in the analyses were based on five biological replicate measurements, with three technical replicates for each time point and treatment.

Constructs for transient expression studies

For RNAi, a 536-bp extracellular domain/receptor domain starting at the 66th base pair of *HvCRK1* CDS was PCR amplified and cloned into the pENTR™ TOPO vector using the pENTR™ Directional TOPO Cloning Kit (Invitrogen, Carlsbad, CA, USA). PCR was performed using high-fidelity *pfu* polymerase (Promega). The RNAi fragment was cloned from the pENTR™ TOPO donor vector into the RNAi destination vector pIPKTA30N as inverted repeats by a single LR recombination reaction using the LR clonase and the Invitrogen Gateway system to generate pIPKTA30_CRK1 (Douchkov *et al.*, 2005). Two other constructs, namely pIPKTA36 (an RNAi construct for silencing *Mlo*) and pIPKTA30_Mla6 (an RNAi construct for silencing *Mla6* and *Mla9*) (Douchkov *et al.*, 2005), were used as controls. As a result of the high sequence similarity between *Mla6* and *Mla9* (Seeholzer *et al.*, 2010), pIPKTA30_Mla6 is expected to efficiently silence the expression of both genes. The overexpression construct pIPKTA9_CRK1 was obtained by subcloning a BamHI-ApaI fragment of the full coding sequence of *HvCRK1* under control of the CaMV 35S promoter into the pIPKTA9 vector for overexpression (Dong *et al.*, 2006). The pWIR3 overexpression construct for the peroxidase gene *TaPrx103* and empty pIPKTA9 vector (Johrde and Schweizer, 2008) were included as control. An *HvCRK1* C-terminal GFP fusion for studies of subcellular localization in barley was generated in the pIPKTA9 vector by an overlap extension strategy (Dong *et al.*, 2006; Jensen *et al.*, 2007). *HvCRK1::CFP* fusion proteins for the study of the subcellular localization in *N. benthamiana* were prepared by amplification of the open reading frame without the stop codon using primers Topo r1k FP (5'-CACCATGGCGACCATCGGCGT-3') and Topo r1k ns RP (5'-TCCATGAATTGTCGCAGATATGG-3'). The PCR product was first cloned into the pENTR/D-TOPO vector (Invitrogen). The resulting plasmids were combined with the destination vector pEarleygate 102 (Earley *et al.*, 2006) in an LR reaction to generate the *HvCRK1::CFP* fusion construct.

Microprojectile bombardment and challenge inoculation

TIGS and transient overexpression were performed in 7-day-old susceptible barley plants of cultivar Golden Promise (*mlo9*) or Roland (*Mla9*), as described previously (Douchkov *et al.*, 2005; Ihlow *et al.*, 2008). Briefly, leaf segments were bombarded by gold particles that had been coated with either one of our test constructs or one of the control constructs. In addition, pUbiGUS, containing the GUS gene under the control of the maize *Ubiquitin* promoter, was included and co-transformed as a reporter for transformed epidermal cells (Schweizer *et al.*, 1999). Leaves were bombarded using the PDS-1000/He biolistic particle delivery system (Bio-Rad, Munich, Germany). Leaf segments were inoculated with *Bgh*, 3 days after bombardment for the RNAi experiments and 4 h after bombardment for the overexpression experiments. After another 40 h of incubation, cells were stained for GUS activity. Leaves were screened for the infection of transformed cells using an automated analysis system (Ihlow *et al.*, 2008).

The fraction of successfully formed haustoria (infection index) in GUS-stained cells was calculated as:

$$\text{Infection index} = \frac{\text{number of haustoria}}{\text{total number of transformed cells}}$$

and the data were analysed for significance using the Wilcoxon rank-sum test.

Subcellular localization

The pPKTA9 plasmid (Dong *et al.*, 2006) harbouring the CaMV 35S promoter was used to express GFP and *HvCRK1::GFP* transiently in barley epidermal cells, as described previously (Jensen *et al.*, 2007). A total of 10 µg of each expression construct was coated onto 27.5 mg/mL 1-µm gold particles and transferred into the cells, as described for transient gene silencing experiments. For the subcellular localization of *HvCRK1::CFP* in *N. benthamiana*, plants were transformed by the infiltration of *Agrobacterium tumefaciens* (Liu *et al.*, 2009). Transformed cells were visualized using a Leica TCS SP2/MP confocal laser scanning microscope (Leica Microsystems, Mannheim, Germany).

Bioinformatic tools

Amino acid sequences from poplar, rice and *Arabidopsis* duf26-containing RLKs were extracted from RefSeq (<http://www.ncbi.nlm.nih.gov/RefSeq/>). The batch extraction largely overlapped with the annotation table from Lehti-Shiu *et al.* (2009). Kinase domain sequences were extracted and aligned using SMART (<http://smart.embl-heidelberg.de/>) (Schultz *et al.*, 2000) and CLUSTALX (Thompson *et al.*, 1997). A rooted phylogenetic tree was built using NJPlot software (Perriere and Gouy, 1996) using neighbour joining (gap open and gap extension penalties of 10 and 0.2, respectively) with 1000 bootstrap trials. Alignment of the deduced transmembrane domain of *HvCRK1* with *Arabidopsis* CRK (*AtCRK6*) was carried out using a high-performance multiple alignment program: KALIGN2 (<http://msa.sbc.su.se/cgi-bin/msa.cgi>) (Lassmann *et al.*, 2009). PSORT (<http://wolfpsort.org/>) (Horton *et al.*, 2007) was used to predict the subcellular localization of *HvCRK1*. The hydrophobicity index of the transmembrane domain was calculated using ProtScale and following the method of Kyte and Doolittle (1982) with a window size of 21 (<http://expasy.org/tools/protscale.html>).

Accession number

The isolated *HvCRK1* cDNA clone described in this article has been deposited in the EMBL database with the following accession number: FR717136

ACKNOWLEDGEMENTS

This work was supported by the Danish Council for Independent Research, Technology and Production Sciences (projects 23-03-0167 and 274-07-0173) and the Villum Foundation.

REFERENCES

Acharya, B.R., Raina, S., Maqbool, S.B., Jagadeeswaran, G., Mosher, S.L., Appel, H.M., Schultz, J.C., Klessig, D.F. and Raina, R. (2007) Overexpression of CRK13, an

- Arabidopsis* cysteine-rich receptor-like kinase, results in enhanced resistance to *Pseudomonas syringae*. *Plant J.* **50**, 488–499.
- Bhat, R.A., Miklis, M., Schmelzer, E., Schulze-Lefert, P. and Panstruga, R. (2005) Recruitment and interaction dynamics of plant penetration resistance components in a plasma membrane microdomain. *Proc. Natl. Acad. Sci. USA*, **102**, 3135–3140.
- Brandizzi, F., Frangne, N., Marc-Martin, S., Hawes, C., Neuhaus, J.M. and Paris, N. (2002) The destination for single-pass membrane proteins is influenced markedly by the length of the hydrophobic domain. *Plant Cell*, **14**, 1077–1092.
- Bretscher, M.S. and Munro, S. (1993) Cholesterol and the Golgi-apparatus. *Science*, **261**, 1280–1281.
- Buschges, R., Hollricher, K., Panstruga, R., Simons, G., Wolter, M., Frijters, A., van Daelen, R., van der Lee, T., Diergaarde, P., Groenendijk, J., Topsch, S., Vos, P., Salamini, F. and Schulze-Lefert, P. (1997) The barley *Mlo* gene: a novel control element of plant pathogen resistance. *Cell*, **88**, 695–705.
- Carver, T.L.W., Zeyen, R.J., Robbins, M.P., Vance, C.P. and Boyles, D.A. (1994) Suppression of host cinnamyl alcohol-dehydrogenase and phenylalanine ammonia-lyase increases oat epidermal-cell susceptibility to powdery mildew penetration. *Physiol. Mol. Plant Pathol.* **44**, 243–259.
- Chen, K.G., Du, L.Q. and Chen, Z.X. (2003) Sensitization of defense responses and activation of programmed cell death by a pathogen-induced receptor-like protein kinase in *Arabidopsis*. *Plant Mol. Biol.* **53**, 61–74.
- Chen, K.G., Fan, B.F., Du, L.Q. and Chen, Z.X. (2004) Activation of hypersensitive cell death by pathogen-induced receptor-like protein kinases from *Arabidopsis*. *Plant Mol. Biol.* **56**, 271–283.
- Chen, Z.X. (2001) A superfamily of proteins with novel cysteine-rich repeats. *Plant Physiol.* **126**, 473–476.
- Collinge, D.B., Gregersen, P.L. and Thordal-Christensen, H. (2002) The nature and role of defence response genes in cereals. In: *The Powdery Mildews: A Comprehensive Treatise* (Belanger, R.R., Bushnell, W.R., Dik, A.J. and Carver, T.L.W., eds), pp. 146–160. St. Paul, MN: American Phytopathological Society Press.
- Devoto, A., Piffanelli, P., Nilsson, I., Wallin, E., Panstruga, R., von Heijne, G. and Schulze-Lefert, P. (1999) Topology, subcellular localization, and sequence diversity of the *Mlo* family in plants. *J. Biol. Chem.* **274**, 34 993–35 004.
- Dievart, A. and Clark, S.E. (2004) LRR-containing receptors regulating plant development and defense. *Development*, **131**, 251–261.
- Dong, W.B., Nowara, D. and Schweizer, P. (2006) Protein polyubiquitination plays a role in basal host resistance of barley. *Plant Cell*, **18**, 3321–3331.
- Douchkov, D., Nowara, D., Zierold, U. and Schweizer, P. (2005) A high-throughput gene-silencing system for the functional assessment of defense-related genes in barley epidermal cells. *Mol. Plant–Microbe Interact.* **18**, 755–761.
- Du, L.Q. and Chen, Z.X. (2000) Identification of genes encoding receptor-like protein kinases as possible targets of pathogen- and salicylic acid-induced WRKY DNA-binding proteins in *Arabidopsis*. *Plant J.* **24**, 837–847.
- Dykema, P.E., Sipes, P.R., Marie, A., Biermann, B.J., Crowell, D.N. and Randall, S.K. (1999) A new class of proteins capable of binding transition metals. *Plant Mol. Biol.* **41**, 139–150.
- Earley, K.W., Haag, J.R., Pontes, O., Opper, K., Juehne, T., Song, K.M. and Pikaard, C.S. (2006) Gateway-compatible vectors for plant functional genomics and proteomics. *Plant J.* **45**, 616–629.
- Glazebrook, J. (2001) Genes controlling expression of defense responses in *Arabidopsis*—2001 status. *Curr. Opin. Plant Biol.* **4**, 301–308.
- Gregersen, P.L. and Collinge, D.B. (2001) Penetration attempts by the powdery mildew fungus into barley leaves are accompanied by increased gene transcript accumulation in the epidermal cell layer. In 5th Congress of the European Foundation for Plant Pathology. *J. Plant Pathol.* **82**, 257–260. Edizioni/ETS.
- Gregersen, P.L., Thordal-Christensen, H., Forster, H. and Collinge, D.B. (1997) Differential gene transcript accumulation in barley leaf epidermis and mesophyll in response to attack by *Blumeria graminis* f.sp. *hordei* (syn. *Erysiphe graminis* f.sp. *hordei*). *Physiol. Mol. Plant Pathol.* **51**, 85–97.
- Horton, P., Park, K.J., Obayashi, T., Fujita, N., Harada, H., Adams-Collier, C.J. and Nakai, K. (2007) WOLF PSORT: protein localization predictor. *Nucleic Acids Res.* **35**, W585–W587.
- Huckelhoven, R., Fodor, J., Preis, C. and Kogel, K.H. (1999) Hypersensitive cell death and papilla formation in barley attacked by the powdery mildew fungus are associated with hydrogen peroxide but not with salicylic acid accumulation. *Plant Physiol.* **119**, 1251–1260.
- Ihlow, A., Schweizer, P. and Seiffert, U. (2008) A high-throughput screening system for barley/powdery mildew interactions based on automated analysis of light micrographs. *BMC Plant Biol.* **8**, Article 6.

- Jensen, M.K., Rung, J.H., Gregersen, P.L., Gjetting, T., Fuglsang, A.T., Hansen, M., Joehnk, N., Lyngkjaer, M.F. and Collinge, D.B. (2007) The HvNAC6 transcription factor: a positive regulator of penetration resistance in barley and Arabidopsis. *Plant Mol. Biol.* **65**, 137–150.
- Jiang, J.F., Li, J.H., Xu, Y.Y., Han, Y., Bai, Y., Zhou, G.X., Lou, Y.G., Xu, Z.H. and Chong, K. (2007) RNAi knockdown of *Oryza sativa* root meander curling gene led to altered root development and coiling which were mediated by jasmonic acid signaling in rice. *Plant Cell Environ.* **30**, 690–699.
- Johrde, A. and Schweizer, P. (2008) A class III peroxidase specifically expressed in pathogen-attacked barley epidermis contributes to basal resistance. *Mol. Plant Pathol.* **9**, 687–696.
- Jørgensen, J.H. (1994) Genetics of powdery mildew resistance in barley. *Crit. Rev. Plant Sci.* **13**, 97–119.
- Kim, M.C., Panstruga, R., Elliott, C., Muller, J., Devoto, A., Yoon, H.W., Park, H.C., Cho, M.J. and Schulze-Lefert, P. (2002) Calmodulin interacts with MLO protein to regulate defence against mildew in barley. *Nature*, **416**, 447–450.
- Kölster, P., Munk, L., Stølen, O. and Løhde, J. (1986) Near-isogenic barley lines with genes for resistance to powdery mildew. *Crop Sci.* **26**, 903–907.
- Kyte, J. and Doolittle, R.F. (1982) A simple method for displaying the hydrophobic character of a protein. *J. Mol. Biol.* **157**, 105–132.
- Lassmann, T., Frings, O. and Sonnhammer, E.L.L. (2009) Kalogn2: high-performance multiple alignment of protein and nucleotide sequences allowing external features. *Nucleic Acids Res.* **37**, 858–865.
- Lehti-Shiu, M.D., Zou, C., Hanada, K. and Shiu, S.H. (2009) Evolutionary history and stress regulation of plant receptor-like kinase/Pelle genes. *Plant Physiol.* **150**, 12–26.
- Liu, J., Elmore, J.M., Fuglsang, A.T., Palmgren, M.G., Staskawicz, B.J. and Coaker, G. (2009) RIN4 functions with plasma membrane H⁺-ATPases to regulate stomatal apertures during pathogen attack. *PLoS Biol.* **7**, e100013.
- Lyngkjaer, M.F., Newton, A.C., Atzema, J.L. and Baker, S.J. (2000) The barley *mlo*-gene: an important powdery mildew resistance source. *Agronomie*, **20**, 745–756.
- Michelsen, K., Schmid, V., Metz, J., Heusser, K., Liebel, U., Schwede, T., Spang, A. and Schwappach, B. (2007) Novel cargo-binding site in the beta and delta subunits of coatamer. *J. Cell Biol.* **179**, 209–217.
- Overmyer, K., Brosche, M. and Kangasjarvi, J. (2003) Reactive oxygen species and hormonal control of cell death. *Trends Plant Sci.* **8**, 335–342.
- Panstruga, R. and Schulze-Lefert, P. (2003) Corruption of host seven-transmembrane proteins by pathogenic microbes: a common theme in animals and plants? *Microb. Infect.* **5**, 429–437.
- Perriere, G. and Gouy, M. (1996) WWW-query: an on-line retrieval system for biological sequence banks. *Biochimie*, **78**, 364–369.
- Pfaffl, M.W., Horgan, G.W. and Dempfle, L. (2002) Relative expression software tool (REST (c)) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res.* **30**, e36.
- Piffanelli, P., Zhou, F.S., Casais, C., Orme, J., Jarosch, B., Schaffrath, U., Collins, N.C., Panstruga, R. and Schulze-Lefert, P. (2002) The barley MLO modulator of defense and cell death is responsive to biotic and abiotic stress stimuli. *Plant Physiol.* **129**, 1076–1085.
- Prats, E., Mur, L.A.J., Sanderson, R. and Carver, T.L.W. (2005) Nitric oxide contributes both to papilla-based resistance and the hypersensitive response in barley attacked by *Blumeria graminis* f. sp. *hordei*. *Mol. Plant Pathol.* **6**, 65–78.
- Ren, X.R., Reiter, E., Ahn, S., Kim, J., Chen, W. and Lefkowitz, R.J. (2005) Different G protein-coupled receptor kinases govern G protein and beta-arrestin-mediated signaling of V2 vasopressin receptor. *Proc. Natl. Acad. Sci. USA*, **102**, 1448–1453.
- Runions, J., Brach, T., Kuhner, S. and Hawes, C. (2006) Photoactivation of GFP reveals protein dynamics within the endoplasmic reticulum membrane. *J. Exp. Bot.* **57**, 43–50.
- Schultz, J., Copley, R.R., Doerks, T., Ponting, C.P. and Bork, P. (2000) SMART: a web-based tool for the study of genetically mobile domains. *Nucleic Acids Res.* **28**, 231–234.
- Schweizer, P., Pokorny, J., Abderhalden, O. and Dudler, R. (1999) A transient assay system for the functional assessment of defense-related genes in wheat. *Mol. Plant-Microbe Interact.* **12**, 647–654.
- Seeholzer, S., Tsuchimatsu, T., Jordan, T., Bieri, S., Pajonk, S., Yang, W.X., Jahoor, A., Shimizu, K.K., Keller, B. and Schulze-Lefert, P. (2010) Diversity at the *Mla* powdery mildew resistance locus from cultivated barley reveals sites of positive selection. *Mol. Plant-Microbe Interact.* **23**, 497–509.
- Shiu, S., Karlowski, W.M., Mayer, K.F.X., Pan, R., Tzeng, Y. and Li, W. (2004) Comparative analysis of the receptor-like kinase family in *Arabidopsis thaliana* and rice. *Plant Cell*, **2004**, 1220–1234.
- Shiu, S.H. and Bleeker, A.B. (2001) Receptor-like kinases from Arabidopsis form a monophyletic gene family related to animal receptor kinases. *Proc. Natl. Acad. Sci. USA*, **98**, 10 763–10 768.
- Singh, K.B., Foley, R.C. and Onate-Sanchez, L. (2002) Transcription factors in plant defense and stress responses. *Curr. Opin. Plant Biol.* **5**, 430–436.
- Szczesna-Skorupa, E. and Kemper, B. (2000) Endoplasmic reticulum retention determinants in the transmembrane and linker domains of cytochrome P450C1. *J. Biol. Chem.* **275**, 19 409–19 415.
- Teasdale, R.D. and Jackson, M.R. (1996) Signal-mediated sorting of membrane proteins between the endoplasmic reticulum and the Golgi apparatus. *Annu. Rev. Cell Dev. Biol.* **12**, 27–54.
- Thomas, C.L., Bayer, E.M., Ritzenthaler, C., Fernandez-Calvino, L. and Maule, A.J. (2008) Specific targeting of a plasmodesmal protein affecting cell-to-cell communication. *PLoS Biol.* **6**, 180–190.
- Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25**, 4876–4882.
- Thordal-Christensen, H., Zhang, Z.G., Wei, Y.D. and Collinge, D.B. (1997) Subcellular localization of H₂O₂ in plants. H₂O₂ accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. *Plant J.* **11**, 1187–1194.
- Tichtinsky, G., Vanoosthuysse, V., Cock, J.M. and Gaude, T. (2003) Making inroads into plant receptor kinase signalling pathways. *Trends Plant Sci.* **8**, 231–237.
- Wrzaczek, M., Brosche, M., Salojarvi, J., Kangasjarvi, S., Idanheimo, N., Mersmann, S., Robatzek, S., Karpinski, S., Karpinska, B. and Kangasjarvi, J. (2010) Transcriptional regulation of the CRK/DUF26 group of receptor-like protein kinases by ozone and plant hormones in Arabidopsis. *BMC Plant Biol.* **10**(95), 1–19.
- Zeyen, R.J., Carver, T.L.W. and Lyngkjaer, M.F. (2002) Epidermal cell papillae. In: *The Powdery Mildews: A Comprehensive Treatise* (Belanger, R.R., Bushnell, W.R., Dik, A.J. and Carver, T.L.W., eds), pp. 107–125. St. Paul, MN: American Phytopathological Society Press.

SUPPORTING INFORMATION

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

Fig. S1 Cellular localization of *Hordeum vulgare* cysteine-rich receptor-like protein kinase (*HvCRK1*) is mainly associated with the endoplasmic reticulum (ER). (A) *HvCRK1::GFP* (top row) and the ER marker *YFP::HDEL* (Runions *et al.*, 2006) (bottom row), co-expressed under the control of the CaMV 35S promoter after biolistic delivery of vector DNA to barley epidermal cells, and then analysed for green fluorescent protein/yellow fluorescent protein (*GFP/YFP*) fluorescence by confocal microscopy. Panels I and III show fluorescent close-up images of the edge of the transformed cell and panels II and IV show fluorescent images of the central part of the transformed cell. The fluorescence signal patterns appear to be identical, although *YFP::HDEL* gives a stronger signal than *HvCRK1::GFP* inside the nucleus. (B) *HvCRK1::CFP* and the plasma membrane-associated transporter *AtAHA2::CFP* (Liu *et al.*, 2009) in *Agrobacterium*-transformed *Nicotiana benthamiana* leaves. Panels I and IV show fluorescent images, panels III and VI show light images and panels II and V show overlay images of the same *N. benthamiana* cells. *HvCRK1::CFP* is localized in a polygonal structured ER-like network traversing the cytoplasm (arrows in II), whereas *AtAHA2::CFP* is only localized around the cell periphery.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.