Mycosphaerella graminicola LysM effector-mediated stealth pathogenesis subverts recognition through both CERK1 and CEBiP homologues in wheat

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Key words: chitin elicitor, LysM effector, Virus-induced gene silencing (VIGS), *Barley stripe mosaic virus* (BSMV), *Mycosphaerella graminicola*, *Septoria tritici*, *Zymoseptoria tritici*, *Triticum aestivum* (wheat), plant defense against fungal pathogens

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ABSTRACT

Fungal cell wall chitin is a well-recognized pathogen-associated molecular pattern. Recognition of chitin in plants by pattern recognition receptors activates pathogen triggered immunity (PTI). In Arabidopsis this process is mediated by a plasma membrane receptor kinase CERK1, whereas in rice a receptor-like protein CEBiP in addition to CERK1 is required. Secreted chitin-binding lysin motif (LysM) containing fungal effector proteins such as Ecp6 from the biotrophic fungus *Cladosporium fulvum* have been reported to interfere with PTI. Here we identified wheat homologs of *CERK1* and *CEBiP* and investigated their role in the interaction with the non-biotrophic pathogen of wheat *Mycosphaerella graminicola*. We show that silencing of either *CERK1* or *CEBiP* in wheat using *Barley stripe mosaic virus*-mediated Virus Induced Gene Silencing (BSMV-VIGS) is sufficient in allowing leaf colonization by the normally nonpathogenic *M. graminicola Mg3LysM* (homolog of *Ecp6*) deletion mutant, while the *Mg1LysM* deletion mutant was fully pathogenic toward both silenced and wild type wheat leaves. These data indicate that Mg3LysM is important for fungal evasion of PTI in wheat leaf tissue and that both CERK1 and CEBiP are required for activation of chitin-induced defenses, a feature conserved between rice and wheat, and also perhaps in other cereal species.

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INTRODUCTION

Plants have evolved the ability to detect potentially pathogenic microorganisms by recognizing conserved pathogen-derived signals known as pathogen-associated molecular patterns (PAMPs), usually via pattern-recognition receptors (PRRs) that are expressed on the surface of plant cells (Jones & Dangl, 2006). Recognition of PAMPs by the PRRs triggers downstream signaling, which results in the activation of plant defense responses, a process referred to as PAMP-triggered immunity (PTI). Pathogenic microbes, on the other hand, have evolved specialized secreted effector proteins that are able to interfere with or suppress the activation of PTI, thus enabling the pathogen to successfully evade this first layer of plant defense (Göhre *et al*., 2008; Gimenez-Ibanez *et al*., 2009; Zhang *et al*., 2010). These secreted effectors are typically relatively small proteins and have in many cases been shown to function as virulence factors (de Jonge *et al*., 2011). Plants have also evolved disease resistance proteins that recognize either directly or indirectly pathogen effectors, or the activity of these effectors, which results in activation of a second layer of defense known as effector-triggered immunity (ETI) (Boller & He, 2009).

One of the fungal PAMPs recognized by both plants and animals is chitin, a major component of fungal cell walls. In *Arabidopsis thaliana*, chitin recognition and signaling is mediated by CERK1 (Chitin Elicitor Receptor Kinase 1). Arabidopsis CERK1 contains three lysin motifs (LysM) that mediate binding to chitin-derived oligosaccharides (Miya *et al*., 2007). In rice (*Oryza sativa*), a second LysM-containing protein called CEBiP (Chitin Elicitor Binding Protein) is required in addition to CERK1 for chitin elicitor perception and signaling (Fig. 1A) (Kaku *et al*., 2006; Shimizu *et al*., 2010). This is in contrast to Arabidopsis, where CERK1 alone appears to be sufficient for chitin-induced activation of defenses (Shinya *et al*., 2012). However, recently it has been suggested that in this plant species a homologue of rice

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CEBiP called LYM2 mediates a decrease in molecular flux between cells in the presence of chitin via a pathway which is independent of PTI and not requiring CERK1 (Faulkner *et al.,* 2013). Another recent report suggests that Arabidopsis LYM2 is not involved in chitin signaling but contributes to CERK1-independent resistance against a necrotrophic fungus *Alternaria brassicicola* via a novel disease resistance mechanism (Narusaka *et al.,* 2013). Several pathogenic fungi have been shown to secrete LysM-containing effector proteins that are able to interfere with chitin-induced plant defense responses, such as the Ecp6 protein from the biotrophic fungal pathogen of tomato *Cladosporium fulvum,* which binds chitin with ultra-high affinity (de Jonge & Thomma, 2009; de Jonge *et al* 2010; Mentlak *et al.,* 2012; Sánchez-Vallet *et al.,* 2013). Ecp6 homologue-expressing fungal species include the economically important fungal pathogen *Mycosphaerella graminicola* [*Mg*, anamorph *Septoria tritici,* recently renamed *Zymoseptoria tritici* (Quaedvlieg *et al*., 2011)], the causal agent of Septoria tritici blotch (STB) disease of wheat.

STB is recognized as one of the most economically important diseases of wheat in the UK and Western Europe, and a threat to wheat crop yields worldwide (Dean *et al.*, 2012). *Mg* infects only the leaves of wheat (*Triticum* spp.) plants, entering the leaf via open stomata, after which the fungal hyphae grow apoplastically between the mesophyll cells in an extended symptomless infection phase of between 7 to 28 days depending on the particular wheat genotype - fungal isolate combination. This extended symptomless phase is followed by rapid induction of host cell death and a subsequent necrotrophic feeding phase which is required for fungal asexual sporulation (Kema *et al*., 1996; Dean *et al*., 2012). Notably, fungal biomass does not increase significantly until the latter necrotrophic phase (Keon *et al*., 2007). Previous studies indicate that two homologs of *C. fulvum* Ecp6 identified in the *Mg* genome, Mg3LysM and Mg1LysM, are capable of binding chitin and are also able to protect fungal hyphae against plant-derived chitinases (Marshall *et al*., 2011). In *C. fulvum* the latter

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function is fulfilled by a separate effector protein Avr4 rather than by Ecp6 (van den Burg *et al.,* 2006). Analyses of *Mg* single gene deletion mutant strains demonstrated that only Mg3LysM, but not Mg1LysM, is important for fungal virulence through a role in preventing activation of chitin–triggered plant defenses. This indicated that chitin-induced PTI is important in STB disease development (Marshall *et al.*, 2011), although the components of chitin recognition and downstream signaling pathways in wheat have not yet been characterized. It is worth noting that even for the well studied tomato – *C. fulvum* pathosystem the nature of the tomato receptor(s) that competes for chitin binding with fungal Ecp6 remains unknown (Sánchez-Vallet *et al.,* 2013).

In this study we demonstrated that *Barley stripe mosaic virus*-mediated virus-induced gene silencing (BSMV-VIGS) is relatively long-lasting and therefore can be applied for functional analysis of wheat genes involved in the interaction with plant pathogens that have a long symptomless infection stage, such as *Mg*. Using *in silico* analyses we then identified the most likely wheat (*Tricitum aestivum*) orthologs of *CERK1* and *CEBiP* and then explored whether these two genes play a role during *Mg* infection of wheat. Our results indicate that Mg3LysM is important for fungal evasion of PTI activation in the host tissue and that both *CERK1* and *CEBiP* are required for chitin recognition and signaling in wheat.

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RESULTS

Identification of rice homologs of *CERK1* and *CEBiP* in wheat

We searched the NCBI and DFCI Wheat Gene Index databases to find potential homologues of the rice *CERK1* and *CEBiP* genes in wheat. Only one wheat candidate unigene was identified for *CERK1* (Ta.25461) and *CEBiP* (Ta.58242), respectively. The sequences of wheat and rice CERK1 proteins share 86% amino acid identify and are predicted to contain the same structural features, namely a N-terminal signal peptide, three LysM motifs, a transmembrane domain and a C-terminal kinase domain. The sequences of wheat and rice CEBiP proteins are also very similar and share 81% amino acid identify. Both contain a signal peptide at the N terminus, two LysM motifs and a putative glycosylphosphatidylinositol (GPI) anchor site at the C-terminus (Supporting Information Fig. S1-S3).

Gene expression analysis revealed that both predicted chitin receptor genes, *CERK1* and *CEBiP,* were significantly up-regulated in susceptible wheat leaves at approximately 9 days post inoculation (dpi) with *Mg* isolate IPO323 when the fungus begins to transition from biotrophic to necrotrophic growth (Supporting Information Fig. S4). This is consistent with the function of these genes in activation of the PTI immune responses during the infection phase transition (Rudd *et al*., 2008 and unpublished).

Virus-induced gene silencing of *CERK1* and *CEBiP* in wheat

In order to study the role of *CERK1* and *CEBiP* in the wheat-*Mg* interaction, we used BSMV-

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VIGS to target each of these two genes for silencing. Three different fragments for each target gene were selected for VIGS construct preparation, aided by the siRNA-Finder (Si-Fi) software (http://labtools.ipk-gatersleben.de/index.html) (Fig. 1B-C). Off-target silencing was not predicted to arise from any of the gene fragments. Efficient knock-down of *CERK1* transcript levels was achieved with all three of the VIGS constructs designed, with a reduction in target transcript levels of around 35% with construct 'a' to 56% with constructs 'b' and 'c' in the third leaves of wheat plants subjected to BSMV-VIGS when sampled at 14-dpi (Fig. 1D). The relative degree of silencing induced by each construct agreed well with the Si-Fi software predictions (Fig. 1C). Similar data were obtained in three independent experiments although the exact degree of silencing varied slightly depending on when samples were taken and ranged from 20% to 40% reduction in *CERK1* transcript levels in construct 'a'-infected plants, and from 45% to 70% reduction in construct 'b' or 'c'-infected plants when sampled at different times between 14-dpi and 25-dpi (data not shown).

Only two of the three BSMV-VIGS constructs designed to target *CEBiP* were effective. Constructs 'b' and 'c' induced 15% and 67% reduction of wheat *CEBiP* transcript levels in the third leaves of infected plants (Fig. 1E). This was again consistent with the Si-Fi software predictions as construct 'a' was not predicted to generate any effective small interfering RNAs (siRNAs) (Fig. 1C). Similar degree of silencing with each *CEBiP*-specific VIGS construct were seen in three independent experiments (data not shown).

In all subsequent experiments involving fungal inoculations we used only the two VIGS constructs for each target gene, *CERK1* and *CEBiP*, that provided the greatest degree of silencing.

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Optimization of BSMV-VIGS for studying wheat leaf-*Mg* interactions

We proceeded to explore the effect of transiently silencing *CERK1* or *CEBiP* on various types of *Mg*-wheat interactions. Plants were pre-inoculated with the different BSMV-VIGS constructs at the two-leaf seedling stage, and between 10-14 days later the third leaves inoculated with the wild-type *Mg* strain IPO323 in an attached leaf bioassay (Rudd *et al.*, 2008). Very few or no fungal pycnidia formed on either the *CERK1*- or *CEBiP*-silenced or control BSMV:*asGFP* inoculated wheat cv. Cadenza, which is resistant to *Mg* strain IPO323, indicating that pre-infection with BSMV did not compromise resistance to *Mg* in these plants (Supporting Information Fig. S5). There was also no significant difference in pycnidial coverage ($F_{15, 70} = 1.04$, $p = 0.427$) in leaves of wheat cv. Riband (susceptible to *Mg* strain IPO323) pre-inoculated with the different BSMV-VIGS constructs (Fig. 2A-B). Pycnidial coverage of the majority of cv. Riband leaves was more than 60% for all treatments and therefore indicated that the compatible wheat-*Mg* interaction also remained unaltered. This experiment was repeated three more times with both cv. Cadenza and cv. Riband with consistent results.

Silencing either *CERK1* or *CEBiP* allows wheat leaf colonization by the normally nonpathogenic *M. graminicola ΔMg3LysM* mutant

We previously showed that the *Mg* mutant strain *ΔMg1LysM* is fully virulent on leaves of wheat cv. Riband and also lacked the ability to block chitin-elicited defense responses (Marshall *et al*., 2011). When we inoculated *CERK1- / CEBiP*-silenced and control cv. Riband plants with the *ΔMg1LysM* mutant strain similar levels of disease were observed on silenced and non-silenced leaves (Fig. 2C-D), with no significant difference in pycnidial

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coverage on plants pre-inoculated with the different BSMV-VIGS constructs ($F_{15, 40} = 0.91$, $p = 0.564$).

We then investigated whether silencing either *CERK1* or *CEBiP* would fully or partially restore the disease-causing ability of the *Mg* mutant strain *ΔMg3LysM*. When the cv. Riband leaves were examined at 21 days post-fungal-inoculation, significant differences were found between the pycnidial coverage profiles on leaves of plants that received treatments with the different BSMV-VIGS constructs ($F_{15, 80} = 3.67$, $p < 0.001$). As expected, few or no pycnidia were detected on leaves of most of the virus-free or BSMV:*asGFP*-infected control plants (Fig. 2E). By contrast, a significant proportion of the *CERK1*- or *CEBiP*-silenced leaves displayed high (>60%) *ΔMg3LysM* pycnidial coverage ($p < 0.05$, Fisher's Least Significant Difference test) (Fig. 2E-F).

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DISCUSSION

We have previously shown that Mg3LysM is required for full virulence of *Mg* on wheat leaves, and that this effector protein is able to block the induction of chitin-elicited defenses in tomato cell cultures (Marshall *et al.,* 2011). In this study we demonstrate that Mg3LysM is required during the early asymptomatic phase of STB disease on wheat and has the role of shielding fungal chitin from recognition by the wheat chitin receptor proteins CERK1 and CEBiP. This is the first study to show that both CERK1 and CEBiP are involved in chitin perception in wheat, and also that BSMV-VIGS can be used in wheat for identification of host defense components in the wheat-*Mg* interaction. We show that infection of wheat leaves with the control viral vector does not in itself interfere with the outcome of either the incompatible (resistance) or compatible (disease) interaction with *Mg*. These new data indicate that this transient gene silencing approach is suitable for investigating and functionally characterizing the plant genes involved in both interaction outcomes.

In Arabidopsis, the receptor kinase CERK1 alone is sufficient for chitin perception, whereas in rice CERK1 and CEBiP co-operatively regulate chitin signaling (Shimizu *et al*., 2010; Shinya *et al*., 2012). It has previously been shown that Slp1, a homolog of *C. fulvum* Ecp6 from the rice blast fungus *Magnaporthe oryzae* is able to directly compete with the rice CEBiP receptor for chitin binding (Mentlak *et al*., 2012), and that the *M. oryzae Δslp1* mutant was able to cause full rice blast disease in stable transgenic rice lines in which *CEBiP* had been silenced by RNAi. It remains unknown whether CERK1 also plays a role in the *M. oryzae* – rice interaction. However this is conceivable because rice CEBiP and CERK1 have been shown to interact with each other through the extracellular LysM domains in the yeast two-hybrid system assays and are speculated to form functional heteroduplexes *in planta* (Shimizu *et al.*, 2010). Whether chitin signaling is co-regulated by CERK1 and CEBiP in

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other monocotyledonous species has not yet been reported. It has however been shown that barley *CEBiP* is involved in resistance to the mutant strain of rice blast pathogen *M. oryzae,* called *mossd1*, that lacks the function of the *SSD1* gene (a regulator of cell wall assembly). The *mossd1* mutant was able to form appressoria and primary hyphae but post-invasion was severely restricted in pathogenicity towards leaf sheaths of compatible rice plants due to the activation of a plant hypersensitive cell death response (Tanaka *et al*., 2007). However, when inoculated onto barley plants in which *CEBiP* had been silenced either through stable RNAi or through BSMV-VIGS, this mutant was able to produce small disease lesions at a small proportion of inoculation sites, indicating that *CEBiP* in barley contributes towards the host resistance to *mossd1* (Tanaka *et al*., 2010). Our data suggest that in wheat, as in rice, chitin recognition and signaling is co-regulated by CERK1 and CEBiP and represents an evolutionarily conserved perception mechanism shared between rice and wheat, and also perhaps in other cereal species.

In this study we demonstrate that the *ΔMg3LysM* mutant of *Mg*, which is severely compromised in its disease-causing ability on wheat, is fully pathogenic on wheat leaves in which either *CERK1* or *CEBiP* have been silenced. On the other hand, silencing *CERK1* or *CEBiP* had no effect on the pathogenicity of the *Δ1MgLysM* mutant toward wheat leaves. This is consistent with our previous observation that although both *Mg* LysM effectors Mg1LysM and Mg3LysM are able to bind chitin and protect fungal hyphae against hydrolytic plant enzymes, only Mg3LysM is able to block chitin-induced plant defense responses (Marshall *et al*. 2011). Our new data therefore suggests indirectly that the chitin binding function of Mg3LysM during disease development is likely to be more important than its protective activity against plant chitinases.

Mg has an infection biology which is different to that of another dothideomycete *C. fulvum*

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and to the more distantly related sordariomycete *M. oryzae*. These are the only two fungal species to date that have been investigated with respect to LysM effectors and suppression or evasion of chitin-triggered plant immunity. While *C. fulvum*, like *Mg*, invades host tissue via stomata and grows strictly intercellularly, it is a true biotrophic pathogen that is able to increase its biomass and sporulate in association with living plant cells (Thomma *et al*., 2005). *M. oryzae* is a hemibiotroph which in contrast to *Mg* and *C. fulvum* uses specialized infection structures called appressoria to penetrate inside plant cells. The *M. oryzae* hyphae then undergo an extensive intracellular biotrophic phase during which the fungus is able to obtain nutrition from living plant cells before inducing extensive host cell death and lesions formation (Wilson & Talbot, 2009).

Given the length of the early symptomless phase of *Mg* infection, which persisted for 10-14 days in our experimental system, it is noteworthy that we have been able to use BSMV-VIGS to silence the wheat chitin receptor genes and study the *in planta* role of both in the infection process. Prior to this study, BSMV-VIGS has only been used to study plant-pathogen interactions in which fungus-induced disease symptoms could be scored after between 5 days (in the case of powdery mildew disease; Hein *et al*., 2005) to 12 days (as with leaf or stem rust diseases; Scofield *et al*., 2005; Zhang *et al*., 2009) post-fungal inoculation. However, here we were able to detect and utilize effective gene silencing through VIGS when scoring STB disease symptoms at 21 days post fungal inoculation (corresponding to 31 days post virus inoculation). This indicates that BSMV-VIGS may be suitable for functional gene studies involving other pathogens of monocotyledonous plant species with prolonged life cycles, for example other members of the *Mycosphaerella* genus. Importantly the efficiency of silencing induced by the different VIGS constructs targeting different parts of the same gene agreed well with that predicted by the siRNA-Finder software (http://labtools.ipk-gatersleben.de/index.html). Even though only a small number of constructs were tested in this

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study, this good correspondence between experimental prediction and data generated suggests that siRNA-Finder could be used to speed up the process of identifying effective target gene fragments for VIGS experiments and to minimize the likelihood of off-target silencing.

In summary, our data indicate that CERK1-/CEBiP- co-regulation of chitin elicitor detection has been conserved within cereal species and that evasion of this plant immune response has been conserved between fungi with very different *in planta* lifestyles. Putative LysM-containing effector proteins have been identified in both pathogenic and saprophytic fungal species across five different phyla (de Jonge & Thomma, 2009), and it is possible that in many of these pathogenic species at least, LysM-containing effector proteins are similarly involved in subversion of host recognition of essential fungal cell wall components.

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MATERIALS AND METHODS

Sequence analysis, alignment and domain predictions

To identify *CERK1* and *CEBiP* homologues in wheat, the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nim.nih.gov/BLAST.cgi) and the DFCI Wheat Gene Index (http://compbio.dfci.harvard.edu/tgi/tgipage.html) resources were used. As the genome of wheat has not been fully sequenced, bioinformatic analysis was carried out on the available wheat transcripts databases. The nucleotide and amino acid sequences of rice CERK1 and CEBiP proteins were used to search for their homologues in wheat using the BLASTN and TBLASTN programs. Only one candidate full length coding DNA sequence with high homology to the corresponding rice genes was identified for each of wheat *CERK1* (Ta.25461) and *CEBiP* (Ta.58242), whereas searches for wheat sequences with LysM motifs did not identify any other candidate LysM-containing sequences in the available databases. ClustalW (http://www.genome.jp/tools/clustalw/) was used for multiple sequence alignment of all known rice LysM receptor-like kinases and wheat CERK1, and for alignment of the rice and corresponding wheat candidate CEBiP amino acid sequences. The multiple sequence alignment was used to construct an unrooted Maximum Likelihood phylogenetic tree in MEGA5 (Tamura *et al.*, 2011). Protein domain predictions were made using InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan/) and using the PROSITE database resource (http://prosite.expasy.org). All predictions were inspected and adjusted manually. GPI modification sites were predicted using the big-PI Plant Predictor (Eisenhaber *et al.*, 2003; http://mendel.imp.ac.at/gpi/plant_server.html).

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Plant growth conditions

N. benthamiana plants for preparation of BSMV sap inoculum and seedlings of wheat (*Triticum aestivum*) cultivars Riband and Cadenza were grown in a controlled environment chamber at 23^{0}C, 60% relative humidity, with a 16 h photoperiod (approximately 120 µmol.m^{-2}.s^{-1} light).

Preparation of BSMV VIGS constructs and viral inoculations

The BSMV-VIGS system described by Yuan and associates (2011), comprising three T-DNA binary plasmids, pCaBS-α, pCaBS-β and pCa-□bLIC, was used. Gene silencing constructs were created by cloning fragments of wheat *CERK1* and *CEBiP* fragments into pCa-□bLIC in antisense orientation using a ligation-independent cloning strategy. *In silico* predictions by Si-Fi software (http://labtools.ipk-gatersleben.de/) were used to select the most effective gene-specific fragments for silencing ranging from 254 to 325 bp in size, and also to ensure the selected fragments were not likely to direct off-target silencing. The cDNA fragments were generated by standard RT-PCR from total RNA extracted from wheat cv. Cadenza leaf tissue using primers described in Supporting Information Table S1. A 236 bp fragment of *GFP* was amplified by PCR from the GFP-expression cassette plasmid pBIN35S::GFP4 (Haseloff *et al.*, 1997) using the primer pair LIC asGFP F / LIC asGFP R (Table S1) and inserted into pCa-□bLIC in antisense orientation to generate the negative control construct for VIGS.

The BSMV pCaBS-α, pCaBS-β and pCa-□bLIC derivatives were transformed separately into *Agrobacterium tumefaciens* strain GV3101 by electroporation. For agroinfiltration, single colonies were grown for 20-22 h at 28^{0}C with constant shaking in 5 ml of LB containing 50

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μg.ml^{-1} kanamycin. Bacterial cells were pelleted by centrifugation at 2400 *g* for 15 min at 4^{0}C and resuspended in agroinfiltration buffer [10 mM $MgCl_2$, 10 mM 2-(*N*-morpholino) ethanesulfonic acid (MES), pH 5.6 and 0.1 mM acetosyringone] to a final OD_{600} of 1.5. After 3 h incubation at room temperature, agrobacteria containing pCaBS-α, pCaBS-β and the relevant pCa-□bLIC derivative were mixed in 1:1:1 ratio and infiltrated into the abaxial side of the leaves of 3-4 week-old *N. benthamiana* plants with a 1 ml needleless syringe. The infiltrated leaves were harvested at 3-4 days post-infiltration, ground using a mortar and pestle in 10 mM potassium phosphate buffer (pH 6.8) containing 1 % celite, and the sap used to mechanically inoculate the first leaf of 11-day-old wheat plants.

Fungal strains and inoculations

The *M. graminicola* wild-type isolate IPO323 and its mutant derivatives *ΔMg3LysM* and *ΔMg1LysM* (Marshall *et al*., 2011) were used in this study. Attached wheat leaf infection assays were done as described previously (Rudd *et al*., 2008) with slight modifications. The third leaves of 23-day-old wheat plants (corresponding to 12-days post viral-inoculation where BSMV-infected plants were used) were fastened, adaxial side up, to a metal platform using double-sided sticky tape. The leaves were then inoculated evenly with fungal spores at a density of 5 x 10^5 cells.ml^{-1} water containing 0.1 % (v/v) Silwet L-77 (GE Silicones) using cotton swabs. The plants were covered with transparent boxes to retain high humidity for the first 72 h, and then returned to 60% relative humidity for up to 20 days. Disease was assessed at 21 dpi by scoring the area of *Mg*-inoculated leaf tissue that was covered by fungal pycnidia. The scores were sorted into six pycnidial coverage classes: 0, 1-20, 21-40, 41-60, 61-80 and 80-100%. All disease assessments were made by the same person.

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RNA extraction and Quantitative RT-PCR

Where qRT-PCR on *Mg*-infected tissue was carried out, tissues were excised at various time points after inoculation, with 5 leaves pooled for each biological replicate, with each leaf harvested from a separate plant. All leaf bioassays were performed in triplicate.

Total RNA was extracted from freeze-dried leaves using TRI Reagent (Sigma Aldrich) following the manufacturer's protocol. One microgram of total RNA was treated with RQ1 RNase-free DNase I (Promega) and used for cDNA synthesis using High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturers' instructions. The resulting cDNA was diluted 1:20 with sterile deionized water and analyzed using SYBR Green Jumpstart ReadyMix (Sigma Aldrich). A 5 µl aliquot of diluted cDNA was used in a 20 µl PCR reaction, with an annealing temperature of 60^0C. Quantification of gene expression was carried out in an ABI 7500 Real-Time PCR system (Applied Biosystems). The primers and final primer concentrations used for real-time PCR are described in Supporting Information Table S2. The wheat *CDC48* housekeeping gene (Paolacci *et al*., 2009) was used for expression normalization.

Statistical analyses

GenStat (release 14.1, 2011, VSN International Ltd, Hemel Hempstead, UK) was used for the statistical analyses. Differences in normalized *CERK1* and *CEBiP* transcript levels between mock- and *Mg* IPO323-inoculated wheat leaves were analyzed using REML linear mixed modeling. Significance of difference between mean transcript levels was determined using

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least significant differences (LSD) at the 5% ($p < 0.05$) level of significance. Means and LSD values are displayed in Supporting Information Tables S3-S6. Pycnidial coverage data were analyzed using generalized linear modeling (GLM) assuming a Poisson distribution with a logarithm link function. The variate modeled was the number of leaves within each pycnidial coverage class for each BSMV-VIGS construct treatment group, accounting for the three experiments and seedling trays nested within experiments as blocking terms in the model. Significance of model terms was assessed using change in deviance, invoking approximate F-tests. Calculated mean values and standard errors were output and are displayed in Supporting Information Tables S7-S9. Significance of difference between calculated means was determined using LSD at the 5% level of significance.

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ACKNOWLEDGEMENTS

We thank Stephen Powers (Rothamsted Research) for help with statistical analyses. The work was supported by the Biotechnology and Biological Sciences Research Council of the UK (BBSRC) through the Institute Strategic Program 20:20 Wheat®. This research was carried out under the Fera agency of the UK Department for Environment, Food and Rural Affairs plant health license PHSI 181/6786.

AUTHOR CONTRIBUTIONS

W-S.L., J.J.R., K.E.H-K. and K.K. conceived the experimental plan. W-S.L. and K.K. performed the experiments. W-S.L., K.E.H-K. and K.K. wrote the manuscript. W-S.L., J.J.R., K.E.H-K. and K.K. were all involved in revising the manuscript and in approving the final version.

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FIGURE LEGENDS

Figure 1. Silencing predicted components of the chitin signalling pathway in wheat. (**A**) Recognition of chitin fragments by rice CERK1 and CEBiP is thought to trigger downstream signaling resulting in defense activation. (**B**) Diagrammatical representation of wheat CERK1 and CEBiP. Bars extending from the termini of the predicted protein structures indicate non-translated cDNA regions up- and down-stream of the coding regions. Bars above each gene model indicate regions amplified to generate different BSMV-VIGS constructs targeting these genes. (**C**) Numbers of effective siRNA hits predicted by Si-Fi software to arise from each of the gene fragments used in BSMV-VIGS. (**D and E**) qRT-PCR analysis of *CERK1* and *CEBiP* transcript levels in BSMV-infected wheat plants at 14 days post-inoculation, respectively. Data shown is representative from one of three independent experiments. Error bars in (**D-E**) represent mean ± s.e.m of three biological replicates.

Figure 2. The effect of silencing *CERK1* and *CEBiP* on disease caused by wild-type *Mycosphaerella graminicola* (wt *Mg*) strain IPO323 and the *ΔMg1LysM* and *ΔMg3LysM* mutants in wheat. The control virus treatments (no virus and BSMV:asGFP) and silencing constructs BSMV:asCERK1b, BSMV:asCERK1c, BSMV:asCEBiPb and BSMV:asCEBiPc were used for these experiments. Disease symptoms and pycnidial coverage on (**A-B**) wt *Mg*, (**C-D**) *ΔMg1LysM*- and (**E-F**) *ΔMg3LysM*-inoculated leaves of wheat cv. Riband. Photographs of leaves and pycnidial coverage scores were taken at 21 days post-inoculation (dpi). Ten leaves per *Mg* strain-BSMV VIGS construct combination were scored in each experiment. The data plotted are the mean numbers of leaves within each of the six pycnidial

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coverage score class, calculated using data pooled from three independent experiments.

Error bars represent mean ± s.e.

SUPPORTING MATERIALS

Fig. S1. Sequence alignment of wheat and rice CERK1 proteins.

Fig. S2. Sequence alignment of wheat and rice CEBiP proteins.

Fig. S3. Phylogenetic relationship between predicted wheat CERK1 and all known LysM receptor-like kinases in rice.

Fig. S4. *CERK1* and *CEBiP* transcript expression levels during early stage of *Mycosphaerella graminicola* infection of wheat leaves.

Fig. S5. BSMV-mediated silencing of *CERK1* or *CEBiP* does not compromise resistance of wheat cv. Cadenza to wild-type *Mycosphaerella graminicola* isolate IPO323.

Table S1. PCR primers used for generating gene fragments for VIGS.

Table S2. Primers used for quantitative real-time RT-PCR.

Table S3. Mean *CERK1* transcript levels in mock- and *M. graminicola*-inoculated wheat leaves during the infection time-course.

Table S4. Matrix of least significant differences used to assess variation in *CERK1* transcript levels in mock- vs. *M. graminicola*-infected wheat leaves.

Table S5. Mean *CEBiP* transcript levels in mock- and *M. graminicola*-inoculated wheat

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leaves during the infection time-course.

Table S6. Matrix of least significant differences used to assess variation in *CEBiP* transcript levels in mock- vs. *M. graminicola*-infected wheat leaves.

Table S7. Calculated means and standard errors of *Mg* isolate IPO323 pycnidial coverage scores for leaves of silenced and non-silenced wheat plants.

Table S8. Calculated means and standard errors of *ΔMg3LysM* mutant pycnidial coverage scores for leaves of silenced and non-silenced plants.

Table S9. Calculated means and standard errors of *ΔMg1LysM* mutant pycnidial coverage scores for leaves of silenced and non-silenced plants.

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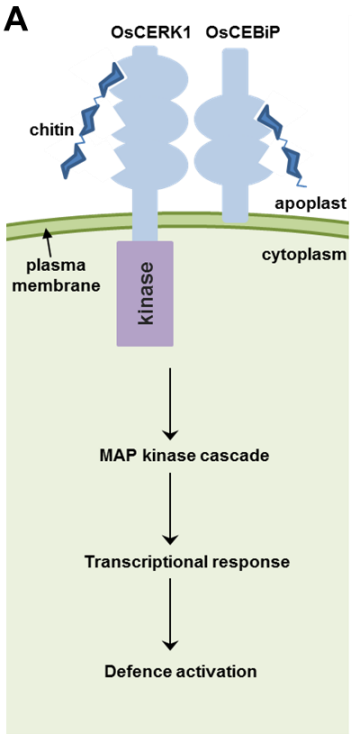
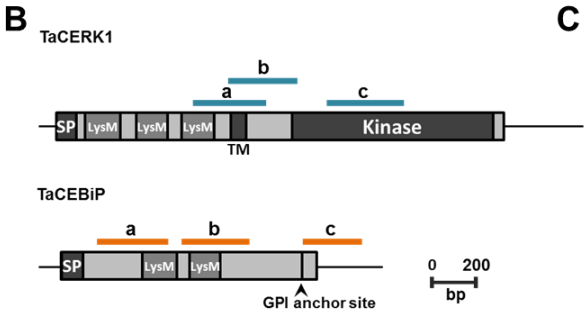
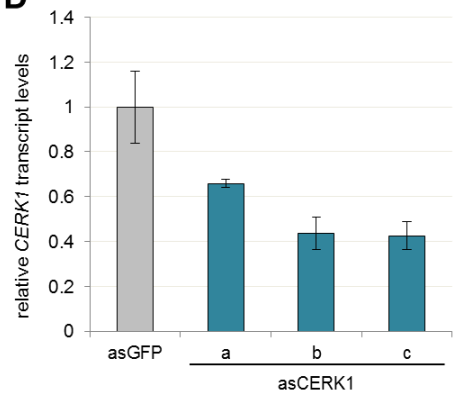
Figure 1





C

Target gene	VIGS fragment	Effective siRNA hits
TaCERK1	a	12
	b	36
	c	36
TaCEBiP	a	0
	b	25
	c	39



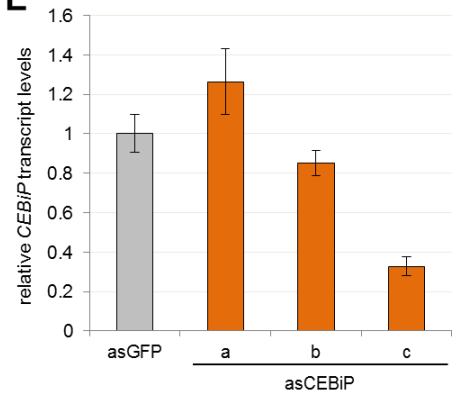
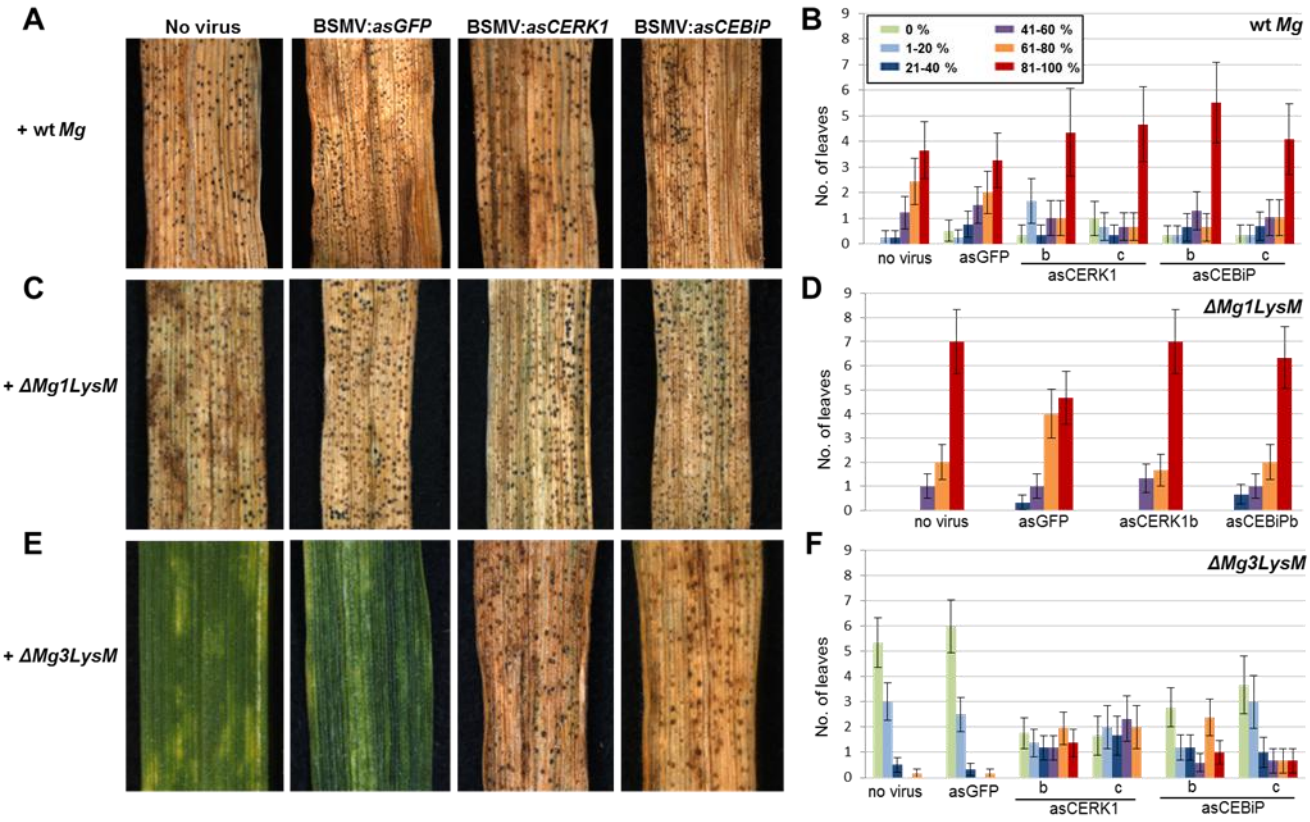


Figure 2


A
No virus
BSMV:asGFP
BSMV:asCERK1
BSMV:asCEBiP
+ wt Mg
B
wt Mg
0 %
1-20 %
21-40 %
41-60 %
61-80 %
81-100 %
No. of leaves
no virus
asGFP
b
c
asCERK1
b
c
asCEBiP
C
+ ΔMg1LysM
D
ΔMg1LysM
No. of leaves
no virus
asGFP
asCERK1b
asCEBiPb
E
+ ΔMg3LysM
F
ΔMg3LysM
No. of leaves
no virus
asGFP
b
c
asCERK1
b
c
asCEBiP

Supplemental e-Xtras (Figures S1-S5 and Tables S1-S9)

Figure S1. Sequence alignment of wheat and rice CERK1 proteins

Black boxes highlight identical amino acids and gray boxes highlight similar amino acids. Predicted protein motifs and domains are boxed. The predicted wheat (*Triticum aestivum*) CERK1 amino acid sequence shows 86 % identity to rice (*Oryza sativa*) CERK1. SP, signal peptide; LysM 1/LysM 2/LysM 3, LysM motifs; TM, transmembrane region.

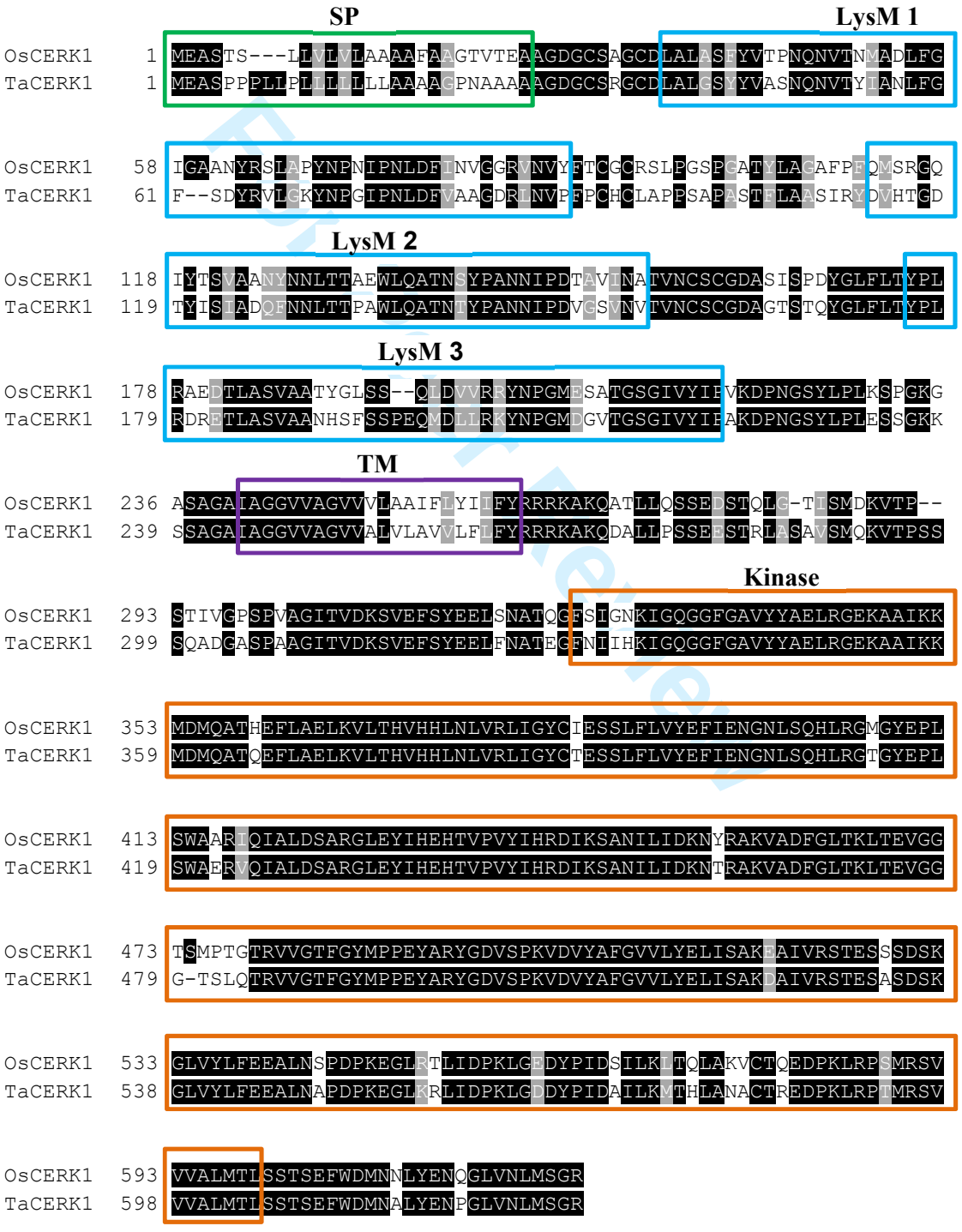


Figure S2. Sequence alignment of wheat and rice CEBiP proteins

Black boxes highlight identical amino acids and gray boxes highlight similar amino acids. Predicted protein domains are boxed. The predicted wheat (*Triticum aestivum*) CEBiP amino acid sequence shows 81 % identity to rice (*Oryza sativa*) CEBiP. SP, signal peptide; LysM 1/LysM 2, LysM motifs; GPI anchor site, putative glycosylphosphatidylinositol modification site.

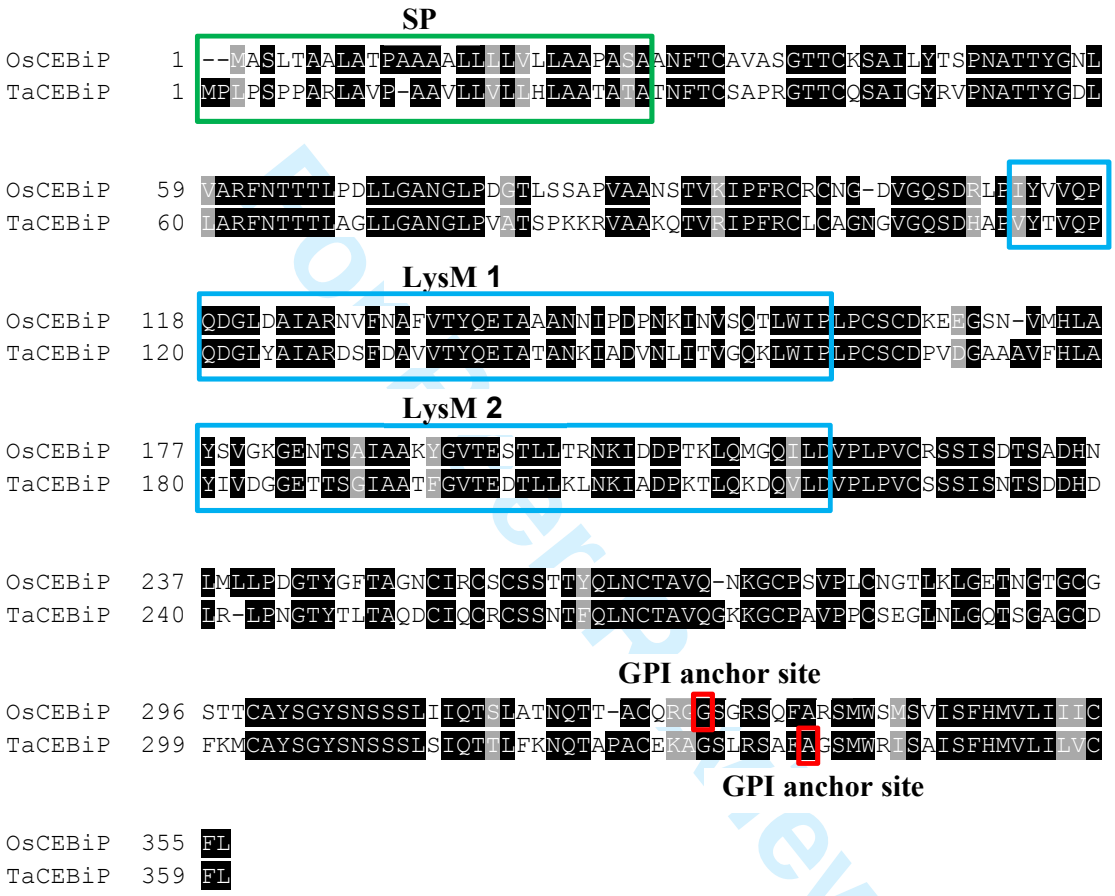


Figure S3. Phylogenetic relationship between predicted wheat CERK1 and all known LysM receptor-like kinases in rice.

Unrooted tree generated using the MEGA5 program by Maximum Likelihood method is shown. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was less than 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

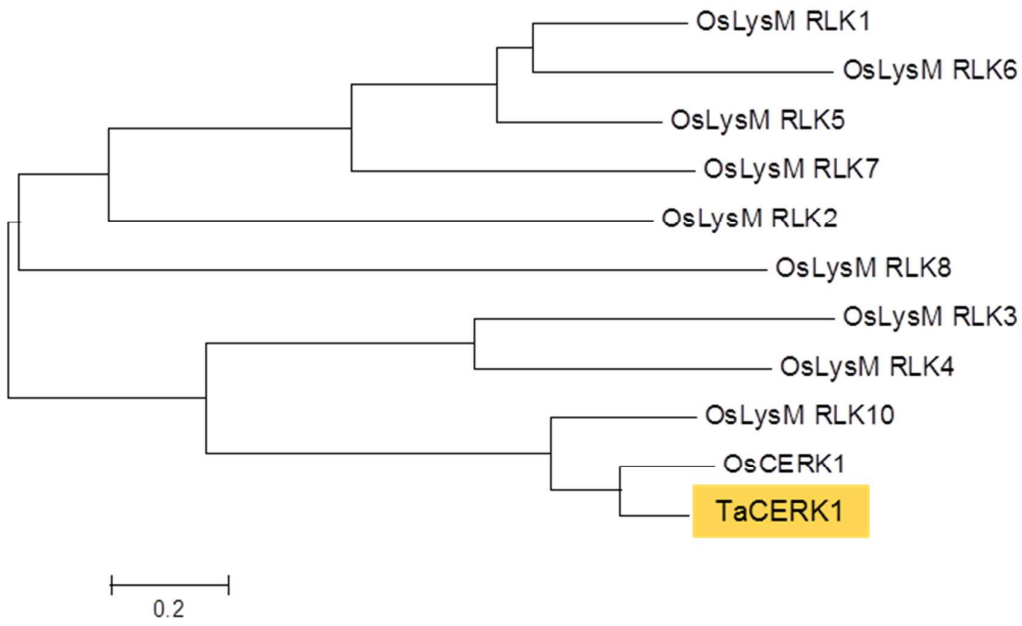


Figure S4. *CERK1* and *CEBiP* transcript expression during early stages of *Mycosphaerella graminicola* infection of wheat leaves

Data shown are ratios of transcript abundance in *Mycosphaerella graminicola*-infected tissue relative to transcript abundance in mock-inoculated tissue at the corresponding numbers of days post-inoculation as determined by quantitative RT-PCR. Error bars indicate mean ± s.e. of the ratio of infected to mock-inoculated samples ($n = 3$). Asterisks indicate significant fold change in transcript abundance in infected tissue when compared to mock-inoculated tissue ($p < 0.05$, Fisher's Least Significant Difference test).

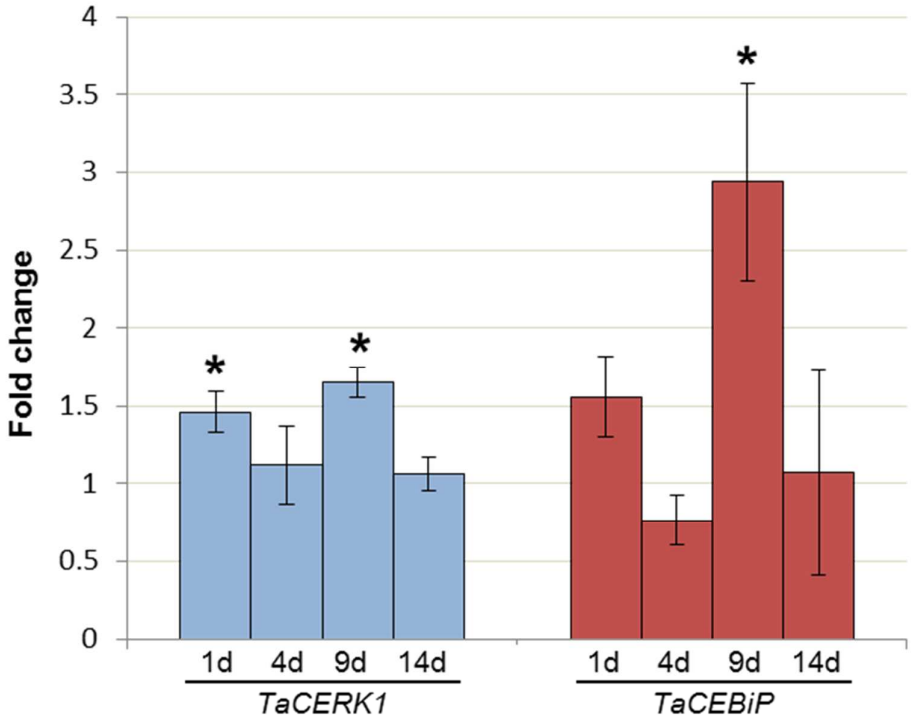


Figure S5. BSMV-mediated silencing of *CERK1* or *CEBiP* does not compromise resistance of wheat cv. Cadenza to wild-type *Mycosphaerella graminicola* isolate IPO323.

Mycosphaerella graminicola isolate IPO323 (wt *Mg*) was unable to induce lesions or pycnidia formation on leaves of control-treated (no virus or BSMV:*asGFP*-infected) or silenced (BSMV:*asCERK1*- or BSMV:*asCEBiP*-infected) Cadenza plants. Photographs were taken at 21 days post-inoculation with *Mg*.

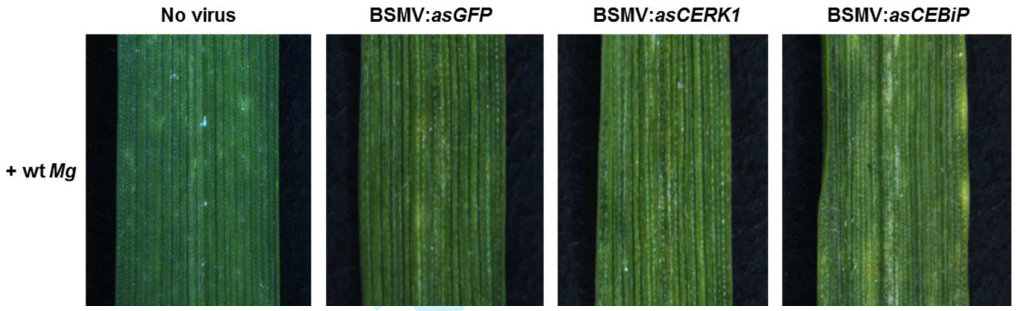


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Table S1. PCR primers used for generating gene fragments for VIGS

Target gene	Primer name	Sequence (5' to 3')[a]
TaCERK1	LIC asCERK1a F	**AAGGAAGTTTAA***GAAGATGGAAGCAAAGCGTCCT*
	LIC asCERK1a R	**AACCACCACCACCGT**ATACCCGCTGAGGGACAGAGAG
	LIC asCERK1b F	**AAGGAAGTTTAA**ACAGCACCAAAACCACCTTGT
	LIC asCERK1b R	**AACCACCACCACCGT**TGTTGTGGCTGGTGTAGTTGC
	LIC asCERK1c F	**AAGGAAGTTTAA**GGCATGTAACCGAATGTACCAA
	LIC asCERK1c R	**AACCACCACCACCGT**TTGGTTATTGCACGGAGAGTTC
TaCEBiP	LIC asCEBIPa F	**AAGGAAGTTTAA**CCGACGGTGATGAGGTTGAC
	LIC asCEBIPa R	**AACCACCACCACCGT**ACCACCTACGGGGACCTCCT
	LIC asCEBIPb F	**AAGGAAGTTTAA**CGGTGCAATTTAGCTGGAAGG
	LIC asCEBIPb R	**AACCACCACCACCGT**CGTCTTCCACCTCGCCTACAT
	LIC asCEBiPc F	**AAGGAAGTTTAA**GATGCAAACCTTACGCATATCA
	LIC asCEBiPc R	**AACCACCACCACCGT**TATCTCCTTCCACATGGTGTTG
GFP	LIC asGFP F	**AAGGAAGTTTAA**AATCCCAGCAGCAGTTACAAA
	LIC asGFP R	**AACCACCACCACCGT**ACATCACGGCAGACAAACAA

[a]Sequences complementary to target gene cDNA sequence are underlined. Ligase-independent cloning (LIC) adaptor sequences are shown in bold.

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Table S2. Primers used for quantitative real-time RT-PCR.

Target gene	Primer name	Final primer concentration (nM)	Sequence (5' to 3')
TaCERK1	CERK1 QPCR F	400	TTACCCCATCGACGCCATTC
	CERK1 QPCR R	400	TCTGCCGGACATGAGGTTCA
TaCEBiP	CEBiP QPCR F	400	AAAAGGGATGCCCAGCAGTG
	CEBiP QPCR R	600	TTTCTCGCAAGCTGGTGCTG
TaCDC48	CDC48 QPCR F	400	AAATACGCCATCAGGGAGAACATCGAG
	CDC48 QPCR R	400	CTCGCTGCCGAAACCACGAGAC

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Table S3. Mean *TaCERK1* transcript levels in mock- and *Mycosphaerella graminicola*-inoculated wheat leaves during the infection time course.

	1 dpi	4 dpi	9 dpi	13 dpi
IPO323	1.710	1.037	1.058	1.030
mock	1.169	0.924	0.639	0.970

Table S4. Matrix of least significant differences used to assess variation in transcript levels in mock- vs. *M. graminicola*-infected wheat leaves.[a]

Matrix of least significant differences calculated at the 5 % level of significance using REML.

IPO323 - 1 dpi	1	*							
IPO323 - 4 dpi	2	0.3225	*						
IPO323 - 9 dpi	3	0.5586	0.5586	*					
IPO323 - 13 dpi	4	0.5586	0.5586	0.3225	*				
Mock - 1 dpi	5	0.2944	0.2944	0.5429	0.5429	*			
Mock - 4 dpi	6	0.2944	0.2944	0.5429	0.5429	0.2633	*		
Mock - 9 dpi	7	0.5429	0.5429	0.2944	0.2944	0.5267	0.5267	*	
Mock - 13 dpi	8	0.5429	0.5429	0.2944	0.2944	0.5267	0.5267	0.2633	*
		1	2	3	4	5	6	7	8

[a]When the actual difference in mean transcript levels for two samples is greater than the corresponding least significant difference the difference is deemed significant ($P < 0.05$)

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Table S5. Mean *CEBiP* transcript levels in mock- and *M. graminicola*-inoculated wheat leaves.

	1 dpi	4 dpi	9 dpi	13 dpi
IPO323	1.706	0.745	2.881	1.699
mock	1.095	0.975	0.981	2.001

Table S6. Matrix of least significant differences used to assess variation in transcript levels in mock- vs. *M. graminicola*-infected wheat leaves[a]

Matrix of least significant differences calculated at the 5 % level of significance using REML.

IPO323 - 1 dpi	1	*						
IPO323 - 4 dpi	2	0.746	*					
IPO323 - 9 dpi	3	1.293	1.293	*				
IPO323 - 13 dpi	4	1.293	1.293	0.746	*			
Mock - 1 dpi	5	0.681	0.681	1.256	1.256	*		
Mock - 4 dpi	6	0.681	0.681	1.256	1.256	0.609	*	
Mock - 9 dpi	7	1.256	1.256	0.681	0.681	1.219	1.219	*
Mock - 13 dpi	8	1.293	1.293	0.746	0.746	1.256	1.256	0.681	*
		1	2	3	4	5	6	7	8

[a]When the actual difference in mean transcript levels for two samples is greater than the corresponding least significant difference the difference is deemed significant ($P < 0.05$)

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Table S7. Calculated means and standard errors of *Mg* isolate IPO323 pycnidial coverage scores for leaves of silenced and non-silenced wheat plants

	Pycnidial coverage classes											
	0 %		1-20 %		21-40 %		41-60 %		61-80 %		81-100 %	
Virus construct	**Mean[a]**	**s.e.**	**Mean**	**s.e.**	**Mean**	**s.e.**	**Mean**	**s.e.**	**Mean**	**s.e.**	**Mean**	**s.e.**
No virus control	0.000	0.0075	0.244	0.2833	0.244	0.2833	1.219	0.6351	2.438	0.9010	3.656	1.1070
BSMV:asgfp	0.503	0.4136	0.252	0.2923	0.755	0.5069	1.509	0.7181	2.012	0.8302	3.270	1.0614
BSMV:asCERK1b	0.335	0.3888	1.673	0.8707	0.335	0.3888	1.004	0.6739	1.004	0.6739	4.350	1.4083
BSMV:asCERK1c	1.000	0.6714	0.667	0.5480	0.333	0.3873	0.667	0.5480	0.667	0.5480	4.667	1.4570
BSMV:asCEBiPb	0.324	0.3766	0.324	0.3766	0.648	0.5330	1.296	0.7548	0.648	0.5330	5.509	1.5693
BSMV:asCEBiPc	0.341	0.3962	0.341	0.3962	0.682	0.5606	1.023	0.6870	1.023	0.6870	4.091	1.3815

[a]Mean values represent the mean number of leaves within each pycnidial coverage class calculated using data from three independent experiments. Ten leaves per *Mg* strain-BSMV VIGS construct combination were scored in each experiment.

Table S8. Calculated means and standard errors of *ΔMg3LysM* mutant pycnidial coverage scores for leaves of silenced and non-silenced plants.

	Pycnidial coverage classes											
	0 %		1-20 %		21-40 %		41-60 %		61-80 %		81-100 %	
Virus construct	**Mean[a]**	**s.e.**	**Mean**	**s.e.**	**Mean**	**s.e.**	**Mean**	**s.e.**	**Mean**	**s.e.**	**Mean**	**s.e.**
No virus control	0.000	0.0015	0.000	0.0015	0.000	0.0015	1.000	0.5049	2.000	0.7140	7.000	1.3358
BSMV:asgfp	0.000	0.0015	0.000	0.0015	0.333	0.2915	1.000	0.5049	4.000	1.0097	4.667	1.0907
BSMV:asCERK1b	0.000	0.0015	0.000	0.0015	0.000	0.0015	1.333	0.583	1.667	0.6518	7.000	1.3358
BSMV:asCEBiPb	0.000	0.0015	0.000	0.0015	0.667	0.4122	1.000	0.5049	2.000	0.7140	6.333	1.2706

[a]Mean values represent the mean number of leaves within each pycnidial coverage class calculated using data from three independent experiments. Ten leaves per *Mg* strain-BSMV VIGS construct combination were scored in each experiment.

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Table S9. Calculated means and standard errors of *ΔMg1LysM* mutant pycnidial coverage scores for leaves of silenced and non-silenced plants.

	Pycnidial coverage classes											
	0 %		1-20 %		21-40 %		41-60 %		61-80 %		81-100 %	
Virus construct	**Mean[a]**	**s.e.**	**Mean**	**s.e.**	**Mean**	**s.e.**	**Mean**	**s.e.**	**Mean**	**s.e.**	**Mean**	**s.e.**
No virus control	5.333	0.9864	3.000	0.7368	0.500	0.2995	0.000	0.0020	0.167	0.1728	0.000	0.0020
BSMV:asgfp	6.000	1.0474	2.500	0.6721	0.333	0.2445	0.000	0.0020	0.167	0.1728	0.000	0.0020
BSMV:asCERK1b	1.764	0.6111	1.372	0.5386	1.176	0.4985	1.176	0.4985	1.960	0.6444	1.372	0.5386
BSMV:asCERK1c	1.667	0.7727	2.000	0.8464	1.667	0.7727	2.333	0.9142	2.000	0.8464	0.000	0.0029
BSMV:asCEBiPb	2.783	0.7735	1.193	0.5054	1.193	0.5054	0.596	0.3571	2.385	0.7158	0.994	0.4613
BSMV:asCEBiPc	3.667	1.1461	3.000	1.0366	1.000	0.5985	0.667	0.4887	0.667	0.4887	0.667	0.4887

[a]Mean values represent the mean number of leaves within each pycnidial coverage class calculated using data from three independent experiments. Ten leaves per *Mg* strain-BSMV VIGS construct combination were scored in each experiment.

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