Studies on the mechanism of resistance to *Bipolaris sorokiniana* in the barley lesion mimic mutant *bst1*

MATTIAS PERSSON*, ANDERS FALK AND CHRISTINA DIXELIUS

Department of Plant Biology and Forest Genetics, Uppsala BioCenter, SLU, PO Box 7080, 750 07 Uppsala, Sweden

SUMMARY

The *Bipolaris sorokiniana* tolerant 1 (*bst1*) barley mutant is derived from fast neutron-irradiated seeds of wild-type Bowman(*Rph3*). The induced mutation was genetically localized to a position on chromosome 5HL distal to the centromere using amplified fragment length polymorphism markers. In addition, the defence responses and related gene expression in the *bst1* mutant after fungal challenge were compared with those occurring in wild-type plants. Hydrogen peroxide generation, determined by 3,3-diaminobenzidine staining, revealed a clearly reduced level of *bst1*, compared with the wild-type, during the entire experimental time: 8–120 h post-inoculation (hpi). At 48 hpi, the wild-type samples displayed twice as much fungal mass and three times greater H₂O₂ production than *bst1*. At the same time, staining of *B. sorokiniana* showed less fungal growth in the spontaneous lesions of *bst1* compared with the wild-type. Monitoring of defence-related genes at 48 hpi demonstrated strong expression of *PR-1a*, *PR-2*, *PR-5* and *PR-10* in *bst1*. A gene coding for a unique oxidoreductase enzyme, designated as *HCP1*, was expressed at much higher levels in inoculated leaves of the *bst1* mutant than in those of the wild-type plant. Taken together, the results suggest that the defence to *B. sorokiniana* largely relies on salicylic acid-responsive pathogenesis-related (*PR*) genes, as well as selected reactive oxygen species and unknown *HCP1*-associated factors.

INTRODUCTION

Plants have developed a variety of strategies, including molecular, chemical and physical barriers, to oppose infection. With regard to molecular barriers, it is known that plants have evolved two classes of immune receptors to detect nonself molecules. One class consists of membrane-resident pattern recognition receptors (PRRs) that detect microbe-associated molecular pat-

terns (MAMPs). Plant resistance (R) proteins define a second, mainly intracellular, immune receptor class that has the capacity to detect, directly or indirectly, isolate-specific pathogen effectors, encoded by avirulence (*Avr*) genes (reviewed by Chisholm *et al.*, 2006; Jones and Dangl, 2006). Pathogen recognition, through interactions between plant R proteins and the corresponding *Avr* gene products, may induce localized cell death, the so-called hypersensitive response (HR). HR may also be triggered in nonhost resistance interactions (Alfano and Collmer, 2004). In addition to restricting the spread of a pathogen, especially biotrophic organisms and their containment to the infection site, HR also triggers local and systemic signalling for the activation of various defences in noninfected tissues. Early signalling associated with HR involves the rapid production of highly reactive molecules, such as nitric oxide and reactive oxygen species (ROS) (Besson-Bard *et al.*, 2008). The latter molecules play a role as antimicrobial compounds and in cell wall reinforcements, in addition to signalling (Gechev and Hille, 2005; Gechev *et al.*, 2006). Several other components take part in the subsequent defence signalling that activates resistance responses, such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET) and other plant hormones (Glazebrook, 2005; Robert-Seilanianantz *et al.*, 2007). SA is a positive regulator of HR and is also implicated in systemic acquired resistance (SAR) and the expression of pathogenesis-related (*PR*) genes (Raffaele *et al.*, 2006). It is generally believed that SA is associated with biotrophic resistance, whereas necrotrophs benefit from host cell death and their resistance responses are activated by JA and ET (Glazebrook, 2005).

Plants that spontaneously develop necrotic lesions during development or in response to changes in the environment, without the presence of any pathogen, are denoted as disease lesion mimics or lesion mimic mutants (LMMs). LMM genotypes have been observed in many plant species, such as maize (Johal *et al.*, 1995; Walbot *et al.*, 1983), rice (Marchetti *et al.*, 1983; Takahashi *et al.*, 1999; Yin *et al.*, 2000) and barley (Kjaer *et al.*, 1990; Rostoks *et al.*, 2006; Wolter *et al.*, 1993). In *Arabidopsis thaliana*, screening of similar mutants has resulted in the identification of accelerated cell death (*acd*), constitutive expresser of *PR* genes (*cpr*), lesion simulating disease resistance (*lsd*) and

*Correspondence: E-mail: mattias.persson@vbsg.slu.se

other groups of cell death-related mutants (reviewed by Lorrain *et al.*, 2003). Much of our current understanding about the control and execution of HR comes from the analysis of these mutants, but many details remain to be elucidated.

The *Bipolaris sorokiniana* tolerant 1 (*bst1*) barley mutant has previously been identified as a genetic source for resistance against fungal spot blotch disease (Persson *et al.*, 2008). *bst1* exhibits conspicuous black or brown spots that appear on all above-ground parts of the plant, including the bristles, with the lesion phenotype most prominent on the leaves. The *bst1* mutant is classified as belonging to the initiation group of LMMs. This mutant class, on which locally determinant necrotic lesions are formed, is in contrast with propagation mutants that are unable to control lesion formation (Dangl *et al.*, 1996). In this work, we genetically define the chromosomal region of the *bst1* mutation and enhance our understanding of important defence signalling molecules induced on fungal challenge on *bst1*.

RESULTS

Localization of *bst1* to chromosome 5HL

The *bst1* mutant was identified in a population composed of 5000 M₂ sown spikes derived from fast neutron-irradiated seeds of the wild-type cultivar Bowman (Persson *et al.*, 2008). The wild-type genotype in this study contains the leaf rust resistance gene *Rph3* located on chromosome 7HL (Jin *et al.*, 1993). The *Rph3* gene has not been found to possess any impact on the barley–*Bipolaris sorokiniana* interaction in our previous assessments (data not shown).

The *bst1* line was crossed with the barley cultivars Proctor and Nudinka, on which amplified fragment length polymorphism (AFLP)-based marker information was available (Castiglioni *et al.*, 1998). Segregation of *bst1* was analysed in the F₂ generations, and the analysis showed that the mutation is recessive ($\chi^2 = 116$, $P < 0.005$). From segregating F₂ progeny, 20 lesion mimic phenotypic plants and 20 wild-type plants derived from the *bst1* and Nudinka cross were assessed with 72 AFLP primer combinations. Five markers were found that showed polymorphisms of the *bst1* mutation on chromosome 5HL distal to the centromere. E40M38-7 was the most closely linked marker at 2.1 cM distal to *bst1* (Fig. 1a). The flanking markers from the Proctor × Nudinka map, Xcln.WG644 and Xcln.BC0298, were used as a link to the Barley BinMap 2005 and to the rice sequence information (Fig. 1b,c).

In order to obtain more markers in the chromosomal area of interest, an F₂ population (610 lesion mimic phenotypic plants and 48 plants with wild-type phenotype) derived from the *bst1* and Proctor cross was analysed by combinations of *Pst*I primers P11–P26 and *Mse*I primers M31–M78. In total, seven linked AFLP markers were found, with the two closest markers P26M65

and P26M77 at a distance of 0.25 and 0.23 cM from the mutation, respectively (Fig. 1d).

Fungal infection and *bst1* mutant characteristics

A fungal isolate constitutively expressing the *GUS* (β -glucuronidase) gene, driven by the glyceraldehyde 3-phosphate dehydrogenase (*GPD-1*) promoter (Liljeroth *et al.*, 1993), was introduced to facilitate analysis. At 1 day after point inoculation, a clear difference in lesion size between the moderately susceptible genotypes Bowman(*Rph3*) and ND B112, compared with the susceptible ND 5883, was observed (Fig. 2). However, fungal growth on *bst1* was reduced after the first day ($F = 25.52$, $P < 0.001$). The phenotypic difference between the occurrence of small spontaneous necrotic spots of *bst1* and pathogen-induced necrosis was time dependent. At 72 h after spray inoculation, a clear difference between mock- and *B. sorokiniana*-inoculated leaves of both the wild-type and *bst1* was seen (Fig. 3).

In order to assess the differences in the level of hydrogen peroxide (H₂O₂) in *bst1*, spray-inoculated leaves were stained with 3,3-diaminobenzidine (DAB) and compared with the wild-type. The *bst1* mutant exhibited a distinctly low generation of H₂O₂ induced by the fungus (Fig. 4). Less than 1% of the leaf area was stained with DAB during the first 72 h post-inoculation (hpi); thereafter, an increase to 3% occurred at 120 hpi (Fig. 4). Bowman(*Rph3*), however, exhibited 2% DAB-stained leaf area at 8 and 24 hpi, with a rapid increase to 8% at 48 hpi. At 120 hpi, 12% of the leaf area was stained with DAB.

In parallel, the fungal mass in the inoculated samples was determined by GUS quantification. The most striking difference between the two genotypes occurred at 48 hpi, when Bowman(*Rph3*) contained more than double the increase in fungal mass in the leaves (value $\pm$ standard deviation, 1.26 ± 0.23 nmol/mg protein/min) compared with *bst1* (0.48 ± 0.12 nmol/mg protein/min) ($P < 0.05$). This difference in fungal growth was also visualized by lactophenol trypan blue and GUS staining of inoculated leaves (Fig. 5). No mycelia could be detected in spontaneously occurring lesions in the leaves of *bst1* (Fig. 5c). The roots of *bst1* did not show any spontaneous lesions. No fungal spread from the leaves to the roots could be detected in the plants 4 weeks after inoculation, with only a few exceptions in Bowman(*Rph3*) with a frequency of less than 1%.

Expression of defence- and cell death-related genes

To further investigate the events taking place in the *bst1* mutant, several genes linked to cell death processes and defence were monitored at the transcriptional level and compared with the wild-type at 48 hpi. Primers were designed using barley sequences from orthologous genes in *Arabidopsis* or rice (Table 1).

Fig. 1 Genetic linkage data on *bst1* and genome comparisons. (a) Genetic map constructed on mutant *bst1* on barley chromosome 5HL bin11 using five different amplified fragment length polymorphism (AFLP) primer combinations on F_2 offspring from a cross between *bst1* and Nudinka. (b) Genes located on barley BinMap 2005 at the positions between flanking markers Xcnl.WG644 and Xcnl.BCD0298 from the Proctor $\times$ Nudinka map. (c) A selection of defence-related genes located between the flanking markers Xcl.WG644 and Xcl.BCD298 in rice. (d) Genetic map constructed using seven additional AFLP markers on F_2 offspring from a cross between *bst1* and Proctor. Genetic distances are shown in cM.

In the genetic region to which *bst1* is localized on 5HL, the cyclic nucleotide-gated ion channel 2 (*CNGC2*) gene, showing high similarity to *Arabidopsis DND1* (defence no death 1), has been mapped (Rostoks *et al.*, 2006). *CNGC2* expression was generally low in both the wild-type and *bst1* genotypes, independent of treatment, but the *bst1* mutant showed significantly lower expression of this gene compared with wild-type plants (Fig. 6a). To further assess the importance of *CNGC2*-related processes in defence to *B. sorokiniana*, *Arabidopsis* LMM *dnd1* (Clough *et al.*, 2000) and corresponding wild-type Col-0 plants were inoculated. It was found that the *dnd1* mutant exhibited clear susceptibility, whereas Col-0 showed no symptoms

(Fig. 7a–d). Fungal colonization and growth in the *dnd1* mutant were, however, not as fast as those observed on the An-1 accession (Fig. 7e). Two genes encoding for antioxidant enzymes involved in ROS scavenging/detoxification were analysed: the barley ascorbate peroxidase 1 (*APX1*) and the barley gene orthologue to copper superoxide dismutase (*CSD2*) from *Arabidopsis* (At2g28190). No differences in the expression levels of *APX1* were found in the different samples. In comparison, the expression of *CSD2* increased four-fold in fungal-challenged plants of both genotypes (Fig. 6b). To follow up this result, a respiratory burst oxidase homologue B (*RBOHB*) gene was analysed. Interestingly, it was found to be expressed at low levels in *bst1*

Fig. 2 Lesion size after point inoculation with *Bipolaris sorokiniana* in different barley genotypes: Bowman(*Rph3*), *bst1* and the two reference lines ND B112 and ND 5883. At every time point, *bst1* exhibited significantly smaller lesions formed by the fungal infection. Bars indicate pooled standard deviation using Hsu's Multiple Comparisons with the Best (MCB) test.

Fig. 3 Leaves of wild-type Bowman(*Rph3*) and mutant *bst1* after spray inoculation with H₂O or *Bipolaris sorokiniana*, 72 h post-inoculation (hpi). Clear disease symptoms are evident in the wild-type samples after inoculation. *bst1* exhibits spontaneous necrotic spots in the mock-treated leaves that are similar to infection symptoms. On *B. sorokiniana*-inoculated *bst1* plants, symptom development is not as pronounced as seen in the wild-type.

Fig. 4 Hydrogen peroxide detection using 3,3-diaminobenzidine (DAB) staining of *Bipolaris sorokiniana* spray-inoculated *bst1* and wild-type Bowman(*Rph3*) leaves. The software AP5 assess was used to quantify the stained leaf surface area. In order to determine the proportion of fungal-induced cell death, the mock-inoculated leaf data (spontaneous lesions) were subtracted from the fungal-inoculated samples. The ratio of stained leaf surface area was calculated as a percentage of the total leaf surface area. Error bars indicate standard deviations for triplicate measurements.

independent of treatment, but significantly up-regulated in fungal-inoculated wild-type plants (Fig. 6c). A marker for the ET biosynthesis pathway, a barley orthologue to the *Arabidopsis* 1-aminocyclopropane-1-carboxylate synthase (*ACS2*), was also studied. In this case, inoculated *bst1* and wild-type plants showed a down-regulation when compared with water-inoculated samples (Fig. 6d). The *PR-1a*, *PR-2*, *PR-5* and *PR-10* genes, all markers for activation of the SA defence response pathway, were expressed at the same high level in mock or inoculated *bst1* plants (Fig. 6e–h). However, *PR-2* and *PR-5* expression in inoculated wild-type plants could not be distinguished from the expression in any *bst1* samples. This observation was opposite to that found for *PR-1a* and *PR-10*, where transcript levels in wild-type materials were significantly lower than those in the *bst1* mutant (Fig. 6e,h). For comparison of the results on SA-related *PR* genes, the barley orthologue to the *Arabidopsis* nonexpressor of *PR* genes 1, *NH1*, was included. In this case, a significant increase in gene expression in fungal-inoculated Bowman(*Rph3*), compared with *bst1*, was found (Fig. 6i). Finally, we included the *HCP1* barley gene encoding for an iron/ascorbate-dependent oxidoreductase. Elevated transcript levels were found in mock-treated and inoculated *bst1* plants (12 and 46 relative expression units, respectively), whereas the transcript levels were lower in the wild-type (Fig. 6j). However, an increase in *HCP1* expression was also observed in wild-type samples after fungal challenge.

Fig. 5 Histological staining of *Bipolaris sorokiniana* in barley. Lactophenol trypan blue staining showing fungal growth in different barley genotypes 72 h post-inoculation (hpi). (a) Limited numbers of infected cells in *bst1*. (b) Large areas of several cells infected with *B. sorokiniana* in the wild-type Bowman(*Rph3*). β-Glucuronidase (GUS) staining of GUS-tagged *B. sorokiniana* at 48 hpi in *bst1* (c) and wild-type Bowman(*Rph3*) (d) leaves.

Table 1 List of primers used in quantitative real-time polymerase chain reaction analysis.

Gene	Forward sequence	Reverse sequence	Product size (bp)
<i>ACS2</i> *	CTGCTCATCCATCGACCTTT	CTCCACTCCTCGATCAGGTC	244
<i>APX</i> †	CCAAGGGTTCTGACCACTA	TCAAAGGGTTCCTTGTCAG	150
<i>CSD</i> ‡	CATGAGTTCGGTGACACGAC	TGTCTCTGCCACACCTTCAG	150
<i>RBOHB</i> §	GATCGAGCTCCACAACCATT	CAAGAACAGGCTCACCACAA	160
<i>HPC1</i> ¶	ACCGTCTGAGGAGAAGCAGA	ATCTTCGGGCTCAACTCTCA	151
<i>PR1-a</i> **	ACACCAAACCCAGAATGGAG	TGGGGTGAAAGGTAGTCCTG	118
<i>PR-2</i> ††	GACCTGGCAACCCTCACTAA	CAGCCCAAAGTTCCTCTCTG	162
<i>PR-5</i> ‡‡	CTTCAACCTTGCCATGGACT	CTTCATGGGCAGAAGGTGAT	158
<i>PR-10</i> §§	GGTCGAGATGAAGCTTGAGG	CAAACAACACCGGGCTACTT	320
<i>NH1</i> ¶¶	TCCACCTCCGGAGATAACAA	AGCCAGCTCAGGTTTCATCAT	175
<i>CNGC2</i> ***	GAAAATGCAGAAGGTGACAGG	CAACACCCACCTGCAATATG	104
<i>GADPH</i> †††	CGTTCATCACCACCGACTAC	CAGCCTTGTCCTTGTCAGTG	225
<i>CesA1</i> ‡‡‡	CAGGCAAAACCGCACAC	ATCAACACAAACCCACAGCA	85
<i>Cyclophilin</i> §§§	AGCAGGAACAACAGGCAGAGA	GGGAGCATCACCAAGGAGG	140

**ACS2* PlantGDB-assembled PUT-161a-*Hordeum vulgare*-52696, orthologue to *ACS2* from *Arabidopsis*.

†*APX* GENBANK accession AF411227.

‡*CSD2* GENBANK accession AK248474, orthologue to *CSD2* from *Arabidopsis*.

§*RBOHB* GENBANK accession AK249349, orthologue to *RBOHB* from *Oryza sativa*.

¶*HPC1* GENBANK accession AF527606.

***PR1-a* GENBANK accession X74939.

††*PR-2* GENBANK accession AY239039.

‡‡*PR-5* GENBANK accession AM403331.

§§*PR-10* GENBANK accession AY220734.

¶¶*NH1* GENBANK accession AM050559.

****CNGC2* GENBANK accession AY972628.

†††*GADPH* (Horvath *et al.*, 2003).

‡‡‡*CesA1* GENBANK accession AY483150 (Bengtsson, 2008).

§§§*Cyclophilin* Plexdb Barley1_13715 (Bengtsson, 2008).

Fig. 6 Transcript levels of 10 genes in *Bipolaris sorokiniana*-inoculated and H₂O-treated barley genotypes Bowman(*Rph3*) (wild-type) and *bst1* mutant. The expression at 48 h post-inoculation (hpi) was analysed by real-time polymerase chain reaction with primer sequences for the cyclic nucleotide-gated ion channel 2 (*CNGC2*) (a), copper superoxide dismutase (*CSD2*) (b), the respiratory burst oxidase (*RBOHB*) (c), 1-aminocyclopropane-1-carboxylate synthase (*ACS2*) (d), the pathogenesis-related *PR-1a* (e), *PR-2* (f), *PR-5* (g) and *PR-10* (h), *Hordeum vulgare* *NH1* gene for NPR1-like 1 protein (*NH1*) (i) and *HCP1* encoding an iron/ascorbate-dependent oxidoreductase (j). Relative transcript levels were calculated with barley *GADPH*, *CesA1* and *Cyclophilin* expression as reference. The experiment was based on 10 leaves per genotype and repeated twice.

DISCUSSION

A number of LMMs have been identified and characterized, not least in barley (Lundqvist *et al.*, 1997; Rostoks *et al.*, 2003, 2006). One of the most well studied is the *mlo* mutant, conferring broad resistance to the powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (Wolter *et al.*, 1993). In this case, the plasma membrane-localized MLO proteins are required for successful invasion by the fungus, whereas barley genotypes lacking functional MLO, either as a result of natural variation (Piffanelli *et al.*, 2002) or induced lesions in the *Mlo* gene (Büschges *et al.*, 1997; Piffanelli *et al.*, 2002), are resistant.

The genetic mapping of the *B. sorokiniana*-tolerant *bst1* mutation revealed a linkage to chromosome 5HL and bin11–13. No barley LMM has been reported in this chromosomal region previously. During the identification of the barley homologue to the *Arabidopsis* *HLM1* (an HR-like lesion mimic) gene, which

belongs to the nucleotide-gated ion channel (*CNGC*) gene family, other members of the barley *CNGC* gene family were identified (Rostoks *et al.*, 2006). Three of 10 unique genes, represented by EST unigenes, were located to chromosome 5H, and the putative barley *CNGC* orthologue of the *Arabidopsis* *DND1* gene (EST unigene abc22879) was positioned on bin11. This information led us to study the expression of the *CNGC2* gene corresponding to *DND1*. The transcript level in wild-type barley was reduced following challenge with *B. sorokiniana*, whereas the transcript level in the *bst1* mutant was even lower and remained unaltered following inoculation. The transcript levels are overall relatively low in both genotypes and do not generate clear evidence to link *DND1* to the putative *BST1* gene. Sequence analysis of the *CNGC2* gene in the *bst1* mutant did not reveal any mutations in the coding region. However, we cannot exclude that mutations may have occurred in upstream regions or in other components that could impact upon *CNGC2* transcription.

Fig. 7 Phenotype responses in *Arabidopsis* Col-0 (wild-type) plants, the *dnd1* mutant and the An-1 accession to *Bipolaris sorokiniana*, 10 days post-inoculation. (a) Col-0 exhibits no disease symptoms. The *dnd1* mutant displays a large difference in symptom development between mock- (b) and *B. sorokiniana*-inoculated (c) plants. (d) The fungal growth in *dnd1* is not as pronounced as seen on the highly susceptible *Arabidopsis* accession An-1, visualized by β -glucuronidase (GUS) staining (e). Conidiophores and conidia can also be formed in An-1 within 10 days. Ten plants of each genotype were inoculated and the experiment was repeated twice.

The susceptible phenotype of the *Arabidopsis dnd1* mutant to *B. sorokiniana* showed, however, that this gene plays an important role.

The mapped region in Nudinka has a corresponding chromosomal region in the barley BinMap from 2005, which contains several defence-related genes: for example, genes regulating resistance to the cereal cyst nematode (*Rha4*) and to the stem rust *Puccinia graminis* (*RpgQ*). The corresponding area in rice includes more than 78 genes, a selection of which is shown in Fig. 1. The peroxidase precursors Os03g0762300 and Os03g0762400 are interesting, as malfunction of peroxidase produces a toxic environment that could incite cell death. However, in our DAB staining experiment of *bst1*, we detected less H_2O_2 production than in the wild-type, suggesting an alternative functional explanation.

Enhanced resistance responses in LMMs have been observed in many cases (Dietrich *et al.*, 1994), in particular to biotrophic pathogens. In our attempts to further characterize the *bst1* mutant, it became clear that *B. sorokiniana* has a very slow growth on this genotype compared with the wild-type. This observation is in accordance with a more extensive study in which lesion formation on leaves caused by a larger set of fungal isolates was compared on 22 different barley accessions (Persson *et al.*, 2008). In many LMMs, an accumulation of superoxide and H_2O_2 in or around necrotic tissues occurs. The current understanding suggests that high doses trigger cell death, whereas low doses induce protective mechanisms and eventually ROS detoxification (Vranova *et al.*, 2002). Based on DAB staining, it can be concluded that no rapid production of H_2O_2 occurred in *bst1* compared with the wild-type samples, and no fungal growth was observed in the spontaneous lesions formed

on *bst1* leaves. The spontaneously appearing lesions are not only spatially, but also temporally, separated from disease lesions, and occur 2 days later than the first disease symptoms. *Bipolaris sorokiniana* has a short biotrophic phase and successful tissue infection in the necrotrophic growth phase has been correlated previously with the amount of H_2O_2 produced (Kumar *et al.*, 2002). The delayed disease development seen on *bst1* could be linked to reduced establishment of the fungus and its initial biotrophic growth, but this phase is not succeeded by rapid mycelial growth later in pathogenesis, which is to be expected by a necrotroph surrounded by dead leaf tissue. One explanation of this phenomenon could be the induction of cell wall reinforcement via rapid peroxidase activity and the formation of covalent cross-links between cell wall constituents, or lignification processes (Cona *et al.*, 2006). No enhanced or changed lignification could, however, be seen after lignin staining of fungal-inoculated leaves (data not shown), excluding a major impact of lignification and a possible involvement of the barley orthologue on the lignin biosynthesis-related (Os03g0809000) rice gene.

Interestingly, although the *Arabidopsis AtrbohD* knock-out mutants exhibited reduced levels of ROS, they showed increased cell death when introduced into an *Isd1-1* background challenged with avirulent bacteria (Torres *et al.*, 2005). One interpretation, together with more recent work (Rusterucci *et al.*, 2007; Zago *et al.*, 2006), suggests that nitric oxide and ROS levels may work in synergy and govern whether cell death is initiated or suppressed. In the case of the *bst1* mutant, the *RBOHB* gene was down-regulated independent of treatment, pointing to a disturbed *RBOHB* function. Likewise, a low transcript level of a barley gene orthologue of *Arabidopsis* nitric oxide synthase 1 (*NOS1*) was found in the different samples (data not shown).

These observations imply that these ROS and NO-associated genes are not main actors in this particular response. However, in studies of the interaction between *Arabidopsis* and *B. sorokiniana* (Persson, 2008), it was shown that some mutants impaired in ROS processes are susceptible, such as *Isd1*, *AtrbohD* and *AtrbohF*, but not *ran1-1* (responsive-to-antagonist 1) and *rcd1-1* (radical-induced cell death 1), further demonstrating the complexity of ROS-related responses.

NPR1 is a key component of the SA signalling pathway and *npr1* plants do not show *PR* gene induction (Cao *et al.*, 1994). The *NPR1* orthologue *NH1* was down-regulated in *bst1*, whereas elevated transcript levels of the SA-activated *PR-1a*, *PR-2*, *PR-5* and *PR10* genes were obvious. The latter is common among mutants which develop necrotic lesions spontaneously, for example *cpr* mutants in *Arabidopsis* (Yoshioka *et al.*, 2001). Analyses of additional *Arabidopsis* mutants have, however, shown evidence for the existence of an SA-dependent, but NPR1-independent pathway (Lorrain *et al.*, 2004; Shah *et al.*, 2001; Shirano *et al.*, 2002). The interactions between HR, SA, JA and ET are known to be complex, and both antagonistic and agonistic cross-talk can take place. Elevated levels of ET are commonly observed among different LMMs. However, neither SA nor ET alone can trigger HR (Bouchez *et al.*, 2007; Verberne *et al.*, 2000). Both SA and ET are regarded as positive regulators of HR, in contrast with JA which displays a negative role in programmed cell death regulation (Devadas *et al.*, 2002). Current evidence suggests that components such as enhanced disease susceptibility 1 (EDS1) and phytoalexin deficient 4 (PAD4) amplify ET and SA signals by processing ROS-derived molecules that are essential for immunity to biotrophic pathogens (Brodersen *et al.*, 2006; Rusterucci *et al.*, 2001).

A relationship between senescence and HRs has been found in several pathosystems. For example, the *Arabidopsis* double mutant *hys1cpr5* shows accelerated senescence and constitutively high levels of defence responses (Yoshida *et al.*, 2002). It has also been demonstrated recently by global transcript profiling that viruses induce large sets of senescence-associated genes (Espinoza *et al.*, 2007). One class of genes involved in multiple pathways, such as those linked to ROS and senescence, are oxidoreductases (Jamet *et al.*, 2008). In our survey of barley defence-related sequences, the oxidoreductase HCP1 protein, previously shown to interact with *Brome mosaic virus* coat protein (Okinaka *et al.*, 2003), was found. When *HCP1* gene expression was monitored on *bst1* and wild-type barley materials, it became clear that it was significantly up-regulated in *bst1* samples. The family of oxidoreductases exhibits a great variety of functions, with those having an impact on antioxidant processes and antimicrobial activity via metabolites derived from flavonoid, catechin and anthocyanidin biosynthesis pathways possibly being among the most important in a defence context

(Lukacin and Britsch, 1997; Schweizer, 2008; van Damme *et al.*, 2008). We hypothesize that HCP1 may possess antimicrobial properties, which, together with enhanced *PR* gene expression, could explain the enhanced resistance to *B. sorokiniana* without an increase in H₂O₂. However, much of the defence response in this pathosystem remains unclear. To achieve a fuller understanding of which defence components to *B. sorokiniana* are activated, and their function and interaction, it seems logical to further explore the *Arabidopsis* system, followed by a comparison in barley to reveal any discrepancies between the two plant species.

EXPERIMENTAL PROCEDURES

Genetic localization

The *bst1* mutant was initially crossed with the barley mapping cultivar Nudinka. The barley plants were grown without inoculation in soil (Weibull's K-soil, Svalöv Weibull AB, Sweden) in a glasshouse at a temperature of 15 ± 5 °C day/12 ± 5 °C night, with a 16-h/8-h light/dark regime. From this cross, 20 F₂ plants with the lesion mimic phenotype and 20 F₂ plants with the parent cultivar phenotypes were selected. Genomic DNA preparations from 10 plants were combined to form a DNA bulk (Michelmore *et al.*, 1991). AFLP analysis was carried out as described previously (Vos *et al.*, 1995), but ³²P-dATP was used for the labelling of primers. The DNA bulk samples were subjected to AFLP analyses using the 72 primer combinations developed by Castiglioni *et al.* (1998). For further fine-scale mapping, bulk samples from a *bst1* × Proctor F₂ population were screened with 768 *Pst*I–*Mse*I AFLP primer combinations. The generation of *Pst*I primers was essentially the same as described by Vos *et al.* (1995), with two selective nucleotides. Primers relevant for polymorphism detection are shown in Table S1 (see Supporting Information). The primer combination P25M44 detected a marker that was assessed on a population of 610 F₂ segregants, resulting in the identification of 52 recombinants. The individual recombinants were used to map the other six markers, P26M33, P26M65, P13M72, P13M61, P24M69 and P16M70. Linkage was calculated using the Kosambi mapping function (Kosambi, 1944).

DNA sequencing

Fragments of the four closest AFLP markers, P26M65, P26M77, P13M72 and P13M61, were cut out from the AFLP gel. Extracted DNA was amplified using AFLP primers (Table S1), cloned into pJET1.2 using a CloneJET™ PCR Cloning Kit (Fermentas GmbH, St. Leon-Rot, Germany), followed by sequencing (Macrogen Inc., Seoul, South Korea). Sequence data are shown in Table S2 (see Supporting Information).

Fungal growth and inoculations

The *GUS*-tagged *B. sorokiniana* isolate (Liljeroth *et al.*, 1993) was grown on potato dextrose agar medium (Difco, Sparks, MD, USA) for 14 days in a 12-h dark/12-h near-ultraviolet (near-UV) light regime. Conidia were released by sterile water and the solution was filtered through a 100- μ m Teflon filter to exclude hyphae fragments. For point inoculations, plants were kept at 23 °C, 70% relative humidity (RH), 160 μ mol light for 16 h, and 18 °C, 80% RH, darkness for 8 h. The third leaf was carefully detached in a horizontal position, 21 days after sowing, and inoculated with 5 $\times$ 5 μ L containing 5000 conidia. Five leaves of the genotypes Bowman(*Rph3*), *bst1*, ND 5883 and ND B112 were inoculated and the experiment was repeated twice. After inoculation, the humidity was raised to 100% for 18 h. The lesion sizes were measured with a sliding calliper at 24-h intervals, starting after 24 hpi. For quantitative real-time polymerase chain reaction and for histological analyses, the first leaves of Bowman(*Rph3*) and *bst1* were spray inoculated 7 days after sowing with a concentration of 10 000 conidia/mL (Ma *et al.*, 2004). Plants were sown in Weibull's K-soil, and grown at 22 °C, 230 μ mol light for 16 h and 8 h of darkness. After inoculations, plants were covered with transparent plastic to retain high humidity until the leaf material was harvested at 8, 24, 48, 72 and 120 hpi. As controls, plants were inoculated with water. The *Arabidopsis* accession Col-0, An-1 and mutant *dnd1* (Yu *et al.*, 1998) were cultured as described by Kaliff *et al.* (2007). Inoculation and screening were performed on 10 plants at the five to six leaf stage and repeated twice using the barley spray inoculations described above.

Histochemical staining and quantification of GUS activity

For DAB detection of the H₂O₂ level, the method described by Thordal-Christensen *et al.* (1997) was used, and de-staining was performed in 95% boiling ethanol for 20 min. As a control, active catalase was co-infiltrated simultaneously with DAB, as described by Able (2003). The percentage of DAB-stained leaf area was quantified from photographs of leaves from Bowman(*Rph3*) and *bst1* using APS assess software (L. Lamari, University of Manitoba, Winnipeg, MB, Canada) and compared with water-inoculated samples. For each time point, five leaves were photographed and analysed. The spray inoculation, staining and quantification were repeated twice. GUS quantification of the *GUS*-tagged *B. sorokiniana* isolate was assessed at 48 hpi and based on 10 inoculated leaves of Bowman(*Rph3*) and *bst1*. The GUS activity was measured using 4-methylumbelliferyl β -D-glucuronide (4-MUG) (Weigel and Glazebrook, 2002), and analysed in a DyNA Quant™ 200 fluorometer (Hoefer Pharmacia Biotech Inc., Minnesota, USA). The GUS quantification was

repeated twice with different biological replicates. Protein concentrations were determined as described by Bradford (1976). Histochemical GUS staining was performed at 48 and 72 hpi on 10 leaves, as described in Jefferson *et al.* (1987), and the experiment was repeated twice. Leaves of Bowman(*Rph3*) and *bst1* were spray inoculated and fungal growth was visualized at 48 hpi by lactophenol trypan blue staining (Koch and Slusarenko, 1990). The staining procedure was repeated twice, each with seven individual leaves.

Quantitative real-time polymerase chain reaction

Spray-inoculated wild-type Bowman(*Rph3*) and *bst1*, treated with either H₂O or *B. sorokiniana*, were harvested at 48 hpi. For each biological replicate, 10 individual leaves were used. Total plant RNA was isolated using the Aurum Total RNA Mini Kit (Bio-Rad, Hercules, CA, USA). For cDNA syntheses, the iScript cDNA Synthesis Kit (Bio-Rad) was used with 500 ng of total RNA, as described by the manufacturer. For the expression analysis, 10 μ L Absolute™ QPCR SYBR® Green Fluorescein Mix (Thermo Fisher Scientific Inc., Epsom, UK) was used, together with 3 μ M of primers and 5 ng of cDNA, in a total reaction volume of 20 μ L. All amplifications were performed in an iQ5 cyclor (Bio-Rad) with the conditions of 15 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, and 60 s at 60 °C, followed by a melt curve analysis. Reactions were analysed in triplicate and experiments were repeated twice. As reference, the barley *GADPH* (Horvath *et al.*, 2003), *CesA1* and *Cyclophilin* (Bengtsson, 2008) genes were used. Primer sequences used in the transcript analysis (Table 1) were designed using the software Primer3 v. 0.4.0 (<http://frodo.wi.mit.edu/cgi-bin/primer3/primer3-www.cgi>).

Statistical analysis

Statistical analyses were carried out using MINITAB® Release 14.20 (Minitab Inc., State College, PA, USA). The data for mapping were evaluated using χ^2 analyses. Hsu's Multiple Comparisons with the Best (MCB) test (Hsu, 1984) was used to evaluate the fungal growth in point-inoculated ND B112, ND 5883, Bowman(*Rph3*) and *bst1* leaves. Student's *t*-test was used to assess gene expression and GUS quantification in Bowman(*Rph3*) and *bst1* inoculated plants at 48 hpi.

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SUPPORTING INFORMATION

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

Table S1 List of selected amplified fragment length polymorphism (AFLP) primers used in the genetic mapping of *bst1*. *MseI* primers (M numbers) were used together with *PstI* primers (P numbers) on the F₂ population between *bst1* and Proctor, whereas *EcoRI* (E numbers) and *MseI* primers were employed on the F₂ population between *bst1* and Nudinka.

Table S2 Sequence information of the four closest amplified fragment length polymorphism (AFLP) markers to *bst1*.

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