HvCEBiP, a gene homologous to rice chitin receptor CEBiP, contributes to basal resistance of barley to Magnaporthe oryzae

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Abstract

Background: Rice CEBiP recognizes chitin oligosaccharides on the fungal cell surface or released into the plant apoplast, leading to the expression of plant disease resistance against fungal infection. However, it has not yet been reported whether CEBiP is actually required for restricting the growth of fungal pathogens. Here we evaluated the involvement of a putative chitin receptor gene in the basal resistance of barley to the ssd1 mutant of Magnaporthe oryzae, which induces multiple host defense responses.

Results: The mossd1 mutant showed attenuated pathogenicity on barley and appressorial penetration was restricted by the formation of callose papillae at attempted entry sites. When conidial suspensions of mossd1 mutant were spotted onto the leaves of HvCEBiP-silenced plants, small brown necrotic flecks or blast lesions were produced but these lesions did not expand beyond the inoculation site. Wild-type M. oryzae also produced slightly more severe symptoms on the leaves of HvCEBiP-silenced plants. Cytological observation revealed that these lesions resulted from appressorium-mediated penetration into plant epidermal cells.

Conclusions: These results suggest that HvCEBiP is involved in basal resistance against appressorium-mediated infection and that basal resistance might be triggered by the recognition of chitin oligosaccharides derived from M. oryzae.

Background

To resist attack by microbial pathogens, plants have evolved to recognize them, triggering the expression of diverse defense reactions. The currently accepted model is that plants recognize conserved pathogen-associated molecular patterns (PAMPs) through corresponding pattern recognition receptors (PRRs) which in turn trigger plant immune responses [1-3]. The involvement of PRRs in disease resistance against bacterial pathogens is well-documented. For example, the N-terminal amino acid sequence of bacterial flagellin (designated as flg22) can be recognized through the corresponding receptor FLS2 in Arabidopsis thaliana [4,5]. In addition, the N-terminal sequence of bacterial translational elongation factor Tu (designated as elf18) can be recognized through the corresponding receptor EFR [6,7].

In contrast to bacterial PAMP receptors, much less is known about the role of fungal PAMP receptors in plants. It is conceivable that oligosaccharides derived from chitin or glucan may function as PAMPs because they are major structural components of fungal cell walls and can induce the expression of several defense-related genes when they are applied to plants [8,9]. The rice plasma membrane glycoprotein CEBiP (Chitin Elicitor Binding Protein) was shown to be an important component for chitin-derived signaling and is thought to be a receptor for fungal PAMPs [10]. CEBiP was identified as a chitin-binding protein from suspension cultured rice cells and contains two LysM (lysin) domains which mediate binding to oligosaccharides. Physiological experiments suggest that CEBiP is required for the production of reactive oxygen species by rice plants in response to treatment with chitin elicitor [10].
It is assumed that CEBiP recognizes chitin oligosaccharides present on the fungal cell surface or released into the plant apoplast, leading to the expression of plant disease resistance against fungal infection. However, it has not yet been reported whether CEBiP is actually required for restricting the growth of fungal pathogens in rice.

*Magnaporthe oryzae* is an ascomycete fungus that causes the devastating blast disease in rice [11]. In the previous report, we have generated ssd1 mutants in *M. oryzae* and the cucumber anthracnose fungus *Colletotrichum orbiculare*, in which infection of their respective host plants was restricted by cellular defense responses [12]. Subsequently, by inoculating the *C. orbiculare ssd1* mutant onto *Nicotiana benthamiana* plants in which defense-related genes were silenced, we evaluated the involvement of those genes in basal defense. These experiments revealed that plants in which genes encoding specific MAPKK (MEK2) and MAPKs (SIPK/WIPK) had been silenced were susceptible to the ssd1 mutant, as well as the wild-type strain [13]. Furthermore, we revealed that these MAPKs were activated by fungal cell surface components during infection and that the level of MAPK activation induced by the ssd1 mutant was higher than by the wild-type strain, suggesting that MAPK signaling is required for enhanced basal defense and restriction of fungal infection. In addition, use of the ssd1 mutant together with gene-silenced plants allowed us to critically evaluate the involvement of specific defense-related genes in basal resistance by assessing whether the ssd1 mutant could produce disease lesions on the silenced plants.

In plants, RNA interference (RNAi) is a powerful tool for the evaluation of gene function [14]. For RNAi, it is necessary to generate transgenic plants that express a partial fragment of the target gene, but considerable time is required to obtain seeds from T1 transformants. In contrast, virus-induced gene silencing (VIGS) is a simple, rapid method to transiently generate knockdown plants that avoids the need for stable transformation [15]. Although procedures for VIGS are not yet established for rice, there are reports that VIGS is applicable to barley through the use of barley stripe mosaic virus (BSMV) [16,17]. Barley is a susceptible host plant for *M. oryzae*, so that interactions between *M. oryzae* and barley provide a model for the molecular analysis of compatible interactions between monocot plants and fungal pathogens [18].

In this study, we have exploited the barley-*Magnaporthe* pathosystem to evaluate the involvement in basal resistance of genes encoding a putative PAMP receptor, namely *HvCEBiP*, which is homologous to the rice chitin receptor CEBiP. For this, we used the *M. oryzae ssd1* mutant and BSMV-mediated gene silencing. We present evidence that *HvCEBiP* contributes to basal defense against appressorium-mediated infection by *M. oryzae* in barley.

Results

*Magnaporthe oryzae SSD1* is required for infection of barley

In previous work we showed that the SSD1 gene of *M. oryzae* is essential for the successful infection of susceptible rice plants, and that the failure of *mossd1* mutants to infect was associated with the accumulation of reactive oxygen species (ROS) by host cells [12]. First, we examined whether the SSD1 gene is also essential for the infection of barley (*Hordeum vulgare*). When conidial suspensions of the wild-type strain Hoku-1 were inoculated onto leaves, necrotic lesions similar to those of rice blast disease could be observed at 4 days post inoculation (dpi). In contrast, leaves inoculated with the *mossd1* mutants K1 and K4 did not show visible disease symptoms (Figure 1A). When conidial suspensions were spotted onto intact leaf blades of barley, mutant K1 did not produce any disease symptoms, although the wild-type Hoku-1 forms typical blast lesions at inoculation sites at 4 dpi (Figure 1B). To test whether the K1 mutant retained invasive growth ability, conidial suspensions were spotted onto wound sites on the surface of barley leaves. The mutant produced brown necrotic flecks at wound sites but disease symptoms did not spread further, in contrast to the wild-type Hoku-1 which could form typical blast lesions after infection through wounds (Figure 1B). Overall, the pathogenicity of the *M. oryzae ssd1* mutants was severely attenuated on barley, producing an infection phenotype similar to that seen previously on rice [12].

Microscopic analysis showed that the *mossd1* mutant formed appressoria on the plant surface indistinguishable from those of the wild-type strain Hoku-1 (Figure 2A). However, while Hoku-1 produced intracellular infection hyphae inside host epidermal cells, mutant K1 had formed no infection hyphae at 48 hpi (Figure 2A). To observe the responses of *H. vulgare* cells to attempted infection by the mutant, inoculated leaves were stained with 3,3′-diaminobenzidine (DAB) to detect H$_2$O$_2$ accumulation. However, no significant accumulation of H$_2$O$_2$ was detectable in host cells after inoculation with Hoku-1 or K1 at 48 hpi (data not shown). Next, we attempted to detect the formation of autofluorescent papillae under appressoria using epi-fluorescence microscopy [18]. At sites of attempted penetration by the *mossd1* mutant, autofluorescent papilla-like structures could be observed beneath approximately 80-90% of mutant appressoria (Figure 2B), and intracellular infection hyphae were only rarely observed inside host cells (Figure 2C). On the other hand, the frequency of papilla formation under appressoria of Hoku-1 was only 20%
and infection hyphae developed from 60% of appressoria (Figure 2C). These results suggest that the localized deposition of cell wall material (papillae) at attempted fungal entry sites forms part of the basal defense response of barley epidermal cells to appressorial penetration by *M. oryzae*.

**Virus-induced gene silencing of HvCEBiP using barley stripe mosaic virus**

Chitin is a major structural component of fungal cell walls and is therefore likely to function as a PAMP [10]. We therefore searched for a gene homologous to the *CEBiP* chitin receptor of rice using a barley EST database (TIGR plant transcript assemblies; http://blast.jcvi.org/euk-blast/plannta_blast.cgi) and found an assembled sequence TA30910_4513 which contains the putative full-length coding sequence. The predicted amino acid sequence showed 66% identity to rice *CEBiP*. Furthermore, this sequence contained a signal peptide at the N-terminus, and two LysM motifs and a transmembrane region in the C-terminal region, which are all present in

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**Figure 1** Pathogenicity of *M. oryzae* ssd1 mutant against barley

(A) Pathogenicity assay by spray inoculation of the wild-type strain Hoku-1, and *mossd1* mutants K1 and K4. Conidial suspension (1 × 10^6 conidia/ml) was sprayed onto barley leaves and incubated at 24°C. Typical blast lesions were observed on the inoculated leaves with Hoku-1 but not K1 and K4. Photographs were taken 5 days post inoculation. (B) Pathogenicity assay by droplet inoculation of the wild-type Hoku-1 and *mossd1* mutant K1. Conidial suspensions (1 × 10^5 conidia/ml) were spotted onto leaf blades and incubated at 24°C. On intact leaves, severe blast lesions were observed at sites inoculated with Hoku-1, but not K1. On wounded leaves, brown deposition were observed at inoculated sites with both Hoku-1 and K1 but spreading of the lesions only occurred with Hoku-1.

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**Figure 2** Cytology of infection of barley leaf tissue by the *M. oryzae* ssd1 mutant

(A) Infection phenotypes of the wild-type Hoku-1 and *mossd1* mutant K1. Inoculated leaves at 48 hpi were decolorized and observed with light microscopy. The wild-type strain Hoku-1 formed infection hyphae from appressoria on the plant surface but *mossd1* mutant K1 did not show infection hyphae inside plant cell. Ap, appressorium; Ih, infection hypha; Bar = 5 μm. (B) Formation of papilla-like structures under appressoria of *ssd1* mutant K1. At 48 hpi, the decolorized leaves were observed with epi-fluorescence microscopy. Autofluorescent papillae were visible beneath appressoria. Ap, appressorium; Pa, papilla; Bar = 5 μm. (C) Frequency of appressorial penetration and host papilla formation. Leaves sprayed with conidial suspension (1 × 10^5 conidia/ml) were observed at 48 hpi. Infection phenotypes were classified as follows; Ih, infection hyphae under appressoria; Pa, papilla under appressoria; Ap, appressorium without papilla or infection hyphae. Appressoria of the wild-type strain Hoku-1 penetrated with high frequency to form infection hyphae, but those of *ssd1* mutant K1 induced papillae with high frequency.
The expression of selected defense-related genes during infection. Genes homologous to phenylalanine ammonia lyase, respiratory burst oxidase homologue A and pathogenesis-related proteins 1, 2, and 5 were searched from the barley EST database, and designated as HvPAL, HvRBOHA, HvPR-1, HvPR-2a and HvPR-5, respectively. As shown in Figure 3C, transcripts of HvPAL, HvRBOHA and HvPR-5 could be detected at all time points, suggesting they are constitutively expressed. However, it should be noted that both PAL and PR5 generally belong to multi-gene families and we cannot exclude that gene members other than those evaluated in this experiment may be inducible by fungal infection. HvPR-1 and HvPR-2a expression could not be detected at 0 hpi (no inoculation) but was detected from 6 hpi, suggesting the expression of HvPR-1 and HvPR-2a was induced by inoculation with M. oryzae. However, there were no major differences in plant defense gene expression induced by the wild type and mossd1 mutant K1.

Next, to evaluate the involvement of HvCEBiP in basal resistance of barley, we attempted to perform virus-induced gene silencing (VIGS) using the barley stripe mosaic virus (BSMV) [17]. Before silencing HvCEBiP, we first confirmed the efficiency of BSMV-mediated gene silencing in barley by silencing a gene encoding phytoene desaturase (PDS). After BSMV:PDS genomic RNA was inoculated into the first developed leaves of barley plants, a photobleaching phenotype typical of PDS deficiency was visible on the third developed leaves of all inoculated plants, indicating that BSMV-mediated gene silencing of PDS was effective in barley (see Additional file 1: Figure S1). For silencing of HvCEBiP, we first amplified a 298 bp partial fragment of HvCEBiP from barley leaf cDNA and introduced it into plasmid pSL038-1 which carries the γ genome of BSMV. The resulting construct, in which a fragment of the target gene is introduced in the antisense orientation, was designated as pγ:HvCEBiPAs (Figure 4A). The sequence used for silencing HvCEBiP did not contain either of the two LysM motifs (Figure 3A). In the EST database background, we selected unique sequences to HvCEBiP, although without access to the complete barley genome, we could not exclude that there might be other
potential CEBiP homologs that are silenced. Next, we attempted to evaluate the silencing effect of HvCEBiP by RT-PCR. After inoculation of BSMV:HvCEBiP onto first-developed barley leaves, total RNA was extracted from the third-developed leaves and used for reverse transcription. Typical viral disease symptoms were observed in the third leaves of plants treated with BSMV (control) or BSMV:HvCEBiP genomic RNA (Figure 4B). In these leaves, the expression of both BSMVCP, encoding the BSMV coat protein, and HvEF1α, encoding barley translational elongation factor, was detectable (Figure 4C). On the other hand, the third leaves of plants treated with BSMV:HvCEBiP showed reduced transcription levels of HvCEBiP compared to control plants treated with BSMV (Figure 4C). These results indicate that the transcript level of HvCEBiP was down-regulated by BSMV:HvCEBiP-mediated gene silencing in barley.

HvCEBiP contributes to restricting infection by mossd1 mutants

To examine whether HvCEBiP is involved in the basal resistance of barley to Magnaporthe, we inoculated the mossd1 mutant K4 onto the third-developed leaves of barley plants after inoculation of BSMV:HvCEBiP onto the first-developed leaves. To quantify the severity of disease symptoms produced by the mossd1 mutant, we classified disease symptoms as follows; Type I, no visible symptoms; Type II, brown necrotic flecks; Type III, blast lesions without brown necrotic flecks (Figure 5A). On the leaves of BSMV-treated plants, most symptoms produced by mossd1 mutant K4 were classified as Type I (Figure 5B), whereas on leaves of BSMV:HvCEBiP-treated plants Type II symptoms were produced at approximately half of the sites inoculated with K4 (Figure 5B). This tendency was confirmed in three independent experiments. When the wild-type strain Hoku-1 was inoculated onto leaves of BSMV:HvCEBiP-treated plants, the frequency of Type III symptoms was slightly but consistently higher compared to the control plant, although these effects were not statistically significant (Figure 5B). When conidial suspensions were inoculated onto wound sites on the leaves of BSMV:HvCEBiP-treated plants, there was no significant difference in disease symptoms produced by Hoku-1 and K4 (data not shown), suggesting that the silencing of HvCEBiP does not affect invasive growth ability through wounds. Taken together, these results suggest that HvCEBiP is involved in basal defense responses of susceptible barley plants to appressorial penetration by M. oryzae.

To determine whether the mossd1 mutant was able to develop infection hyphae and colonize barley tissues, we
could penetrate into HvCEBiP-silenced plants but subsequent growth of the infection hyphae became restricted by host defense responses. However, at the few inoculation sites showing severe lesions (Type III), infection hyphae were seen to develop from appressoria without visible host cell death (Figure 5C). Taken together, these results suggest that HvCEBiP contributes to host defense responses expressed after invasion of epidermal cells by M. oryzae infection hyphae.

To evaluate whether HvCEBiP is also involved in non-host resistance, we inoculated conidia of the non-adapted maize anthracnose pathogen C. graminicola onto the third leaves of BSMV:HvCEBiP-treated plants. Although C. graminicola formed appressoria on the leaves of both BSMV- and BSMV:HvCEBiP-treated plants, intracellular infection hyphae were not observed, and no disease symptoms were produced (Figure 6). This suggests that HvCEBiP does not play a critical role in resistance to non-adapted pathogens such as C. graminicola.

Next, we evaluated the possible role in basal defense of selected barley genes required for penetration

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**Figure 5** Pathogenicity of M. oryzae ssd1 mutant on the third leaves of BSMV:HvCEBiP-treated barley plants. (A) Disease symptom index on barley leaves inoculated with M. oryzae Type I, no visible disease symptoms; Type II, brown necrotic flecks; Type III, severe blast lesion with less brown necrotic flecks. (B) Quantification of disease symptoms at 7 dpi according to the disease index shown in (A). Conidial suspensions (1 × 10⁵ conidia/ml) of the wild-type strain Hoku-1 or ssd1 mutant K4 were spotted onto the third leaves of BSMV- or BSMV:HvCEBiP-treated plants. Mutant K4 produced a greater frequency of Type II and Type III infections on BSMV:HvCEBiP-treated plants than on BSMV-treated plants. On BSMV, HvCEBiP-treated plants, the wild-type Hoku-1 also produced slightly more severe symptoms (type III) than on BSMV-treated plants. Twenty droplet inoculations were performed in each experiment with three biological replicates. Data represent mean numbers of inoculation sites and error bars = 1 standard deviation. (C) Cytology of appressorium-mediated infection by ssd1 mutant K4 on leaves of BSMV:HvCEBiP-treated plants. In Type II lesions, infection hyphae emerging from appressoria were observed inside only one epidermal cell, without further hyphal growth into adjacent cells. Formation of infection hyphae was associated with death of the penetrated cell. In Type III lesions, infection hyphae developed further, colonizing neighboring cells, without visible host cell death. Ap, appressorium; Ih, infection hypha; Bar = 10 µm.

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**Figure 6** Pathogenicity of nonadapted pathogen Colletotrichum graminicola on barley. (A) photographs of the inoculated leaves of BSMV:HvCEBiP-treated plants. Droplets of conidial suspension of C. graminicola were applied onto the leaves and photographs were taken at 96 hpi. (B) Microscopy showed that C. graminicola could form appressoria on BSMV:HvCEBiP-treated plants but could not penetrate epidermal cells to form infection hyphae. Bar = 10 µm.
resistance and R-gene mediated resistance to the powdery mildew fungus, *Blumeria graminis* f. sp. *hordei*. For this, we used barley mutant lines deficient in *Ror1* and *Ror2* (required for *mlo*-specified resistance) [19,20], *Rar1* (required for *Mla12*-specified resistance) [21] and *Rom1* (restoration of *Mla12*-specified resistance) [22]. After inoculating conidial suspension of *mossd1* mutant K4 onto leaves of these barley mutants, no significant differences in symptom severity were observed compared to the respective wild-type barley cultivars (Figure 7). It therefore appears that none of these genes are involved in restricting infection by the *mossd1* mutant.

Expression profiling of defense-related genes in *HvCEBiP*-silenced plants

To identify plant defense-related genes that may be regulated by HvCEBiP-mediated signaling, we evaluated the expression patterns of selected barley defense genes in the leaves of BSMV:HvCEBiP-treated plants (Figure 8). Total RNAs were extracted at 0 h (no inoculation), 24 h and 48 h after inoculation of the wild-type Hoku-1 or *mossd1* mutant K4 onto leaves of BSMV- or BSMV: HvCEBiP-treated plants. The expression of *HvEF1α* and BSMVCP was detected at all time points. In contrast, the expression of *HvCEBiP* was clearly down-regulated in BSMV:HvCEBiP-treated plants compared to BSMV-treated plants. The expression of *HvPAL*, *HvPR-2a* and *HvPR-5* was also down-regulated in BSMV:HvCEBiP-treated plants compared to BSMV-treated plants. In contrast, the expression levels of *HvPR-1* and *HvRBOHA* in BSMV:HvCEBiP-treated plants were similar to those in BSMV-treated plants.

Discussion

Barley expresses two layers of basal defense in response to infection by *Magnaporthe oryzae*

In our previous study, we generated an *ssd1* mutant of *M. oryzae*, in which the infection of rice plants was restricted by a defense response involving death of the initially infected epidermal cell [12]. This cell death reaction expressed by rice in response to compatible isolates of *M. oryzae* has been termed ‘whole-plant specific resistance’ (WPSR), and is independent of R-gene mediated resistance in rice [23,24]. In the present study, infection assays revealed that the *mossd1* mutant also showed attenuated pathogenicity on barley. However, the host defense responses expressed in barley to appressorial penetration by the *mossd1* mutant took the form of papilla deposition at attempted fungal entry sites rather than host cell death. The phenomenon of papilla formation during *M. oryzae* infection of barley has also been reported by other authors [18]. In rice, papilla-like wall appositions were also observed beneath appressoria of *M. oryzae*, although these appeared small and thin with electron microscopy [25]. Therefore, the formation of papillae appears to be a general form of basal defense against attempted appressorial penetration by *M. oryzae* in barley. However, the efficiency of
papillae in restricting appressorial penetration seems to be weak because the wild-type strain could successfully penetrate into plant cells with high frequency, as shown in Figure 2C. Apart from papilla formation, a localized cell death reaction was also observed in the initially penetrated host cells in which infection hyphae had developed. This cell death reaction was observed in the leaves of BSMV:HvCEBiP-treated barley plants after infection by both the ssd1 mutant and the wild-type strain of *M. oryzae*. The cell death reaction was associated with inhibition of fungal growth because infection hyphae had not developed beyond the first infected epidermal, even after 7 days. The barley cell death reaction resembles WPSR in rice [23] and conceivably it represents a basal defense response triggered after successful penetration by *M. oryzae* appressoria. It therefore appears that barley deploys two distinct layers of basal defenses against appressorium-mediated infection by *M. oryzae*, namely papilla formation and localized cell death. Two similar layers of plant defense were also shown to operate during non-host resistance of *Arabidopsis* to powdery mildew fungi [26].

**HvCEBiP is involved in basal resistance to appressorial penetration by *M. oryzae***

In our recent work, we used the *C. orbiculare ssd1* mutant to show that a specific MAPK pathway in *N. benthamiana* plays a critical role in host basal defense but genes required for *R*-gene mediated resistance (*RAR1, SGTT* and *HSP90*) do not [13]. Here, we used the *M. oryzae ssd1* mutant to examine the role in basal defense of genes required for penetration resistance and *R*-gene mediated resistance. *Ror1* and *Ror2* were identified as genes required for *mlo*-specific resistance against the barley powdery mildew fungus *Blumeria graminis* f. sp. *hordei* and *Ror2* shows functional homology to syn- taxin AtSYP121 in *Arabidopsis* [27]. *Rar1* was originally shown to be required for race-specific resistance trig- gered by resistance gene *Mla12* against *B. graminis* f. sp. *hordei* expressing the avirulence gene *AvrMla12* [28,29]. *Rom1* was identified as a restoration of *Mla12*-specified resistance (*rom1*) mutant that restores disease resistance to *B. graminis* f. sp. *hordei* carrying the avirulence gene *AvrMla12* [22]. However, infectivity of the ssd1 mutant was not significantly enhanced on any of these barley mutants compared to wild-type plants, suggesting that genes required for *R*-gene mediated resistance do not play a role in basal defense against *M. oryzae*, consistent with findings from the *C. orbiculare-N. benthamiana* interaction [13].

In contrast to mutations in these barley genes, the knock-down of HvCEBiP did enhance infection by the mossd1 mutant. Thus, on BSMV:HvCEBiP-treated plants mutant K4 produced more severe (Type II) symptoms, i.e. brown necrotic flecks, compared to BMSV-treated control plants (Figure 5B). The silencing of HvCEBiP also increased the frequency of successful appressorial penetration by the mossd1 mutant. However, the formation of infection hyphae inside penetrated epidermal cells appeared to trigger localized host cell death, resulting in brown necrotic symptoms. These results suggest that HvCEBiP is involved in basal defense against appressorial penetration by *M. oryzae*. In contrast to the mossd1 mutant, infectivity of the wild-type strain was not significantly enhanced on HvCEBiP-silenced plants but there was a slight increase in symptom severity. This suggests that although HvCEBiP contributes to basal defense in barley, the level of its contribution may be low, so that with the highly pathogenic wild-type strain differences in symptoms between non-silenced and HvCEBiP-silenced plants were hard to distinguish. One plausible explanation of these findings is that basal defense against appressorial penetration involves multi- ple PAMP receptors and signaling pathways, of which signaling via HvCEBiP is only one. A working model for the contribution of HvCEBiP to the dual-layered basal defense responses of barley to *M. oryzae* is presented in Figure 9.

In addition to the increased frequency of brown necrotic fleck symptoms induced by the mossd1 mutant on BSMV:HvCEBiP-treated plants, a few inoculation sites also showed formation of severe blast lesions (Type III symptom) as shown in Figure 5A. Lesion formation was not associated with localized cell death reactions and infection hyphae developed extensively, colonizing many host cells. This suggests that in some cases the mossd1 mutant was able to infect HvCEBiP-silenced plants without triggering cell death-associated defense responses. This raises the possibility that HvCEBiP might be involved in mediating the localized cell death response of barley epidermal cells to invasion by *M. oryzae* infection hyphae. Thus, HvCEBiP might contribute not only to papilla-based defenses but also to the hypersensitive cell death response to cell invasion. HvCEBiP does not appear to play a central role in non-host resistance because the non-adapted pathogen *C. graminicola* produced no symptoms on silenced plants. In contrast, the LysM domain receptor kinase CERK1 was reported to contribute weakly to the resistance of *Arabidopsis thaliana* against the incompatible pathogen *Alternaria brassicicola* [30].

**Is HvCEBiP a specific receptor for components of the mossd1 mutant?***

In the interaction between cucumber anthracnose pathogen *C. orbiculare* and *N. benthamiana*, we reported previously that the altered fungal cell wall composition conferred by ssd1 gene disruption triggers
plant basal resistance through the activation of a specific plant MAPK cascade [13]. We hypothesized that activation of the MAPK pathway might result from recognition of fungal PAMP(s) by corresponding plant receptor protein(s). In this study, we attempted to determine whether HvCEBiP is a specific receptor for PAMPs expressed uniquely by the mossd1 mutant, in which case pathogenicity of the wild-type strain should not be affected by the silencing of HvCEBiP. However, the wild-type strain Hoku-1 showed a slight increase in pathogenicity on HvCEBiP-silenced plants, suggesting that HvCEBiP is a receptor for component(s) shared by both the wild-type M. oryzae and mossd1 mutant.

Rice CEBiP is a receptor-like protein containing two LysM domains, which was originally identified in enzymes that degrade the bacterial cell wall component peptidoglycan [31]. Recent biochemical analysis showed that the LysM domain can also mediate binding to chitin oligosaccharides [32]. The genome of Arabidopsis contains five LysM domain-containing receptor-like kinases [33], among which CERK1 (At3g21630) was identified as a receptor-like protein required for chitin signaling in Arabidopsis [30]. Although the function of the other LysM domain-containing receptor-like kinases is unknown, it is tempting to speculate that plants possess multiple receptor proteins for the perception of particular classes of pathogen-derived oligosaccharides. It is likely that other PAMP receptors, in addition to HvCEBiP, are conserved in barley and contribute to basal resistance to M. oryzae.

**Conclusions**

Rice CEBiP recognizes chitin oligosaccharides derived from fungal cells leading to the expression of plant disease resistance against fungal infection. We evaluated the involvement of putative chitin receptor gene HvCEBiP in barley basal resistance using the mossd1 mutant of Magnaporthe oryzae, which enhances host basal defense responses. The mossd1 mutant showed attenuated pathogenicity on barley and appressorial penetration was restricted by the formation of papillae at attempted entry sites. On HvCEBiP-silenced plants, the mutant produced small brown necrotic flecks or blast lesions accompanied by appressorium-mediated penetration into plant epidermal cells. Wild-type M. oryzae also produced slightly more severe symptoms on the leaves of HvCEBiP-silenced plants. These results indicated that HvCEBiP is involved in basal resistance against appressorium-mediated infection and that basal resistance could be triggered by the recognition of chitin oligosaccharides derived from M. oryzae.

**Methods**

**Plant growth conditions and fungal strains**

Hordeum vulgare wild-type cultivars Fiber-snow, Ingrid and Sultan5, and genetic mutants mlo5, mlo5ror1, mlo5ror2, rar1 and rom1 were grown in a controlled environment chamber (16 h photoperiod, 24°C). Magnaporthe oryzae Hoku-1 was used as the wild-type strain in this study. The mossd1 mutants K1 and K4 were generated as reported previously [12]. These fungal cultures were maintained at 24°C on oatmeal agar medium (6.0 g
powder oatmeal, 1.25 g agar per 100 ml distilled water) under continuous light. *Colletotrichum graminicola* isolate MAFF236902 was described previously [13].

Pathogen inoculation and cytological assays
To induce conidiation, two week-old cultures of *M. oryzae* were washed with sterile water to remove aerial mycelia and then incubated for a further 3 days. For inoculation, conidial suspension was sprayed (5 ml; 1 × 10^6 conidia/ml) or spotted (10 μl; 1 × 10^5 conidia/ml) onto the third leaves of *H. vulgare* and incubated in a humid plastic box at 24°C. For evaluation of invasive growth ability, the surface of barley leaves was scratched with a sterile plastic pipette tip and droplets of conidial suspensions were placed directly onto the wound sites. Cytological observations and the detection of papillae were performed as follows. Inoculated leaves were cut to 1 cm × 1 cm size and decolorized with a 3:1 mixture of ethanol:chloroform and mounted under a coverslip in lactophenol solution. Autofluorescent papillae formed beneath appressoria were visualized by epifluorescence. The accumulation of H_2O_2 in host cells was detected by staining with 3,3′-diaminobenzidine [13].

RT-PCR
Total RNA was extracted from barley leaves using TRIzol Reagent (Invitrogen) following the manufacturer’s protocol. RT-PCR was performed using ReverTra Dash RT-PCR kit (Toyobo) following the manufacturer’s protocol. The primers used for RT-PCR are listed in Additional file 1: Table S1. The sequence data of HvPAL, HvPR-1, HvPR-2a, HvPR-5, HvRB0HA and HvEF1α can be found in GenBank with accession numbers Z49147, Z21494, AY612193, AF355455, AJ871131 and Z50789, respectively.

Vector construction
A 298 bp partial fragment of HvCEBiP was amplified by primer pairs HvCBP1-S1 (5′-CCAAAGACCTCAAGAGGGA-3′) and HvCBP1-AS1 (5′-AGCCGTTGGAA-TAACCTAGT-3′) from cDNA of *H. vulgare* and subcloned into the pGEM-T easy vector (Promega). The resulting construct was digested by NotI and a fragment containing the amplified sequence of HvCEBiP was introduced into the NotI site of pSL038-1 in the anti-sense orientation. This construct was designated as pY-HvCEBiPas.

Virus-induced gene silencing
BSMV genomic RNAs were transcribed *in vitro* as previously described with some modifications [17]. The reaction was performed at 37°C for 60 min in 50 μl of reaction buffer containing 1 μg of linearized plasmids, 1 μl of T7 RNA polymerase (Takara), 10 μl of 50 mM DTT, 6 μl of 10 mM NTPs (rATP, rCTP, rUTP), 0.4 μl of 10 mM rGTP and 5 μl of 5 mM m7G(PPp)G RNA cap structure analog (New England Biolabs). After the reaction, 1.62 μl of 10 mM rGTP and 1 μl of T7 RNA polymerase were added to the reaction mixture, and further incubated at 37°C for 60 min. Transcribed α, β, γ genomic RNAs were mixed in a 1:1:1 ratio with 20 μl FES and inoculated onto the first-developed leaves of *H. vulgare* plants with gentle rubbing. The third-developed leaves were used for evaluating fungal infections.

**Additional material**

**Additional file 1: Figure S1** Efficiency of BSMV-mediated gene silencing in barley. (A) photobleaching by gene silencing of phytoene desaturase (PDS) in barley BSMV:PDS was inoculated onto the first developed leaf (1). After 10 days, photobleaching was observed in the third developed leaf (2). (B) close-up photograph of third- and fourth-developed leaves shown in A. (C) photobleaching phenotypes in five individual plants treated with BSMV:PDS. Third leaves of all five plants showed photobleaching. Table S1 Primers used for RT-PCR.

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Authors’ contributions
ST designed the experiments, performed the gene silencing study and wrote the manuscript. AI performed the sample preparations and vector construction. KY performed the inoculation assay for barley mutant lines. GT participated in experimental procedures for PCR analysis. HK participated in cytological analysis of barley infection assay. YHM participated in barley gene silencing and data analysis. TN participated in barley infection assay and data analysis. NY participated in experimental procedures concerning CEBiP and data analysis. RO supervised the study and critically revised the manuscript. YK conceived and directed the whole study, and participated in the writing of the manuscript. All authors read and approved the final manuscript.

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**References**
1. Thordal-Christensen H: Fresh insights into processes of nonhost resistance. *Current Opinion in Plant Biology* 2003, 6:351-357.
2. Numbert B, Brunn L, Kemmerling B, Piater L. Innate immunity in plants and animals: striking similarity and obvious differences. Immunological Reviews 2004, 198:249-266.
3. Jones JD, Dangl JL. The plant immune system. Nature 2006, 444:323-329.
4. Felix G, Duran JD, Volko S, Bolter T. Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. Plant Journal 1999, 18:265-276.
5. Gómez-Guzmán L, Bauer Z, Bolter T. Both the extracellular leucine-rich repeat domain and the kinase activity of FLS2 are required for flagellin binding and signaling in Arabidopsis. Plant Cell 2001, 13:1155-1165.
6. Kunze C, Zipfel C, Gabrich D, Cariard A, Jones JD, Bolter T, Felix G. The N terminus of bacterial elongation factor Tu elicits innate immunity in Arabidopsis plants. Plant Cell 2004, 16:3496-3507.
7. Zipfel C, Kunze C, Chinchilla D, Cariard A, Jones JD, Bolter T, Felix G. Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated transformation. Cell 2006, 125:749-760.
8. Zhang B, Ramonell K, Somerville S, Stacey G. A LysM receptor-like kinase plays a critical role in chitin binding and signaling in Arabidopsis. Molecular Plant-Microbe Interactions 2002, 15:963-970.
9. Shinya M, Minard R, Kozono I, Matsuoka H, Shibuya N, Kauffmann S, Matsuoka K, Saito M. Novel beta-1,3-1,6-oligosaccharide elicitor from Alternaria alternata 102 for defense responses in tobacco. FEBS Journal 2006, 273:2431-2439.
10. Kaku H, Nishizawa Y, Ishii-Minami N, Akimoto-Tomiyama C, Dohmae N, Takio K, Minami E, Shibuya N. Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. Proceedings of National Academy of Sciences USA 2006, 103:11086-11091.
11. Wilson RA, Talbot NJ. Under pressure: investigating the biology of plant infection by Magnaporthe oryzae. Nature Reviews Microbiology 2009, 7:185-193.
12. Tanaka S, Yamada K, Yabumoto K, Fujii S, Huser A, Tsuji G, Koga H, Dohi K, Mori M, Shiroma T, O’Connell R, Kubo Y. Saccharomyces cerevisiae SSD1 orthologues are essential for host infection by the ascomycete plant pathogens Colletotrichum lagenarium and Magnaporthe grisea. Molecular Microbiology 2007, 64:1332-1349.
13. Tanaka S, Ishihama N, Yoshioka H, Huser A, O’Connell R, Tsuji G, Tsuge S, Kubo Y. The Colletotrichum orbiculare orb1 mutant enhances Nicotiana benthamiana basal resistance by activating a mitogen-activated protein kinase pathway. Plant Cell 2009, 21:2517-2526.
14. Weisley SV, Hellwell CA, Smith NA, Wang MB, Rouse DT, Liu Q. Gooding PS, Singh SP, Abbott D, Stoutjesdijk PA, Robinson SP, Gleave AP, Green AG, Waterhouse PM. Construct design for efficient, effective and high-throughput gene silencing in plants. Plant Journal 2001, 27:581-590.
15. Ruiz MT, Voinnet O, Baulcombe DC. Initiation and maintenance of virus-induced gene silencing. Plant Cell 1998, 10:937-946.
16. Holzberg S, Broos P, Gross C, Pague GP. barley stripe mosaic virus induced gene silencing in a monocot plant. Plant Journal 2002, 30:315-327.
17. Scofield SR, Huang L, Brandt AS, Gill BS. Development of a virus-induced gene-silencing system for hexaploid wheat and its use in functional analysis of the L227-mediated leaf rust resistance pathway. Plant Physiology 2005, 138:2165-2173.
18. Jarosch B, Collins NC, Zellerhoff N, Schaffrath U. RAR1, ROR1, and the actin cytoskeleton contribute to basal resistance to Magnaporthe grisea in barley. Molecular Plant-Microbe Interactions 2005, 18:397-404.
19. Freialdenhoven A, Peterhansel C, Kurth J, Kreuzaler F, Schulze-Lefert P. Identification of genes required for the function of non-race-specific mlo resistance to powdery mildew in barley. Plant Cell 1996, 8:15-41.
20. Collins NC, Lahaye T, Peterhansel C, Freialdenhoven A, Corbett M, Schulze-Lefert P. Sequence haplotypes revealed by sequence-tagged site fine mapping of the Ror1 gene in the centromeric region of barley chromosome 1H. Plant Physiology 2001, 125:1236-1247.
21. Shirasu K, Lahaye T, Tan MW, Zhou F, Azevedo C, Schulze-Lefert P. A novel class of eukaryotic zinc-binding proteins is required for disease resistance signaling in barley and development in C. elegans. Cell 1999, 99:355-366.
22. Freialdenhoven A, Orme J, Lahaye T, Schulze-Lefert P. barley Ror1 reveals a potential link between race-specific and nonhost resistance responses to powdery mildew fungi. Molecular Plant-Microbe Interactions 2005, 18:291-299.
23. Koka H, Dohi K, Mori M. Absciscic acid and low temperatures suppress the whole-plant-specific resistance reaction of rice plants to the infection of Magnaporthe grisea. Physiological and Molecular Plant Pathology 2004, 65:3-9.
24. Koka H, Dohi K, Yoshimoto R, Mori M. Resistance in leaf blades assessed by counting conidia correlates with whole-plant-specific resistance in leaf sheaths in a compatible rice-Magnaporthe oryzae interaction. Journal of General Plant Pathology 2008, 74:246-249.
25. Koka H. Hypersensitive death, autofluorescence, and ultrastructural changes in cells of leaf sheaths of susceptible and resistant near-isogenic lines of rice (Ph2a) in relation to penetration and growth of Pyricularia oryzae. Canadian Journal of Botany 1994, 72:1463-1477.
26. Lipka V, Dittgen J, Bednarek P, Bhrt R, Wiermer M, Stein M, Landig J, Brandt W, Rosal S, Scheel D, Lorette F, Molina A, Parker J, Somerville S, Schulze-Lefert P. Pre- and post-induction defenses both contribute to nonhost resistance in Arabidopsis. Science 2005, 305:1180-1183.
27. Collins NC, Thordal-Christensen H, Lipka V, Bau S, Kombirink E, Qu JU, Hückelhoven R, Stein M, Freialdenhoven A, Somerville SC, Schulze-Lefert P. SNARE-protein-mediated disease resistance at the plant cell wall. Nature 2003, 425:973-977.
28. Freialdenhoven A, Scherag B, Hoffrich K, Collinge D, Christensen HT, Schulze-Lefert P. Recognition specificity and RAR1/SGT1 independence in barley Mlo disease resistance genes to the powdery mildew fungus. Plant Cell 2003, 15:732-744.
29. Miy a A, Albert P, Shinya T, Desaki Y, Ichimura K, Shiraishi K, Narusaka Y, Kawamaki N, Kaku H, Shibuya H. CEK1, a LysM receptor kinase, is essential for chitin elicitor signaling in Arabidopsis. Proceedings of National Academy of Sciences USA 2007, 104:19613-19618.
30. Jones JD, Dangl JL. The plant immune system. Nature 2001, 418:881-888.
31. Holzberg S, Broos P, Gross C, Pague GP. barley stripe mosaic virus induced gene silencing in a monocot plant. Plant Journal 2002, 30:315-327.
32. Ohnuma T, Onaga S, Murata K, Taira T, Kato K, Aono S, Harada T, Kato E. LysM domains from Pteris rufyaenesis chitinase-A: a stability study and characterization of the chitin-binding site. Journal of Biological Chemistry 2008, 283:5178-5187.
33. Jay S, Zhang XC, Neece D, Ramonell K, Clough S, Kim SY, Stacey MG, Stacey G. A LysM receptor-like kinase plays a critical role in chitin sensing and fungal resistance in Arabidopsis. Plant Cell 2008, 20:471-481.