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GmRmd1 encodes a TIR-NBS-BSP protein and confers resistance to powdery mildew in soybean

Dear editor,

Powdery mildew (PMD) is a widespread, fungal-borne disease that impacts crop yield worldwide. In soybean, PMD is caused by the fungal pathogen, *Microsphaera diffusa.* The most efficient and economic strategy for PMD management with minimal environmental impact is through the deployment of resistance genes (Dangl et al., 2013; Hafeez et al., 2021). Although resistant genes against PMD have been identified in some crops, identification of those in soybean remains elusive. Several independent reports have consistently mapped the PMD-resistance locus to the end of Chr 16 (Kang and Mian, 2010; Jun et al., 2012; Jiang et al., 2019), however, the underlying gene that confers PMD resistance in soybean has yet to be cloned. Identification of the *resistance-to-M. diffusa 1* (*Rmd1*) gene is critical for the breeding of resistant soybean varieties, and thus control of PMD in this important crop.

Previously, the *Rmd1* locus was mapped to a 188.06-kb genomic region at the end of Chr 16 (Jiang et al., 2019). To further identify the gene underlying *Rmd1*-mediated resistance, a genome-wide association study (GWAS) was performed to catalog genetic polymorphisms associated with PMD resistance in soybean. Using 2,176,969 single-nucleotide polymorphisms (SNP) from 467 soybean accessions (Supplemental Table 1), a single region associated with PMD resistance was identified. These results are consistent with the previously mapped region (*Rmd_B13*) to the end of Chr 16 (Figure 1A). To further identify the potential resistance gene, 471 F_8 recombinant inbred lines derived from Guizao1 (susceptible) × B13 (resistant) were genotyped using molecular markers, narrowing down the *Rmd1* locus to a 124.2-kb region between markers M5 and M8. This region harbors 19 tightly linked putative NLR-like genes (Figure 1B; Supplemental Table 2). It has been reported that NLR gene clusters can cause genome structural variants (SVs) through unequal crossing-over (Wang et al., 2021). To explore SVs in the *Rmd1* region, and to identify the *Rmd1* gene, *de novo* assembly of the Guizao1 and B13 draft genomes was conducted using long reads generated by single-molecule long-read sequencing technology, an approach that is frequently used for reliable detection of complex SVs (Sedlazeck et al., 2018). The assembled genome size and contig N50s for Guizao1 and B13 were: 976, 10.4, and 960, 9.23 Mb, respectively. Comparison of the ~124-kb *Rmd1* region from Guizao1, B13, Williams 82, and 27 high-quality public genomes (Shen et al., 2019; Liu et al., 2020) revealed 13 different types of genomic structures (Supplemental Figure 1; Supplemental Table 3), thus confirming significant SVs in the *Rmd1* region.

Based on a comparison of the assemblies and annotations from the two genomes, it was determined that the *Rmd1* region of B13 consists of 14 genes (*B1–B14*), whereas the corresponding region from Guizao1 consists of 16 (*G1–G16*) (Supplemental Figure 2). The comparison also revealed seven insertion or deletion (indel) markers between M5 and M8, which enabled the identification of additional recombinants in the segregating population that were derived from selfed heterozygous plants. Genotyping of these new recombinants narrowed the *Rmd1* locus to a 23.73-kb region, which contains only two genes (*B5* and *B6*) in the B13 genome, located between the indel3 and indel7 markers (Figure 1B).

Comparison of the Guizao1 and B13 genomes with 16 known-phenotype, high-quality genomes from the soybean pangenome (Liu et al., 2020) revealed that *B5* is present only in the genomes of PMD-resistant varieties, whereas *B6* is present in all genomes examined (Figure 1C; Supplemental Table 3). This suggested that *B5* is the likely candidate for the *Rmd1* gene. To investigate this hypothesis, 3531 soybean accessions were collected and 1441 were genotyped for the *B5* gene. *B5* was consistently present in 1018 PMD-resistant accessions but absent in 423 PMD-susceptible accessions (Supplemental Table 4), providing strong evidence that *B5* is the long-sought *Rmd1* gene.

Molecular cloning and sequence analysis of the full-length coding sequence revealed that *B5* encodes a TIR-NBS protein with a basic secretory protein domain at the C-terminus (Supplemental Figure 3). To further validate *B5 as the Rmd1 gene,* CRISPR-Cas9-based genome-editing was employed to generate *b5 mutants* in Young, a PMD-resistant variety. Four independent homozygous mutants (Figure 1D and Supplemental Figure 4) were obtained that showed similar susceptibility to *M. diffusa as those varieties lacking the B5 gene, all of which were highly susceptible* (Figures 1E and 1F). Next, the genomic fragment of *B5* and its native promoter from Young were introduced into a PMD-susceptible variety, Huaxia3. Compared with the negative control, the two positive *B5*-transgenic lines (Com-1 and Com-2) showed immunity to *M. diffusa* (Figure 1G and 1H). Collectively, these loss- and gain-of-function experiments demonstrated that *B5* is indeed the *Rmd1* gene. Because of these data, and the fact that this is the first-described PMD-resistance gene in soybean, *B5* was named *GmRmd1*.

In addition to the presence/absence variation of *GmRmd1,* sequence variations in the *GmRmd1* coding region were also investigated. *GmRmd1* from 232 PMD-resistant accessions were sequenced by Sanger sequencing. As a result, eight SNPs in the coding region were identified, which define three

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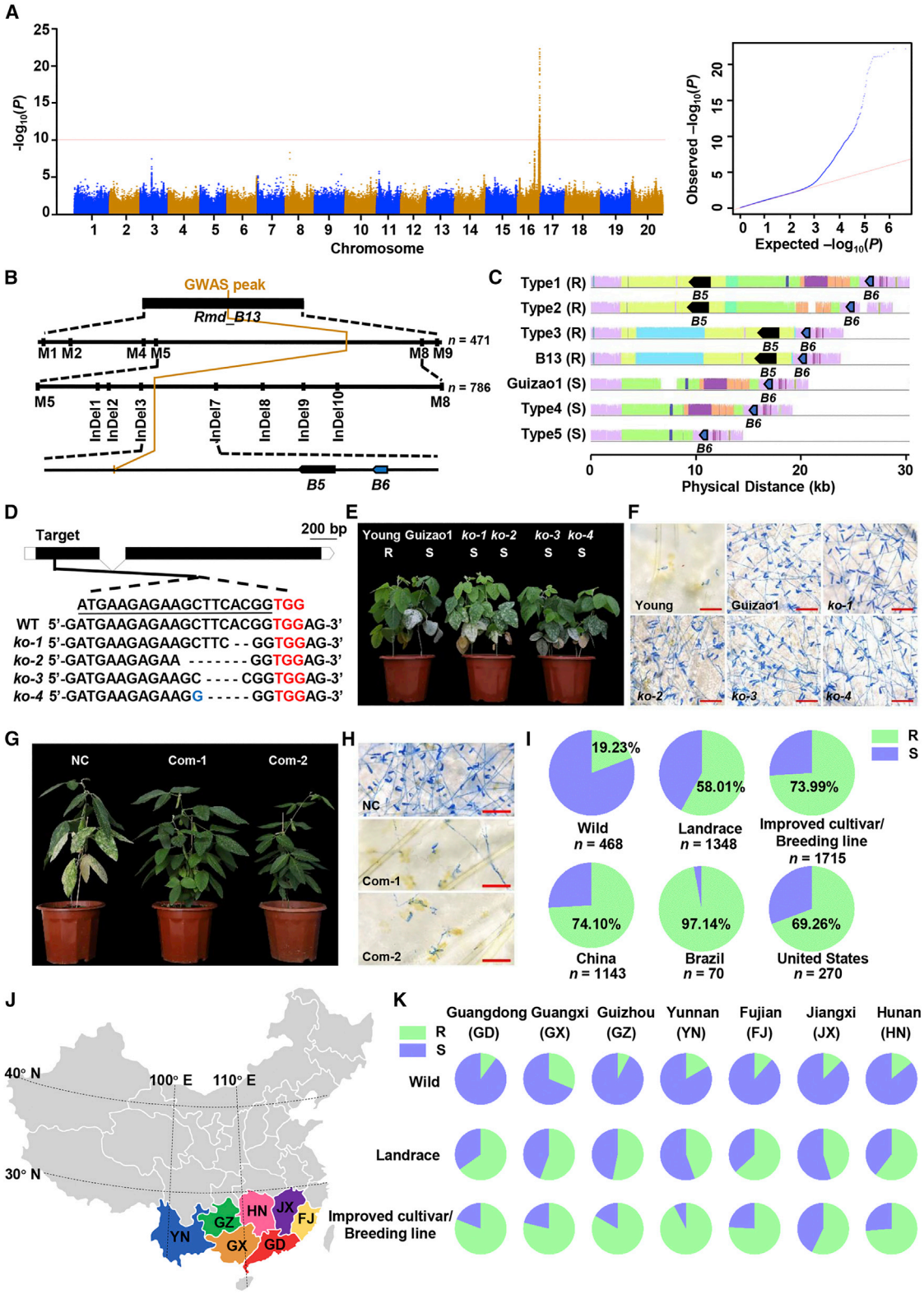

Figure 1. Identification and characterization of *GmRmd1*.

(A) Manhattan (left) and the quantile-quantile (right) plots showing the association between PMD resistance and SNPs.

(B) Fine mapping of *GmRmd1*.

(C) Alignment of the genomic region between markers 'indel3' and 'indel7' from seven different genotypes of soybean. *R* and *S* indicate resistant and susceptible to PMD, respectively. Same color indicates homologous fragments identified by the Mauve program.

(legend continued on next page)

haplotypes of *GmRmd1* (Supplemental Table 5). Among the eight SNPs, six result in amino acid changes (Supplemental Table 5), however, these amino acid changes might not have a significant effect on the resistance to *M. diffusa* as they are all from the PMD-resistant accessions.

To further explore the role of *GmRmd1* in resistance against PMD, the response to *M. diffusa* infection for all 3531 soybean accessions was evaluated. These accessions originated from 17 countries and include 468 wild soybeans, 1348 landraces, and 1715 improved cultivars/breeding lines (Supplemental Table 4). Although a majority (60.63%) displayed resistance to PMD, the distribution of the PMD-resistant accessions in wild soybean (*Glycine soja*) and cultivated soybean differed. Overall, the proportion of PMD-resistant accessions was lowest in wild soybeans, followed by landraces, with the greatest proportion of resistance in improved cultivars/breeding lines (Figure 1I; Supplemental Table 6), demonstrating selection for resistance during soybean domestication and breeding. In Brazil, PMD was a minor disease before the outbreak in the 1996/1997 growth season, and it has since become a major threat to soybean production (Almeida et al., 2008). This has driven the expedited breeding of PMD-resistant varieties, explaining how approximately 97% of accessions are PMD resistant in Brazil, far higher than that in China and the United States (Figure 1I; Supplemental Table 7). Similarly, an analysis of the geographic distribution of the Chinese soybean accessions revealed that the PMD-resistant accessions are more common in improved cultivars/breeding lines than in landraces and wild soybeans, especially in the southern region of China where PMD is most prevalent (Figure 1J and 1K; Supplemental Table 8). Notably, *GmRmd1* is present in all improved, PMD-resistant soybean cultivars from Brazil and South China (Supplemental Table 9).

In summary, the combinatorial approach of GWAS, gene mapping, *de novo* genome sequencing, analysis of a large number of soybean accessions, and gain/loss-of-function experiments resulted in the successful identification of the first soybean PMD-resistance gene, *GmRmd1*. Because of the worldwide threat of PMD to soybean yield, PMD resistance has been one of the major goals of soybean breeding. The identification of *GmRmd1* fills a critical genomic gap between traditional breeding practice and gene-focused development of PMD-resistance in improved varieties of soybean.

SUPPLEMENTAL INFORMATION

Supplemental information is available at *Plant Communications Online*.

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AUTHOR CONTRIBUTIONS

H.N., L.G., and Y.W. conceptualized the study. P.X., Z.C., B.J., Q.X., and L.G. performed genetic-linkage mapping and gene cloning. P.X., Y.Y., and Z.C. conducted the soybean transformation experiments, gene sequence analysis, and gene editing. P.X., C.Z., B.J., Y.C., Y.Y., Q.Z., T.L., and Q.M. conducted phenotyping and part of the genotyping analyses. P.X., Z.C., and L.G. analyzed the experimental data. H.N. acquired funding. P.X., Z.C., Y.W., L.G., and H.N. wrote the manuscript.

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(D) CRISPR-Cas9-mediated knockout of *GmRmd1*. The single guide RNA target sequence is underlined in black, and the protospacer-adjacent motif (PAM) sequences are shown in red. 'Wild type' refers to the sequence from the resistant variety, Young. The labels *ko-1* to *ko-4* refer to the two different types of knockout mutants. Base substitutions and deletions are shown in blue letters and short lines, respectively.
(E–H) The phenotypes of knockout mutants and complementation lines. Plants were inoculated with *M. diffusa* **(E and G)**. The spores and colonies of *M. diffusa* on the surfaces of leaves were stained with Coomassie blue **(F and H)**. Scale bar indicates 100 μm. **(I)** Top panel: distribution of R and S accessions within three germplasm groups, wild soybean, landraces, and improved cultivars/breeding lines, for 3531 soybean accessions. Bottom panel: distribution of R and S accessions in improved cultivar/breeding lines within China, Brazil, and the United States.
(J–K) The geographic distribution of soybean accessions from seven provinces in the south of China **(J)** and the distribution of R and S accessions within three germplasm groups **(K)**. The map does not show the complete territory of China.

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