A Genetic Screen Identifies a Requirement for Cysteine-Rich–Receptor-Like Kinases in Rice NH1 (OsNPR1)-Mediated Immunity

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Abstract

Systemic acquired resistance, mediated by the Arabidopsis NPR1 gene and the rice NH1 gene, confers broad-spectrum immunity to diverse pathogens. NPR1 and NH1 interact with TGA transcription factors to activate downstream defense genes. Despite the importance of this defense response, the signaling components downstream of NPR1 NH1 and TGA proteins are poorly defined. Here we report the identification of a rice mutant, snim1, which suppresses NH1-mediated immunity and demonstrate that two genes encoding previously uncharacterized cysteine-rich-receptor-like kinases (CRK6 and CRK10), complement the snim1 mutant phenotype. Silencing of CRK6 and CRK10 genes individually in the parental genetic background recreates the snim1 phenotype. We identified a rice mutant in the Kitaake genetic background with a frameshift mutation in crk10; this mutant also displays a compromised immune response highlighting the important role of crk10. We also show that elevated levels of NH1 expression lead to enhanced CRK10 expression and that the rice TGA2.1 protein binds to the CRK10 promoter. These experiments demonstrate a requirement for CRKs in NH1-mediated immunity and establish a molecular link between NH1 and induction of CRK10 expression.

Author Summary

To survive, plants and animals must resist microbial infection. Plants employ an immune response called systemic acquired resistance that confers long-lasting resistance to a broad-spectrum of pathogens. Researchers have previously identified two key proteins (NPR1/NH1 and TGA) that control this immune response. Despite these advances, there remain many gaps in our knowledge and understanding of this important immune response. We have identified a new gene (CRK10) required for this immune response;
without it, plants are more susceptible to infection. These findings advance basic knowledge of systemic acquired resistance and open the door to a new avenue of research on this exciting and important resistance mechanism.

### Introduction

Despite the lack of circulating immune cells, plants share similarities with animals in their defense responses against pathogens; they both use innate immune systems to counter attacks. For example, plants and animals utilize membrane-localized receptors that detect conserved molecular patterns derived from microbes [1], including peptidoglycan, flagellin, chitin, and sulfated peptides. Another component of the immune system consists of cytoplasmic nucleotide-binding-domain, leucine-rich repeat receptors (NLR) [2] that recognize cognate effector proteins secreted by the microbe into the cell. Plants have a third type of defense system, absent in animals, called systemic acquired resistance (SAR) [3,4]. Activation of this system leads to a long lasting, broad-spectrum defense response.

SAR is induced by the plant hormone salicylic acid (SA), and its analogues 2,6-dichloroisonicotinic acid (INA), probenazole, and benzothiadiazole (BTH) [5–10]. In Arabidopsis, NPR1 (nonexpressor of pathogenesis-related genes 1; also known as NIM1 and SAII) is the key regulator of SAR [11–15]; SA induces NPR1 expression and activates the NPR1 protein leading to immunity against diverse pathogens [16–19]. NPR1 interacts with TGA transcription factors, which are required for the SAR response [20–24]. NPR1 contains an ankyrin-repeat domain, a BTB/POZ domain, and a C-terminal transcription activation domain. The ankyrin-repeat domain interacts with TGA proteins [20]; the BTB/POZ domain regulates the C-terminal activation domain [25,26]. SA binds NPR1 and may regulate NPR1 directly [27]; another model suggests that SA regulates NPR1 indirectly through NPR3 and NPR4, which bind SA with high affinities [28].

Arabidopsis NIMIN (NIM1 interacting) and rice NRR (negative regulator of resistance) family proteins are the second class of proteins that interact with Arabidopsis NPR1/NIM1 and rice NH1 (NPR1 homolog 1; also known as OsNPR1) [29,30], respectively. Arabidopsis NIMINs [31] and rice NRR members (NRR, RH1, RH2, and RH3) [32] negatively regulate NPR1 and NH1, respectively [31–33].

In rice, elevated expression of Arabidopsis NPR1 or the rice ortholog NH1 or the NH1 paralog NH3 [22,29,30,34] results in enhanced resistance to Xanthomonas oryzae pv. oryzae (Xoo) and Magnaporthe oryzae, the causal agents of rice bacterial leaf blight and rice blast, respectively. Like Arabidopsis NPR1, rice NH1 also interacts with TGA transcription factors [29]. The enhanced disease resistance of NH1 overexpression (NH1ox) rice plants is accompanied by and correlated with a spontaneous cell death phenotype, commonly referred to as a lesion mimic phenotype [29,35,36]. Application of BTH enhances the formation of necrotic spots from the spontaneous cell death on the NH1ox plants [29], indicating a tight association between this lesion mimic phenotype and enhanced resistance to Xoo.

Although Arabidopsis NPR1 and rice NH1 bind to TGA proteins and act as transcriptional co-activators [26,37], the downstream components required for the NPR1/NH1-mediated response remain largely uncharacterized. To identify such proteins, we screened a fast-neutron-induced rice mutant population of NH1ox plants treated with BTH and identified a mutant called suppressor of NH1-mediated immunity 1 (snim1) that no longer responds to BTH. Here, we report the identification of two previously uncharacterized cysteine-rich receptor-like kinases (encoded by CRK6 and CRK10) that complement the snim1 phenotype and are...
required for the BTH-induced immune response. We demonstrate that elevated NH1 levels induce CRK10 expression and that the rice TGA2.1 protein binds to the CRK10 promoter, indicating that both NH1 and TGA proteins regulate CRK10 expression.

Results
Identification of the snim1 mutant and an 88-kb deletion associated with the suppressor phenotype

In our screen for suppressors of NH1-mediated immunity [38], we isolated the snim1 mutant. After inoculation with Xanthomonas oryzae pv. oryzae (Xoo), snim1 develops long water-soaked lesions, characteristic of the disease. In contrast, the parental control NH1ox plants are resistant to infection, resulting in short lesions (Fig 1A). Furthermore, the NH1ox parent displays a lesion mimic spontaneous cell death phenotype after application of BTH, which is a plant defense activator [38], but snim1 lacks this phenotype (Fig 1B). Bacterial population measurements show that snim1 harbors 18 times more Xoo than NH1ox plants (P = 0.0052) (Fig 1C). These results indicate that the snim1 mutation impairs NH1-mediated immunity.

To expedite isolation of the gene(s) responsible for the snim1 phenotype, we conducted comparative genome hybridization (CGH) analysis using a NimbleGen 2.1-million-probe rice tiling array [38] comparing snim1 and NH1ox DNA (Fig 2A). These experiments identified a single large deletion (from approximately 21,297,000 to 21,385,000) on chromosome 7 of snim1 (Fig 2B). This 88kb deletion was confirmed by PCR. The deleted region contains 11 genes: six CRKs, one encoding development and cell death-kelch motif protein, three encoding expressed proteins, and one Ty3-gypsy retrotransposon gene. Their MSU gene IDs and relative positions are shown in S1 Fig.

Analysis of F2 progeny derived from a cross between snim1 and the parent reveals a complete association of the 88kb deletion with the snim1 phenotype (Fig 3). F2 progeny that contain the 88kb region exhibit levels of Xoo resistance similar to that of the NH1ox parent (Fig 3). In contrast, F2 progeny lacking the 88kb region are susceptible to Xoo. T-test yields P<0.0001, indicating the difference between the two groups is highly significant.

CRK6 and CRK10 complement the snim1 phenotype and silencing of CRK6 and CRK10 individually compromises NH1-mediated immunity

To assess the involvement of the 10 non-retrotransposon genes deleted in snim1, we conducted complementation experiments. We ligated each isolated gene into the binary vector C4300, and used the resulting construct to transform snim1. Following inoculation with Xoo, we found that only the CRK6 and CRK10 constructs restore resistance to the snim1 mutant (Fig 4). None of the other 8 genes restore resistance to Xoo (S2 Fig).

To investigate the relationship between the rice CRKs and their Arabidopsis paralogs, we constructed a phylogenetic tree for the 45 CRKs and included the Arabidopsis CRKs to display their relative positions (S3 Fig).

To further validate the requirement of CRK6 and CRK10 for NH1-mediated immunity to Xoo, we silenced CRK6 and CRK10 individually in the NH1ox background using RNA interference (Ri) and inoculated the resulting six transgenic lines with Xoo. Real-time quantitative reverse transcription (qRT)-PCR experiments revealed that the CRK6Ri and CRK10Ri lines are effectively silenced for CRK6 and CRK10 expression (70%-90% reduction) (S4 Fig). Accumulation of CRK6 and CRK g35580 RNAs are also reduced in the CRK10Ri lines. In contrast, RNA levels of CRK g35650, g35660 and g35680 are not reduced in the CRK10Ri lines. These results indicate that silencing of CRK10 also affects CRK6 and CRK g35580 expression levels.
Based on the low sequence similarity of CRK6 and CRK g35580 with the region of CRK10 used for silencing, we hypothesize that the observed reduced expression is indirect and not due to co-silencing. In contrast to the resistant NH1ox parent plants, which display spontaneous cell death, lesion mimic spots following BTH treatment (Fig 5A) and are resistant to Xoo (Fig 5B), the CRK6Ri and CRK10Ri lines lack the spontaneous cell death phenotype (Fig 5A) and are susceptible to Xoo (Fig 5B and 5C). The presence of the CRK10Ri and CRK6Ri transgenes...
cosegregates with susceptibility to Xoo in T1 progeny (S5 and S6 Figs). Bacterial growth curve analyses reveal that CRK6Ri (lines #3&10) and CRK10Ri (#4&13) plants harbor 5–8 times and 11–12 times more Xoo than the NH1ox parent, respectively (Fig 5D). Statistical analysis of bacterial populations at day 12 (Fig 5D) indicated significant differences between NH1ox parent, CRK6Ri, and CRK10Ri lines. These results confirm the requirement of CRK6 and CRK10 for NH1-mediated immunity.

Fig 2. Comparative genome hybridization identifies a deletion in snim1. (A) A composite CGH graph. Each color represents a chromosome; the downward peak represents a deletion. The codes 4009202–635 vs 532 represent snim1 vs NH1ox DNA samples. Each dot represents a segment of 1500 bp. The Y-axis is in log2 scale. (B) An enlarged view of part of chromosome 7 that contains the deleted region. Expression level is in log2 scale. Each dot represents an actual microarray probe.

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Fig 3. The 88-kb deletion cosegregates with the snim1 phenotype. A segregating progeny population derived from a cross between the snim1 mutant and the LG parent was genotyped for the presence of the Ubi-NH1 gene (NH1ox) and the CRK10 gene (representing the 88-kb region) and scored for resistance to Xoo. Only progeny containing the Ubi-NH1 gene are shown. CRK10 positive and negative progeny plants are separated into two groups. The letters above each bar show the statistical groupings using the student T-test on each pair based on the 5% significance level.

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Fig 4. CRK6 and CRK10 complement snim1. The snim1 complementation lines containing either CRK6 or CRK10 were inoculated with Xoo. Each bar represents the average and standard deviation of at least three leaves from an independent transgenic line. Letters show statistical groupings (P<0.05).

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Fig 5. Silencing of CRK6 or CRK10 re-creates the snm1 phenotypes. CRK6Ri and CRK10Ri plants were treated with 1 mM BTH before Xoo inoculation. Two lines each of CRK6Ri and CRK10Ri are shown. (A) Lesion mimic formation after BTH treatment. Lesion mimic spots on NH1ox leaves are indicated by white arrowheads. (B) Xoo-induced water-soaked lesions. The bottom boundary of the lesion on each leaf is marked with an arrowhead to indicate the lesion lengths. Leaves were taken 14 days post inoculation. (C) Lesion length development after Xoo inoculation. Each time point contains 6 leaves. (D) Bacterial growth curves. Each time point represents three replicates. Statistical groupings: A = CRK10Ri-4, AB = CRK10Ri-13, BC = CRK6Ri-3, C = CRK6Ri-10, D = NH1ox.

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The snim1 and CRK10 mutations compromise BTH-induced resistance to Xoo independent of ectopic NH1 over-expression

We next tested whether the snim1 mutation affects resistance to Xoo in the absence of the NH1ox transgene. For this purpose, we crossed snim1 with the parental rice cultivar LiaoGeng (LG), which lacks the NH1ox transgene. We identified 27 individual progeny (snim1/LG) from this cross that carry homozygous snim1 88kb deletion and lack the NH1ox transgene. We pre-treated the snim1/LG progeny and the LG control with 1mM BTH and then inoculated with Xoo. The snim1/LG individuals display significantly (P<0.0001) longer lesions (9.0±1.9 cm) than the LG control (6.0±1.5 cm) (Fig 6A). These results indicate that the snim1 mutation also affects BTH-induced resistance to Xoo in the absence of the NH1ox transgene.

We further verified the role of the CRK10 gene with a crk10 rice mutant. We identified a homozygous crk10 mutant from a fast neutron mutagenized rice population in the Kitaake genetic background [39]. This mutant carries a two-base deletion (S7 Fig) in the third exon of CRK10 causing a frameshift, resulting in a predicted truncated CRK10 protein missing the kinase domain (see Methods). To assess the effect of the CRK10 mutation in this second genetic background, we inoculated 27 progeny plants of this crk10 mutant along with 25 wild type Kitaake control plants with Xoo following 1mM BTH treatment. We observed that the crk10/Kitaake mutant plants develop significantly (P = 0.0009) longer lesions (~12.5 cm) than the control Kitaake plants (~10.5 cm) (Fig 6B). These results further support the conclusion that the CRK10 gene is required for resistance to Xoo.

Elevated expression of CRK10 enhances resistance to Xoo

To test the hypothesis that higher levels of CRK10 enhance resistance, we generated an inducible construct (GVG-CRK10) using the GVG-dexamethasone (DEX) inducible system [40,41] and obtained 10 healthy, independently transformed lines. These plants display enhanced resistance to Xoo after DEX induction (Fig 7A–7C). The enhanced resistance cosegregates with the GVG-CRK10 transgene in T1 segregating progeny (S8 Fig). Further analysis indicates that higher levels of CRK10 lead to higher levels of resistance to Xoo (Fig 7D). Overexpression of CRK6 did not confer enhanced resistance to Xoo (S9 Fig).

Expression levels of CRK10 are dependent on NH1 levels and BTH induction

To determine if NH1 regulates expression of CRK genes, we assessed CRK10 and CRK6 expression levels in Kitaake and NH1 overexpression plants (nNH1 in Kitaake genetic background). We treated plants with 1 mM BTH and collected leaf samples 0, 1, 4, 8, 24, and 48 hours after treatment. BTH treatment induces NH1 expression levels in both Kitaake (labeled Kit) and nNH1 plants, reaching peaks at 8 hours (Fig 8A). BTH treatment induces 3-fold higher levels of CRK10 mRNA (at 8 hr) in Kitaake compared with the untreated control (0 hr); this induction is higher (4.5-fold) in the nNH1 plants (at 8 hr; Fig 8A). In the absence of BTH pretreatment, the CRK10 mRNA level is 2.5-fold higher in nNH1 plants compared with control plants. BTH treatment also induces CRK6 expression (by 4 fold at 8hr). We observed a slight delay in CRK6 induction in nNH1 plants. This delay does not significantly change the basal and peak levels of CRK6 expression (S10 Fig).

To further investigate the observation that NH1 regulates CRK10 expression, we assessed CRK10 expression levels in transgenic Kitaake plants carrying an NH1 RNAi construct (NH1Ri), under control of the DEX-inducible promoter. The NH1 mRNA levels are significantly reduced in the NH1Ri plants (NH1Ri-6A and -7A) silenced for expression of NH1.
compared with the Kitaake control, 6 and 8 hours after DEX and BTH treatment (Fig 8B). Importantly, we also observed that CRK10 mRNA levels are also significantly reduced in the NH1Ri plants compared with the Kitaake control (Fig 8B). These results indicate that BTH and NH1 regulate CRK10 expression.

The CRK10 promoter contains a TGA binding site

To assess the mechanism by which CRK10 expression is activated, we examined the CRK10 promoter for potential transcription factor binding sites. We identified the sequences TGACGT (-793 from the TATA box) and TGACG (-164) in the CRK10 and CRK6 promoters, respectively, that match the consensus sequence binding sites for TGA transcription factors. We synthesized an oligonucleotide containing this putative CRK10 TGA binding site and employed an electrophoresis mobility shift assay (EMSA) to test its interaction with the rice TGA2.1 protein, which has been shown to bind to an SA-responsive element and interact with NH1 [42,43]. We found that rice TGA2.1 binds to the synthetic oligonucleotide and that the observed gel shift can be competed with higher concentrations of wild-type oligonucleotides but not by oligonucleotides carrying a mutation in the TGA binding site (Fig 9A). These results demonstrate that the CRK10 promoter contains a cognate TGA binding site.
CRK6 is an active kinase

CRK6 and CRK10 carry conserved kinase motifs. To assess the kinase activity of CRK6, we fused the CRK6 kinase domain (CRK6K) to the His:Nus protein, expressed the fusion construct in E. coli and purified the fusion protein using Ni-NTA resins. As a negative control, we generated a kinase-dead mutant of the CRK6 kinase by mutating the conserved, required aspartate at amino acid 488 to asparagine (CRK6DN). The two fusion proteins were subject to kinase activity assay (see Methods). The Nus:CRK6K protein shows clear autophosphorylation, whereas the Nus:CRK6DN protein did not (S11A Fig). These results indicate that CRK6 is an active kinase.
active kinase. We were unable to express and purify the CRK10 kinase. However, the kinase domains of CRK6 and CRK10 are highly conserved sharing 76% similarity (S11B Fig), including all amino acids known to be critical for kinase activity suggesting that CRK10 is likely also an active kinase.

In addition to the kinase domain, we have also analyzed the CRK6 and CRK10 proteins for other conserved protein domains with the SMART program, which is specialized in detecting protein domains. Both CRK6 (amino acids 1–31) and CRK10 (aa. 1–27) contain a predicted signal peptide. Both also contain a predicted transmembrane region: amino acids 295–317 for CRK6 and 284–306 for CRK10. These results strongly predict that CRK6 and CRK10 are membrane-localized proteins.

Discussion

In this manuscript we demonstrate that the previously uncharacterized proteins, CRK6 and CRK10, are required for BTH-inducible, NH1-mediated immunity in rice. There are 43 additional CRK genes in rice (http://rice.plantbiology.msu.edu) and at least 44 CRK members in Arabidopsis [44,45]. In both rice and Arabidopsis, many of the CRK genes are clustered together in the genome. This structure may facilitate recombination and accelerate evolution of resistance. A similar mechanism for generating diversity has been postulated for plant resistance genes encoding both nucleotide-binding site leucine-rich repeats proteins and leucine rich repeat receptor kinases [46,47].

Fig 8. NH1 regulates CRK10 expression. (A) BTH and NH1 induce CRK10 expression. Kitaake (Kit) and nNH1 plants, which contain an extra copy of NH1 and overexpress NH1, were treated with 1 mM BTH and leaf samples taken at time points 1, 4, 8, 24, and 48 hours after treatment. The nNH1 samples were compared to the Kit control at each time point for statistical analysis. (B) NH1-silencing reduces CRK10 expression. Leaves were treated with 1 mM BTH and 100 μM DEX. Each sample represents two biological and three technical replicates. The NH1Ri samples were compared to the Kit control at each time point for statistical analysis. One * indicates P<0.05 and two indicates P<0.01.

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Although altered expression of CRK genes has been observed in several datasets in response to biotic stress [48–53], the biological functions of CRK genes have not previously been well elucidated for any of the Arabidopsis or rice CRK genes for their involvement in the immune response. In particular, the requirement of these genes in NPR1- or NH1-mediated immunity...
has not previously been demonstrated. The observed functional redundancy of CRK genes in monocots and dicots has likely hindered their characterization and prevented an unambiguous assignment of their function [53]. Because of these complications, previous analyses of CRK proteins relied almost exclusively on overexpression experiments. For example, overexpression of AtCRK4, AtCRK5, AtCRK6, AtCRK13, and AtCRK45 resulted in cell death, activation of defense genes, and/or increased resistance to *Pseudomonas syringae pv. tomato* DC3000. Of the knockout analyses conducted, only the *Atcrk45* mutant displayed a slight alteration in response to *Pst* DC3000 [50]. Here we provide direct and robust genetic evidence that the CRK6 and CRK10 genes mediate BTH-induced immune response.

We have demonstrated that the *snim1* mutant and the *crk10* knockout mutant compromise resistance to *Xoo* in two genetic backgrounds in the absence of *NH1* overexpression (Fig 6). Furthermore, CRK10 is likely broadly involved in resistance to pathogens in a quantitative manner because higher levels of CRK10 correlate with enhanced cell death and immune response (Fig 7). This result is consistent with the observations that CRK genes are induced by diverse pathogens [48–53]. CRK10 may play a more significant role in the immune response than other rice CRK genes because our results clearly show that silencing (Fig 5) and knockout (Fig 6B) of CRK10 itself both cause obvious phenotypes in the immune response; CRK6-silenced lines show a less susceptible phenotype.

Our genetic data reveal that elevated expression of NH1 results in enhanced CRK10 expression. Conversely, a reduction in NH1 expression leads to a reduction of CRK10 expression. These results unambiguously demonstrate that CRK10 expression is regulated by the NH1 protein. The observations that TGA proteins bind to the CRK10 promoter (Fig 9A) and interact with NH1 [43] suggest that NH1 and TGA proteins function together to activate expression of CRK10. Fig 9B presents a model for NH1 and CRK10-mediated activation of defense responses. Based on the observation that NPR1 or NPR3/NPR4 proteins bind SA [27,28], we hypothesize that rice NH1 also senses BTH/SA and that the activated NH1 protein translocates to the nucleus where it interacts with TGA transcription factors. The TGA/NH1 protein complex then activates expression of downstream genes, including CRK6/CRK10.

**Methods**

**Plant materials, screening, and Xoo inoculation**

The rice mutant population in the NH1ox-54 genetic background (in the rice LiaoGeng variety carrying the *Ubi-NH1* gene) was generated by irradiation with fast neutrons at 20 Grays as previously described [38]. Xoo inoculation was carried out in a growth chamber, set at 26°C with 80% humidity. Inoculation of rice plants with Xoo strain PXO99 was carried out with the scissors-dip method [54] with absorbance (600 nm) at OD = 0.5. The *crk10*/*Kitaake* mutant was generated in the *Xa21* background and was inoculated with the ΔraxST/PXO99 strain, which evades *Xa21*-mediated immunity [55].

**Comparative genome hybridization**

Comparative genome hybridization was carried out at the Roche NimbleGen facility (Madison, WI) using the Roche NimbleGen rice whole genome tiling array as described before [38].

**Cloning of individual genes on chromosome 7 for test to complement the snim1 mutant**

A Qiagen Long Range PCR kit was used for amplification of each gene including the promoter, the coding region, and the 3’ sequence, from chromosome 7. Amplification of g35580 used
primers G580-1 and G580-3, g35600 used primers G600-1 and G600-2, g35610 used primers G610-1 and G610-3, g35620 used primers G620-1a and G620-2a, g35630 used primers G630-1 and G630-2, g35640 used primers G640-1 and G640-2, g35650 used primers G650-1 and G650-2, g35660 used primers G660-1 and G660-2, g35680 used primers G680-1 and G680-3, g35690 used primers G690-1 and G690-3, and g35700 used primers G700-1 and G700-2.

Amplification of g35600, g35610, g35640, g35650, g35660, g35690, g35700 used Liaogeng genomic DNA as the PCR template. The remaining genes used PAC clone P0458H05 as template. PCR products were cloned into the pCR8/GW/TOPO vector (Invitrogen) and confirmed by sequencing. Each gene was subcloned into the C4300 vector by Gateway recombination.

Genotyping of plants carrying the 88-kb deletion was carried out with primers targeting genes CRK6, CRK10, or Os07g35610. CRK6 genotyping used primers G690-RT1 and G690-RT2. CRK10 genotyping used primers G700-RT3 and G700-RT4. Os07g35610 genotyping used primers G610-10 and G610-2.

**crk10 mutant in kitaake**

A two-base deletion in exon 3 of the CRK10 gene (Os07g35700) is present (line # FN892-S, M2) compared to the Kitaake parent that causes a frame shift disrupting the CRK10 open reading frame. M3 progeny plants were used for Xoo inoculation.

**Plasmid construction for gene silencing and overexpression**

To generate an RNAi construct targeting CRK6 (Os07g35690), we used primers G690-SiRI and G690-SiBam to amplify a 500bp fragment from the 5’-end of CRK6. This fragment was digested with EcoRI and BamHI and cloned into plasmid pENTR/L16, modified from pENTR/D to contain multiple cloning sites. The clone was confirmed by sequencing. The fragment was excised with EcoRI and BamHI and subcloned into pBluescript SK- pre-cut with BamHI and phosphatase-treated, jointly with the Xa21 intron (precut with EcoRI). The resulting clone (dsG690/SK) contained two pieces of the CRK6 fragment head-to-head with the Xa21 intron in between to serve as a spacer to stabilize the clone in bacteria. The dsG690 insert was excised with BamHI and subcloned back to the pENTR/L16 vector using the BamHI site and recombined with a Gateway compatible Ubi-C4300 binary vector (Ubi-C4300/GA) to yield construct Ubi-dsG690/C4300. To generate an RNAi construct targeting CRK10, we used primers G700-SiRI and G700-SiBam to amplify a 500-bp fragment from the 5’-end of CRK10. The PCR product was processed the same way as the CRK6 fragment for generating the end product Ubi-dsG700/C4300 construct. These constructs were also used to transform the NH1ox-11 line. Genotyping of CRK6Ri plants used primers G690-SiRI and Ubi-1 primers; genotyping of CRK10Ri plants used G700-SiRI and Ubi-1 primers.

For silencing of NH1, an RNAi construct was generated using the GVG-DEX inducible vector. A 500 bp NH1 5’-cDNA fragment was excised from NH1 cDNA with EcoRI and SalI. This fragment was ligated with a Gus spacer digested with EcoRI into the pENTR/L16 vector. The resulting construct was recombined with a Gateway compatible pTA7002 binary vector to generate RNAi construct GVG-NH1Ri targeting NH1.
Real time quantitative RT-PCR

Total RNA was extracted using the Trizol reagent (Invitrogen) and purified with spin-column (NucleoBond). One to five μg of total RNA each sample was used to synthesize cDNA for real time RT-PCR.

To assess the expression level of CRK6, primers G690-Q1a and G690-Q2 or primers G690-Q1b and G690-Q2 were used. These primers were determined to be specific to the CRK6 gene. For CRK10, primers G700-RT3 and G700-RT5 were used for real time RT-PCR.

BTH and DEX applications to plants

BTH was applied to rice leaves in a greenhouse in the form of a foliar spray at a concentration of 1 mM in the form of Actigard (Syngenta). DEX was dissolved in DMSO and diluted to 100 μM in 0.05% Tween 20 and applied by foliar spray.

Electrophoresis mobility shift assay (EMSA)

For the EMSA assay, a probe was generated via annealing two oligonucleotides containing the putative TGA binding site. The top oligonucleotide contains biotin at the 5’end. Detection of biotin on the probe by streptavidin was carried out using a Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific, Rockford, IL).

Statistical analysis

Statistical analysis was carried out using the JMP Pro 10 statistics program.

Protein expression in E. coli and purification

Protein expression in E. coli BL21 cells and purification of the fusion protein was carried out according to Chen et al. [56].

Kinase activity assay

Kinase activity assay was conducted as described by Chen et al. [56].

Supporting Information

S1 Table. The nucleotide sequences of the primers used in this study are provided in the table.

S1 Fig. Genes in the 88-kb region deleted in the snim1 mutant. A schematic diagram depicting the positions of the 11 annotated, expressed genes contained in the 88 kb region on chromosome 7 that is deleted in mutant snim1. The six CRK-encoding genes are highlighted as filled bars. CRK6 is g35690 and CRK10 is g35700.

S2 Fig. Complementation with genes in the snim1 deletion. Each gene, including the coding region, promoter (approximately 1.5 kb upstream of the start codon), and 3’ (500 bp) regions, was amplified, confirmed by sequencing, and cloned into binary vector C4300. T0 transgenic plants were generated by transforming mutant snim1 with each individual gene in the 88-kb region deleted in snim1. T0 plants were inoculated with Xoo and lesion lengths measured and recorded 14 days after inoculation. Each bar represents the average and standard deviation of 2
to 8 leaves.

(PPT)

S3 Fig. A phylogenetic tree of rice and Arabidopsis CRK proteins. Forty-five rice CRK protein sequences were retrieved from the MSU rice database RGAP V7. Forty four Arabidopsis CRK protein sequences are included in the tree construction. Multiple alignments with the ClustalX program were performed; bootstrapping was performed 1000 times to generate the tree. The tree is viewed using FigTree v1.4.2.

(PPT)

S4 Fig. CRK6 and CRK10 are silenced in the CRK6Ri and CRK10Ri lines. RNA was extracted from independent CRK6Ri and CRK10Ri lines as labeled under each bar. CRK6 RNA levels were determined by running real time RT-PCR with primers G690-Q1a and G690-Q2, which are specific to the CRK6 gene. Real time RT-PCRs were also carried out with primers G690-Q1b and G690-Q2 to confirm the above PCR results. CRK10 RNA levels were assessed with primers G700-RT3 and G700-RT5, which are specific to the CRK10 gene. RNA levels of g35580 (G580), g35650 (G650), g35660 (G660), and g35680 (G680) were also assessed in CRK6Ri #3 & #10 and CRK10Ri #4 & #13. Each bar represents the average and standard deviation of three replicates. The letters above each bar show the statistical groupings using the student T-test on each pair based on the 5% significance level.

(PPT)

S5 Fig. The CRK10Ri construct cosegregates with enhanced susceptibility in progeny. Segregating progeny were genotyped for the presence of the CRK10Ri transgene. Those containing the transgene colored in green and the null segregants colored in orange. Progeny plants and the NH1ox parent were inoculated with Xoo. Lesion lengths were measured two weeks after inoculation. The inoculation results of three parental lines are presented. Each bar represents the average lesion length and standard deviation of all inoculated leaves from one plant. The letters above each bar show the statistical groupings using the student T-test on each pair based on the 5% significance level within the progeny of each line plus control.

(PPT)

S6 Fig. The CRK6Ri construct cosegregates with enhanced susceptibility. Segregating progeny were genotyped for the presence of the CRK6Ri transgene. Those containing the transgene colored in green and the null segregants colored in orange. Progeny plants were inoculated with Xoo together with the NH1ox parent. Lesion lengths were measured two weeks after inoculation. The inoculation results of four lines are presented. Each bar represents the average lesion length and standard deviation of all inoculated leaves from one plant. The letters above each bar show the statistical groupings using the student T-test on each pair based on the 5% significance level within the progeny of each line plus control.

(PPT)

S7 Fig. A 2-nucleotide deletion causes a frameshift in the CRK10 gene of the crk10/Kitaake mutant. The sequences of the crk10/Kitaake mutant and the wild type Kitaake parent are aligned in the Integrative Genome Viewer program. A 2-nucleotide deletion is revealed in the reads of the crk10/Kitaake mutant.

(PPT)

S8 Fig. The GVG-CRK10 construct cosegregates with enhanced resistance. Segregating progeny were genotyped for the presence of the GVG-CRK10 transgene. Those containing the transgene are presented in filled bars and the null segregants in open bars. Progeny plants were inoculated with Xoo together with the Kitaake control after DEX induction. Lesion lengths
were measured two weeks after inoculation. The results of four lines from one inoculation and another line from another inoculation are presented. Each bar represents the average lesion length and standard deviation of all inoculated leaves from one plant. Progeny of lines #3, #4, #16, and #32 are compared together with the Kitaake control. Progeny of line #21 were compared with its own Kitaake control separately due to the different inoculation time. The letters above each bar show the statistical groupings using the student T-test on each pair based on the 5% significance level within the progeny of each line plus control.

S9 Fig. Xoo inoculation results of transgenic rice plants overexpressing CRK6. CRK6 overexpression transgenic lines carrying the CRK6 gene driven by the maize Ubi-1 promoter were generated in the Kitaake genetic background. (A) T0 plants were inoculated with Xoo and lesion lengths recorded 14 days after inoculation. Fourteen T0 lines are presented. Each bar represents the average and standard deviation of at least 5 leaves. (B) Four CRK6 overexpression lines were tested for CRK6 expression levels compared to the Kitaake control. Each bar represents three replicates.

S10 Fig. BTH treatment, but not elevated NH1 levels, induces CRK6 expression. Kitaake (Kit) and nNH1 plants were treated with 1mM BTH and leaf samples taken at time points 1, 4, 8, 24, and 48 hours after treatment. The nNH1 samples were compared with the Kit control at each time point for analysis.

S11 Fig. CRK6 kinase activity and CRK6 and CRK10 kinase domain sequence alignment. (A) The CRK6 kinase domain was fused to the His:Nus protein and expressed in E coli BL21 cells. The fusion protein was purified using Ni-NTA resins. A negative control containing a change from aspartate to asparagine at amino acid 488 was also expressed and purified. Kinase activity assay was performed in parallel for the two proteins. (B) The kinase domains of CRK6 and CRK10 are aligned using Geneious to display their similarity.

Author Contributions
Conceived and designed the experiments: MC PCR. Performed the experiments: MC QX RSB WB DR WHST PEC RJ. Analyzed the data: MC XC PCR. Contributed reagents/materials/analysis tools: MC RSB XC. Wrote the paper: MC PCR.

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