New Phytologist

ZmWAK02 encoding an RD-WAK protein confers maize resistance against gray leaf spot

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Received: *15 June 2023*
Accepted: *21 November 2023*

New Phytologist (2024) **241:** 1780–1793
doi: 10.1111/nph.19465

Key words: gray leaf spot, maize, resistance, *ZmWAK02*.

Summary

- Gray leaf spot (GLS) caused by *Cercospora zeina* or *C. zeae-maydis* is a major maize disease throughout the world. Although more than 100 QTLs resistant against GLS have been identified, very few of them have been cloned.
- Here, we identified a major resistance QTL against GLS, *qRglsSB*, explaining 58.42% phenotypic variation in SB12×SA101 BC_1F_1 population. By fine-mapping, it was narrowed down into a 928 kb region. By using transgenic lines, mutants and complementation lines, it was confirmed that the *ZmWAK02* gene, encoding an RD wall-associated kinase, is the responsible gene in *qRglsSB* resistant against GLS.
- The introgression of the *ZmWAK02* gene into hybrid lines significantly improves their grain yield in the presence of GLS pressure and does not reduce their grain yield in the absence of GLS.
- In summary, we cloned a gene, *ZmWAK02*, conferring large effect of GLS resistance and confirmed its great value in maize breeding.

Introduction

Maize is one of the most important crops in the world. But it is vulnerable to diseases that cause serious reduction in grain yield and quality (Balint-Kurti & Johal, 2009; Zhu *et al.*, 2021). The best way to control those diseases is to plant-resistant maize hybrids (Carson *et al.*, 2002) while cloning of resistance genes is the primary basis to achieve that goal by using biotechnology. Although a lot of work on maize resistance against diseases has been done during the past 10 yr, only few maize resistance genes have been cloned, compared with resistance genes cloned from other crops such as rice and wheat.

Gray leaf spot (GLS) is one of major maize diseases throughout the world and yield losses due to GLS range from 11% to 69%, depending on the hybrid cultivars and environmental conditions (Ward *et al.*, 1999; Mueller *et al.*, 2020). Gray leaf spot was first reported in Alexander County, IL in 1925, showing rectangle lesions on leaves. Until the 1970s, it became prevalent in the eastern United States, resulting in serious economic losses (Donahue *et al.*, 1991; Coates & White, 1998). Up to now, GLS has spread throughout most of maize planting areas and has become one of the most important yield-limiting maize diseases in the world (Ward *et al.*, 1999; Mueller *et al.*, 2016, 2020; Chen *et al.*, 2021). Although multiple *Cercospora* species have been isolated from maize leaves showing GLS lesions, only two of them (*C. zeae-maydis* and *C. zeina*) are the causal agents of GLS (Wang *et al.*, 1998; Crous *et al.*, 2006). *Cercospora zeae-maydis* is generally distributed in the United States, Brazil and Northern China (Wang *et al.*, 1998; Balestre *et al.*, 2012; Q-K. Liu *et al.*, 2013) while *C. zeina* is spread in the United States, Southwest China and African (Wang *et al.*, 1998; Q-K. Liu *et al.*, 2013; Nsibo *et al.*, 2021).

Based on the genetic analysis, it is known that the GLS resistance is a quantitative trait and is inherited additively (Clements *et al.*, 2000). Up to now, more than 100 resistance QTLs against GLS have been reported (Du *et al.*, 2020). And five loci were repeatedly identified from different maize inbred lines by different research groups: bin 1.05–1.06 (Lehmensiek *et al.*, 2001; Balint-Kurti *et al.*, 2008; Pozar *et al.*, 2009; Sun *et al.*, 2021), bin 2.03–2.05 (Zwonitzer *et al.*, 2010; Zhang *et al.*, 2012; Lennon *et al.*, 2016), bin 4.05–4.08 (Gordon *et al.*, 2004; Balint-Kurti *et al.*, 2008; Lennon *et al.*, 2016), bin 5.03–5.06 (Clements *et al.*, 2000; Xu *et al.*, 2014; Lennon *et al.*, 2016) and bin 7.02–7.03 (Pozar *et al.*, 2009; Benson *et al.*, 2015; Mammadov *et al.*, 2015;

Du *et al.*, 2020). Although none of these QTLs have been cloned, two cloned maize multiple disease resistance (MDR) genes (*ZmCCoAOMT2* and *ZmMM1*) were found to positively regulate GLS resistance (Yang *et al.*, 2017; Wang *et al.*, 2021). The resistance mechanisms of *ZmCCoAOMT2* is related to the increased levels of lignin and other metabolites, and these of *ZmMM1* are associated with ROS accumulation (Yang *et al.*, 2017; Wang *et al.*, 2021). But the detailed molecular mechanisms of them are still unknown. In summary, our understanding of maize GLS resistance is limited.

To fight against pathogens, plants largely depend on the innate immunity consisting of two branches: pattern-triggered immunity (PTI) and effector-triggered immunity (ETI; Jones & Dangl, 2006). Pattern-triggered immunity is activated by cell-surface pattern recognition receptors (PRRs) after detecting microbe-associated molecular patterns (MAMPs) or damage-associated molecular patterns (DAMPs); MAMPs are microbe-derived conserved elicitors such as chitin, and DAPMs are plant-derived molecules released after pathogen invasion, such as AtPep1 (Dodds & Rathjen, 2010; Liu *et al.*, 2012; Z. Liu *et al.*, 2013). While ETI is activated when pathogen-derived effectors are recognized by plant proteins such as nucleotide-binding leucine-rich repeat proteins (NLRs), following with strong immune responses such as hypersensitive response (HR; Ngou *et al.*, 2022). Cell wall-associated kinase (WAK) proteins possess an extracellular domain, a transmembrane domain and an intercellular kinase domain. Their extracellular domain is responsible for binding small pectic oligosaccharides (pectic products of cell wall degradation; Kohorn *et al.*, 2014), and the intercellular kinase domain is responsible for activating downstream signaling pathways (Amsbury, 2020). So, WAK proteins were taken as kinase receptor-activating PTI, which contributes basal resistance and has no race-specificity (Bacete *et al.*, 2018). While, the race-specific resistance gene *Xa4* in rice encodes a WAK protein (Hu *et al.*, 2017). It indicates that WAK proteins also play important roles in ETI. Based on the presence or absence of the conserved arginine (R) residue adjacent to the aspartate (D) residue in the kinase domain, WAK proteins are classified into two subgroups: RD (arginine-aspartate) WAK and non-RD (non-arginine-aspartate) WAK (Dardick *et al.*, 2012). In crops, RD WAKs are normally associated with roles in growth and development, whereas non-RD WAKs play roles in disease resistance (Stephens *et al.*, 2022).

Here, we report the identification and cloning of a major resistance QTL against GLS: *qRglsSB*. It was identified in bin 1.06, explaining 58.42% phenotypic variation of GLS resistance in SB12×SA101 BC_1F_1 population. Based on map-based cloning strategy, *qRglsSB* was narrowed into 817 kb region, containing 12 candidate genes. Three of them encoding WAKs (*ZmWAK02*, *ZmWAK03* and *ZmWAK04*) were selected as the best candidates, and their genomic DNA was transferred into susceptible inbred line KN5585. Only transgenic plants containing *ZmWAK02* showed resistance phenotype against GLS. Also, the disease phenotypes of mutants and complementary lines confirmed that the *ZmWAK02* gene is responsible for GLS resistance in *qRglsSB*. Unlike most of resistance *WAK* genes in crops encoding non-RD WAKs, *ZmWAK02* encodes an RD WAK.

Materials and Methods

Plant materials

Maize inbred line SB12, highly resistant to GLS, was used as the donor parent and maize inbred line SA101, highly susceptible to GLS, was taken as the recurrent parent. Both of them were provided by Hubei Tenglong Seed Co. Ltd. SB12 was crossed with SA101 to generate F_1 plants, and F_1 was backcrossed with SA101 to generate BC_1F_1 population. The BC_1F_1 population containing 217 individual plants was used for the initial QTL analysis. All of those BC_1F_1 plants were self-crossed to generate BC_1F_2 families. And those BC_1F_2 families were used to confirm the results of the initial QTL analysis. BC_3F_1 population was generated via marker-associated selection (MAS) through backcrossing resistant BC_1F_1 plants with SA101 twice and BC_5F_1 population was generated via MAS through backcrossing resistant BC_3F_1 plants with SA101 twice. Resistant BC_5F_1 plants were self-crossed to generate BC_5F_5 families via MAS. And NIL plants were derived from resistant BC_5F_5 plants.

Evaluation of GLS resistance phenotype

For primary QTL analysis, the BC_1F_1 population was planted at Badong (Hubei Province, China), and the BC_1F_2 families were planted at Badong and Dehong (Yunnan Province, China) under natural infection in two replicates at each location. For the fine-mapping, all plants were planted at Badong under natural infection. *Cercospora zeina* is prevalent in Badong and Dehong, and GLS happens every year in those two regions.

The GLS disease phenotype was examined at 2–3 wk after anthesis, and the disease severity was evaluated by using the five-class scale (Supporting Information Fig. S1), in which '1' means highly resistant phenotype to GLS and '9' means highly susceptible phenotype to GLS.

Marker development and genotyping

For primary QTL analysis, SSR and insertions or deletions (InDels) polymorphism (IDP) markers were obtained from the Maize Genetics and Genomics Database (http://www.maizegdb.org), and markers showing polymorphism in SB12×SA101 population were used to genotype all individual plants in the SB12×SA101 BC_1F_1 population and to generate the genetic map. For fine-mapping, IDP markers were developed based on the sequence difference between SB12 and SA101, which was detected based on sequence difference of specific fragments in the QTL region.

For genotyping individual plants, the Fragment Analyzer system (Fragment Analyzer™ Automated CE System) was used. This system is a multiplexed capillary electrophoresis (CE) instrument for performing automated, high-throughput separation and

quantification of double-stranded DNA, and it can separate DNA fragments that differ in size > 5 base pairs (bp).

Construction of a linkage map and QTL analysis

The linkage map was generated by using the MAPMAKER 3.0 software (Lander *et al.*, 1987). The input data included markers for the BC_1F_1 population and their corresponding genotype. The 'group' command was used to produce linkage groups with a logarithm of odds (LOD) score of 3.0, and the Kosambi mapping function was applied to covert recombination frequency into centiMorgen (cM).

QTL detection was done by using Windows QTL CARTOGRAPHER 2.5 software with composite interval mapping (CIM) method (Silva *et al.*, 2012). The walk speed is 1 cM, and a significant threshold for QTLs was determined by 1000 permutation tests at the level $P = 0.05$.

Fine-mapping for *qRglsSB* and the progeny test

To fine-map *qRglsSB*, recombinants in the QTL regions were identified from BC_3F_1 populations and BC_5F_1 populations by using molecular markers. And those recombinants were self-pollinated to generate progenies (BC_3F_2 families or BC_5F_2 families), which were planted at Badong for disease phenotype examination.

More than 100 progenies from the self-cross of each recombinant were planted in the field for genotyping and phenotyping evaluation. Progenies derived from the same recombinant were genotyped by markers. Based on the presence or absence of SB12 donor region, three genotypes (SB12/SB12 homozygous, SA101/SA101 homozygous and SB12/SA101 heterozygous genotypes) are present in those progenies. And the significant difference of disease phenotypes between SB12/SB12 homozygous and SA101/SA101 homozygous genotypes in the same recombinant-derived progenies was examined by using a Student's *t*-test (two-tailed). If the SB12 donor region in the recombinant contains the resistance QTL, plants with the SB12/SB12 homozygous genotype is expected to be significantly more resistant ($P < 0.05$) than plants with the SA101/SA101 homozygous genotype. On the contrary, if the SB12 donor region in the recombinant does not contain the resistance QTL, there is no significant difference ($P > 0.05$) in disease phenotypes between plants with the SB12/SB12 homozygous genotype and plants with the SA101/SA101 homozygous genotype.

Genome sequencing, assembling and gene annotation

Sequencing, optical map generation and assembling were performed as previously described (Hufford *et al.*, 2021), with a few modifications. SB12 seedlings were grown in growth room to three-leaf stage. Leaf tissue of seedings was harvested and frozen in liquid nitrogen. Ultra-high molecular weight (uHMW) DNA was isolated from *c.* 1 g frozen leaf tissue grinded in liquid nitrogen using the Bionano Plant Tissue DNA isolation kit as previously described (Hufford *et al.*, 2021). The resulting DNA was used for Bionano DLS genome optical mapping, the PacBio CLR sequencing and illumina library construction.

Approximately 1 μg uHMW DNA was labelled by using the DLS kit (Cat. 80005; Bionano Genomics, San Diego, CA, USA) as per the manufacturer's instructions (Bionano Genomics). The labelled sample was loaded onto a Bionano chip flow cell, where molecules were separated, imaged and digitized in the Saphyr System (https://bionanogenomics.com/support-page/saphyr-system/). In total, 1120 634 molecules with a minimum length of 150 and 306 kb N50 were assembled at effective coverage of 95.6× and optical genome map N50 of 102.6 Mb from 33 maps.

For Illumina libraries, Chromium 10× libraries were constructed using a Chromium 10× system as per the manufacturer's recommendations (10× Genomics, Pleasanton, CA, USA) and sequenced in one Illumina HiSeq sequencer using a pair end 2 × 150 configuration. The chromium libraries yielded a total of 98 859 309 600 bp in 328 749 165 PE150 clusters, for an expected coverage of 44×.

For PacBio sequencing, sequencing libraries were constructed following PacBio's template prep protocols for the Express Template Prep Kit 2.0. PacBio CLR sequencing yielded 72.5-fold coverage of sequence (162.5 Gb; 6.887 million subreads; N50 read length: 26.3 kb). PacBio reads were trimmed and assembled with CANU (v.1.8; Koren *et al.*, 2017), and the resulting contigs were filtered to a minimum contig length of 30 kb. The PacBio sequence assembly was merged with the optical map using the hybrid scaffolding module of BIONANOSOLVE (v.3.4.0) and BIONANO ACCESS (v.1.3.0). The *de novo* assembly yielded 1101 contigs with contig N50 of 43.5 Mb and total assembly size of 2.242 Gb. Hybrid scaffolding yielded 31 hybrid scaffolds with an N50 of 103.9 Mb. Polished hybrid scaffolds were ordered and oriented into 10 pseudomolecules relative to B73_v4 with a total length of 2.152 Gb.

For gene annotation, RNA-seq data were generated by using RNA isolated from leaf samples of SB12 seedlings at three-leaf stage. Then, paired-end reads from the generated Illumina RNA-seq libraries were aligned to the assembled SB12 genome using TOPHAT2 v.2.0.4, with default parameters (https://genomebiology.biomedcentral.com/articles/10.1186/gb-2013-14-4-r36). The aligned RNA-seq reads were then utilized to assemble the SB12 transcriptome with STRINGTIE v.2.1.1, with default parameters (https://www.nature.com/articles/nbt.3122). The SB12 genome was annotated using a hybrid evidence and *ab initio*-based gene prediction pipeline (Li *et al.*, 2021). Evidence-based predictions were directly inferred from the assembled transcripts generated by using STRINGTIE v.2.1.1. For generating assembled transcripts, RNA-seq reads were mapped to SB12 genome using STAR (v.2.5.3a), and those mapped reads were organized by using SAMTOOLS (v.1.9) to generate input for transcript assembly programs (Li *et al.*, 2009). *Ab initio* predictions were done by using BRAKER (v.2.1.2) with both evidence-based predicted proteins and mapped RNA-seq reads as inputs.

Transgenic validation of *ZmWAK02*, *ZmWAK03* and *ZmWAK04*

The genomic fragments of *ZmWAK02*, *ZmWAK03* and *ZmWAK04* were amplified from the genomic DNA of the

resistant line SB12 and were cloned into the binary vector *pZZ01523* to generate *pZZ01523-ZmWAK02*, *pZZ01523-ZmWAK03* and *pZZ01523-ZmWAK04* constructs. The insertions in these constructs were sequenced to confirm the accuracy of the fragments. Construct *pZZ01523-ZmWAK02* contains the genomic sequence of *ZmWAK02* (9335 bp) including 3060 bp promoter, 3503 bp gene body and 2772 bp downstream region; construct *pZZ01523-ZmWAK03* contains the genomic sequence of *ZmWAK03* (8149 bp) including 3050 bp promoter, 3637 bp gene body and 1462 bp downstream region; and construct *pZZ01523-ZmWAK04* contains the genomic sequence of *ZmWAK04* (10 182 bp) including 4020 bp promoter, 4538 bp gene body and 1624 bp downstream region. To generate three overexpression constructs (*pZZ01523-Ubi::ZmWAK02-GFP*, *pZZ01523-Ubi::ZmWAK03-GFP* and *pZZ01523-Ubi::ZmWAK04-GFP*), the coding sequences of *ZmWAK02*, *ZmWAK03* and *ZmWAK04* were amplified from SB12 cDNA library and were fused with *GFP* gene, and the fused genes were driven by the promoter of maize *Ubiquitin* gene.

The six constructs were transformed into the susceptible maize inbred line KN5585 by the gene transformation platform of Weimi Biotechnology Co. Ltd Positive T_1 plants were identified and self-crossed twice to produce homozygous positive and negative T_3 plants, which were planted at Enshi (Hubei Province, China) for evaluating the GLS disease phenotype. The two-tailed Student's *t*-test was conducted to test the significant difference on GLS phenotype between positive and negative transgenic plants.

Mutant generation and screening

More than 5000 seeds of SB12 were radiated with ^{60}Co-γ ray with 200 Gy at the Institute of Biological and Nuclear Technology, Hubei Academy of Agricultural Sciences, China. A total of 1290 M_2 families were generated after self-crossing 2000 M_1 plants. Those M_2 families were planted at Badong for GLS phenotype evaluation. Susceptible plants were self-crossed to generate M_3 families. And those M_3 families were planted at Badong to test the GLS resistance phenotype again to get stable mutant plants showing susceptible phenotype to GLS.

RT-qPCR analysis

Maize inbred line SB12 at five-leaf stage was inoculated with *C. zeina* spores under high-humidity conditions. Leaf samples were collected at different time points before and after inoculation, then RNAs were isolated from those leaf samples by using TRIZOL (Invitrogen). RT-qPCR was done by using SYBR premix (TaKaRa, Dalian, China) on real-time PCR (CFX96 Real-Time System; Bio-Rad). Primers used to detect the expression levels of *ZmWAK02*, *ZmWAK03*, *ZmWAK04* and *ZmUbiquitin* are listed in Table S1.

Molecular breeding of the *ZmWAK02* gene

The *ZmWAK02* gene was used to improve commercial maize hybrid lines XianYu335 (PH4CV × PH6WC) and ZhengDan958 (Zheng58 × Chang7-2), widely planted in China.

The resistant inbred line SB12 was crossed with two parental inbred lines of XianYu335, respectively. The BC_5F_3 lines containing *ZmWAK02* were generated through maker-associated selection. The PH4CV^{ZmWAK02} and PH6WC^{ZmWAK02} lines identified from the BC_5F_3 population were crossed to generate the improved hybrid line XianYu335^{ZmWAK02} (PH4CV^{ZmWAK02}×PH6WC^{ZmWAK02}).

Introgression of the *ZmWAK02* gene into Zheng58 was done by crossing SB12 with Zheng58. Finally, The Zheng58^{ZmWAK02} lines identified from the BC_5F_3 population were crossed with Chang7-2 to generate the improved hybrid ZhengDan958^{ZmWAK02} (Zheng58^{ZmWAK02}×Chang7-2).

The improved hybrid lines and wild-type (WT) hybrid lines were planted in fields for yield evaluation under two conditions: in the absence of GLS: field in Huanggang (29°N, 114°E), China, in 2022; and in the presence of GLS: natural GLS nursery field in Enshi (30.5°N, 109.6°E), China, in 2022. In Huanggang, each line was planted in four subplots and each subplot contains three rows, each row was 3 m long with 11 plants. In Enshi, each line was planted in three subplots, and each subplot contains three rows, each row was 4 m long with 14 plants. All ears were harvested and dried to uniform moisture for scoring ear and grain traits. The two-tailed Student's *t*-test was done to test the significant difference of grain yield, plant height, days to tassel, days to silking, days to anthesis and ear height between the improved hybrid lines and WT hybrid lines.

Results

Identification of a major resistance QTL, *qRglsSB*, against GLS

Maize inbred line SB12 is highly resistant to GLS, while inbred line SA101 is highly susceptible to GLS (Fig. 1a). In order to identify resistance QTLs against GLS, a BC_1F_1 population derived from a cross between SB12 and SA101 was generated for genotyping and phenotyping examinations. In 2015, 217 plants in the BC_1F_1 population were planted at Badong in Hubei province for GLS disease evaluation (15Badong), based on the five-class disease scale (Fig. S1). And the genetic linkage map for the SB12×SA101 population was developed by using 166 SSR markers (Fig. S2). As determined by the analysis of the association between the genetic data and the phenotypic data, QTL analysis identified one major resistance QTL against GLS, *qRglsSB*, in the region between molecular markers C1–56 and TF104 on chromosome 1. The resistance allele at *qRglsSB* was derived from the resistant inbred line SB12 (Fig. 1b). And, it could account for 58.42% of the total phenotypic variation with 4.087 additive effect on the disease scale (Figs 1b, S2a).

In order to confirm the QTL analysis results, those SB12×SA101 BC_1F_1 plants were self-crossed to generate $BC_1F_{1:2}$ families, which were planted at Badong in Hubei Province and Dehong in Yunnan Province (two repeats for each location) for GLS disease evaluation in 2016. The correlation coefficients between the disease phenotypes of the BC_1F_1 population and the $BC_1F_{1:2}$ population were 0.74–0.79, $P < 0.0001$

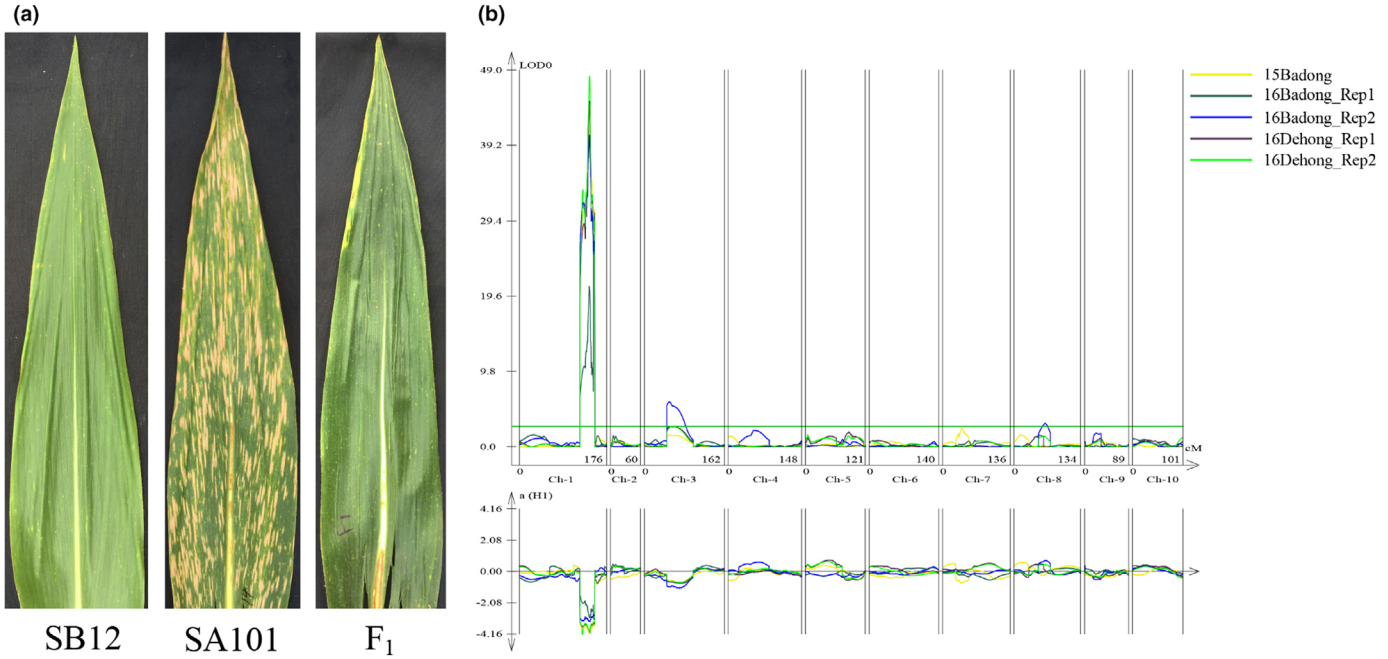


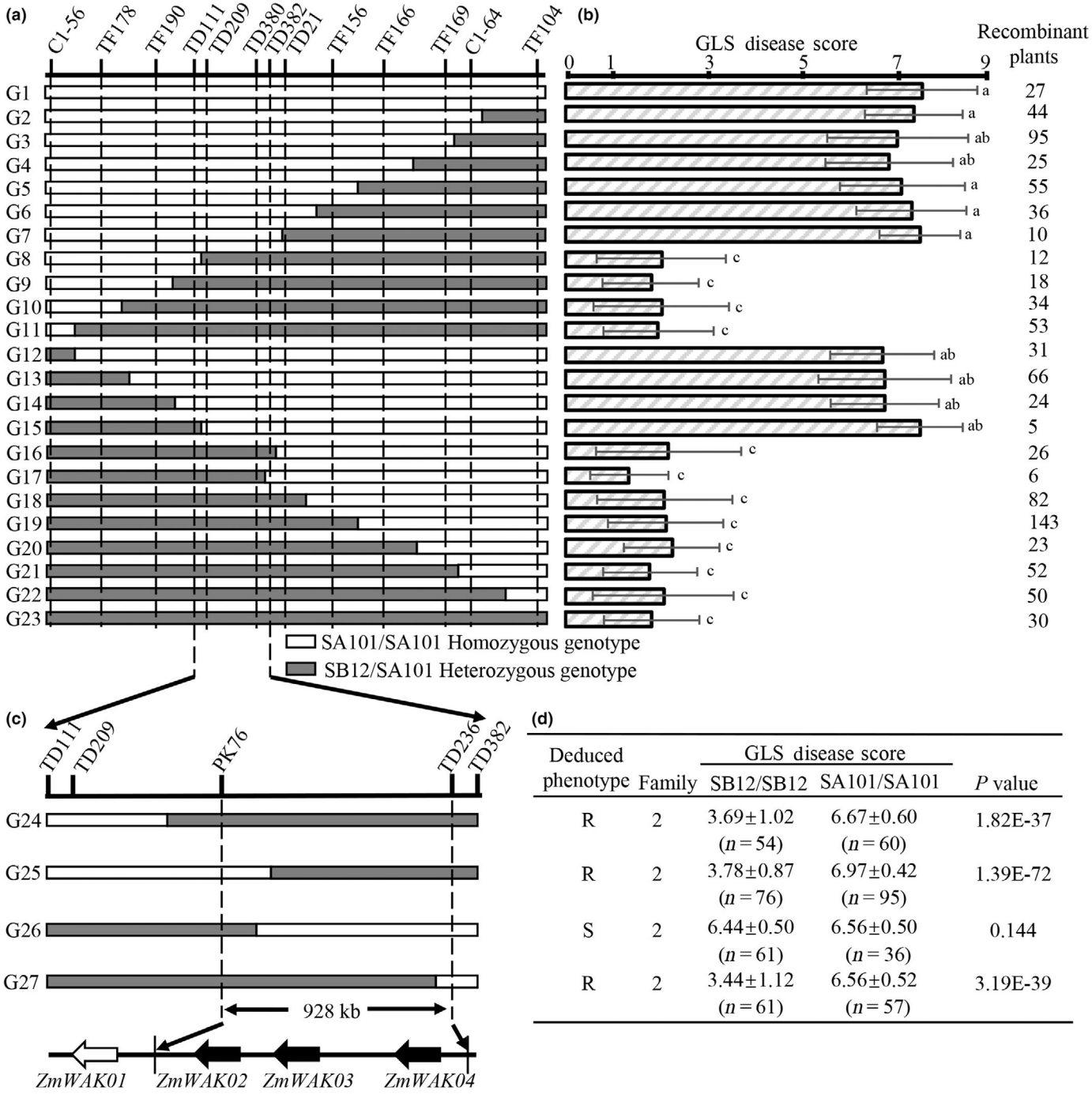
Fig. 1 Identification of a major QTL, *qRglsSB*, resistant against gray leaf spot (GLS). (a) GLS disease phenotypes of maize inbred lines SB12, SA101 and F_1 (SB12×SA101). (b) Log-of-odd (LOD) profiles and additive genetic effects of the QTL for resistance against GLS. Disease phenotypes were collected from BC_1F_1 population grown at Badong in 2015 and were collected from BC_1F_2 population in four replicate plots grown at Badong and Dehong in 2016. a(H1), additive effect under alternative hypothesis (H1).

(Fig. S3). And the major QTLs detected from the $BC_1F_{1:2}$ population in the four repeats were exactly the same as *qRglsSB*, confirming the reliability of the QTL mapping results in BC_1F_1 population (Fig. 1b).

Fine-mapping of *qRglsSB*

In order to fine-map *qRglsSB*, resistant BC_1F_1 plants with heterozygous genotype at the QTL region were identified by using molecular markers C1–56 and TF104; then, those BC_1F_1 plants were backcrossed twice to SA101 to generate the BC_3F_1 population. Next, 9600 BC_3F_1 plants were planted at Badong for GLS disease evaluation. By using molecular markers C1–56 and TF104, 947 recombinant plants were identified from the BC_3F_1 population. Furthermore, all recombinant plants were genotyped by 11 molecular markers, resulting in 23 different genotypes (G1–G23) in those recombinant plants (Fig. 2a). Based on the disease phenotype data and the genotype data of recombinant BC_3F_1 plants, the QTL region was narrowed down in a region between molecular marker TD111 and TD382 (Fig. 2b), which is *c.* 2.7 Mb based on the B73 genome sequence (RefGenV4).

Since the recombination frequency between molecular markers TD111 and TD382 was very low, we needed a larger population to isolate enough recombinants between these two molecular markers. So, resistant genotype G17 and G18 plants in BC_3F_1 population were backcrossed twice to SA101 to develop a big BC_5F_1 population and > 50 000 BC_5F_1 plants were screened by using molecular markers TD209 and TD382 to identify recombinants. Finally, 220 recombinant plants were identified. In order to genotype those recombinant plants, more markers in the region between TD209 and TD380 were required. Based on the published genomic DNA sequence data online (http://www.maizegdb.org) and resequencing results of some genes in the QTL region, we designed multiple candidate SSR and KASP markers in the region between TD209 and TD382. However, only two of them (PK76 and TD236) can clearly identify SB12/SA101 heterozygous genotype and SA101/SA101 homozygous genotype in the BC_5F_1 population, which indicates that there might be a big structure variation or low DNA polymorphism in the QTL region between SB12 and SA101. Based on the genotyping data, four genotypes (G24–G27) were identified in these recombinants (Fig. 2c). The progenies (> 100 progeny plants) from the self-cross of each recombinant were planted for GLS phenotype evaluation. In the progenies of G24, G25 and G27 genotype recombinants, SB12/SB12 homozygous plants were significantly more resistant against GLS than SA101/SA101 homozygous plants. It indicates that G24, G25 and G27 genotype recombinants contain the corresponding resistance gene. While, all plants in the progenies of G26 genotype recombinants were susceptible to GLS and no significant difference on the disease phenotype was observed between SB12/SB12 homozygous plants and SA101/SA101 homozygous plants. It indicates that G26 genotype recombinants do not contain the corresponding resistance gene. Together, we concluded that *qRglsSB* is located in the region between molecular markers PK76 and TD236, which is *c.* 1.5 Mb based on the B73 genome sequence (RefGenV4).



(d)

Deduced phenotype	Family	GLS disease score SB12/SB12	GLS disease score SA101/SA101	*P* value
R	2	3.69±1.02 (*n* = 54)	6.67±0.60 (*n* = 60)	1.82E-37
R	2	3.78±0.87 (*n* = 76)	6.97±0.42 (*n* = 95)	1.39E-72
S	2	6.44±0.50 (*n* = 61)	6.56±0.50 (*n* = 36)	0.144
R	2	3.44±1.12 (*n* = 61)	6.56±0.52 (*n* = 57)	3.19E-39

Fig. 2 Fine-mapping of *qRglsSB* resistant against gray leaf spot (GLS) based on recombinants. (a) The genetic structures of 23 genotypes (G1–G23). 947 BC_3F_1 recombinants were classified into 23 genotypes based on 13 molecular markers. The genetic structure for each genotype is depicted as gray rectangles and white rectangles, corresponding to heterozygous SB12/SA101 alleles and homozygous SA101/SA101 alleles, respectively. (b) GLS disease phenotypes of 23 genotypes. The number of recombinant plants for each genotype was presented. The disease score for each genotype was presented as mean ± SD. Statistical analysis was done based on the two-tailed Student's *t*-test. Different letters indicate significant differences at $P < 0.05$. (c) The genetic structures of four genotypes (G24–G27). BC_5F_1 recombinant plants were analyzed by using five molecular markers. *qRglsSB* was narrowed down into an interval between PK76 and TD236, which is 926 kb based on the genome sequence of SB12 and contains three *ZmWAK* genes. (d) The deduced disease phenotypes of four genotypes (G24–G27). For each genotype, two BC_5F_2 families were planted for disease evaluation. The disease scores of homozygous SB12/SB12 and homozygous SA101/SA101 in progeny are presented with mean ± SD. '*n*' is the number of plants. Significant difference ($P < 0.05$) between homozygous SB12/SB12 and homozygous SA101/SA101 indicates the presence of *qRglsSB* in the donor region, and the genotype is deduced to be resistant to GLS; otherwise, it indicates the absence of *qRglsSB* in the donor region and the genotype is deduced to be susceptible to GLS.

ZmWAK02, encoding an RD WAK, is responsible for *qRglsSB* resistant against GLS

In order to clone the resistance gene in *qRglsSB* locus, we assembled the whole genome of the resistance parent line SB12 with PacBio CLR sequencing data integrated with Bionano optical map and illumina short reads, and obtained 10 pseudomolecules of the maize inbred line SB12 with a total length of 2.152 Gb. Synteny analysis showed that the genomic DNA sequence of the QTL region in SB12 was not colinear with that in B73 (Fig. S4), which indicates that there is a big structure variation in the QTL regions between SB12 and B73.

Based on the genomic DNA sequence of SB12, the QTL region between PK76 and TD236 is 928 kb, containing 12 predicted protein-coding genes (Fig. S5). Three of them (Gene 1, Gene 4 and Gene 9) encode RD WAKs and they were named *ZmWAK02* (Gene 1), *ZmWAK03* (Gene 4) and *ZmWAK04* (Gene 9), respectively. Gene 2 encodes a protein only containing a GUB binding domain; Gene 3, Gene 6, Gene 7 and Gene 10 encode unknown proteins; Gene 5 encodes a B-lectin kinase; Gene 8 and Gene 11 encode proteins containing two EFh domains; and Gene 12 encodes glycerophosphodiester phosphodiesterase 4 (GDPD4).

Since *WAK* genes play critical roles in plant defense, we considered *ZmWAK02*, *ZmWAK03* and *ZmWAK04* as the most promising candidate genes. ZmWAK02 and ZmWAK04 proteins contain a signal peptide, two GUB_WAK binding domains, two epidermal growth factor-like (EFG) domains, a transmembrane domain and a cytoplasmic kinase domain; while, ZmWAK03 contains a signal peptide, a GUB_WAK binding domain, an internal repeat1 domain, an EFG domain, a transmembrane domain and a cytoplasmic kinase domain (Fig. S6a). Protein sequence alignment showed that the three WAK proteins share > 80% amino acid identity with each other (Fig. S6b). At first, we examined whether their transcription levels could be induced by *C. zeina* infection. RT-qPCR results showed that the transcription level of *ZmWAK03* was barely detected before or after pathogen infection; while, the transcription levels of *ZmWAK02* and *ZmWAK04* were strongly induced by *C. zeina* infection at 3, 24 and 48 h after inoculation and that of *ZmWAK02* was much higher than that of *ZmWAK04* after *C. zeina* infection (Fig. 3a). It indicates that the *ZmWAK02* gene might be the best candidate gene in the QTL region.

To determine the function of these three *WAK* genes in the GLS resistance, we cloned genomic DNA fragments of *ZmWAK02* (9.3 kb), *ZmWAK03* (8.1 kb) and *ZmWAK04* (10.1 kb) from SB12, respectively; these fragments contained their genomic DNA sequence including native promoters, gene coding region and native terminator sequences (Figs 3b, S7a,b). The three fragments were then transformed into the maize inbred line KN5585, which is susceptible to GLS. Two independent T_3 families of *ZmWAK02*, *ZmWAK03* and *ZmWAK04* transgenic plants were tested for disease phenotype to natural infection by *C. zeina* in the field at Badong over 2 yr. Only *ZmWAK02* transgenic-positive plants from two independent families showed stronger resistance to GLS than their corresponding nontransgenic siblings (Fig. 3c,d). It indicated that the introgression of the *ZmWAK02* gene can enhance maize resistance against GLS caused by *C. zeina*. No difference on GLS resistance phenotype was observed between transgenic positive plants and their corresponding nontransgenic siblings in *ZmWAK03* and *ZmWAK04* transgenic T_3 families, and all of them showed susceptible phenotype to GLS (Fig. S7c–f). It means that the *ZmWAK03* gene and the *ZmWAK04* gene do not contribute resistance against GLS caused by *C. zeina*.

In order to test whether the three *ZmWAK* genes contribute resistance against *C. zeae-maydis*, the causal agent of GLS in Northern China. We planted these transgenic plants in Gongzhuling at Jilin Province for natural inoculation, where *C. zeae-maydis* is the only agent for GLS. It was observed that *ZmWAK02* transgenic-positive plants but not *ZmWAK03* or *ZmWAK04* transgenic-positive plants showed stronger resistance against GLS than their corresponding nontransgenic siblings (Figs 3e,f, S7g–j). These results indicate that the introgression of the *ZmWAK02* gene but not the *ZmWAK03* gene or the *ZmWAK04* gene increases maize resistance against GLS caused by *C. zeae-maydis*. So, we made a conclusion that *ZmWAK02* confers maize resistance against GLS caused by both of *C. zeina* and *C. zeae-maydis*.

We also generated overexpression constructs (*Ubi::ZmWAK02-GFP*, *Ubi::ZmWAK03-GFP* and *Ubi::ZmWAK04-GFP*) and transformed them into KN5585 (Fig. S8a,b). However, we only got *Ubi::ZmWAK03-GFP*/KN5585 and *Ubi::ZmWAK04-GFP*/KN5585 transgenic plants (Fig. S8c,d). No *Ubi::ZmWAK02-GFP*/KN5585 transgenic plants were gotten, although the company tried the transformation assay twice. It is possible that overexpression of *ZmWAK02* caused strong immunity and killed transgenic-positive calli. Next, two independent T_3 families of *Ubi::ZmWAK03-GFP*/KN5585 and *Ubi::ZmWAK04-GFP*/KN5585 transgenic plants were tested for resistance against natural infection by *C. zeina* in the field at Badong. All transgenic-positive and -negative plants showed susceptible phenotype to GLS and no difference on the GLS resistance was observed between transgenic-positive plants and their corresponding nontransgenic siblings (Fig. S8e–h). It confirmed that the *ZmWAK03* gene and the *ZmWAK04* gene do not contribute GLS resistance.

ZmWAK02 rescues the phenotype of the mutant to resistance against GLS

In order to confirm the resistance function of *ZmWAK02*, we tried to generate mutation of *ZmWAK02* in SB12 background. A total of 5000 SB12 seeds were treated with ^{60}Co-γ ray to generate a mutant library. A total of 1290 M_2 families of SB12 mutants were evaluated for resistance against GLS at Badong, and three different mutant plants (427M, 472M and 1538M) from different M_2 families showed more susceptible phenotype to GLS than SB12 (Fig. 4a). And, their progenies (M_3 family seeds) showed more susceptible phenotype to GLS than the WT plants (SB12) (Fig. 4b). Therefore, 427M, 472M and 1538M were stable mutants showing susceptible phenotype to GLS. Since ^{60}Co-γ ray normally causes large fragment deletion, we tried to examine whether the 12 genes in the QTL region were deleted in the three

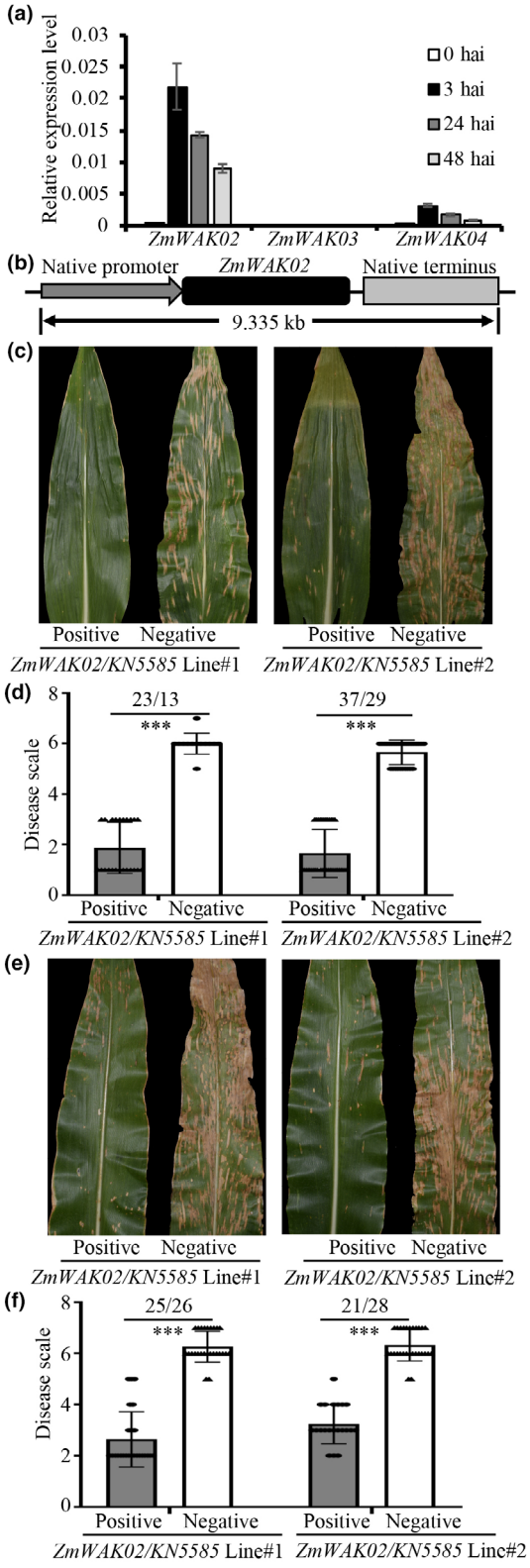


Fig. 3 *ZmWAK02* is responsible for *qRglsSB* resistant against gray leaf spot (GLS). (a) The transcription levels of *ZmWAK02*, *ZmWAK03* and *ZmWAK04* at 0, 3, 24 and 48 h after *C. zeina* inoculation measured by RT-qPCR. (b) The structure of the *ZmWAK02* genomic sequence construct used for generating transgenic maize plants. The construct contains the entire *ZmWAK02* genomic DNA sequence, including its 3 kb promoter region and a 2.772 kb downstream region. (c) The disease phenotypes of two *ZmWAK02*/KN5585 transgenic lines resistant against GLS caused by *C. zeina*. The *ZmWAK02* genomic DNA construct was transferred into susceptible maize inbred line KN5585. Two independent T_3 families were used to test disease phenotype against GLS. 'Positive' means transgenic-positive plants and 'negative' means transgenic-negative plants. (d) The disease scores of two *ZmWAK02*/KN5585 transgenic lines resistant against GLS caused by *C. zeina*. (e) The disease phenotypes of two *ZmWAK02*/KN5585 transgenic lines resistant against GLS caused by *C. zeae-maydis*. The *ZmWAK02* genomic DNA construct was transferred into susceptible maize inbred line KN5585. Two independent T_3 families were used to test disease phenotype against GLS. 'Positive' means transgenic-positive plants and 'negative' means transgenic-negative plants. (f) The disease scores of two *ZmWAK02*/KN5585 transgenic lines resistant against GLS caused by *C. zeae-maydis*. The number of plants for each genotype was presented. The disease score for each genotype was presented as mean ± SD. Statistical analysis was done based on the two-tailed Student's *t*-test. *** means $P < 0.001$.

mutants by PCR amplification. And the results showed that five genes (Gene 1 to Gene 5) were deleted in 427M and 11 genes (Gene 1 to Gene 11) were deleted in 472M and 1538 M (Figs 4c, S9). It indicates that *ZmWAK02* (Gene 1) and *ZmWAK03* (Gene 4) were deleted in 427M, and *ZmWAK02* (Gene 1), *ZmWAK03* (Gene 4) and *ZmWAK04* (Gene 9) were deleted in 472M and 1582M.

In order to evaluate the resistance function of the three *ZmWAK* genes, we tried to test whether they could complement the susceptible phenotype of 472M which does not contain the three *ZmWAK* genes. *ZmWAK02*/KN5585, *ZmWAK03*/KN5585 or *ZmWAK04*/KN5585 transgenic plants were crossed with 472M respectively to generate *ZmWAK02*/472M, *ZmWAK03*/472M and *ZmWAK04*/472M F_1 plants. Since those transgenic plants used for crossing were all heterozygous for their transgenic gene, we got plants with and plants without the transgenic gene in each F_1 family. The three F_1 families were planted for disease evaluation. And the results showed that *ZmWAK02*/472M F_1 plants containing the transgenic *ZmWAK02* gene exhibited stronger resistance phenotype against GLS than those without the *ZmWAK02* gene (Fig. 4d,e). And no difference on GLS phenotype was observed between plants with and without the *ZmWAK03* gene or the *ZmWAK04* gene in *ZmWAK03*/472M F_1 plants or *ZmWAK04*/472M F_1 plants (Fig. S10). It indicates that the introgression of the *ZmWAK02* genomic DNA can significantly enhance the resistance phenotype of 472M against GLS; whereas, the introgression of the *ZmWAK03* or the *ZmWAK04* genomic DNA cannot increase the resistance phenotype of 472M against GLS. Taken together, the results obtained in the transgenic plants and complementation assay demonstrated that the *ZmWAK02* gene is responsible for the resistant effect at *qRglsSB* against GLS.

Evaluation of the effect of the *ZmWAK02* gene on agricultural traits

In order to explore the natural *ZmWAK02* variation in maize accessions, we used a *ZmWAK02*-specific molecular marker (PK537 + PK538) to screen 358 maize inbred lines by PCR

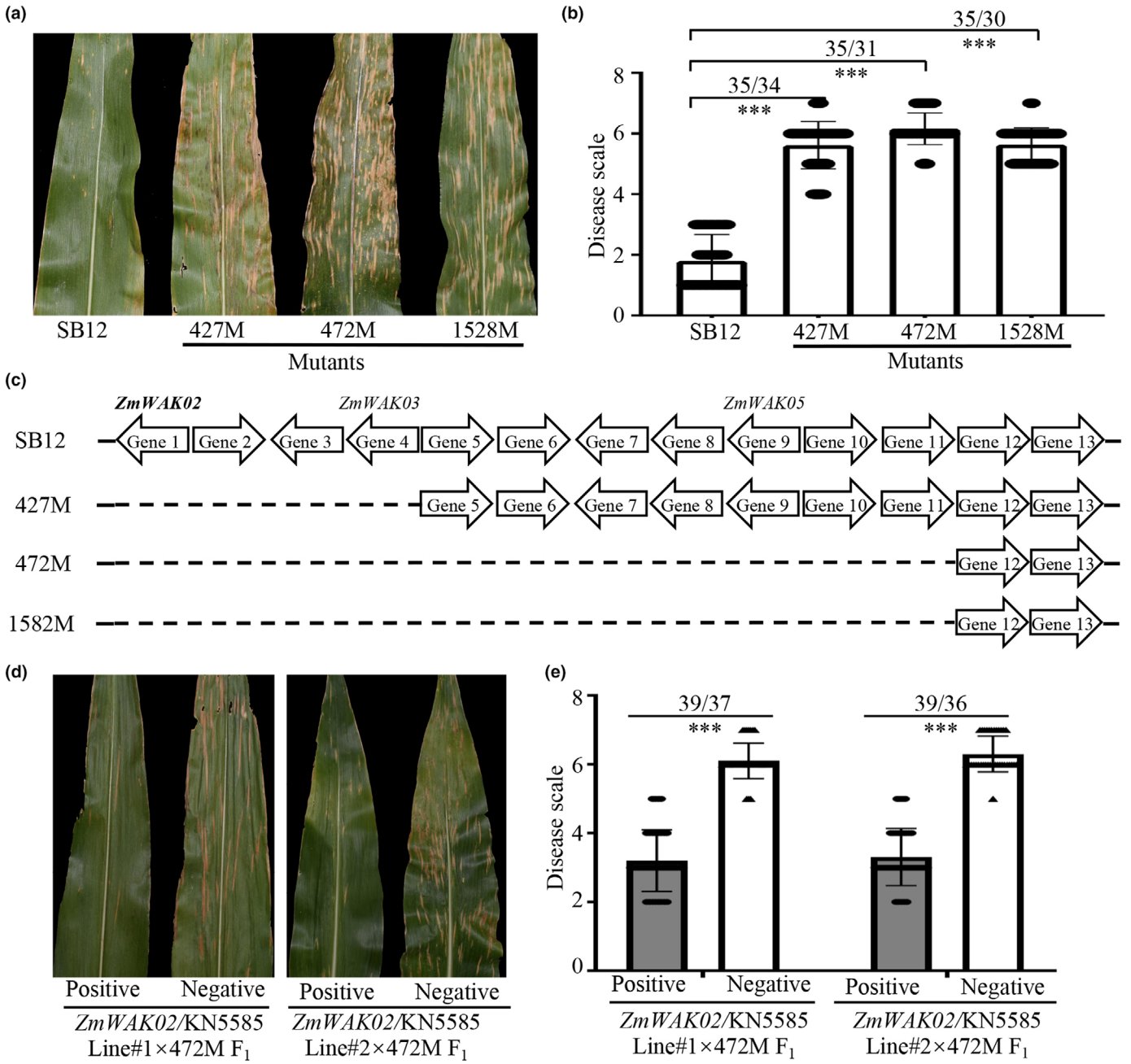


Fig. 4 Validation of *ZmWAK02* function in resistance against (gray leaf spot) GLS. (a) Three mutants (427M, 472M and 1538M) are susceptible to GLS. (b) The disease phenotypes of M_3 populations of 427M, 472M and 1538M. (c) The diagram to show the genotypes of 427M, 472M and 1538M. (d) The introgression of *ZmWAK02* enhances 472M resistance against GLS. Two F_1 populations were generated by crossing two independent *ZmWAK02*/KN5585 lines with SB12 mutant 472M. And they were used to examine whether *ZmWAK02* is co-segregated with the disease resistance against GLS. 'Positive' means plants with *ZmWAK02* gene and 'negative' means plants without *ZmWAK02* gene. (e) The disease phenotypes of *ZmWAK02*/KN5585 line#1×472 F_1 population and *ZmWAK02*/KN5585 line#2×472 F_1 population. The number of plants for each genotype was presented. The disease score for each genotype was presented as mean ± SD. Statistical analysis was done based on the two-tailed Student's *t*-test. *** means $P < 0.001$.

amplification and sequencing. The results showed that only six of them (BY4944, BY4960, CIMBL49, CIMBL86, CIMBL93 and CIMBL364) might contain the *ZmWAK02* gene (Table S2). Also, we sequenced the *ZmWAK02* genomic DNA sequences of the six maize inbred lines and found that they shared > 91% identity with the genomic DNA sequence of $ZmWAK02^{SB12}$, respectively (Fig. S11a). We also predicted their mRNA sequences and their encoding protein sequences based on those of $ZmWAK02^{SB12}$ and found that the predicted $ZmWAK02^{BY4944}$ and $ZmWAK02^{BY4960}$ proteins were only 143 aa and 120 aa, which were shorter than $ZmWAK02^{SB12}$ protein (960 aa; Fig. S11b). It indicates $ZmWAK02^{BY4944}$ and $ZmWAK02^{BY4960}$ genes might encode

truncated proteins, and they do not have the same function as *ZmWAK02*^{SB12}. That might explain why BY4944 and BY4960 were susceptible to GLS (Table S2). Further, we screened 188 teosinte lines and 288 landrace accessions by using the molecular marker (PK537 + PK538). After sequencing the PCR products, we found six teosintes (Table S3) and nine landrace accessions (Table S4) might contain the *ZmWAK02* gene (Fig. S12). Furthermore, we examined 80 commercial maize hybrid lines by using the molecular marker (PK537 + PK538) and found that only one of them might contain the *ZmWAK02* gene (Table S5; Fig. S12). The low frequency of the *ZmWAK02* gene in the collected maize hybrids indicates that the *ZmWAK02* gene has not been broadly used in commercial hybrids and the *ZmWAK02* gene is a rare allele, which highlight its potential value for maize breeding.

Next, we examined whether the introgression of the *ZmWAK02* gene affects five agricultural traits (plant height, days to tassel, days to silking, days to anthesis and ear height) by using *ZmWAK02* transgenic plants and no difference on those agricultural traits was observed between transgenic-positive plants and their nontransgenic sibling plants (Fig. S13). Also, we tested the phenotypes of the five agricultural traits in two SB12×SA101 NIL lines, and no significant difference on them was observed (Fig. S14). Those indicate that the introgression of the *ZmWAK02* gene does not affect the five agricultural traits.

Furthermore, we introduced the *ZmWAK02* gene into the two parental lines of commercial maize hybrid Xianyu335 (PH6WC×PH4CV) by repeated backcrossing and molecular assisted selection. The PH4CV^{ZmWAK02} and PH6WC^{ZmWAK02} lines identified from the BC_5F_3 progenies were crossed to generate the improved hybrid XianYu335 ^{ZmWAK02} (PH4CV^{ZmWAK02}× PH6WC^{ZmWAK02}). In the field, the improved hybrid Xianyu335^{ZmWAK02} (PH6WC^{ZmWAK02}×PH4CV^{ZmWAK02}) plants were more resistant to GLS than the original Xianyu335 hybrid plants (Fig. 5a,b). As a result, the yield of Xianyu335^{ZmWAK02} was 3.9% higher than that of the original Xianyu335 hybrid in the presence of GLS, and no significant difference on the five agronomic traits were observed (Figs 5c,d, S15). It indicates that the introgression of the *ZmWAK02* gene can reduced GLS-caused yield loss and does not affect five major agricultural traits under the pressure of GLS. In the absence of GLS, no significant changes were observed on grain yield and other five agronomic traits between Xianyu335^{ZmWAK02} and Xianyu335 (Figs 5c,d, S15). It means the introgression of the *ZmWAK02* gene does not affect grain yield and other five major agricultural traits in the absence of GLS. We also improved the father parent line (Zheng58) of the commercial hybrid ZhengDan958 (Zheng58×Chang7-2) by using the similar strategy. And we got similar results as Xianyu335^{ZmWAK02}. In the presence of GLS, the improved hybrid ZhengDan958^{ZmWAK02} (Zheng58^{ZmWAK02}× Chang7-2) was more resistant against GLS than the original hybrid ZhengDan958; the grain yield of ZhengDan958^{ZmWAK02} was 8% more than that of the original hybrid ZhengDan958; and, no significant difference on yield and other five major agronomic traits was observed between ZhengDan958^{ZmWAK02} and ZhengDan958 (Figs 5e–h, S16). In the absence of GLS, no significant changes were observed on grain yield and other agronomic traits between ZhengDan958^{ZmWAK02} and ZhengDan958 (Figs 5g,h, S16). These results confirmed the breeding value of the *ZmWAK02* gene in maize.

Discussion

In this study, we identified a major QTL, *qRglsSB*, conferring maize resistance against GLS. By combining map-based cloning strategy and transgenic functional complementation, we confirmed that *ZmWAK02* gene encoding an RD WAK is responsible for the resistance of *qRglsSB*. The introgression of the *ZmWAK02* gene can significantly enhance GLS resistance and increase grain yield in the presence of GLS; and it does not cause yield loss in the absence of GLS.

ZmWAK02 has a large effect on GLS resistance

Previously, only two genes resistant to GLS have been cloned: *ZmCCoAOMT2* from $qMdr_{9.02}$ and *ZmMM1* from *qLM7* and the introgression of $qMdr_{9.02}$ or *qLM7* slightly enhanced GLS resistance (Yang *et al.*, 2017; Wang *et al.*, 2021). While, *qRglsSB* explained 58.42% phenotypic variation of the resistance against GLS in SB12×SA101 population, which indicates that *qRglsSB* might confer qualitative rather than quantitative resistance against GLS. The additive effects of *qRglsSB* in SB12×SA101 population at different repeats were between 3.1914 and 4.087 (Fig. S3c). The introgression of the *ZmWAK02* genomic DNA into susceptible maize inbred line KN5585 or susceptible large-fragment deletion mutants significantly enhances their GLS resistance. And the difference of GLS disease scale between those plants carrying *ZmWAK02* and plants without *ZmWAK02* were between 3 and 4.3 (Figs 3b–f, 4a,b). Similar results were observed between Xianyu335^{ZmWAK02} and Xianyu335 and between ZhengDan958^{ZmWAK02} and ZhengDan958 (Fig. 5a,b,e,f). All of these results indicate the *ZmWAK02* gene has a large effect on the GLS resistance.

ZmWAK02 presents great value for maize breeding

One of the major goals for resistance study is to isolate resistance genes that can be used for crop resistance breeding. However, that goal has been ignored for a while. Most of work on resistance genes has been focused on exploring molecular mechanisms of them, and only a few resistance genes were evaluated for their value on breeding (Zhang *et al.*, 2018). Here, we proved that the *ZmWAK02* gene has great value for maize breeding. First, *ZmWAK02* confers large effect on GLS resistance in different backgrounds (SA101, KN5585, Xuanyu335 and ZhengDan958; Figs 3–5). Second, *ZmWAK02* contributes strong resistance against both of *C. zeae-maydis* and *C. zeina*, the two causal agents of GLS (Fig. 3c–f). Third, the introgression of the *ZmWAK02* gene increased yield in the presence of GLS pressure and did not cause yield loss in the absence of GLS (Fig. 5). Fourth, the introgression of the *ZmWAK02* gene did not affect five major agricultural traits (plant height, days to tassel, days to silking, days to anthesis, and ear

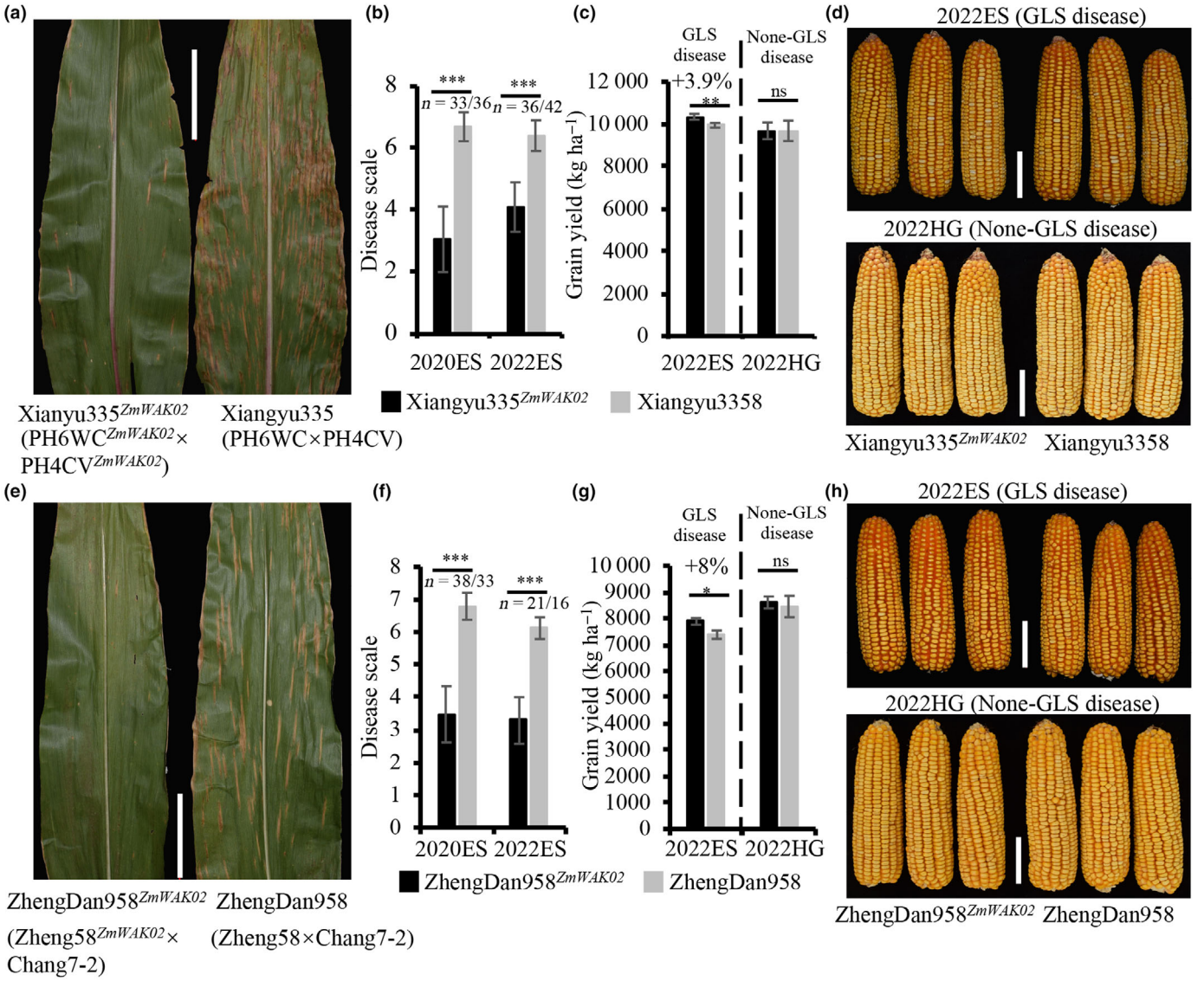


Fig. 5 Introgression of *ZmWAK02* into maize hybrids significantly enhances their resistance against GLS without fitness cost. (a) The GLS disease phenotypes of Xianyu335^{ZmWAK02} and Xianyu335. Bar: 10 cm. (b) The GLS disease phenotypes of Xianyu335^{ZmWAK02} and Xianyu335 at Enshi (ES) in 2020 and 2022. (c) The grain yield phenotypes of Xianyu335^{ZmWAK02} and Xianyu335 at Enshi (ES) in the presence of GLS disease) and Huanggang (HG) in the absence of GLS disease in 2022. (d) Ear phenotypes of Xianyu335^{ZmWAK02} and Xianyu335 at Enshi (ES) in the presence of GLS disease and at Huanggang (HG) in the absence of GLS disease in 2022. Bar: 5 cm. (e) The GLS disease phenotypes of ZhengDan958^{ZmWAK02} and ZhengDan958. Bar: 10 cm. (f) The GLS disease phenotypes of ZhengDan958^{ZmWAK02} and ZhengDan958 at Enshi (ES) in 2020 and 2022. (g) The grain yield phenotypes of ZhengDan958^{ZmWAK02} and ZhengDan958 at Enshi (ES) in the presence of GLS disease) and Huanggang (HG) in the absence of GLS disease in 2022. (h) Ear phenotypes of ZhengDan958^{ZmWAK02} and ZhengDan958 at Enshi (ES) in the presence of GLS disease and at Huanggang (HG) in the absence of GLS disease in 2022. Bar: 5 cm. The number of plants for each genotype was presented. The disease score for each genotype was presented as mean ± SD. Statistical analysis was done based on the two-tailed Student's *t*-test. * means $P < 0.05$; ** means $P < 0.01$; *** means $P < 0.001$; ns means no significant difference.

height; Figs S15, S16). Fifth, *ZmWAK02* has not been wildly used in maize breeding. Only one of 81 tested commercially cultured hybrids carries *ZmWAK02* gene (Table S5).

ZmWAK02 encodes an RD wall-associated kinase

Wall-associated kinase proteins are a group of receptor-like kinases (RLKs), playing important roles in plant development and stress resistance (Verica & He, 2002). They are classified into two groups: RD WAKs and non-RD WAKs (de Oliveira *et al.*, 2014). In crops, non-RD WAK proteins play critical roles in disease resistance. *ZmWAK/qHSR1* contributes resistance against head smut (Zuo *et al.*, 2015); *ZmWAK-RLK1/HtN1* and its alleles (*Ht2* and *Ht3*) are responsible for resistance against northern corn leaf blight (Hurni *et al.*, 2015; Yang *et al.*, 2021); rice *Xa4*, encoding a cell WAK, confers race-specific resistance against rice bacterial blight at all stages (Hu *et al.*, 2017); cotton *GhWAK7A* contributes resistance against *Verticillium* and *Fusarium* wilts (Wang *et al.*, 2020). However, *ZmWAK02* gene conferring GLS resistance (Fig. 3b–f) encodes an RD WAK protein but

not a non-RD WAK protein (Fig. S6b). Recently, another RD WAK protein, TaStb6, was identified to contribute resistance against *Septoria tritici* blotch on wheat (Saintenac *et al.*, 2018). It indicates that RD WAK proteins might also be a major group in disease resistance. Compared with non-RD WAKs, the molecular mechanisms of RD WAKs in disease resistance are largely unknown. In the future, more work should be done on RD WAKs to explore their molecular mechanisms and identify the difference between RD WAKs and non-RD WAKs.

Acknowledgments

We thank Dr Jianwei Zhang at Huazhong Agricultural University for critical editing of the manuscript. This work was supported by grants from the National Natural Science Foundation of China (32072007 and 31761143008), HZAU-AGIS Cooperation Fund (SZYJY2023017), the fellowship of China Postdoctoral Science Foundation (2022M721272) and Postdoctoral Creative Research Positions of Hubei Province of China (2022).

Competing interests

Patents with application no. ZL 2022 10642762.6 (*ZmWAK02* related to the GLS resistance and application; Inventors: Zhibing Lai, Zhikang Dai, Long Hu, Bingchen Li) is pending. Other authors claim no competing interests.

Author contributions

ZD performed QTL analysis, fine-mapping, and disease evaluation. LH and BL screened recombinant plants. QP, WL and BZ did pollination and crossing. YL did resequencing of *ZmWAK02* gene in different maize accessions. VL and KF did genome sequencing and assembling. ST did gene annotation within the QTL interval. SG did *ZmWAK02* variation analysis in maize accessions. YW, MJ, XQ, ZL, XL and XF did field management. ZL initiated the project, performed the experiment design and manuscript writing and editing.

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Data availability

The data that support the findings of this study are available in the Supporting Information. The genomic DNA sequence, CDS and protein sequence are available at GenBank under accessions OR474082. The whole-genome sequence data of SB12 have been deposited in the Genome Warehouse in National Genomics Data Center, Beijing Institute of Genomics, Chinese Academy of Sciences/China National Center for Bioinformation, under accession no. GWHEQHS00000000.

References

Amsbury S. 2020. Sensing attack: the role of wall-associated kinases in plant pathogen responses. *Plant Physiology* **183**: 1420–1421.

Bacete L, Melida H, Miedes E, Molina A. 2018. Plant cell wall-mediated immunity: cell wall changes trigger disease resistance responses. *The Plant Journal* **93**: 614–636.

Balestre M, Von Pinho RG, Brito AH. 2012. Bayesian inference to study genetic control of resistance to gray leaf spot in maize. *Genetics and Molecular Research* **11**: 17–29.

Balint-Kurti PJ, Johal GS. 2009. Maize disease resistance. In: Bennetzen JL, Hake SC, eds. *Handbook of maize: its biology*. New York, NY, USA: Springer New York, 229–250.

Balint-Kurti PJ, Wisser R, Zwonitzer JC. 2008. Use of an advanced intercross line population for precise mapping of quantitative trait loci for gray leaf spot resistance in maize. *Crop Science* **48**: 1696–1704.

Benson JM, Poland JA, Benson BM, Stromberg EL, Nelson RJ. 2015. Resistance to gray leaf spot of maize: genetic architecture and mechanisms elucidated through nested association mapping and near-isogenic line analysis. *PLoS Genetics* **11**: e1005045.

Carson ML, Goodman MM, Williamson SM. 2002. Variation in aggressiveness among isolates of *Cercospora* from maize as a potential cause of genotype–environment interaction in gray leaf spot trials. *Plant Disease* **86**: 1089–1093.

Chen L, Liu L, Li Z, Zhang Y, Kang MS, Wang Y, Fan X. 2021. High-density mapping for gray leaf spot resistance using two related tropical maize recombinant inbred line populations. *Molecular Biology Reports* **48**: 3379–3392.

Clements MJ, Dudley JW, White DG. 2000. Quantitative trait loci associated with resistance to gray leaf spot of corn. *Phytopathology* **90**: 1018–1025.

Coates ST, White DG. 1998. Inheritance of resistance to gray leaf spot in crosses involving selected resistant inbred lines of corn. *Phytopathology* **88**: 972–982.

Crous PW, Groenewald JZ, Groenewald M, Caldwell P, Braun U, Harrington TC. 2006. Species of *Cercospora* associated with grey leaf spot of maize. *Studies in Mycology* **55**: 189–197.

Dardick C, Schwessinger B, Ronald P. 2012. Non-arginine-aspartate (non-RD) kinases are associated with innate immune receptors that recognize conserved microbial signatures. *Current Opinion in Plant Biology* **15**: 358–366.

Dodds PN, Rathjen JP. 2010. Plant immunity: towards an integrated view of plant-pathogen interactions. *Nature Reviews. Genetics* **11**: 539–548.

Donahue PJ, Stromberg EL, Myers SL. 1991. Inheritance of reaction to gray leaf spot in a diallel cross of 14 maize inbreds. *Crop Science* **31**: 926–931.

Du L, Yu F, Zhang H, Wang B, Ma K, Yu C, Xin W, Huang X, Liu Y, Liu K. 2020. Genetic mapping of quantitative trait loci and a major locus for resistance to grey leaf spot in maize. *Theoretical and Applied Genetics* **133**: 2521–2533.

Gordon SG, Bartsch M, Matthies I, Gevers HO, Lipps PE, Pratt RC. 2004. Linkage of molecular markers to *Cercospora zeae-maydis* resistance in maize. *Crop Science* **44**: 628–636.

Hu K, Cao J, Zhang J, Xia F, Ke Y, Zhang H, Xie W, Liu H, Cui Y, Cao Y *et al.* 2017. Improvement of multiple agronomic traits by a disease resistance gene via cell wall reinforcement. *Nature Plants* **3**: 17009.

Hufford MB, Seetharam AS, Woodhouse MR, Chougule KM, Ou S, Liu J, Ricci WA, Guo T, Olson A, Qiu Y *et al.* 2021. *De novo* assembly, annotation, and comparative analysis of 26 diverse maize genomes. *Science* **373**: 655–662.

Hurni S, Scheuermann D, Krattinger SG, Kessel B, Wicker T, Herren G, Fitze MN, Breen J, Presterl T, Ouzunova M *et al.* 2015. The maize disease resistance gene *Htn1* against northern corn leaf blight encodes a wall-associated receptor-like kinase. *Proceedings of the National Academy of Sciences, USA* **112**: 8780–8785.

Jones JD, Dangl JL. 2006. The plant immune system. *Nature* **444**: 323–329.

Kohorn BD, Kohorn SL, Saba NJ, Martinez VM. 2014. Requirement for pectin methyl esterase and preference for fragmented over native pectins for wall-associated kinase-activated, EDS1/PAD4-dependent stress response in Arabidopsis. *Journal of Biological Chemistry* **289**: 18978–18986.

Koren S, Walenz BP, Berlin K, Miller JR, Bergman NH, Phillippy AM. 2017. Canu: scalable and accurate long-read assembly via adaptive k-mer weighting and repeat separation. *Genome Research* **27**: 722–736.

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newberg LA. 1987. MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. *Genomics* **1**: 174–181.

Lehmensiek A, Esterhuizen AM, van Staden D, Nelson SW, Retief AE. 2001. Genetic mapping of gray leaf spot (GLS) resistance genes in maize. *Theoretical and Applied Genetics* **103**: 797–803.

Lennon JR, Krakowsky M, Goodman M, Flint-Garcia S, Balint-Kurti PJ. 2016. Identification of alleles conferring resistance to gray leaf spot in maize derived from its wild progenitor species teosinte. *Crop Science* **56**: 209–218.

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, Subgroup GPDP. 2009. The sequence alignment/map format and SAMTOOLS. *Bioinformatics* **25**: 2078–2079.

Li J, Singh U, Bhandary P, Campbell J, Arendsee Z, Seetharam Arun S, Wurtele ES. 2021. Foster thy young: enhanced prediction of orphan genes in assembled genomes. *Nucleic Acids Research* **50**: e37.

Liu Q-K, Qin Z-H, Zhang X-L, Jiang K, Chen M-G, Wu X-F, He Y-Q, Wang G-Q, Jin Q-M, Wang X-M. 2013. Identification of *Cercospora* species associated with maize gray leaf spot in China. *Scientia Agricultura Sinica* **46**: 4044–4057.

Liu T, Liu Z, Song C, Hu Y, Han Z, She J, Fan F, Wang J, Jin C, Chang J *et al.* 2012. Chitin-induced dimerization activates a plant immune receptor. *Science* **336**: 1160–1164.

Liu Z, Wu Y, Yang F, Zhang Y, Chen S, Xie Q, Tian X, Zhou JM. 2013. BIK1 interacts with PEPRs to mediate ethylene-induced immunity. *Proceedings of the National Academy of Sciences, USA* **110**: 6205–6210.

Mammadov J, Sun X, Gao Y, Ochsenfeld C, Bakker E, Ren R, Flora J, Wang X, Kumpatla S, Meyer D *et al.* 2015. Combining powers of linkage and association mapping for precise dissection of QTL controlling resistance to gray leaf spot disease in maize (*Zea mays* L.). *BMC Genomics* **16**: 916.

Mueller DS, Wise KA, Sisson AJ, Allen TW, Bergstrom GC, Bissonnette KM, Bradley CA, Byamukama E, Chilvers MI, Collins AA *et al.* 2020. Corn yield loss estimates due to diseases in the United States and Ontario, Canada, from 2016 to 2019. *Plant Health Progress* **21**: 238–247.

Mueller DS, Wise KA, Sisson AJ, Allen TW, Bergstrom GC, Bosley DB, Bradley CA, Broders KD, Byamukama E, Chilvers MI *et al.* 2016. Corn yield loss estimates due to diseases in the United States and Ontario, Canada from 2012 to 2015. *Plant Health Progress* **17**: 211–222.

Ngou BPM, Jones JDG, Ding P. 2022. Plant immune networks. *Trends in Plant Science* **27**: 255–273.

Nsibo DL, Barnes I, Omondi DO, Dida MM, Berger DK. 2021. Population genetic structure and migration patterns of the maize pathogenic fungus, *Cercospora zeina* in East and Southern Africa. *Fungal Genetics and Biology* **149**: 103527.

de Oliveira LFV, Christoff AP, de Lima JC, de Ross BCF, Sachetto-Martins G, Margis-Pinheiro M, Margis R. 2014. The wall-associated kinase gene family in rice genomes. *Plant Science* **229**: 181–192.

Pozar G, Butruille D, Silva HD, McCuddin ZP, Penna JC. 2009. Mapping and validation of quantitative trait loci for resistance to *Cercospora zeae-maydis* infection in tropical maize (*Zea mays* L.). *Theoretical and Applied Genetics* **118**: 553–564.

Saintenac C, Lee WS, Cambon F, Rudd JJ, King RC, Marande W, Powers SJ, Berges H, Phillips AL, Uauy C *et al.* 2018. Wheat receptor-kinase-like protein Stb6 controls gene-for-gene resistance to fungal pathogen *Zymoseptoria tritici*. *Nature Genetics* **50**: 368–374.

Silva LDCE, Wang S, Zeng Z-B. 2012. Composite interval mapping and multiple interval mapping: procedures and guidelines for using Windows QTL CARTOGRAPHER. In: Rifkin SA, ed. *Quantitative trait loci (QTL): methods and protocols*. Totowa, NJ, USA: Humana Press, 75–119.

Stephens C, Hammond-Kosack KE, Kanyuka K. 2022. WAKsing plant immunity, waning diseases. *Journal of Experimental Botany* **73**: 22–37.

Sun H, Zhai L, Teng F, Li Z, Zhang Z. 2021. *qRgls1.06*, a major QTL conferring resistance to gray leaf spot disease in maize. *The Crop Journal* **9**: 342–350.

Verica JA, He ZH. 2002. The cell wall-associated kinase (WAK) and WAK-like kinase gene family. *Plant Physiology* **129**: 455–459.

Wang H, Hou J, Ye P, Hu L, Huang J, Dai Z, Zhang B, Dai S, Que J, Min H *et al.* 2021. A teosinte-derived allele of a MYB transcription repressor confers multiple disease resistance in maize. *Molecular Plant* **14**: 1846–1863.

Wang J, Levy M, Dunkle LD. 1998. Sibling species of cercospora associated with gray leaf spot of maize. *Phytopathology* **88**: 1269–1275.

Wang P, Zhou L, Jamieson P, Zhang L, Zhao Z, Babilonia K, Shao W, Wu L, Mustafa R, Amin I *et al.* 2020. The cotton wall-associated kinase GhWAK7A mediates responses to fungal wilt pathogens by complexing with the chitin sensory receptors. *Plant Cell* **32**: 3978–4001.

Ward JMJ, Stromberg EL, Nowell DC, Nutter FW. 1999. Gray leaf spot: a disease of global importance in maize production. *Plant Disease* **83**: 884–895.

Xu L, Zhang Y, Shao S, Chen W, Tan J, Zhu M, Zhong T, Fan X, Xu M. 2014. High-resolution mapping and characterization of *qRgls2*, a major quantitative trait locus involved in maize resistance to gray leaf spot. *BMC Plant Biology* **14**: 230.

Yang P, Scheuermann D, Kessel B, Koller T, Greenwood JR, Hurni S, Herren G, Zhou S, Marande W, Wicker T *et al.* 2021. Alleles of a wall-associated kinase gene account for three of the major northern corn leaf blight resistance loci in maize. *The Plant Journal* **106**: 526–535.

Yang Q, He Y, Kabahuma M, Chaya T, Kelly A, Borrego E, Bian Y, El Kasmi F, Yang L, Teixeira P *et al.* 2017. A gene encoding maize caffeoyl-CoA O-methyltransferase confers quantitative resistance to multiple pathogens. *Nature Genetics* **49**: 1364–1372.

Zhang H, Li Y, Zhu JK. 2018. Developing naturally stress-resistant crops for a sustainable agriculture. *Nature Plants* **4**: 989–996.

Zhang Y, Xu L, Fan X, Tan J, Chen W, Xu M. 2012. QTL mapping of resistance to gray leaf spot in maize. *Theoretical and Applied Genetics* **125**: 1797–1808.

Zhu M, Tong L, Xu M, Zhong T. 2021. Genetic dissection of maize disease resistance and its applications in molecular breeding. *Molecular Breeding* **41**: 32.

Zuo W, Chao Q, Zhang N, Ye J, Tan G, Li B, Xing Y, Zhang B, Liu H, Fengler KA *et al.* 2015. A maize wall-associated kinase confers quantitative resistance to head smut. *Nature Genetics* **47**: 151–157.

Zwonitzer JC, Coles ND, Krakowsky MD, Arellano C, Holland JB, McMullen MD, Pratt RC, Balint-Kurti PJ. 2010. Mapping resistance quantitative trait loci for three foliar diseases in a maize recombinant inbred line population-evidence for multiple disease resistance? *Phytopathology* **100**: 72–79.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Disease scale of gray leaf spot.

Fig. S2 Genetic linkage map from SB12×SA101 BC_1F_1 population.

Fig. S3 Phenotypic distribution and correlation of five repeats from QTL detection.

Fig. S4 Comparison of genome sequences of *qRglsSB* regions between SB12 and B73.

Fig. S5 Information of 12 genes in the 928 kb *qRglsSb* region.

Fig. S6 Alignment analysis of ZmWAK02, ZmWAK03 and ZmWAK04 proteins.

Fig. S7 *ZmWAK03* and *ZmWAK04* do not contribute maize resistance against gray leaf spot.

Fig. S8 Overexpression of *ZmWAK03* or *ZmWAK04* does not enhance maize resistance against GLS.

Fig. S9 Genotyping three SB12 mutants (427M, 472M and 1528M) by PCR amplification.

Fig. S10 *ZmWAK03* and *ZmWAK04* did not recue the susceptible phenotype of 472M to gray leaf spot.

Fig. S11 Sequence alignment among ZmWAK02 genomic DNA sequences and ZmWAK02 amino acid sequences.

Fig. S12 DNA sequence alignment among PCR products (PK537 + PK538).

Fig. S13 No significant difference on major agronomic traits between *ZmWAK02*/KN5585 transgenic-positive lines and transgenic-negative lines.

Fig. S14 No significant difference on major agronomic traits in SB12×SA101 NIL lines.

Fig. S15 Introgression of *ZmWAK02* into hybrid line Xianyu335 does not affect its major agronomic traits.

Fig. S16 Introgression of *ZmWAK02* into hybrid line Zheng-Dan958 does not affect its major agronomic traits.

Table S1 Primers used in this study.

Table S2 Genotype of *ZmWAK02* locus in 358 inbred lines of the association mapping panel.

Table S3 Genotype of *ZmWAK02* locus in 188 teosinte lines.

Table S4 Information of 288 landrace accessions.

Table S5 Genotype of *ZmWAK02* in 81 commercially cultured hybrids in China.

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