

Loss of premature stop codon in the *Wall-Associated Kinase 91* (*OsWAK91*) gene confers sheath  
blight disease resistance in rice

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## Abstract

The genetic arms race between pathogen and host plant is a tug of war that has been ongoing for millennia. The “battles” are those of disruption, restoration of signaling and information transmission on a subcellular level. One such battle occurs between rice an important crop that feeds 50% of the world population and the sheath blight disease (SB) caused by the fungus *Rhizoctonia solani*. It results in 10–30% global yield loss annually and can reach 50% under severe outbreak. Many Receptor-like kinases (RLKs) are recruited as soldiers in these battles. Wall Associated Receptor Kinases (WAKs) a subfamily of receptor-like kinases have been shown to play a role in fungal defense. Here we show that rice gene *OsWAK91*, present in the major SB resistance QTL region on Chromosome 9 is a key component in defense against rice sheath blight. An SNP mutation C/T separates susceptible variety, Cocodrie (CCDR) from the resistant line MCR010277 (MCR). The resistant allele C results in the stop codon loss that results in 68 amino acids longer C-terminus carrying longer protein kinase domain and phosphorylation sites. Our genotype and phenotype analysis of the top 20 individuals of the double haploid SB population shows a strong correlation with the SNP. The susceptible allele appears as a recent introduction found in the japonica subspecies reference genome and a majority of the tropical and temperate japonica lines sequenced by the 3000 rice genome project. Multiple US commercial varieties with japonica background carry the susceptible allele and are known for SB susceptibility. This discovery opens the possibility of introducing resistance alleles into high yielding commercial varieties to reduce yield losses incurred by the disease.

# Introduction

The genetic arms race between pathogen and host plant is a tug-of-war that has been going on for millennia. The “battles” are those of disruption, restoration of signaling and information transmission on a subcellular level. One such battle occurs between rice, an important crop that feeds 50% of the global population<sup>1</sup> and the soil-borne pathogen *Rhizoctonia solani* that causes the leaf sheath blight disease (SB). Leaf sheath blight is a major disease in rice that negatively affects crop yield and quality<sup>2</sup>. Identified by the lesions on the leaf sheath, SB causes leaves and tillers (secondary shoots) to undergo early senescence, drying out and tissue death. Ultimately, the significant loss in leaf area due to infection and death affects the plant’s photosynthetic ability, leading to reductions in biomass and yield. There are no known rice cultivars fully resistant to leaf sheath blight disease<sup>3–5</sup>. In the United States, planting high-production rice varieties that are susceptible to SB, resulted in up to 50% yield losses<sup>3</sup>, in a severe breakout season. As a result, about 72% of the rice planting area in the United States is sprayed with fungicide that incurs unsustainable economic cost to the farmers<sup>6</sup>. The spraying may also pose severe ecological constraints on animals and microbiome that co-inhabit and/or frequently visit rice farms and fresh water bodies. Classified as a soilborne basidiomycete fungus, *R. solani* is a particularly destructive plant pathogen<sup>7</sup>. In addition to its impact on rice, *R. solani* infects other major crops such as soybean, barley, sorghum, tomato, and maize<sup>8</sup>. Although *R. solani* rarely produces spores for a mode of mobility, sclerotia are formed which can survive in the soil for up to two years<sup>9</sup>. Flooding in paddy fields, as common practice or natural occurrence combined with a highly humid environment, the sclerotia spread and attach to the plant, causing SB in the susceptible varieties.

Currently, disease management for SB relies heavily on fungicides. This approach may not be the most sustainable due to potential resistance developing in *R. solani* populations, and pollution of agricultural resources<sup>8</sup>. For example, in 2012, a strain of *R. solani* was isolated in the state of Louisiana, USA which was resistant to the strobilurin class of fungicides used by rice farmers<sup>10,11</sup>. A more sustainable approach to disease management is to breed genetic resistance to SB in commercial varieties. In this study, mRNA sequencing was utilized to compare gene expression profiles of resistant and susceptible rice plants inoculated with *R. solani*. Using the transcriptome profiles of two rice lines, susceptible Cocodrie (CCDR)<sup>12</sup>, and resistant MCR010277 (MCR)<sup>13</sup> (Figure-1A), we examined the response to *R. solani* infection at multiple time points including Day-0 (untreated control), and Day-1, 3, and 5 after inoculation.

The high-quality rice reference genome of *O. sativa japonica* cv Nipponbare (IRGSP v1.0)<sup>14</sup>, bioinformatics analysis tools and sequences from public databases were used to profile variety and time point-specific differential expression of all rice genes with special focus on those overlapping and around the known major SB-resistance QTL on Chromosome 9<sup>15</sup>. We aligned the transcriptome sequence reads to the reference rice genome to identify variety-specific synonymous and non-synonymous SNPs, indels and additionally probed their putative consequences on transcript structure, regulation and protein function *in-silico*. The select set of SNP markers overlapping the differentially expressed candidate genes from the major SB-resistance QTL region on chromosome-9 were confirmed by sequencing. The selected markers were also genotyped in resistant and susceptible individuals derived from a biparental (CCDR x MCR) doubled haploid (DH) population of 197 individuals.

We observed clear gene expression and phenotype differences in response to *R. solani* infection in each of the two rice parental lines. Our findings also reveal plant defense response

genes that are differentially expressed in CCDDR and MCR following inoculation and carry genetic variation that alters the gene function. Based on the gene expression data, genotyping and phenotyping, we present strong evidence supporting a rice *Wall Associated Kinase 91* (OsWAK91) gene as a potential breeding target for developing SB resistant breeding lines.

## Results

### Transcriptome analysis

Twenty-four cDNA libraries were created, with 3 replicates from 4 time points for each rice line, Day 0 (untreated control) and Day-1, 3, and 5 after *R. solani* inoculation. Strand-specific 150bp paired-end sequencing of the cDNA libraries yielded 308,482,684 (CCDDR) and 357,018,002 (MCR) reads. After filtering the low-quality raw reads, we obtained 299,687,236 and 346,498,184 (~98.5% of total) from the CCDDR and MCR samples respectively, with an average of 27.7 million reads per sample. The cDNA sequence reads were aligned to the *O. sativa japonica* cv Nipponbare (IRGSP v1.0) reference genome. We found 16,480 (CCDDR) and 17,793 (MCR) protein coding genes which show normalized expression from a minimum of one time point. Expression of known genes from each line, at each time point, was compared in a pairwise fashion against the untreated, Day-0 sample for differential gene expression analysis. For CCDDR there were 105, 367 and 377 transcripts from 79, 281 and 320 genes at the Day-0 to Day-1, Day-0 to Day-3, and Day-0 to Day-5 comparisons, respectively. Whereas, in the MCR line, there were 148 and 597 transcripts from 119 and 443 genes in the Day-0 to Day-3, and Day-0 to Day-5 comparisons, while there were no statistically significant differences in expression observed at Day 0 to 1 (Figure-1B). In the Day-0 to Day-3 comparison, 17 common genes were up-regulated in both the CCDDR and MCR lines, and one common gene was down-regulated. Similarly, in the

Day 0 to 5 comparison, 63 common genes in the CCDR and MCR lines were up-regulated, while 8 genes were down-regulated (Figure 1B). In the susceptible CCDR line, there was a larger number of differentially-expressed genes at Day-1, and increasing number on Day-3, unlike the MCR line which has larger number of differentially expressed genes on Day-5 (Figure 1C).

Genes up-regulated in the CCDR line include transcription factors, and genes playing a role in the biotic stress response (both at Day-0 to 1), and enrichment of hydrolase function in the Day-0 to Day-3 comparison. One gene (OS02G0129800) which is grass family-specific, was down-regulated in both CCDR and MCR lines. This gene is known to be up-regulated in response to *Burkholderia glumae*<sup>16</sup> the causal agent of rice bacterial panicle blight disease, whereas it is down-regulated in response to *Xanthomonas oryzae* the causal agent of rice bacterial leaf streak disease<sup>17</sup>. The Day-0 to Day-5 comparison shows enrichment of genes associated with catabolism of peptidoglycan, an important cell wall component, those associated with biotic stimulus and defense responses, kinases and those with chitinase enzyme activity required for degrading the fungal pathogen cell walls.

We found 1948 and 1914 genes from the CCDR and MCR lines, respectively, that undergo alternative splicing of transcripts. Of these, 1890 genes were common to both lines. Of all the genes showing alternative splicing under biotic stress, 57 and 20 unique genes were differentially-expressed in the CCDR and MCR lines, respectively. Intron retention was identified as the most prevalent form of transcript splicing event in response to *R. solani* infection (Table-1). This is consistent with known reports from plants, including those under abiotic stress<sup>18</sup>.

Four differentially-expressed candidate genes were identified in the vicinity of the major SB resistance QTL on chromosome-9; a dormancy auxin-associated family protein (OS09G0437500), an elicitor-inducible cytochrome P450 (OS09G0441400), a cysteine peptidase (OS09G0442300) and a *Wall-Associated receptor kinase 91* (*WAK91*; OS09G0561600) with contrasting expression profiles (Figure 1D). The *WAK91* transcript showed down-regulation up to Day-3, followed by up-regulation at Day-5 in the MCR line compared to being constitutively expressed in the CCDR line. OS09G0441400, the elicitor-responsive P450 gene, showed an expression profile opposite that of *WAK91*. OS09G0437500, the dormancy auxin-associated gene, exhibited a similar expression profile between the two lines, except at Day-1 in CCDR when it was down-regulated. In contrast, OS09G0442300, the cysteine peptidase, was highly expressed in MCR at Day-5.

# **SNP marker discovery and variant prediction**

Using the transcriptome sequence reads, we identified 65,121 and 82,293 genetic variants, including single nucleotide changes (SNPs in the form of transitions and transversions and insertions and/or deletions (indels) in the susceptible CCDR and the resistant MCR lines, respectively. A total of 32,401 and 49,573 SNPs were unique to CCDR and MCR, respectively, and they shared 32,720 SNPs present at the same loci. Of the shared loci, 103 SNP sites contained alleles different from the reference and between the two lines (Figure 1E). On average, we observed that the MCR line carries ~2500 transitions (Ts), ~750 transversion (Tv) and ~1000 more indel SNP events compared to the CCDR line (Table-2). In general TvSNPs are have been shown to have larger regulatory effects compared to the TsSNPs<sup>19</sup>, and rice is known to carry

more of the transition types of SNPs<sup>20</sup>. The TsSNPs were greater in number in both lines, but the transition to transversion ratio (Ts/Tv) in the MCR line was 2.8 compared to 2.68 in CCDD. Within the TsSNPs, there were almost the same number of A ↔ G, and T ↔ C transitions in CCDD compared to MCR, whereas there were more G → A and C → T transitions than A → G and T → C, respectively. The transition type G→C was high in CCDD compared to MCR, where the opposite type C→G is more abundant. There were significantly fewer TsSNP C→A in MCR (Table 2).

The identified genetic variants map to 22,448 and 24,343 rice reference protein coding genes in the CCDD and MCR lines respectively, constituting 55% and 66% of the genes in the published reference genome<sup>14</sup>. Because we used the cDNA sequence reads for SNP calling and genetic marker development, we used the Variant Effect Predictor (VEP) tool<sup>21</sup> to predict the causal effect of each SNP on the 5' and 3' UTRs, exons, intron and intron splicing boundaries of the transcribed gene. If the SNP was in an exon, we also computed the synonymous (sSNP) and non-synonymous (nsSNP) variations to predict the putative consequences on transcript structure, regulation, splicing, and peptide structure and function (Table-3).

Based on these VEP results, and the differential gene expression results, we developed genetic markers that co-segregate with the disease resistance phenotype in the population study. The set of 135 SNP genetic markers<sup>22</sup>, including those identified herein, was short-listed by selecting for chromosomal locations overlapping the major and minor SB QTL. Sheath blight QTLs were previously identified on chromosomes 1, 2, 3, 4, 5, 6, 7, 8, 9, 11 and 12<sup>15,23-29</sup>. In the major SB QTL region, we found that the differentially expressed *OsWAK91* (OS09G0561600) gene carried

a non-synonymous SNP (T→C) aligned at position 22,318,449 bp on Chromosome-9 of the Nipponbare rice reference genome (IRGSP v1.0; Figure 1F). The SB susceptible line CCDR carried the same allele T as the reference, whereas the resistant MCR line carried the C allele. The T→C transition results in the loss of a stop codon in MCR WAK91, resulting in a predicted OsWAK91 peptide with an additional 68 amino acids. Sequencing the amplified region of the WAK91 SNP marker confirmed for presence of the susceptible T allele in the parent CCDR, as well as other US elite lines with japonica background, namely, CL53, Cypress, Blue Bonnet, and CL111, whereas in the indica lines namely, IR29, IR64, Jasmine, TeQing, Pokkali, Nonabokra, 93-11, Kasalath and Nagina22 (N22) were confirmed to carry the resistant allele C (Figure-1G).

## Population study

Evaluation of the 10 most-resistant individuals of the SB2 double haploid (DH ) population produced disease ratings between 4.7 to 6.0, while the resistant parent MCR showed a 3.5 rating in the same study. In contrast, the 10 most-susceptible individuals of the population scored between 7.5 to 8.0, while the susceptible parent CCDR was a 7.5 rating (Figure-1A, 1H, 1I, Supplementary Data File 1). The selective genotyping of the parent lines MCR and CCDR and 20 selected DH lines with 135 nsSNP markers and located in the major and minor SB QTL regions on chromosomes 1, 2, 3, 4, 5, 6, 8, 9, 11 and 12. These included the non-synonymous *OsWAK91* SNP (T/C) identified in this study. An ANOVA test was performed, and the best 13 ranked nsSNP markers, based on the ANOVA analysis, F values, False Discovery Rates, and adjusted  $R^2$  values (Table 4), mapped to the bottom of chromosome-9, within the major SB resistance QTL that was identified previously in 12 separate studies using six *indica* lines (reviewed by Zuo et al. 2014<sup>30</sup>). In the major SB QTL region on chromosome-

9, we found that the *OsWAK91* (OS09G0561600) gene carried a nsSNP (T/C) at position 22,318,449 bp on Chromosome-9 of the Nipponbare rice reference genome. Based on the genotyping data we found that, except in the SB2-99 line, the *WAK91* SNP allele C contributed by the MCR parent line always co-segregated with SB resistance phenotype, whereas the allele T from the CCDR parent co-segregates with susceptible phenotype (Figure 1H, Supplementary Data File 1).

## Discussion

In our quest of finding candidate genes in rice that can provide good measure of resistance to *Rhizoctomia solani* and its application for breeding improved disease resistant rice, our study integrated transcriptomics, identifying genetic variation and its causes on the gene function. We identified the rice candidate gene *Wall-Associated Kinase 91* (*OsWAK91*) carrying a T/C mutation at the stop codon. The *OsWAK91* SNP site at position 22,318,449 bp on Chromosome-9 of the reference rice genome is part of stop codon (TAG) common to CCDR and the reference japonica line Nipponbare. Due to the presence of allele C in the resistant line MCR, the stop codon TAG is lost and replaced by the codon CAG in the *OsWAK91* open reading frame. The codon CAG codes for glutamine, a polar uncharged side chain amino acid. The stop codon loss in the MCR line also results in a *OsWAK91* peptide that is 68 amino acids longer compared to that of CCDR and Nipponbare (Figure 1F). These results lead to two hypotheses: (1) The *OsWAK91* SNP allele C is associated with SB resistance that is evolutionarily conserved with *Oryza* ancestors, and (2) the longer *OsWAK91* ORF resulting from the loss of the stop codon results in a peptide with gain in novel molecular function which may play a role in leaf sheath blight resistance.

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228 To test if the association of the *WAK91* SNP allele C with SB resistance is evolutionarily  
229 conserved, we sequenced a PCR-amplified fragment overlapping the *WAK91* SNP loci from  
230 13 additional *O. sativa* lines; five from subspecies *japonica* and eight from subspecies *indica*  
231 (Figure-1G).

232 All US elite rice lines have the *japonica* background, and are known to be SB susceptible<sup>22</sup>.  
233 We predicted, and confirmed that the US *japonica* lines CCDR, CL53, Cypress, Blue Bonnet,  
234 CL111 carry the *OsWAK91* T allele associated with known susceptibility<sup>31,4,32–38</sup>. In contrast,  
235 the *indica* lines (IR29, IR64, Kasalath, Jasmin, TeQing, Nagina 22, Pokkali and Nonabokra)  
236 were confirmed to carry the resistant C allele (Figure-1G), and exhibit some level of known  
237 SB resistance phenotype<sup>35,39–41</sup>. One exception is 93-11, from which the *indica* reference  
238 genome was sequenced<sup>42</sup>, and which is known to be moderately susceptible<sup>39,43</sup>. These results  
239 are consistent with the genotype and phenotype observations of the 20 individuals of the SB2  
240 DH population (with the exception of SB2-99), in which the *OsWAK91* SNP allele C  
241 contributed by the MCR parent always co-segregated with SB resistance phenotype (Figure  
242 1H, 1I, Supplementary Data File 1).

243

244 To gain insights on the origin of the *OsWAK91* SNP in the *Oryza* genus, we used synteny data  
245 provided by the Gramene database<sup>44</sup> to query the *OsWAK91* SNP allele T/C by aligning to the  
246 sequenced genomes of the wild species; *O. longistaminata*, *O. glaberrima*, *O. punctata*, *O.*  
247 *meridionalis*, *O. barthii*, *O. glumaepatula*, *O. rufipogon*, *O. brachyantha* and the outgroup  
248 *Leersia perrieri*. All of these species carry the *indica*-type allele C, and the majority are  
249 known to bear the SB resistance phenotype (Figure-1G)<sup>3,33,45,46</sup>. This suggests that the *indica*-

type resistant C allele is of ancestral origin. Conversely, the japonica-type susceptible T allele is a more recent introduction in the *Oryza* clade, and is restricted to the *O. sativa subspecies japonica* for its origin. We further confirmed its origin by mining the publicly-available genetic variation data from the 3000 Rice Genome Project<sup>47</sup>. The majority of the temperate and tropical japonica and aromatic rice lines carry the japonica T allele, whereas the majority of indica and AUS lines carry the C allele. Only a handful of the lines carry the heterozygous T/C allele (Figure 1J). We did not have access to the SB resistance phenotype data for these lines, however, based on our findings, we predict that the indica lines carrying the C allele may bear some degree of SB resistance.

The second hypothesis suggests that the *OsWAK91* gene from the SB resistant MCR parent encodes a peptide predicted to be 68 amino acid longer due to stop loss in the ORF, and this peptide may have gained a function that plays a role in providing the SB resistance phenotype. The *OsWAK91* gene is a member of the Wall-Associated receptor Kinase (WAK) gene family (Figure 1K) and codes for a plasma membrane protein. The OsWAK91 protein contains a wall-associated receptor kinase galacturonan-binding domain (WAK domain), followed by a calcium-binding epidermal growth factor (EGF)-like domain in the N-terminal half of the protein that is present on the extra-cellular side, in the middle is a single transmembrane domain spanning the plasma membrane, followed by the cytosolic side containing a protein kinase domain and additional phosphorylation sites for serine, threonine and tyrosine residues, towards its C-terminal (Figure 1F). The WAK domain is known to be linked to the pectin fraction of the plant cell wall<sup>48-50</sup>.

Members of the WAK gene family are known to express and function in response to biotic and abiotic stress conditions. Expression of *Arabidopsis thaliana* *AtWAK1* is induced by a pathogen response, by exogenous salicylate or by its analog 2,2-dichloroisonicotinic acid, and requires the positive regulator NPR1/NIM1. The expression of complete *AtWAK1* or just its kinase domain alone, can provide resistance to pathogen *Pseudomonas syringae*<sup>51</sup>. Maize *ZmWAK-RLK* a plasma membrane protein, is known for conferring resistance to northern corn leaf blight disease caused by the fungal pathogen *Setosphaeria turcica* (anamorph *Exserohilum turcicum*; previously known as *Helminthosporium turcicum*), by restricting pathogen entry into the host cells<sup>25</sup>. Similarly wheat WAK gene *Stb6* confers fungal pathogen resistance without a hypersensitive response<sup>52</sup>. Barley, *HvWAK1* is known to play a role in root development; however, when compared to other cereals and *Arabidopsis* sequence divergence in the extracellular domain further verifies the multifunctionality of WAK genes<sup>53</sup>. The WAKs are known to play role in plant cell expansion during seedling development, MAP kinase signaling, and to bind to pectin polymers in the plant cell wall and have higher affinity to bind smaller pectin fibers in response to pathogen attack<sup>48</sup>.

In the reference rice genome, the WAK gene family members are distributed in 23 subfamilies (Figure 1K) and are reported to play roles in development<sup>54</sup>, abiotic responses<sup>55,56</sup> and fungal disease responses<sup>57</sup>. Genes from five WAK subfamilies C, F, L, and Q did not show expression in our study (Figure 1K). The gene *OsWAK1* (OS01G0136400) is known to provide resistance to the rice blast disease pathogen *Magnaporthe grisea*, and its expression is induced by salicylic acid and methyl jasmonate. *OsWAK1* phosphorylates itself and *OsRFP1*, a putative transcription regulator which binds to the *OsWAK1* kinase domain. The rice

*OsWAK91* gene, also known as the *DEFECT IN EARLY EMBRYO SAC1* (*OsDEES1*), and *OsWAK1* are known for playing role in rice sexual reproduction by regulating the development of the female gametophyte (the embryo sac), and in anther dehiscence, respectively<sup>54,58</sup>. Overexpression of *OsWAK25* (OS03G0225700) is known to increase susceptibility to *R. solani* and *Cochliobolus miyabeanus*, and resistance to *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and *Magnaporthe oryzae*<sup>59</sup>, whereas its loss of function compromises Xa21-mediated resistance<sup>60,61</sup>. *OsWAK11* has also been shown to regulate copper detoxification<sup>55,56</sup>.

Increased rice blast resistance and reduced fungal hyphae growth was observed when *OsWAK91* was overexpressed, in contrast to the overexpression of *OsWAK112* (OS10G0180800), which suppressed resistance. The genes *OsWAK14* (OS02G0632800), *OsWAK91* and *OsWAK92* (OS09G0562600) are part of the heterotrimeric WAK protein complex located on the plasma membrane in rice cells<sup>62,63</sup> (Figure 1L). A mutant screening by Delteil et al.<sup>57</sup> reported that the loss of function of *OsWAK14*, *OsWAK91* and *OsWAK92* resulted in reduced basal resistance, though it did not affect the growth and fertility<sup>57</sup>.

Conversely, we concluded that both the CCDR and the Nipponbare rice reference lines carry the susceptible T allele with shorter *OsWAK91* ORF, and are known for SB susceptibility. We hypothesize that if the *OsWAK91* function is knocked out or when the longer C-terminal is not present, the line should become increasingly pathogen susceptible. Therefore, we can expect that the Nipponbare *OsWAK91* knock out line should be highly susceptible. The results published by Delteil et al.<sup>57</sup> support this hypothesis, through testing with the pathogen *Magnaporthe grisea*, causal agent of rice blight. In their study, Tos17-transposon knockout

mutant plants were generated in the japonica reference Nipponbare rice background (susceptible). The wildtype Nipponbare and the Tos-17 mutant plants always showed disease lesions in response to the pathogen, and, as expected, the *OsWAK91* mutant plant showed 2.5 fold increase in lesions.

The ability to detect and fight pathogenic microbes by the host plants is a complex process. The pattern-recognition receptors (PRRs) localized on the surface of plant cells are well-known gene family members of the Receptor-Like Kinase (RLK) proteins that assist in the recognition of PAMP (Pathogen Associated Molecular Patterns) conserved motifs from pathogens and DAMPs (Damage Associated Molecular Patterns) derived from the damage by the microbes development<sup>64,65,63</sup>. Also, in the case of *Magnaporthe grisea* blast disease response in rice, the chitin elicitor binding protein, OsCEBiP and a chitin elicitor receptor kinase, OsCERK, are known to form hetero-dimer complex. Upon chitin binding the CEBiP-CERK receptor complex may in turn induce transphosphorylation followed by activating downstream signaling<sup>66</sup> (Figure 1L). The majority of the OsCEBiP and OsCERK gene family members show positive upregulation in the resistant MCR line (Supplementary file 1). Similarly, the OsWAKs show early transcriptional regulation induced by chitin that is partially under the control of the chitin receptor CEBiP<sup>57</sup>. Query of the publicly available SNP datasets<sup>47,67,44</sup> suggests that *OsWAK91* is known to carry ~400 unique SNP and indels present in the 5' and 3' UTRs, introns, exons, and splice junctions. This includes the stop lost at position 22,318,449 bp identified by us, and indel at 9:22,315,774-22,315,791 bp leading to start lost and 5'UTR variant, and stops gained at positions 22,316,748 bp, 22,317,724 bp, 22,318,006 bp on chromosome 9 (Supplementary Data File-2). These genetic differences did not show up

in our transcriptome-based SNP dataset and are potential genetic markers for further study. Therefore, based upon the supporting evidence from the genotyping, phenotyping, evolutionary ancestry of SB tolerance and the favorably segregating SNP in the SB2 DH population, we propose a working model (Figure 1L) that the candidate gene *OsWAK91* provides broad tolerance to leaf sheath blight similar to when infected with pathogens *R. solani* and *Magnaporthe grisea*. The longer ORF of the *OsWAK91* in the indica lines, or the MCR genotype with allele C, *OsWAK91* provides a complete kinase domain and additional active sites for serine, threonine and tyrosine phosphorylation, which are missing in the susceptible CCDR genotype, or japonica lines with shorter C-terminus (Figure 1F). *OsWAK91* forms a heterotrimeric protein complex with *OsWAK14* and *OsWAK92* proteins, and is an essential member of the WAK protein complex. The shorter kinase domain, and C-terminus is expected to maintain proper embryo sac development and fertility in both the japonica and indica lines. The *OsWAK91* in the japonica background with truncated C-terminus kinase domain is not completely dysfunctional, since, it has been shown to be involved in embryo sac development and the japonica line Nipponbare and others mentioned in this work are all fertile. Whereas, the presence of the ancestral allele C contributes to the stop loss in the candidate gene *OsWAK91*, the longer translated protein kinase domain and additional phosphorylation sites in the C-terminus in indica lines are necessary for their role in actively regulating the WAK protein complex function, and downstream signaling to affect the disease tolerance response.

## Conclusions

We determined and confirmed strong association of the *OsWAK91* SNP marker to the leaf sheath blight resistance phenotype by extending the genotyping and phenotyping study to include 10 best resistant and 10 best susceptible individuals from the double haploid population derived from the CCDR and MCR parents. We also mined the publicly available sequenced genomes of reference and ancestral *Oryza* species and the 3000 rice genome project to confirm the evolutionary source of the mutant SNP. The resistant allele and the trait appears ancestral, whereas the susceptible allele is a more recent acquisition in the *O. sativa japonica* clade. In fact all the US elite rice varieties have japonica genome integration and are known to carry the susceptibility trait. A few of them tested by us confirm the presence of the susceptible SNP allele same as the CCDR line. Our results and inferences, supported by sequencing, genotyping and phenotyping experiment are well complemented by mutant/knockout screening by earlier studies. *OsWAK91* knockout mutants make rice disease susceptible. Therefore *OsWAK91* resistant allele and the genetic marker identified by us is of ancestral origin and is a candidate for integration in the existing and new cultivated rice varieties for leaf sheath blight resistance, which may help improve global rice production by rescuing the crop affected by the pathogen *Rhizoctonia solani*

## Methods

### Plant material and Inoculation

The sheath blight resistant line MCR010277 and the susceptible variety Cocodrie (CCDR) were planted in pots placed in a mist chamber in a greenhouse located on the Louisiana State

University (LSU) campus in Baton Rouge, LA. Plants were inoculated 50 days after germination with a Potato Dextrose Agar PDA medium disc (0.8 cm diameter) containing *Rhizoctonia solani* (strain LR172) mycelia. Discs were placed at the base of the stem and between the leaf blade and leaf sheath on the main culm of each plant.

Temperatures inside the mist chamber ranged from a minimum of 27°C at night to a maximum of 37°C in the day. Natural daylight was used, with day length of approximately 11 hours 30 minutes. Humidity was maintained 80-90% using a cool mist humidifier of 1.2 gallons capacity (Vicks Inc.) that was programmed to function for a two-hour period every six hours. The chamber frame was constructed with ¾ inch PVC pipe (Charlotte Pipe<sup>®</sup>) covered by extra light plastic (0.31 mm) (Painter's Plastic – Poly America). The dimensions of the chamber were: 1.32 m wide by 2.70 m length, and 1.42 m in height, (Figure 4.2) for a total capacity of 48 pots per chamber, each pot containing three plants.

Leaf samples approximately 2 cm in length were collected from the control untreated (day 0) and from the inoculation sites 1, 3 and 5 days after treatment, and placed immediately in liquid nitrogen until transfer to -80°C freezer for storage.

Leaf samples from additional 13 rice lines: CL153, Cypress, Blue Bonnet, CL111 (all japonica), and 93-11, IR29, IR64, Kasalath, Jasmin, TeQing, Nagina 22 (N22), Pokkali and Nonabokra (all indica) were collected for DNA extraction, and SNP marker genotyping by DNA amplification and sequencing.

411

## 412 **RNA extraction and sequencing**

413 Frozen leaf samples from the CCDR and MCR lines collected at LSU were shipped to Oregon  
414 State University on dry ice and stored at -80°C - until further processing. Poly(A)-enriched  
415 mRNA libraries was prepared from total RNA extracted from the leaf samples using RNA Plant  
416 Reagent® (Invitrogen Inc., USA), RNeasy kits (Qiagen Inc., USA) and RNase-free DNase (Life  
417 Technologies Inc., USA). The concentration and quality of the poly(A)-enriched mRNA was  
418 determined using a ND-100 spectrophotometer (Thermo Fisher Scientific Inc., USA) and  
419 Bioanalyzer 2100 (Agilent Technologies Inc., USA), respectively. TruSeq RNA Sample  
420 Preparation kits (Illumina Inc., USA) were used to construct sequencing libraries. An Illumina  
421 HiSeq 3000 (Illumina Inc., USA) at the Center for Genome Research and Biocomputing, Oregon  
422 State University (CGRB, OSU), was used to sequence the 150 bp Paired End cDNA libraries.

423

## 424 **Sequence quality control and read alignments**

425 The program Sickle v1.33 was used to filter all reads based on read quality. Reads under the  
426 phred score 30, and read length under 150bp were rejected. FASTQ files containing quality-  
427 trimmed and filtered reads were generated, yielding MCR and CCDR high-quality reads.  
428 Sequence reads were further filtered by removing any reads showing alignment to the pathogen  
429 *Rhizoctonia solani* AG1-IB isolate (GCA\_000832345.1). Quality-filtered reads were aligned to  
430 the reference *Oryza sativa* japonica cv Nipponbare (IRGSP v1.0) genome and annotation. Reads  
431 from each biological replicate, were aligned with alignment software program STAR v.2.4.1a<sup>68</sup>.  
432 The resulting sequence alignment files were converted to binary alignment files (BAM), sorted  
433 coordinately and indexed using SAMTools v.1.3<sup>69</sup>.

434

## 435 **Differential gene expression (DGE)**

436 The program DESeq2 (v 1.22.2) was used to investigate gene expression levels of the two rice  
 437 lines over the time course<sup>70</sup>. A Python package, HTSeq v.0.6.1p1<sup>71</sup>, was used to index the  
 438 alignment BAM files and generate raw counts, with a minimum quality-score cut-off of 10. The  
 439 DESeq2 R-package was the used to identify differentially expressed genes. First, the raw count  
 440 data was transformed using variance stabilizing transformation (vst) method of DESeq2 to filter  
 441 outliers. The DESeq2 functions namely, estimate size factors and estimate dispersions, were used  
 442 to normalize the aligned read counts. This was followed by the DESeq2 function to fit a negative  
 443 binomial general linear model and Wald test statistics to detect the significance scores (p-value).  
 444 The final set of differentially expressed transcripts were called at the FDR, Benjamin Hoffman,  
 445 corrected cutoff P-value of 0.05. Furthermore, the non-significant events identified by the  
 446 DESeq2 software were filtered out. Principle components (PCA) plots were used to visualize  
 447 cross-sample comparison in each DE pairwise comparison. MA-plots were generated to visualize  
 448 the different between signal intensity between samples in each comparison and dispersion plots  
 449 were generated to visualize the fitted normalized counts.

450

## 451 **SNP discovery and variant prediction**

452 Indexed BAM files were analyzed to identify genetic variations including single nucleotide  
 453 changes (SNPs; transitions and transversions) and insertions and/or deletions (indels).  
 454 Alignments from each variety were pooled using the mpileup function of SAMtools<sup>69</sup>.  
 455 VarScan.v2.3.9 (release 80)<sup>72</sup> was used to identify SNPs and indels with four different  
 456 minimum read coverage of categories of 2, 6, 8, and 20 and default minimum variant

frequency of 0.8 with a p-value of 0.005. A consensus set of SNPs were identified in all four minimum read coverages. A genotype-specific SNP output data file in VCF format was generated. The Variant Effect Predictor (VEP) workflow provided by the Gramene database<sup>21</sup> was used to infer the putative consequences to the structure, splicing, and function of the gene product (transcript and/or peptide) based on synonymous and/or nonsynonymous (ns) changes.

In order to develop genetic markers and build a hypothesis, the significant SNPs which overlapped the major SB-resistance QTL on Chromosome-9 were queried to find association to the observed differentially-expressed candidate genes. These same SNPs were also queried against the 3000 Rice Genome data provided by the Rice SNP Seek project<sup>73,74</sup> to profile allelic nature and distribution in the diverse *O. sativa* lines. Some of the SNPs were also queried on the Gramene database against the sequenced genomes of wild species of the *Oryza* genus to infer presence or absence. Finally, peer-reviewed literature was mined to extract, and confirm information on the known leaf sheath blight resistance phenotype for these rice lines.

### **Scoring alternative splicing events**

SpliceGrapher, a Python-based tool<sup>75</sup> was used to score alternative splicing events. This tool contains splice-site classifier files for each major reference genome, including *Oryza sativa japonica*. These classifiers are used with the first program (sam\_filter.py) in the SpliceGrapher pipeline that intakes aligned experimental files and the provided classifier and outputs alignment files filtered by splice site. Next, the filtered alignment files and the reference gene-annotation file (in the gff file format), is used by the second program (predict\_graphs.py) to produce a gff file for each gene and its spliced forms. To compare isoforms detected in our

samples to the known reference isoforms, we utilized the program (gene\_model\_to\_splicegraph.py) to generate gffs for each known reference gene. The SpliceGrapher pipeline provided statistics and graphical data files. The types of alternative splicing events generated using SpliceGrapher are alternative 5' (5'-Alt) and 3' (3'-Alt) events, exon skipping (ES) and intron retention (IR).

## Population study

The RiceCAP SB2 mapping population was developed as a genetic resource to identify lines containing molecular markers associated with SB resistance<sup>76</sup>. The SB2 population consists of 197 doubled-haploid (DH) lines derived from a cross between the susceptible parent Cocodrie (CCDR)<sup>12</sup> and the resistant parent MCR010277 (MCR)<sup>13</sup>. The population and the two parents were evaluated for leaf sheath blight disease response on a scale of 0-9, where 0 = no disease and 9 = dead plant. Plants were grown and phenotyped across three years at Crowley, LA and Stuttgart, AR, USA<sup>26</sup>. The top 10 SB resistant individuals SB2-03 (GSOR200003), SB2-109 (GSOR200109), SB2-134 (GSOR200134), SB2-158 (GSOR200158), SB2-161 (GSOR200161), SB2-174 (GSOR200174), SB2-206 (GSOR200206), SB2-225 (GSOR200225), SB2-259 (GSOR200206), and SB2-272 (GSOR200272) and the 10 most susceptible individuals, SB2-13 (GSOR200013), SB2-48 (GSOR200048), SB2-88 (GSOR200088), SB2-99 (GSOR200099), SB2-125 (GSOR200125), SB2-144 (GSOR200144), SB2-203 (GSOR200203), SB2-255 (GSOR200255), SB2-276 (GSOR200276), and SB2-314 (GSOR200314) from the SB2 DH population along with the two parents were screened with 135 candidate nsSNP markers (Table 4, Supplementary Data File 3) identified by Silva et al. 2012<sup>22</sup>, including the *OsWAK91* SNP. These markers were located in QTL

regions on chromosomes 1, 2, 3, 4, 5, 6, 7, 8, 9, 11 and 12<sup>15,23-29</sup>. An ANOVA analysis was performed to calculate the F values, Bonferroni Hochberg False Discovery Rates (FDR), p values and adjusted R<sup>2</sup> values.

# **DNA extraction, amplification, sequencing and analysis**

Total DNA was extracted from seedling tissue using a DNeasy Plant Mini Kit (Qiagen Inc., USA). PCR primers were synthesized by Invitrogen Inc., USA for amplification of the region of interest (Table 5). For PCR amplification, we used Ready PCR Mix, 2x (VWR) following the recommended protocol on a C100 Thermocycler (BioRad). The thermocycler was set for 3 minutes at 95C for initial denaturation, followed by 40 cycles of 30 seconds at 95C for denaturation, 30 seconds at 55C for annealing, 30 seconds at 72C for extension, and 5 minutes at 72C for final extension. Amplified DNA fragments were separated on a 1% agarose gel in 1x TAE buffer. These fragments were extracted from the gel using QIAGEN Gel Extraction and QIAGEN PCR product cleanup kits. Isolated PCR products were Sanger sequenced at the CGRB, OSU. The amplified sequences from the PCR products for each experimental line, including MCR and CCDR, were aligned to the reference Nipponbare genome (IRGSP v1.0) using ClustalW for confirmation of SNPs and to identify and confirm the alleles.

# **Data mining and functional annotation**

The reference genome sequences, gene function and gene family annotations were downloaded in bulk and/or queried online at Gramene database ([www.gramene.org](http://www.gramene.org))<sup>44</sup>. The 3000 rice genome project data was mined at Rice SNP-Seek Database (<http://snp-seek.irri.org/>)<sup>73,74</sup>. Gramene database was queried to pull out all rice genes (IRGSP v1.0)

annotated to carry the WAK domain and/or were part of WAK gene family. The protein sequences of the canonical (longest) isoform) were downloaded in fasta format to run the CLUSTALW alignment with default parameters. The gene family tree and the differential gene expression data for the respective rice WAK gene family members were uploaded on the iTOL web portal<sup>77</sup> to generate the graphics (Figure 1K).

**Data Availability:** The raw RNA sequence reads were submitted to the EMBL-EBI ArrayExpress (accession number E-MTAB-6402). The raw sequences from the amplified fragments from various rice lines were submitted to the European Nucleotide Archive (study accession PRJEB28811) under the assigned accession numbers ERS2758541-51.

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## Author Contributions

PJ and JO conceived and designed the experiments. YSG, MG, NAB and AM performed the experiments. NAB, MG, AM, YSG, PJ, JO analyzed the data. Everyone wrote the paper:

## Competing Interest

The Authors declare no competing interests.

# **Figure Legend:**

## **Figure-1:**

(A) The rice leaf sheath blight disease response shown by the resistant parent MCR010277 (MCR), the susceptible parent Cocodrie (CCDR) and the SB2-174 individual of the SB2 double haploid population. The disease response was tested by infecting the plants with the fungal pathogen *Rhizoctonia solani* (strain LR172).

(B) The number of differentially expressed genes between the two parent lines when compared to the Day-0 vs Day-1, Day-3 and Day-5 after infection.

(C) Number of up and down regulated genes shared between the time points within each rice line.

(D) The differential expression of four candidate genes overlapping the major leaf sheath resistance QTL on Chromosome 9 of rice. Rows are each of the four candidate genes and columns are (L-R) first three, MCR Day-0 vs Day-1, Day-0 vs Day-3, Day-0 vs Day-5 and last three, CCDR Day-0 vs Day-1, Day-0 vs Day-3, and Day-0 vs Day-5

(E) Number of unique and shared SNPs and indels identified in the CCDR and the MCR lines.

(F) The OsWAK91 ORF showing the regions of the coded domains (top) and zoom-in view of the CDS and the ORF from the reference Nipponbare, and parents CCDR and MCR rice lines (bottom). The presence of allele C in the leaf sheath blight resistant MCR line results in stop codon loss and 82 amino acid longer WAK91 peptide which carries a longer protein kinase domain(cyan colored) and additional predicted sites for serine (S), threonine (T) and tyrosine (Y) phosphorylation sites (green colored).

753 (G) Genotyping and Phenotyping of the top ten resistant and ten susceptible individuals of the  
 754 SB2 double haploid population. The population and the two parents were evaluated for sheath  
 755 blight response phenotype on a scale of 0-9, where 0 = no disease and 9 = dead plant. The  
 756 OsWAK91 SNP alleles T/C were scored for presence/absence.

757 (H) OsWAK91 SNP allele T/C in 13 rice lines was confirmed by sequencing the SNP.  
 758 Synteny-based sequence data was mined at the publicly available Gramene database for the  
 759 reference lines indica 93-11, japonica Nipponbare, the wild species of *Oryza* genus and its  
 760 outgroup *Leersia Perrieri*. The leaf sheath blight disease response phenotypes were mined  
 761 from the previously published literature.

762 (I) The leaf sheath blight response phenotype of the individuals SB2-276 (susceptible) and  
 763 SB2-03 (resistant) from the DH population.

764 (J) Occurrence of the OsWAK91 SNP allele T/C The genotyping for in the genomes of the  
 765 3000 rice lines and their grouping

766 (K) Phylogenetic tree of the rice Wall associated Kinase gene family members distributed in  
 767 23 Subfamilies. Differential gene expression data from the MSC and CCDD rice lines were  
 768 plotted in the track order (outside to inside) MCR Day-0 Vs Day-1, Day-3, Day-5 and CCDD  
 769 Day-0 Vs Day-1, Day-3 and Day-5.

770 (K) A model of the host-pathogen interaction showing response to fungal chitin binding by the  
 771 chitin binding receptor complex, and potential interactions and functions by the heterotrimeric  
 772 WAK receptor protein complex. The WAK complex is functional in both the MCR and the  
 773 CCDD rice lines, however the MCR OsWAK91 C-terminus carrying the longer protein kinase  
 774 domain and the additional serine, threonine and tyrosine phosphorylation sites is expected to

775 play a role in successfully initiating the downstream signaling response to provide leaf sheath

776 blight resistance.

777

778

# **Tables:**

Table-1

Summary of the number of genes and events undergoing alternative splicing of transcripts in the susceptible CCDR and resistant MCR rice lines at different time points.

Rice line	MCR							
Time points	Day-0		Day-1		Day-3		Day-5	
Splicing type	Genes	Events	Genes	Events	Genes	Events	Genes	Events
Alt 5' UTR	335	392	339	396	335	392	335	392
Alt 3' UTR	553	652	553	652	551	650	551	650
Exon Skipping (ES)	189	204	191	207	185	200	185	200
Intron retention (IR)	1,357	1,651	1,357	1,651	1,350	1,644	1,351	1645
Rice line	CCDR							
Time points	Day-0		Day-1		Day-3		Day-5	
Splicing type	Genes	Events	Genes	Events	Genes	Events	Genes	Events
Alt 5' UTR	337	394	336	393	335	392	336	393
Alt 3' UTR	557	656	553	652	551	650	553	652
Exon Skipping (ES)	186	201	189	204	187	202	185	200
Intron retention (IR)	1,371	1,666	1,369	1,664	1,355	1,649	1,357	1,651

**Table 2**

Counts of SNPs and indels identified in the susceptible CCDR and the resistant MCR rice lines, by aligning the RNA-Seq sequence reads against the reference *O. sativa japonica* cv Nipponbare reference genome (IRGSP v1.0).

SNP	Substitution Type	CCDR	MCR
A → G	Transition	8,703	11,102
T → C	Transition	8,896	11,228
G → A	Transition	8,735	11,420
C → T	Transition	8,878	11,622
G → C	Transversion	2,817	3,523
C → G	Transversion	2,730	3,559
T → G	Transversion	3,274	4,108
G → T	Transversion	3,367	4,158
A → C	Transversion	3,316	3,964
C → A	Transversion	3,339	4,175
T → A	Transversion	3,734	4,428
A → T	Transversion	3,685	4,397
Total SNPs		61,474	77,684
Total Indels		3,647	4,609
Total variants		65,121	82,293

### Table-3

Summary of SNP consequences on gene function and structure based on its overlapping position in the genes identified in the reference *O. sativa japonica* cv Nipponbare reference genome (IRGSP v1.0).

SNP Consequence	CCDR	MCR
splice donor variant	3,207	3,457
splice acceptor variant	1,952	2,167
stop gained	256	325
stop lost	96	124
start lost	26	47
missense variant	12,951	18,211
splice region variant	8,527	9,494
synonymous variant	12,849	17,534
stop retained variant	29	33
coding sequence variant	10	8
5 prime UTR variant	4,222	6,031
3 prime UTR variant	17,605	20,730
non coding transcript exon variant	2,389	2,694
intron variant	19,605	23,266
non coding transcript variant	2,854	3,311
upstream gene variant	99,220	124,576
downstream gene variant	110,185	138,011
intergenic variant	887	1,103

**Table: 4**

Results of the ANOVA analysis. Includes Marker rank, MSU (v7) and IRGSP v1.0 rice gene IDs, F value, Bonferroni Hochberg False Discovery Rate (FDR), p values and R-squared values of 135 nsSNP makers genotyped in the 10 most resistant and 10 most susceptible lines of the RiceCAP project's SB2 double haploid mapping population.

Rank	MSU gene ID	IRGSP gene ID	F value	Raw p value	p value	FDR	R <sup>2</sup> value
1	LOC_Os09g32860	N/A	149.15400	0.00000	0.00000	0.00000	0.89232
2	LOC_Os09g33710	Os09g0511900	61.04000	0.00000	0.00004	0.00000	0.77227
3	LOC_Os09g34180	Os09g0517600	61.04000	0.00000	0.00004	0.00000	0.77227
4	LOC_Os09g36900	Os09g0540600	61.04000	0.00000	0.00004	0.00000	0.77227
5	LOC_Os09g37230	Os09g0544300	61.04000	0.00000	0.00004	0.00000	0.77227
6	LOC_Os09g37240	Os09g0544400	61.04000	0.00000	0.00004	0.00000	0.77227
7	LOC_Os09g37590	N/A	61.04000	0.00000	0.00004	0.00000	0.77227
8	LOC_Os09g37800	Os09g0550600	61.04000	0.00000	0.00004	0.00000	0.77227
9	LOC_Os09g37880	Os09g0551400	61.04000	0.00000	0.00004	0.00000	0.77227
10	LOC_Os09g38700	Os09g0559900	61.04000	0.00000	0.00004	0.00000	0.77227
11	LOC_Os09g38710	Os09g0560000	61.04000	0.00000	0.00004	0.00000	0.77227
12	LOC_Os09g38850	Os09g0561600	61.04000	0.00000	0.00004	0.00000	0.77227
13	LOC_Os09g38970	Os09g0563250	61.04000	0.00000	0.00004	0.00000	0.77227
14	LOC_Os12g10330	Os12g0204600	41.63300	0.00000	0.00052	0.00004	0.69815
15	LOC_Os12g10410	Os12g0205500	41.63300	0.00000	0.00052	0.00004	0.69815
16	LOC_Os12g13100	Os12g0233100	41.63300	0.00000	0.00052	0.00004	0.69815
17	LOC_Os12g15460	Os12g0256900	41.63300	0.00000	0.00052	0.00004	0.69815
18	LOC_Os09g39620	Os09g0569800	37.14500	0.00001	0.00106	0.00008	0.67359
19	LOC_Os12g09710	Os12g0198900	33.11300	0.00002	0.00211	0.00016	0.56581
20	LOC_Os12g10180	N/A	33.11300	0.00002	0.00211	0.00016	0.56581
21	LOC_Os09g32020	Os09g0493500	23.45700	0.00013	0.01449	0.00097	0.48368
22	LOC_Os12g06980	Os12g0167700	23.45700	0.00013	0.01449	0.00097	0.48359
23	LOC_Os12g09000	Os12g0192500	16.85600	0.00066	0.07303	0.00468	0.44991
24	LOC_Os12g07950	Os12g0179800	14.72200	0.00121	0.13166	0.00807	0.35200
25	LOC_Os06g13040	Os06g0237400	9.77800	0.00583	0.62916	0.03699	0.33220
26	LOC_Os06g15170	Os06g0262800	8.95400	0.00781	0.83613	0.04726	0.28421
27	LOC_Os12g03554	Os12g0129550	7.14700	0.01550	0.98350	0.08949	0.26383
28	LOC_Os12g04660	Os12g0140700	7.14700	0.01550	0.98350	0.08949	0.26383
29	LOC_Os02g34490	Os02g0549900	6.28300	0.02201	0.98350	0.10351	0.25873
30	LOC_Os02g34850	Os02g0554000	6.28300	0.02201	0.98350	0.10351	0.25873
31	LOC_Os02g35210	Os02g0558400	6.28300	0.02201	0.98350	0.10351	0.25873

32	LOC_Os12g07800	Os12g0177800	6.45100	0.02053	0.98350	0.10351	0.25598
33	LOC_Os12g06740	Os12g0164300	6.45100	0.02053	0.98350	0.10351	0.24260
34	LOC_Os08g19694	Os08g0293300	6.19300	0.02284	0.98350	0.10361	0.24120
35	LOC_Os08g20020	Os08g0296900	5.72100	0.02788	0.98350	0.11423	0.24120
36	LOC_Os08g30850	Os08g0399300	5.72100	0.02788	0.98350	0.11423	0.18705
37	LOC_Os08g30910	Os08g0400200	5.76500	0.02736	0.98350	0.11423	0.18705
38	LOC_Os06g19110	Os06g0295000	3.85200	0.06535	0.98350	0.20748	0.17626
39	LOC_Os06g22020	Os06g0325900	3.85200	0.06535	0.98350	0.20748	0.17626
40	LOC_Os06g22460	Os06g0330100	3.85200	0.06535	0.98350	0.20748	0.17626
41	LOC_Os06g23530	Os06g0343100	3.85200	0.06535	0.98350	0.20748	0.17626
42	LOC_Os06g28124	Os06g0475400	3.85200	0.06535	0.98350	0.20748	0.17626
43	LOC_Os06g28670	Os06g0481400	3.85200	0.06535	0.98350	0.20748	0.17626
44	LOC_Os06g29700	Os06g0492800	3.85200	0.06535	0.98350	0.20748	0.17626
45	LOC_Os06g29844	Os06g0494400	4.14200	0.05684	0.98350	0.20748	0.17626
46	LOC_Os03g43684	N/A	4.14200	0.05684	0.98350	0.20748	0.16570
47	LOC_Os06g31070	Os06g0507200	3.57500	0.07486	0.98350	0.23189	0.12005
48	LOC_Os06g32350	N/A	2.20400	0.15499	0.98350	0.33529	0.12005
49	LOC_Os04g10460	Os04g0183500	2.20400	0.15499	0.98350	0.33529	0.11751
50	LOC_Os04g11640	Os04g0193200	2.20400	0.15499	0.98350	0.33529	0.11751
51	LOC_Os04g11970	Os04g0196200	2.20400	0.15499	0.98350	0.33529	0.11751
52	LOC_Os04g15650	Os04g0227000	2.39700	0.13899	0.98350	0.33529	0.11751
53	LOC_Os04g20680	Os04g0275100	2.39700	0.13899	0.98350	0.33529	0.11751
54	LOC_Os04g21890	N/A	2.39700	0.13899	0.98350	0.33529	0.11751
55	LOC_Os04g23620	N/A	2.39700	0.13899	0.98350	0.33529	0.11751
56	LOC_Os04g23890	Os04g0304200	2.39700	0.13899	0.98350	0.33529	0.11751
57	LOC_Os09g16540	Os09g0334800	2.39700	0.13899	0.98350	0.33529	0.10953
58	LOC_Os09g17600	Os09g0344800	2.39700	0.13899	0.98350	0.33529	0.10953
59	LOC_Os09g17630	Os09g0345300	2.39700	0.13899	0.98350	0.33529	0.10953
60	LOC_Os03g30130	Os03g0415200	2.45600	0.13451	0.98350	0.33529	0.10907
61	LOC_Os03g37720	Os03g0573500	2.45600	0.13451	0.98350	0.33529	0.10907
62	LOC_Os03g39150	Os03g0588400	2.16600	0.15840	0.98350	0.33529	0.10907
63	LOC_Os03g40250	Os03g0599400	2.16600	0.15840	0.98350	0.33529	0.10907
64	LOC_Os08g10560	Os08g0206500	2.21400	0.15407	0.98350	0.33529	0.10739
65	LOC_Os08g12800	Os08g0224500	2.21400	0.15407	0.98350	0.33529	0.10739
66	LOC_Os08g13870	Os08g0236400	2.21400	0.15407	0.98350	0.33529	0.10739
67	LOC_Os03g63110	Os03g0848400	1.47500	0.24022	0.98350	0.46294	0.07666
68	LOC_Os02g42412	Os02g0636400	1.47300	0.24058	0.98350	0.46294	0.07575
69	LOC_Os02g44730	Os02g0667500	1.47300	0.24058	0.98350	0.46294	0.07564
70	LOC_Os02g45160	Os02g0673100	1.47300	0.24058	0.98350	0.46294	0.07564

71	LOC_Os02g45980	Os02g0684900	1.47300	0.24058	0.98350	0.46294	0.07564
72	LOC_Os02g48210	Os02g0712700	1.49400	0.23730	0.98350	0.46294	0.07564
73	LOC_Os08g42930	Os08g0542200	1.20300	0.28720	0.98350	0.54439	0.06264
74	LOC_Os11g13650	N/A	1.04300	0.32068	0.98350	0.59891	0.06264
75	LOC_Os02g58540	Os02g0832150	0.92900	0.34792	0.98350	0.63123	0.05477
76	LOC_Os01g13300	Os01g0234100	0.92900	0.34792	0.98350	0.63123	0.04907
77	LOC_Os04g56250	Os04g0657600	0.89800	0.35586	0.98350	0.63654	0.04907
78	LOC_Os08g35310	Os08g0454500	0.85100	0.36855	0.98350	0.64118	0.04752
79	LOC_Os02g39590	Os02g0608801	0.85100	0.36855	0.98350	0.64118	0.04513
80	LOC_Os02g51900	Os02g0755500	0.75300	0.39685	0.98350	0.68108	0.04513
81	LOC_Os02g52060	Os02g0757400	0.61500	0.44303	0.98350	0.70947	0.04513
82	LOC_Os02g02650	Os02g0118700	0.53600	0.47334	0.98350	0.70947	0.04017
83	LOC_Os02g43460	Os02g0650800	0.53600	0.47334	0.98350	0.70947	0.03305
84	LOC_Os02g53970	Os02g0780200	0.53600	0.47334	0.98350	0.70947	0.02894
85	LOC_Os02g54330	N/A	0.53600	0.47334	0.98350	0.70947	0.02894
86	LOC_Os02g54500	Os02g0786000	0.53300	0.47484	0.98350	0.70947	0.02894
87	LOC_Os02g55180	Os02g0795000	0.53300	0.47484	0.98350	0.70947	0.02894
88	LOC_Os04g57670	Os04g0672700	0.53300	0.47484	0.98350	0.70947	0.02875
89	LOC_Os04g58720	Os04g0683800	0.53300	0.47484	0.98350	0.70947	0.02875
90	LOC_Os04g58820	Os04g0685000	0.53300	0.47484	0.98350	0.70947	0.02875
91	LOC_Os04g58910	Os04g0685900	0.53300	0.47484	0.98350	0.70947	0.02875
92	LOC_Os04g59060	Os04g0687300	0.50800	0.48531	0.98350	0.71668	0.02875
93	LOC_Os04g59540	Os04g0691900	0.43300	0.51884	0.98350	0.74688	0.02875
94	LOC_Os05g50660	Os05g0583500	0.42400	0.52340	0.98350	0.74688	0.02875
95	LOC_Os09g26300	Os09g0432900	0.42400	0.52340	0.98350	0.74688	0.02743
96	LOC_Os04g05030	Os04g0136900	0.34300	0.56525	0.98350	0.74778	0.02349
97	LOC_Os05g37040	Os05g0442100	0.34300	0.56525	0.98350	0.74778	0.02299
98	LOC_Os05g39760	N/A	0.35600	0.55839	0.98350	0.74778	0.02299
99	LOC_Os08g36320	Os08g0465800	0.36200	0.55499	0.98350	0.74778	0.02063
100	LOC_Os08g36760	Os08g0471800	0.37900	0.54578	0.98350	0.74778	0.02063
101	LOC_Os09g27570	Os09g0448100	0.37900	0.54578	0.98350	0.74778	0.02063
102	LOC_Os06g44820	Os06g0658600	0.37900	0.54578	0.98350	0.74778	0.01971
103	LOC_Os02g49986	Os02g0732600	0.13700	0.71588	0.98350	0.85611	0.01937
104	LOC_Os02g09820	Os02g0191500	0.13700	0.71588	0.98350	0.85611	0.01871
105	LOC_Os02g10120	Os02g0194700	0.13700	0.71588	0.98350	0.85611	0.01871
106	LOC_Os02g56380	Os02g0807900	0.12700	0.72610	0.98350	0.85611	0.00789
107	LOC_Os02g56480	Os02g0809100	0.14300	0.70964	0.98350	0.85611	0.00789
108	LOC_Os02g57960	Os02g0826000	0.14300	0.70964	0.98350	0.85611	0.00789
109	LOC_Os06g35850	Os06g0551800	0.14300	0.70964	0.98350	0.85611	0.00789

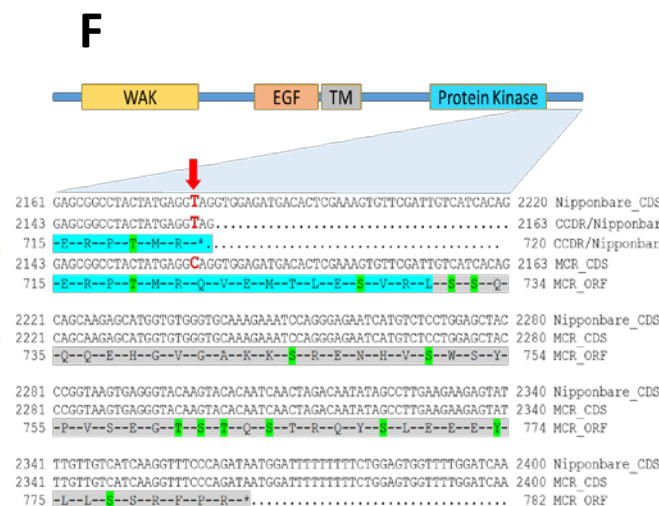
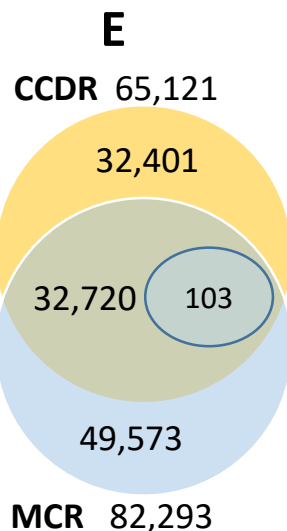
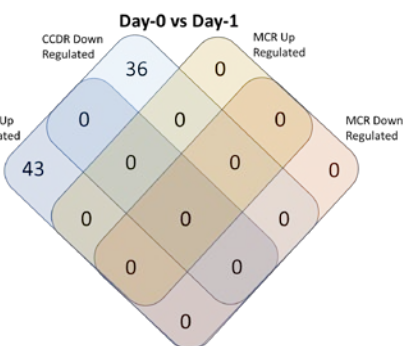
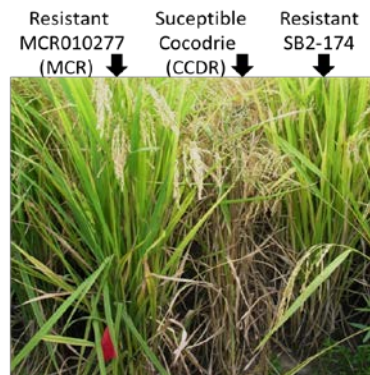
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111	LOC_Os01g52330	Os01g0721300	0.11200	0.74152	0.98350	0.85611	0.00754
112	LOC_Os01g52880	Os01g0729400	0.11200	0.74152	0.98350	0.85611	0.00754
113	LOC_Os01g53420	Os01g0735900	0.11200	0.74152	0.98350	0.85611	0.00754
114	LOC_Os09g25620	Os09g0424300	0.14300	0.70964	0.98350	0.85611	0.00731
115	LOC_Os09g25890	Os09g0427800	0.14300	0.70964	0.98350	0.85611	0.00731
116	LOC_Os02g10900	Os02g0203500	0.13900	0.71698	0.98350	0.85611	0.00699
117	LOC_Os05g40790	Os05g0486300	0.13200	0.72010	0.98350	0.85611	0.00620
118	LOC_Os05g41130	Os05g0490300	0.13200	0.72010	0.98350	0.85611	0.00620
119	LOC_Os05g41290	Os05g0492200	0.13200	0.72010	0.98350	0.85611	0.00620
120	LOC_Os11g19700	Os11g0302500	0.04200	0.83928	0.98350	0.89571	0.00263
121	LOC_Os11g24060	Os11g0427700	0.04200	0.83928	0.98350	0.89571	0.00263
122	LOC_Os11g24180	Os11g0430000	0.04200	0.83928	0.98350	0.89571	0.00263
123	LOC_Os11g24770	N/A	0.04200	0.83928	0.98350	0.89571	0.00263
124	LOC_Os11g28950	Os11g0479100	0.04700	0.83001	0.98350	0.89571	0.00263
125	LOC_Os03g53220	Os03g0743800	0.04700	0.83001	0.98350	0.89571	0.00235
126	LOC_Os03g56400	Os03g0775400	0.04700	0.83001	0.98350	0.89571	0.00235
127	LOC_Os03g57160	Os03g0785300	0.04700	0.83001	0.98350	0.89571	0.00235
128	LOC_Os03g58390	Os03g0798200	0.04700	0.83001	0.98350	0.89571	0.00235
129	LOC_Os04g55760	Os04g0651500	0.03100	0.86218	0.98350	0.91248	0.00172
130	LOC_Os02g11820	Os02g0208900	0.00000	0.98350	0.98350	0.98350	0.00007
131	LOC_Os01g54350	Os01g0747400	0.00000	0.98350	0.98350	0.98350	0.00002
132	LOC_Os01g54515	Os01g0748950	0.00000	0.98350	0.98350	0.98350	0.00002
133	LOC_Os01g55050	Os01g0754500	0.00000	0.98350	0.98350	0.98350	0.00002
134	LOC_Os01g56040	Os01g0765900	0.00000	0.98350	0.98350	0.98350	0.00002
135	LOC_Os01g57230	Os01g0780600	0.00000	0.98350	0.98350	0.98350	0.00002
136	LOC_Os01g57900	Os01g0788900	0.00100	0.97305	0.98350	0.98350	0.00002

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**Table 5** OsWAK SNP Primer Sets and PCR conditions. Primer name with FS and RS suffix were used for amplifying the SNP region and Sanger sequencing. The primer names with suffix FG were used for direct SNP-specific PCR-based genotyping.

Primer	Sequence	Length (nt)	Temperature Degree C	%GC
For PCR and Sanger sequencing				
Forward-FS	CAGGTCAAGGAAGAGGGAGG	20	59	60
Reverse-RS	AGGAGACATGATTCTCCCTGG	20	58	50
For PCR amplification-based genotyping				
Forward-FG1	GAACACTTTTCGAGTGTCATCTC CACCAA	28	62	46
Forward-FG2	ACACTTTTCGAGTGTCATCTCCA CC CG	26	64	54



**G**

Genetic Diversity		
Rice line	Allele	Phenotype
Nipponbare (japonica reference)	T	Susceptible
CL153	T	Susceptible
Cypress	T	Susceptible
Blue Bonnet	T	Susceptible
CL111	T	Susceptible
93-11 (indica reference)	C	Moderate Susceptible
Nonabokra	C	Resistant
Pokkali	C	Resistant
Jasmine	C	Resistant
TeQing	C	Resistant
IR64	C	Resistant
IR29	C	Resistant
Nagina 22 (N22)	C	Resistant
Kasalath (AUS reference)	C	Resistant
<i>O. nivara</i>	C	Resistant
<i>O. rufipogon</i>	C	Resistant
<i>O. barthii</i>	C	Resistant
<i>O. brachyantha</i>	C	N/A
<i>O. glaberrima</i>	C	N/A
<i>O. glumaepatula</i>	C	Resistant
<i>O. longistaminata</i>	C	Resistant
<i>O. meridionalis</i>	C	Resistant
<i>O. punctata</i>	C	N/A
<i>Leersia perrieri</i>	C	N/A

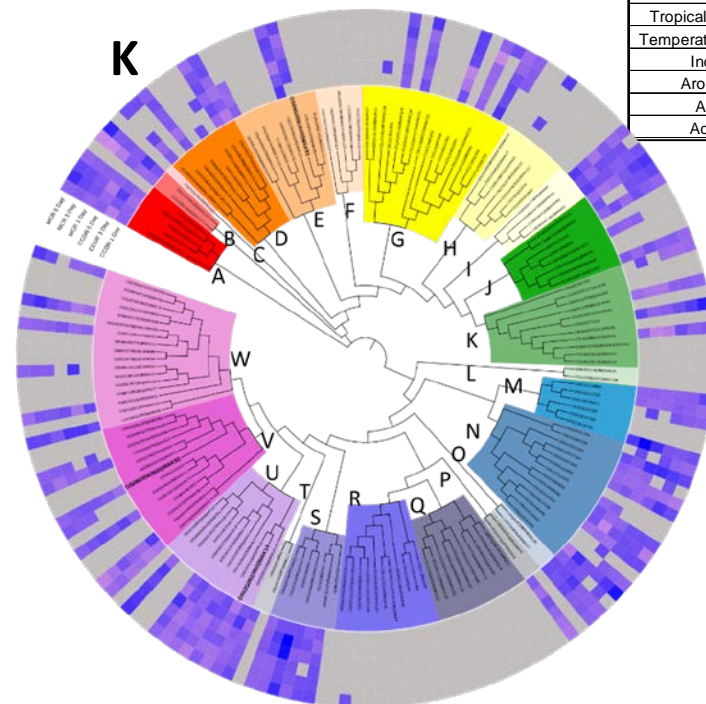
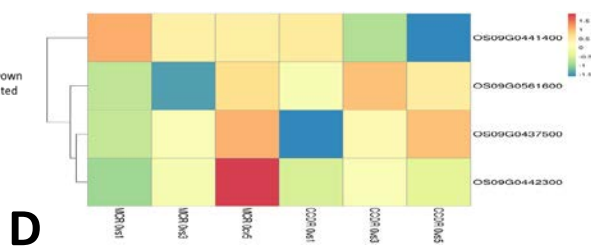
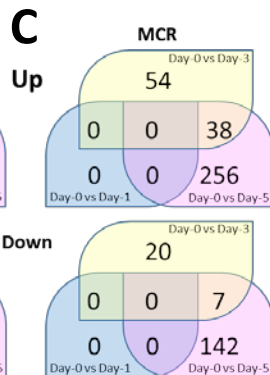
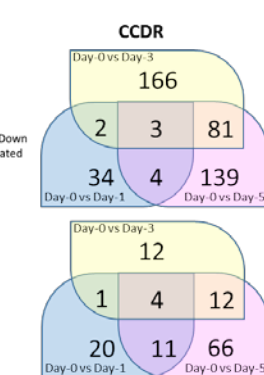
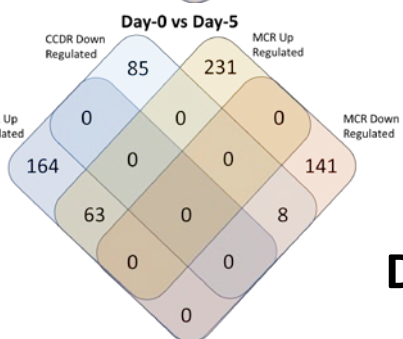
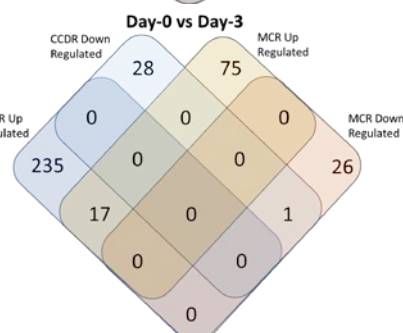
**H**

Individual		Population	
Allele	Phenotype	Score	
CCDR (Parent)	T	Susceptible	7.5
SB2-99	C	Susceptible	7.5
SB2-13	T	Susceptible	7.5
SB2-48	T	Susceptible	8
SB2-88	T	Susceptible	8
SB2-126	T	Susceptible	8
SB2-144	T	Susceptible	8
SB2-203	T	Susceptible	7.5
SB2-255	T	Susceptible	8
SB2-276	T	Susceptible	8
SB2-314	T	Susceptible	7.5
MCR (Parent)	C	Resistant	3.5
SB2-03	C	Resistant	4.7
SB2-109	C	Resistant	5.5
SB2-134	C	Resistant	5.5
SB2-158	C	Resistant	5
SB2-161	C	Resistant	5.7
SB2-174	C	Resistant	4.5
SB2-259	C	Resistant	5.7
SB2-206	C	Resistant	6
SB2-225	C	Resistant	6
SB2-272	C	Resistant	6



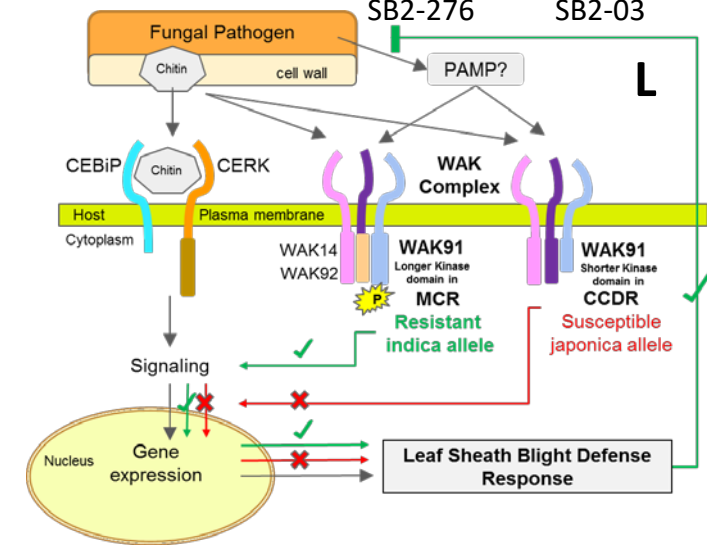
Susceptible SB2-276 Resistant SB2-03

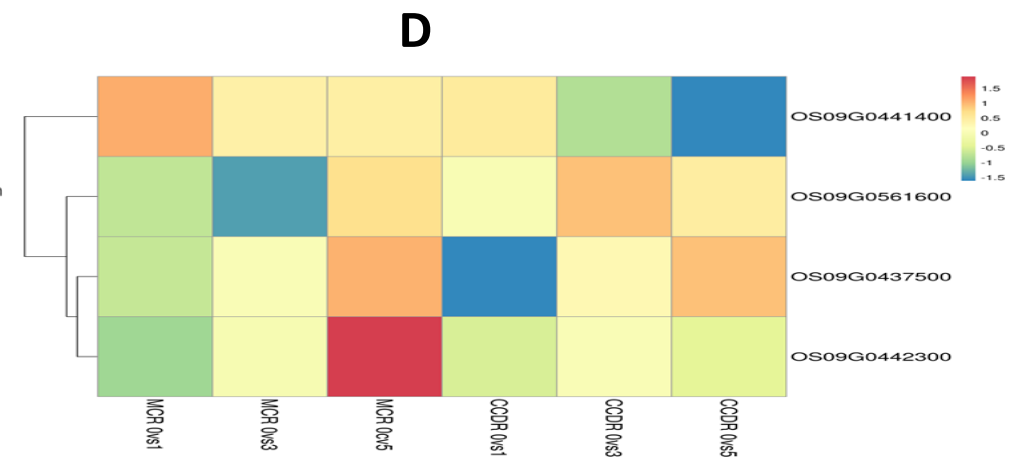
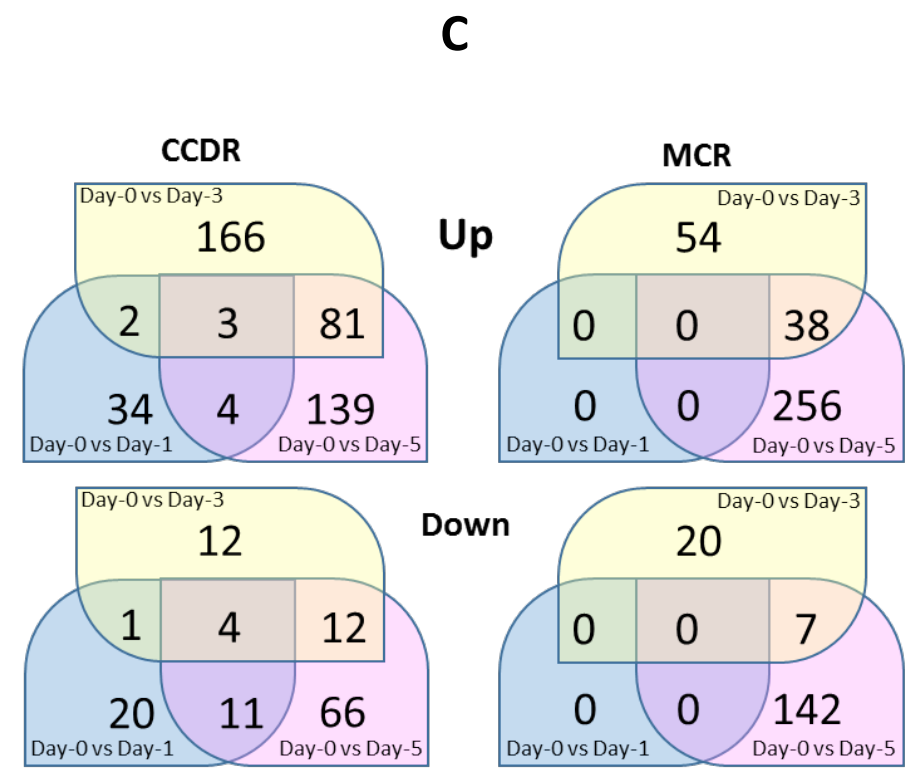
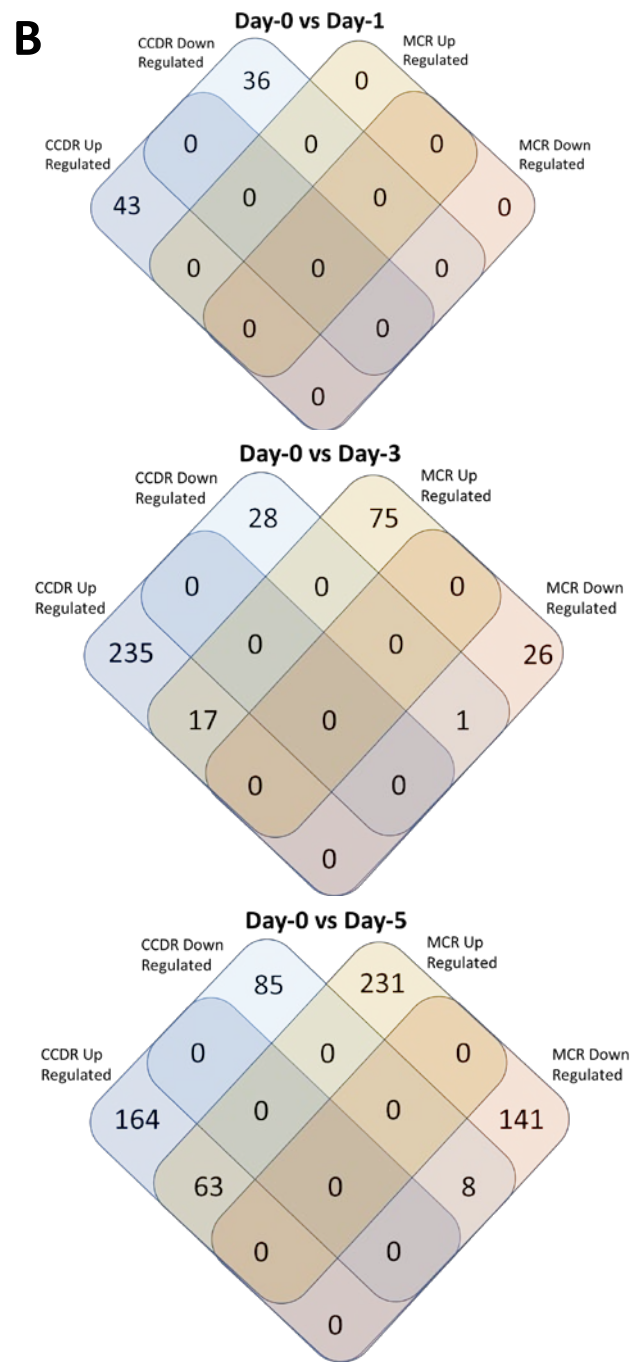
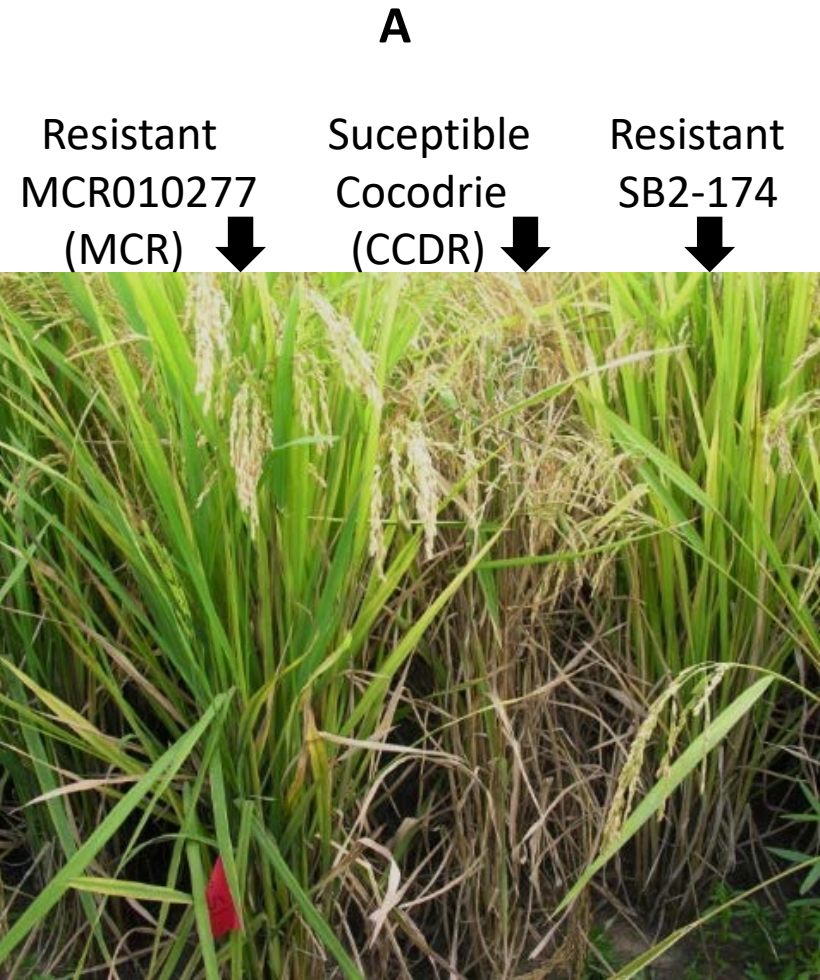
**B**



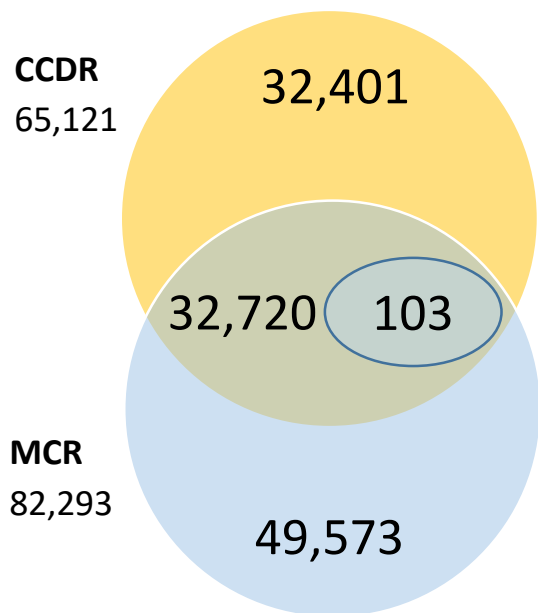
**J**

3K Rice genome project			
Type	Allele		
	T	C	T/C
Tropical Japonica	471	5	0
Temperate Japonica	360	0	3
Indica	18	1742	22
Aromatic	68	4	1
AUS	55	145	1
Admix	35	38	25

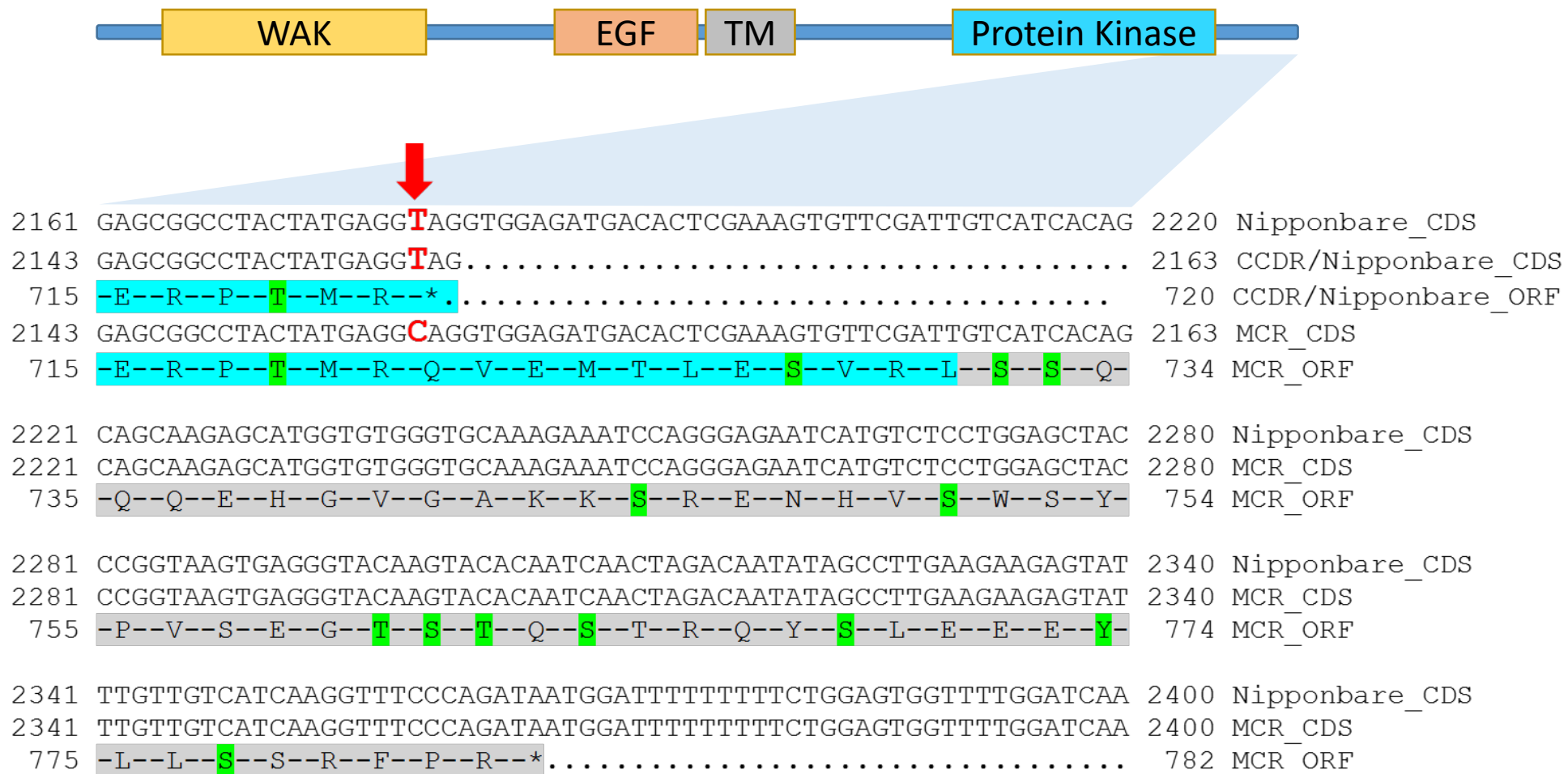




E



F



G

Genetic Diversity		
Rice line	Allele	Phenotype
Nipponbare (japonica reference)	T	Susceptible
CL153	T	Susceptible
Cypress	T	Susceptible
Blue Bonnet	T	Susceptible
CL111	T	Susceptible
93-11 (indica reference)	C	Moderate Susceptible
Nonabokra	C	Resistant
Pokkali	C	Resistant
Jasmine	C	Resistant
TeQing	C	Resistant
IR64	C	Resistant
IR29	C	Resistant
Nagina 22 (N22)	C	Resistant
Kasalath (AUS reference)	C	Resistant
<i>O. nivara</i>	C	Resistant
<i>O. rufipogon</i>	C	Resistant
<i>O. barthii</i>	C	Resistant
<i>O. brachyantha</i>	C	N/A
<i>O. glaberrima</i>	C	N/A
<i>O. glumaepatula</i>	C	Resistant
<i>O. longistaminata</i>	C	Resistant
<i>O. meridionalis</i>	C	Resistant
<i>O. punctata</i>	C	N/A
<i>Leersia perrieri</i>	C	N/A

H

Population			
Individual	Allele	Phenotype	Score
<b>CCDR (Parent)</b>	T	Susceptible	7.5
SB2-99	C	Susceptible	7.5
SB2-13	T	Susceptible	7.5
SB2-48	T	Susceptible	8
SB2-88	T	Susceptible	8
SB2-126	T	Susceptible	8
SB2-144	T	Susceptible	8
SB2-203	T	Susceptible	7.5
SB2-255	T	Susceptible	8
SB2-276	T	Susceptible	8
SB2-314	T	Susceptible	7.5
<b>MCR (Parent)</b>	C	Resistant	3.5
SB2-03	C	Resistant	4.7
SB2-109	C	Resistant	5.5
SB2-134	C	Resistant	5.5
SB2-158	C	Resistant	5
SB2-161	C	Resistant	5.7
SB2-174	C	Resistant	4.5
SB2-259	C	Resistant	5.7
SB2-206	C	Resistant	6
SB2-225	C	Resistant	6
SB2-272	C	Resistant	6

I



Susceptible  
SB2-276

Resistant  
SB2-03

J

3K Rice genome project			
Type	Allele		
	T	C	T/C
Tropical Japonica	471	5	0
Temperate Japonica	360	0	3
Indica	18	1742	22
Aromatic	68	4	1
AUS	55	145	1
Admix	35	38	25

