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Alleles of a wall-associated kinase gene account for three of the major northern corn leaf blight resistance loci in maize

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Abstract: Northern corn leaf blight, caused by the fungal pathogen *Setosphaeria turcica* (anamorph *Exserohilum turcicum*), is one of the most devastating foliar diseases of maize (*Zea mays*). Four genes Ht1, Ht2, Ht3 and Htn1 represent the major sources of genetic resistance against the hemibiotrophic fungus *S. turcica*. Differential maize lines containing these genes also form the basis to classify *S. turcica* races. Here, we show that Ht2 and Ht3 are identical and allelic to the previously cloned Htn1 gene. Using a map-based cloning approach and Targeting Induced Local Lesions in Genomes (TILLING), we demonstrate that Ht2/Ht3 is an allele of the wall-associated receptor-like kinase gene ZmWAK-RLK1. The ZmWAK-RLK1 variants encoded by Htn1 and Ht2/Ht3 differ by multiple amino acid polymorphisms that particularly affect the putative extracellular domain. A diversity analysis in maize revealed the presence of dozens of ZmWAK-RLK1 alleles. Ht2, Ht3 and Htn1 have been described over decades as independent resistance loci with different race spectra and resistance responses. Our work demonstrates that these three genes are allelic, which has major implications for northern corn leaf blight resistance breeding and nomenclature of *S. turcica* pathotypes. We hypothesize that genetic background effects have confounded the classical description of these disease resistance genes in the past.

DOI: <https://doi.org/10.1111/tpj.15183>

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ZORA URL: <https://doi.org/10.5167/uzh-203329>

Journal Article

Accepted Version

Originally published at:

Yang, Ping; Scheuermann, Daniela; Kessel, Bettina; Koller, Teresa; Greenwood, Julian R; Hurni, Severine; Herren, Gerhard; Zhou, Shenghui; Marande, William; Wicker, Thomas; Krattinger, Simon G; Ouzunova, Milena; Keller, Beat (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(2):526-535.

DOI: <https://doi.org/10.1111/tpj.15183>

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Article type : Original Article

Alleles of a wall-associated kinase gene account for three of the major northern corn leaf blight resistance loci in maize

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Running title: ZmWAK-RLK1 variants encode for three NCLB genes

Keywords: WAKs, ZmWAK-RLK1, *Ht2*, *Ht3*, *Htn1*, NCLB, resistance

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/TPJ.15183](https://doi.org/10.1111/TPJ.15183)

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SUMMARY

Northern corn leaf blight (NCLB), caused by the fungal pathogen *Setosphaeria turcica* (anamorph *Exserohilum turcicum*), is one of the most devastating foliar diseases of maize (*Zea mays*). Four genes *Ht1*, *Ht2*, *Ht3*, and *Htn1* represent the major sources of genetic resistance against the hemibiotrophic fungus *S. turcica*. Differential maize lines containing these genes also form the basis to classify *S. turcica* races. Here, we show that *Ht2* and *Ht3* are identical and allelic to the previously cloned *Htn1* gene. Using a map-based cloning approach and Targeting Induced Local Lesions in Genomes (TILLING), we demonstrate that *Ht2/Ht3* is an allele of the wall-associated receptor-like kinase gene *ZmWAK-RLK1*. The *ZmWAK-RLK1* variants encoded by *Htn1* and *Ht2/Ht3* differ by multiple amino acid polymorphisms that particularly affect the putative extracellular domain. A diversity analysis in maize revealed the presence of dozens of *ZmWAK-RLK1* alleles. *Ht2*, *Ht3*, and *Htn1* have been described over decades as independent resistance loci with different race-spectra and resistance responses. Our work demonstrates that these three genes are allelic, which has major implications for NCLB resistance breeding and nomenclature of *S. turcica* pathotypes. We hypothesize that genetic background effects have confounded the classical description of these disease resistance genes in the past.

Introduction

Cell surface immune receptors represent the first line of defense against invading pathogens in plants (Jones and Dangl, 2006, Macho and Zipfel, 2014). Perception of extracellular pathogen-derived molecules or damage-associated molecular patterns by receptor-like kinases (RLKs) and receptor-like proteins (RLPs) results in the initiation of an immune response (Macho and Zipfel, 2014). RLKs have different types of extracellular domains (Dardick *et al.*, 2012; Gomez-Gomez and Boller, 2000; Kanyuka and Rudd, 2019; Macho and Zipfel, 2014), including leucine-rich repeat (LRR), lysine motifs, lectin motifs, epidermal growth factor-like domains, and wall-associated galacturonan-binding (GUB_WAK) domains. In cereals, wall-associated kinases (WAKs) have recently emerged as important players in disease resistance. The WAK genes *ZmWAK/qHSR1* (Zuo *et al.*, 2015), *ZmWAK-RLK1/Htn1* (Hurni *et al.*, 2015), *OsWAK/Xa4* (Hu *et al.*, 2017), *TaWAKL4/Stb6* (Saintenac *et al.*, 2018) and *TaWAK6* (Dmochowska-Boguta *et al.*, 2020) confer

partial or complete resistance against various fungal and bacterial pathogens in maize, rice, and wheat. For example, the wheat TaWAKL4/Stb6 perceives the fungal effector AvrStb6 from *Zymoseptoria tritici*, which activates a race-specific defense response (Kema *et al.*, 2018). Wheat TaWAK/Snn1 is a susceptibility factor that initiates a hypersensitive response (HR) after perceiving the fungal effector SnTox1 from the necrotrophic pathogen *Parastagonospora nodorum*, which supports fungal growth. WAK-mediated resistance is associated with the biosynthesis of phytoalexin (Hu *et al.*, 2017), oxidative bursts (Brutus *et al.*, 2010; Delteil *et al.*, 2016), the expression of defense genes (Zuo *et al.*, 2015), the reduction of benzoxazinoid content (Yang *et al.*, 2019), or callose deposition (Zhang *et al.*, 2020). The cotton GhWAK7A, which itself does not perceive ligands, directly interacts with lysin-motif-containing RLKs GhLYK5/GhCERK1 and promotes chitin-induced GhLYK5-GhCERK1 dimerization to ultimately activate plant defense against pathogenic invasion (Wang *et al.*, 2020). Maize (*Zea mays*) is a globally cultivated crop and ranks first in global grain production (FAO dataset, 2020). Northern corn leaf blight, caused by the hemibiotrophic fungus *Setosphaeria turcica* (anamorph *Exserohilum turcicum*), is one of the most devastating foliar diseases of maize. For example, estimated yield losses in North America between 2010 and 2015 were 27.9 million tons, translating to over five billion US dollars in lost revenue (Mueller *et al.*, 2016). Deployment of genetic resistance has been accepted as the most sustainable strategy to prevent yield loss (Lipps *et al.*, 1997). To date, nine major resistance genes and several quantitative loci have been genetically identified (Galiano-Carneiro and Miedaner, 2017). *Ht1*, *Ht2*, *Ht3* and *Htn1* represent the four major and most widely used resistance genes against NCLB. Differential maize lines containing these genes have served as the basis to classify *S. turcica* races (Dong *et al.*, 2008; Fallah Moghadam and Pataky, 1994; Ferguson and Carson, 2004; Ferguson and Carson, 2007; Leonard *et al.*, 1989; Weems and Bradley, 2018). *Htn1* is the only NCLB resistance gene cloned so far and encodes the wall-associated receptor-like kinase *ZmWAK-RLK1* (Hurni *et al.*, 2015). This gene was detected with a delayed formation of NCLB lesions, which correlates with reduced benzoxazinoid content (Yang *et al.*, 2019). *Ht1*, *Ht2* and *Ht3* are qualitative resistance genes and have been associated with the formation chlorotic lesions following infection (Welz and Geiger, 2000). *Ht1* has been mapped to the long arm of chromosome 2, while *Ht2* is found on the long arm of chromosome 8 (Simcox and Bennetzen, 1993; Yin *et al.*, 2003). *Ht3* was introgressed from the wild maize relative *Tripsacum floridanum* (Hooker, 1981) and its genetic position remained elusive.

Results and Discussions

Ht2* and *Ht3* were mapped to the same interval as *Htn1

Ht2 was first described in the Australian maize landrace NN14 (Hooker, 1977) and was mapped to genetic bins 8.05/8.06 on chromosome 8 (Simcox and Bennetzen, 1993; Yin *et al.*, 2003). The introgression inbred line A619Ht2 (USDA-ARS genebank: Ames25220) served as the donor to generate the near-isogenic line (NIL) RP5Ht2, by crossing and backcrossing with the susceptible inbred line RP5 (Figure 1a). Using a maize 12K SNP array the polymorphisms between both parents were detected with 2, 3, 28 and 5 markers on chromosomes 1, 3, 8 and 9, respectively. By deployment of quantitative trait locus (QTL) mapping using 38 polymorphic markers in a BC₂F₂ population consisting of 720 single plants, a single association peak was detected with a LOD value of 44.02, close to the physical position 158.17 Mb of the B73 reference genome (AGPv04), flanked by the genetic markers ZM012083-0873 and PZE-108100090 (Table S1). By fine mapping with the BC₂F₈ recombinants derived from the same population, the *Ht2* gene was mapped to a 0.3 cM genetic interval with the markers PZE-108096469 and PZE-108092843 (Figure 1a), which flank a 2.81 Mb region on chromosome 8 of the B73 reference genome (AGPv04).

The same strategy was applied for genetic mapping of the resistance gene *Ht3*, which was reported to be crossed into cultivated maize from Florida gamagrass (*T. floridanum*) (Hooker, 1981). The donor line A619Ht3 (USDA-ARS genebank: Ames25221) was crossed with the susceptible parental line RP6 to produce the resistance parent RP6Ht3 (Figure 1b; Figure S1). Using a 12K SNP array, the segments in RP6Ht3 differing from RP6 were found on chromosomes 2, 3, 4 and 8, revealed by 3, 3, 3 and 28 markers, respectively. These markers were used for QTL mapping using 720 BC₂F₂ plants, and a single association peak with a LOD value of 168.77 was found at position of 158.17 Mb on chromosome 8 (Table S2). By further analysis of the BC₂F₈ recombinants for delimiting *Ht3*, this resistance gene was flanked by markers PZE-108096469 and PZE-108092843, exactly the same interval as *Ht2* (Figure 1b). Interestingly, these two genetic intervals coincide with the previously identified *Htn1* locus (Hurni *et al.*, 2015).

***Ht2* and *Ht3* are identical and represented by a unique genetic segment**

To test this hypothesis, we investigated a collection of original donor and introgression lines that were reported to carry either *Ht2*, *Ht3*, or *Htn1* (Table S3). This collection included NN14, the original donor line of *Ht2* (Hooker, 1977), as well as the original *Ht3* introgression line Oh43Ht3 (Hooker, 1981). First, southern blot probes targeting *ZmWAK-RLK1* and *ZmWAK-RLK2* (a second WAK gene present in the *Htn1* target interval) (Hurni *et al.*, 2015) revealed identical band patterns for *Ht2* and *Ht3* containing lines (Figure S2). Second, re-sequencing of the *ZmWAK-RLK1* full-length coding sequence (CDS) revealed that all but one *Ht2* and *Ht3* carrying maize line had identical *ZmWAK-RLK1* CDS (Table 1). The only exception was B37Ht3, which carried a *ZmWAK-RLK1* allele identical to *Htn1*. Third, we investigated the 0.66 cM target region on chromosome 8 identified by genetic fine-mapping with a 600K SNP array (corresponding to the sequence 154.456 - 157.266 Mb of the B73 reference genome AGPv04 and containing 675 SNP markers) (Figure 1c and Table S4). A pairwise comparison between B37Ht2 and B37Htn1 revealed 214 SNPs (31.7%), while only one SNP (0.15%) was detected between B37Ht2 and A619Ht3, suggesting a unique chromosomal segment being present in genotypes carrying *Ht2* and *Ht3* (Table S4). Moreover, the *S. turcica* race 2 (virulent on *Ht2*, but avirulent on other *Ht* genes), which differs from the proposed race 3 (virulent on *Ht3*, but avirulent on other *Ht* genes) was not detected in 497 *S. turcica* isolates (Ferguson and Carson, 2004; Ferguson and Carson 2007), implying that *Ht2* and *Ht3* have identical resistance spectra.

Collectively, our data indicate that the physical interval (according to 2.81 Mb on B73 reference AGPv04) of *Ht2* and *Ht3* is identical (Figure 1c and Table 1). We propose the designation *Ht2/Ht3* for the two formerly independent genes.

Ht2/Ht3* is an allele of *ZmWAK-RLK1

To conduct high-resolution mapping of *Ht2/Ht3*, additional recombinants derived from the original *Ht2* and *Ht3* fine-mapping populations were evaluated. This strategy allowed us to reduce the *Ht2/Ht3* interval (between the markers MA0021 and MA0025) to 0.16 cM, corresponding to 431 kb on B73 reference genome (Figure 1d). Using gene-based probes designed on the *Htn1* physical interval, several bacterial artificial chromosome (BAC) clones were identified from five BAC libraries derived from maize lines RP6, RP5Ht2, RP6Ht3, B37Ht2 and B37Ht3 (*Htn1*) (Table S5). Sequencing of these BAC clones

revealed a common *Ht2/Ht3* haplotype in RP5Ht2, RP6Ht3 and B37Ht2. In *Ht2/Ht3* genotypes, the physical interval between MA0021 and MA0025 spans 236 kb, which contains four candidate genes *ZmWAK-RLK2*, *ZmWAK-RLK1*, *RET1* and *CYT1* as well as a pseudogene *ZmWAK-RLP1* (Figure 1e-f). Considering that *ZmWAK-RLK1* encodes for the resistance gene *Htn1* (Hurni *et al.*, 2015), we thus considered this gene as the strongest candidate for *Ht2/Ht3*.

To test if *Ht2/Ht3* is encoded by *ZmWAK-RLK1*, we developed a TILLING population in the resistant parental line RP6Ht3. Three non-synonymous mutations (k, n and q) located in the kinase domain were identified (Figure 2a and Figure S3). Homozygous lines were generated carrying mutation k (*ht2/ht3-k*), n (*ht2/ht3-n*) or q (*ht2/ht3-q*) as well as the corresponding sibling lines (*Ht2-k-sib*, *Ht2-n-sib* and *Ht2-q-sib*). In a greenhouse seedling assay inoculated with *S. turcica* isolate Passau-1 (race 0), the three mutant lines were more susceptible than the corresponding sibling lines (Figure 2b-c). Field infection assays were conducted at three locations and artificially inoculated with *S. turcica* races 0, 1, and N, respectively (all avirulent on *Ht2/Ht3*). Similar to the greenhouse assay, the mutant lines were more susceptible compared to the sibling lines in all three field trials (Figure 2d-f). In addition, we conducted PCR amplification and Sanger sequencing of the *ZmWAK-RLK2*, *RET1* and *CYT1* coding sequences in RP6Ht3 and three mutant lines as well as the sibling lines. No mutations were detected in these genotypes. These results demonstrate that *Ht2/Ht3* encodes a functional resistance allele of *ZmWAK-RLK1*, which differs from *Htn1* with respect to the resistance spectrum.

The N-terminal sequence of *Ht2/Ht3* contains a cysteine-rich galacturonan binding domain, followed by a wall-associated receptor kinase domain and a transmembrane domain, while the C-terminal sequence contains a typical cytoplasmic non-RD serine/threonine protein kinase domain. Compared to *Htn1*, the deduced protein sequence of *Ht2/Ht3* shows 49 amino acid substitutions, four insertions and three deletions (Figure S3). Prediction with web-based tool PROVEAN (Choi and Chan, 2015) suggested four variants causing deleterious effect. Most of these polymorphisms were located in the putative extracellular domain. Both introgression lines B37Ht2 and B37Htn1 showed NCLB resistance when infected in the greenhouse and in the field (Figure S5a-c). A delayed lesion appearance was observed in *Ht2/Ht3* and *Htn1* (Figure S5a), while a chlorosis was further observed in *Ht2/Ht3* lesion in line with

previous reports (Hooker, 1977; Hooker, 1981; Welz and Geiger, 2000). However, the introgression lines contained Mb-based chromosomal introgressions which were much larger than the coding region of *ZmWAK-RLK1* (Figure 1c). Therefore, it is possible that the chlorotic phenotype in *Ht2/Ht3* has been conferred by a factor that is independent of, but linked with the *ZmWAK-RLK1* gene. Similarly, in wheat, three previously characterized resistance loci showing different phenotypes upon yellow rust infection were eventually demonstrated to be encoded by an identical genomic sequence of a single gene (Klymiuk *et al.*, 2020).

Recently, an effector named 'AvrHt2' derived from *S. turcica* isolate (race-23N, virulent on *Ht2*, *Ht3* and *Htn1*) was genetically mapped (Mideros *et al.*, 2018). The PRR FLS2 in *Arabidopsis* perceives microbial universal PAMP effector flg22 and results in basal and dynamic resistance (Gomez-Gomez *et al.*, 2000). It's plausible that the extracellular domain of the plasma membrane localized *ZmWAK-RLK1* might perceive *S. turcica*-derived effectors and then modulate the immune response. Whether mutations at the extracellular domain of *Ht2/Ht3* and *Htn1* specify the different races remains to be elucidated.

Compared to *ZmWAK-RLK1* from the susceptible line B37, the protein sequence of *Ht2/Ht3* shows 37 amino acid polymorphisms and seven insertions as well as seven deletions (Figure S4). RT-qPCR analyses in the introgression lines with *Ht2*, *Htn1*, and the susceptible line B37 revealed no significant difference in *ZmWAK-RLK1* transcript levels prior to *S. turcica* inoculation. A significantly lower expression was detected in B37Ht2 compared to B37 at 12 days post-inoculation (dpi) (Figure S5d), which is consistent with what was previously reported for *Htn1* (Yang *et al.*, 2019).

Since the 1980s, B37 and derived introgression lines with *Ht1*, *Ht2*, *Ht3*, and *Htn1* were widely deployed in disease surveys and the nomenclature of *S. turcica* isolates (Leonard *et al.*, 1989). We have shown that B37Ht3 and B37Htn1 both contain the *Htn1* allele of *ZmWAK-RLK1* (Table 1), however these lines are reported to show different disease symptoms (Ceballos and Gracen, 1989; Chung *et al.*, 2010; Leath *et al.*, 1990; Leonard *et al.*, 1989; Simcox and Bennetzen, 1993; Thakur *et al.*, 1989). We used the 600K SNP array to determine the genetic differentiation among the various B37 introgression lines. Surprisingly, we found considerable genetic variation (8.69 - 61.03%) between B37 and the 'near isogenic' lines B37Ht1, B37Ht2, B37Ht3, and B37Htn1 (Figure S6a). Similarly the two lines B37Ht3 and B37Htn1 (both containing

Htn1) showed a large amount (71.83%) of polymorphic chromosomal segments (Figure S6b). These results show that the various B37 introgression lines differ considerably for the recurrent parent, which might be the result of gene flow and seed mix-up over the years. We also observed that *Ht2/Ht3* was ineffective in the genetic background of maize line Pa91. *Ht2/Ht3* introgression lines in Pa91 showed similar levels of susceptibility as the susceptible parent Pa91 (Table 1). This observation can be explained by genetic modifiers that influence NCLB resistance. For example, the locus *Sht1* was reported to be a suppressor of *Ht2/Ht3* and *Htn1* mediated NCLB resistance (Ceballos and Gracen, 1989). Moreover, it has been described that NCLB resistance mediated by the various *Ht* loci is influenced by genetic background and environmental factors (e.g. temperature and light) (Leath *et al.*, 1990, Thakur *et al.*, 1989).

Diversity analysis of *ZmWAK-RLK1* in the maize gene pool

We further wanted to determine sequence diversity of *ZmWAK-RLK1* and to identify additional haplotypes of *ZmWAK-RLK1* in the maize gene pool. We characterized the genomic variation in the *Ht2/Ht3* target region by analyzing >200kb contig sequences that cover the flanking region of *ZmWAK-RLK1* from B73 and maize lines containing *Ht2/Ht3* and *Htn1* (Figure 3a). The orientation and sequence organization of *ZmWAK-RLK1*, *ZmWAK-RLP1* and *ZmWAK-RLK2* differed between the three haplotypes. For example, *ZmWAK-RLK1* showed opposite orientation in *Htn1* and *Ht2/Ht3*, and the length of the first *ZmWAK-RLK1* intron varied from 8.3 kb to 58.7 kb in the various maize lines. Moreover, *ZmWAK-RLK1* was found in the conserved target interval of 28 maize genomes (https://www.maizegdb.org/NAM_project) based on a gene-based collinearity analysis (Figure S7), suggesting that *Ht2/Ht3* and *Htn1* are allelic variants of the same gene.

The diversity of *ZmWAK-RLK1* was surveyed by amplifying the full-length coding sequence from 127 historical, geographic-referenced inbred lines (Table S6). A full-length *ZmWAK-RLK1* sequence was amplified from 51 genotypes, which defined 33 haplotypes of *ZmWAK-RLK1* (Figure 3b). None of the alleles were identical to *Ht2/Ht3* or *Htn1*, indicating that both are rare in the historic maize gene pool. Six haplotypes (hap-28p to -33p) contained premature-stop codons or frameshifts. Numerous SNPs and InDels were found, especially in the extracellular domain where the nucleotide diversity (*Pi*) was >3 fold higher compared to that of the kinase domain (Table S7).

NCLB resistance in the modern maize gene pool is controlled by a number of *Ht* genes (Galiano-Carneiro and Miedaner, 2017). Our work demonstrates that two of these genes, *Ht2* and *Ht3*, are identical and allelic to *Htn1*. This has implications for NCLB resistance breeding and highlights the importance of identifying novel sources of NCLB resistance. It is possible that the *Ht2* and *Ht3* loci were discovered independently in different maize genotypes carrying the same unique genetic segment or were introduced more than once into the maize gene pool. The original report of *Ht2* was made in the Australian maize landrace NN14 (Hooker, 1977), whereas *Ht3* was reported to originate from *T. floridanum* (Hooker, 1981). An analysis of *T. floridanum* transcriptome data (Gault *et al.*, 2018) revealed several homologous sequences of *ZmWAK-RLK1*, which form a phylogenetic cluster independent of the maize *ZmWAK-RLK1* haplotypes (Figure S8). This suggests that this wild grass is an unlikely donor of *Ht3*. In the original *Ht3* publication (Hooker, 1981), A. L. Hooker mentions that ‘many years ago Walton Galinat provided me with seed of a corn inbred crossed with *T. floridanum*’. It is possible that this original hybrid contained *Ht3* from a source different than *T. floridanum*. The differences in NCLB resistance reactions and race spectra of *Ht2* and *Ht3* reported in the literature can most likely be attributed to environmental and background dependence of these genes (Ceballos and Gracen, 1989; Leath *et al.*, 1990; Thakur *et al.*, 1989). We also found considerable genetic variation among the B37 near isogenic lines, which highlights the importance to regularly validate genetic stocks with molecular markers. Our work highlights the importance of identifying genes which underlie disease resistance loci in order to streamline and maximize durability in resistance breeding.

Materials and Methods

Plant materials

The maize inbred lines A619Ht2 (USDA-ARS genebank accession name: Ames25220, carrying *Ht2*) and A619Ht3 (Ames25221, carrying *Ht3*) were crossed to two susceptible recurrent parents RP5 and RP6 from the maize breeding program of Germany breeding company KWS SAAT SE & Co KGaA, respectively. Two backcrosses with the same susceptible parental lines were followed to enrich for the recurrent genome and to produce the resistance parental lines RP5Ht2 and RP6Ht3. The populations for QTL mapping and high-resolution mapping were selfed in the crosses of RP5 × RP5Ht2 and RP6 × RP6Ht3. Each BC₂F₂

population contained 720 progeny used for QTL mapping. Heterozygous BC₂F₂ progeny plants in the target region were selfed in generations for assembling the recombinants. These new recombinants (BC₂F₉ to BC₂F₁₁ generations, approximately 2,000 plants) derived from the BC₂F₈ heterozygous plants were genotyped and tested for NCLB resistance and used for high-resolution mapping.

The maize inbred lines with the introgression of resistance genes *Ht2*, *Ht3* or *Htn1* were provided by several research institutions (Table S3). These include ten genotypes from Genebank of United States Department of Agriculture (USDA-ARS), four from Maize Genetics Cooperation Stock Center (MGCSC), one genotype NN14 from Australian Plant Genetic Resource Information Service center (AusPGRIS) as well as two introgression lines and respective recurrent parents and EMS-induced mutants/sibling lines provided by KWS SAAT SE & Co KGaA. In addition, 127 geographically-referenced historic maize inbred lines, which were kindly provided by German *ex-situ* Genebank, were used for allele mining of the *ZmWAK-RLK1* coding sequence diversity (Table S6).

Field test for NCLB resistance

The artificial inoculation in the field and scoring for the disease rates were conducted as previously described (Hurni *et al.*, 2015). The crushed infected leaves were collected from the same location the year before and they were subjected for inoculation. The near isogenic lines (i.e. RP5Ht2 and RP6Ht3) and recurrent parents (i.e. RP5 and RP6) were plated as a control for infection. Experiments were carried out at five locations (Neupotz, Pocking or Passau, Germany; Homburg or Quimperlé, France) and years from 2012 to 2019. The race specificity were determined via planting differential sets carrying major *Ht* genes (Neupotz/Pocking/ Passau = race 0, avirulent on *Ht1*, *Ht2/Ht3* and *Htn1*; Homburg = race 1, virulent on *Ht1* but avirulent on *Ht2/Ht3* and *Htn1*; Quimperlé = race N, virulent on *Htn1* but avirulent on *Ht1* and *Ht2/Ht3*). Except BC₂F₂ populations that were scored by each single plant and used for QTL mapping, a row test with >15 plants was performed in the remaining genotypes (for examples, BC₂F₈ to BC₂F₁₁ recombinants, introgression lines, mutants) and in general three replicates were applied in each location following a complete randomization strategy.

Greenhouse tests for NCLB resistance

The greenhouse seedling assay was performed as previously described (Yang *et al.*, 2017). The *S. turcica* isolate Passau-1 (race 0), was used for typing NCLB resistance. In brief, after the second leaves had fully emerged, the newly emerged leaves were cut and removed until the end of each test experiment. Spore inoculation and culture on PDA medium plate, harvest and quantification of progeny spores was performed (Yang *et al.*, 2017). The maize plants were infected once by spray (sprayer: ø 28mm, Semadeni, Ostermundigen, Switzerland). Every four trays were sprayed with 4 ml of spore suspension with a concentration of 4.5×10^4 spores/ml. A very high humidity growing environment was produced by placing plastic hoods on top of each tray after inoculation. Each plant was scored for disease symptoms between 11 and 25 days and the severity was evaluated by calculating the area under the disease progress curve (AUDPC) or calculating the percentage of diseased leaf area. In general, 15 seedling plants each genotype were analyzed in each experiment following a complete randomization strategy.

RT-qPCR assay

The leaf sampling, RNA extraction, synthesis of the first-strand cDNA were conducted following the established protocol (Yang *et al.*, 2019). The expression of targets was normalized by the endogenous reference genes *FPGS* and *Actin*. The primers for expression analysis are given (Table S8).

BAC-library construction and screening

The library construction and BAC screening completely followed the described method (Hurni *et al.*, 2015). Five BAC libraries for the resistant lines RP5Ht2, RP6Ht3, B37Ht2 and B37Ht3 (carrying *Htn1*) as well as the susceptible parental line RP6 were constructed at INRA-CNRCV and screened by the use of the gene-specific probes that were published.

Genotyping, QTL mapping, marker saturation

Both 12K and 600K SNPs arrays (Unterseer *et al.*, 2014) were deployed for deciphering the polymorphisms in the inbred lines. Polymorphisms in RP5Ht2 and RP6Ht3 to respective recurrent parental lines RP5 and RP6 were revealed by 12K SNP array, and the polymorphic SNPs were converted to produce KASP markers that were subjected for genotyping the BC₂F₂ populations of RP5 x RP5Ht2 and RP6 x RP6Ht3. 38 and 37 KASP markers were obtained in populations of RP5Ht2 x RP5 and RP6Ht3 x RP6, respectively. QTL

mapping using the BC₂F₂ populations was conducted by software PlabMQTL using Composite Interval Mapping (CIM) with a multiple regression approach (Haley and Knott, 1992), with basic parameters: a minimum log of the odds (LOD) threshold of 2.5 and a walking speed of 1 cM. A subset of lines were genotyped with 600K SNP array. The polymorphisms residing in the target interval were used for developing the KASP markers via the Kraken-Software (LGC), followed by the genotyping assay in KWS SAAT SE & Co KGaA, in order to fine map the target genes. Especially for the set B37, B37Ht2, B37Ht3, B37Htn1 as well as the lines which were deployed for analyzing the introgression segments derived from the respective donor lines, all markers with low quality or were missing, are excluded from analysis.

Re-sequencing *ZmWAK-RLK1* in maize inbred lines

20-day-old leaf samples were harvested for total RNA extraction and RNA purification as previously described (Yang *et al.*, 2019). 1 µg of total RNA was used for synthesizing the first-strand cDNA using the SuperScript™ IV Reverse Transcriptase. A nested-PCR procedure was deployed for amplifying the target fragments by using high-fidelity hotstart polymerase. The first PCR amplification in a reaction volume of 10 µl was performed using the described procedure except for the polymerase, while the subsequent PCR was conducted with 2 µl of 100-fold diluted PCR product. The gel-purified PCR products were quantified and then subjected to Sanger sequencing using an ABI 3730XL platform. Sequence analysis was processed with the CLC Workbench 7.5.1. Primers for PCR amplification and Sanger sequencing are listed (Table S8). 105 quality-filtered sequences were obtained. In addition to *ZmWAK-RLK1* with a length of approximately 2.0 kb, a second fragment was sometimes detected with a size of approximately 1.4 kb. Except for the two accessions ZEA272 and ZEA1105 that simultaneously carried both sequences, either one of the two fragments were detected. The shorter fragment contained a complete coding frame with the deduced protein sequence sharing close homology to a receptor-like protein kinase-like 2.5 (NCBI: XM_020542761.1) and NLB17 (Patent application: US2013/0061355A1). This NLB17-like version shows 97% amino acid sequence identity with the kinase domain of *ZmWAK-RLK1* and showed no extracellular domain. 54 genotypes which only produced the smaller of the two fragments were excluded from analysis. The *ZmWAK-RLK1* homologous sequences were identified from *T. floridanum* transcriptome data (Gault *et al.*, 2018), using BlastN by query of *Ht2/Ht3* coding sequence. The phylogenetic tree was generated using

software MEGA 7 (Kumar *et al.*, 2016) with the Neighbor-Joining method, based on the deduced amino acid sequences. Web-based tool ROVEAN was used for predicting the functional effect of amino acid substitutions and indels (Choi and Chan, 2015).

EMS-mutagenesis in RP6Ht3 and mutant identification

The mutant population in the resistance parental line RP6Ht3 was developed by the company Keygene with the KeyPointMB technology. PCR products were sequenced using Illumina sequencing approach, and paired-end short reads were subjected for base-calling against the RP6Ht3 sequence. The screening for mutations at *ZmWAK-RLK1* exon 1 and 3 was performed at the same company and two missense mutants n and q were identified. In addition, another screening for *ZmWAK-RLK1* exon 3 was conducted by Traitgenetics GmbH and resulted in identification of the third missense mutant k. The primer design was adjusted using the genomic sequence of *Ht2/Ht3*. Three mutations in *ZmWAK-RLK1* had been validated by PCR amplification followed by Sanger sequencing. The same strategy was applied for sequencing the genes *ZmWAK-RLK2*, *RET1* and *CYT1* which are resided between the flanking markers. KASP primers to identify the individual mutations were developed at KWS SAAT SE & KGaA (MA005s31 = k; MA005s43 = n; MA005s52= q). The primers used for mutant identification at Traitgenetics GmbH, PCR amplification, Sanger sequencing and KASP markers for mutant identification were listed (Table S8).

Southern blot analysis

The procedure of Southern hybridization was performed as described (Hurni *et al.*, 2015). In brief, 5 µg of genomic DNA was digested with the restriction enzyme *DraI*. The probe for hybridization was obtained by making a 1:1 mixture of PCR amplicons, which were produced by amplification of the extracellular domains of *ZmWAK-RLK1* and *ZmWAK-RLK2* using PCR primers (Table S8). The λDNA digested by *HindIII* served as control of fragment size.

Data availability statement: The sequences reported in this paper have been deposited in the NCBI database (MT145992-MT146024). Otherwise, all relevant data can be found within the manuscript and its supporting materials.

Acknowledgements

We thank German Federal *Ex-situ* Genebank (IPK-Gatersleben), Genebanks of United States Department of Agriculture (USDA-ARS), Maize Genetics Cooperation Stock Center (MGCSC) and Australian Plant Genetic Resource Information Service center (AusPGRIS) for providing the maize accessions, and Mr. Alessandro Artemisio, Mr. Guangqi Gao, Ms. Luxi Yan, Mr. Karl Huwiler, Mr. Thibault Vassor, Ms. Matisse Petit-Prost for technical support. This work was supported by Swiss National Science Foundation Grant 310030B_182833 to B. Keller. S.G.K. is supported by King Abdullah University of Science and Technology. P.Y. is supported by Agricultural Science and Technology Innovation Program of CAAS and Fundamental Research Funds for Central Non-Profit of Institute of Crop Sciences of CAAS, China.

Author contributions

P.Y., D.S., S.G.K., M.O. and B. Keller designed research; P.Y., D.S., B. Kessel, T.K., J.R.G., S. H., W.M. and G.H. performed research; P.Y., D.S., T.K., S.Z., and T.W. analyzed data; and P.Y., T.K., J.R.G., S.G.K., and B. Keller wrote the paper.

Conflicts of interest statement: A patent application has been filed related to this work.

Supplementary Figure legends

Figure S1 Example of resistant (left) and susceptible (right) phenotype of RP6Ht3 and RP6, respectively, in a field infection test at the Neupotz of Germany in September of 2016.

Figure S2 Southern blot analysis by deploying the probes derived from the conserved extracellular domains of *ZmWAK-RLK1* and *ZmWAK-RLK2*. The results of Southern blot in parental lines (a), the introgression lines from KWS breeding company (b) and the donor/introgression lines (c). The genomic DNAs were digested with the restriction enzyme *DraI*. The read-highlighted 1 and 2 indicates the fragments of *ZmWAK-RLK1* and *ZmWAK-RLK2*, respectively. The BAC clones from the genotype RP5Ht2 served as positive control. λ DNA is the control of fragment size.

Figure S3 Alignment of the ZmWAK-RLK1 amino acid sequences from *Ht2/Ht3* and *Htn1*. The functional domains are indicated. The mutations causing amino acid substitutions in three mutants k, n and q are indicated with triangles.

Figure S4 Alignment of the ZmWAK-RLK1 amino acid sequences from *Ht2/Ht3* and the susceptible line B37. The functional domains are indicated.

Figure S5 Tests for NCLB resistance and the expression analysis of *ZmWAK-RLK1* in B37, B37Ht2 and B37Htn1. Statistical analysis was conducted using Tukey's HSD test ($\alpha = 0.05$). Error bars mean $\pm$ SE. (a) The diseased lesion on the mature leaves. These pictures were taken in the Homburg of France in September of 2016. (b) Statistic analysis of diseased datasets in the field, which were collected from four independent experiments. (c) Statistical analysis of disease severity using the greenhouse seedling assay infected with Passau-1 (race = 0, n = 3 to 5 independent experiments). (d) Expression of *ZmWAK-RLK1*. The second leaves (n = 4 or 5) were harvested from the seedling plants in the greenhouse.

Figure S6 Graphic representation of the genetic differences by using 600k SNP array. Colors represent segments with different percentages of polymorphic SNPs. The segment harboring the gene *ZmWAK-RLK1* is indicated. (a) The genomic segments derived from the donor lines in B37Ht1, B37Ht2, B37Ht3 (*Htn1*) and B37Htn1. (b) Pairwise comparison of the genomic segments differing between B37Ht3 (*Htn1*) and B37Htn1.

Figure S7 The diagram represents the homologue genes at the *Ht2/Ht3* interval on 28 maize genomes. The homologue sequences were identified by BLAST search by using the sequences of genes annotated from the B73 reference genome, *Htn1* and *Ht2/Ht3*, as well as the annotated gene *NLB17* (Patent application: US2013/0061355A1).

Figure S8 Phylogenetic analysis of *ZmWAK-RLK1* haplotypes. The phylogenetic tree was generated using software MEGA 7 with the Neighbor-Joining method, based on the deduced amino acid sequences. The haplotypes 28p-33p, because of frameshift or in-frame stop codons, were not shown.

Supplementary Table legends

Table S1 QTL mapping for NCLB resistance in NIL population of RP5 x RP5Ht2 using KASP markers.

Table S2 QTL mapping for NCLB resistance in NIL population of RP6 x RP6Ht3 using KASP markers.

Table S3 Maize inbred lines and introgression lines used in this study.

Table S4 Snapshot of SNP markers in the target interval.

Table S5 The BAC clones identified from five libraries.

Table S6 Passport information of maize inbred lines and haplotypes of *ZmWAK-RLK1*.

Table S7 Statistical analysis of the functional domains in 51 *ZmWAK-RLK1* sequences plus *Ht2/Ht3* and *Htn1*.

Table S8 Primers used in this study.

References

- Brutus, A., Sicilia, F., Macone, A., Cervone, F. and De Lorenzo, G.** (2010) A domain swap approach reveals a role of the plant wall-associated kinase 1 (WAK1) as a receptor of oligogalacturonides. *Proc. Natl. Acad. Sci. U S A* **107**, 9452-9457.
- Ceballos, H. and Gracen, V.E.** (1989) A dominant inhibitor gene inhibits the expression of *Ht2* against *Exserohilum turcicum* race 2 in corn inbred lines related to B14. *Plant Breed.* **102**, 35-44.
- Choi, Y. and Chan, A.P.** (2015) PROVEAN web server: a tool to predict the functional effect of amino acid substitutions and indels. *Bioinformatics* **31**, 2745-2747.
- Chung, C.L., Jamann, T., Longfellow, J., Nelson, R.** (2010) Characterization and fine-mapping of a resistance locus for northern leaf blight in maize bin 8.06. *Theor. Appl. Genet.* **121**, 205-227.
- Dardick, C., Schwessinger, B. and Ronald, P.** (2012) Non-arginine-aspartate (non-RD) kinases are associated with innate immune receptors that recognize conserved microbial signatures. *Curr. Opin. Plant Biol.* **15**, 358-366.

- Delteil, A., Gobbato, E., Cayrol, B., Estevan, J., Michel-Romiti, C., Dievart, A., Kroj, T. and Morel, J.B.** (2016) Several wall-associated kinases participate positively and negatively in basal defense against rice blast fungus. *BMC Plant Biol.* **16**, 17.
- Dmochowska-Boguta, M., Kloc, Y., Zielezinski, A., Werecki, P., Nadolska-Orczyk, A., Karlowski, W.M. and Orczyk, W.** (2020) *TaWAK6* encoding wall-associated kinase is involved in wheat resistance to leaf rust similar to adult plant resistance. *PLoS One* **15**, e0227713.
- Dong, J., Fan, Y., Gui X., An, X., Ma, J. and Dong, Z.** (2008) Geographic Distribution and Genetic Analysis of Physiological Races of *Setosphaeria turcica* in Northern China. *Am. J. Agr. Biol. Sci.* **3**, 389-398.
- Fallah Moghadam, P. and Pataky, J.K.** (1994) Reactions of isolates from matings of races 1 and 23N of *Exserohilum turcicum*. *Plant Dis.* **78**, 767-771.
- Ferguson, L.M. and Carson, M.L.** (2004) Spatial diversity of *Setosphaeria turcica* sampled from the Eastern United States. *Phytopathology* **94**, 892-900.
- Ferguson, L.M. and Carson, M.L.** (2007) Temporal variation in *Setosphaeria turcica* between 1974 and 1994 and origin of races 1, 23, and 23N in the United States. *Phytopathology* **97**, 1501-1511.
- Galiano-Carneiro, A.L. and Miedaner, T.** (2017) Genetics of resistance and pathogenicity in the maize/*Setosphaeria turcica* pathosystem and implications for breeding. *Front. Plant Sci.* **8**, 1490.
- Gault, C.M., Kremling, K.A. and Buckler, E.S.** (2018) *Tripsacum* *de novo* transcriptome assemblies reveal parallel gene evolution with maize after ancient polyploidy. *Plant Genome-US* **11**: 10.3835/plantgenome2018.02.0012.
- Gomez-Gomez, L. and Boller, T.** (2000) FLS2: An LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. *Mol. Cell* **5**, 1003-1011.
- Haley, C.S. and Knott, S.A.** (1992) A simple regression method for mapping quantitative trait loci in line crosses using flanking markers. *Heredity (Edinb)* **69**, 315-324.
- Hooker, A.L.** (1977) A second major gene locus in corn for chlorotic-lesion resistance to *Helminthosporium turcicum*. *Crop Sci.* **17**, 132-135.
- Hooker, A.L.** (1981) Resistance to *Helminthosporium turcicum* from *Tripsacum oridanum* incorporated into corn. *Maize Genet. Coop. Newsl.* **55**, 87-88.

- Hu, K.M., Cao, J.B., Zhang, J. et al.** (2017) Improvement of multiple agronomic traits by a disease resistance gene via cell wall reinforcement. *Nat. Plants* **3**, 17009.
- Hurni, S., Scheuermann, D., Krattinger, S.G. et al.** (2015) The maize disease resistance gene *Htn1* against northern corn leaf blight encodes a wall-associated receptor-like kinase. *Proc. Natl. Acad. Sci. U S A* **112**, 8780-8785.
- Jones, J.D.G. and Dangl, J.L.** (2006) The plant immune system. *Nature* **444**, 323-329.
- Kanyuka, K. and Rudd, J.J.** (2019) Cell surface immune receptors: the guardians of the plant's extracellular spaces. *Curr. Opin. Plant Biol.* **50**, 1-8.
- Kema, G.H.J., Mirzadi Gohari, A., Aouini L. et al.** (2018) Stress and sexual reproduction affect the dynamics of the wheat pathogen effector *AvrStb6* and strobilurin resistance. *Nat. Genet.* **50**, 375-380.
- Kumar, S., Stecher, G. and Tamura, K.** (2016) MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* **33**, 1870-1874.
- Leath, S., Thakur, R.P. and Leonard, K.J.** (1990) Variation in expression of monogenic resistance in corn to *Exserohilum turcicum* race 3 under different temperature and light regimes. *Phytopathology* **80**, 309-313.
- Leonard, K.J., Levy, Y. and Smith, D.R.** (1989) Proposed nomenclature for pathogenic races of *Exserohilum turcicum* on corn. *Plant Dis.* **73**, 776-777.
- Lipps, P.E., Pratt, R.C. and Hakiza, J.J.** (1997) Interaction of *Ht* and partial resistance to *Exserohilum turcicum* in maize. *Plant Dis.* **81**, 277-282.
- Macho, A.P. and Zipfel, C.** (2014). Plant PRRs and the activation of innate immune signaling. *Mol. Cell* **54**, 263-272.
- Mideros, S.X., Chung, C.L., Wiesner-Hanks, T., Poland, J.A., Wu, D., Fialko, A.A., Turgeon, B.G., and Nelson, R.J.** (2018). Determinants of virulence and *in vitro* development colocalize on a genetic map of *Setosphaeria turcica*. *Phytopathology* **108**, 254-263.
- Mueller, D.S., Wise, K.A., Sisson, A.J., Allen, T.W., Bergstrom, G.C., Bosley, D.B., Bradley, C.A., Broders, K.D., Byamukama, E. and Chilvers, M.I.** (2016) Corn yield loss estimates due to diseases in the United States and Ontario, Canada from 2012 to 2015. *Plant Health Prog.* **17**, 211-222.

- Saintenac, C., Lee, W.S., Cambon, F., et al.** (2018) Wheat receptor-kinase-like protein *Stb6* controls gene-for-gene resistance to fungal pathogen *Zymoseptoria tritici*. *Nat. Genet.* **50**, 368-374.
- Simcox, K.D. and Bennetzen, J.L.** (1993) The use of molecular markers to study *Setosphaeria turcica* resistance in maize. *Mol. Plant Pathol.* **83**, 1326-1330.
- Thakur, R.P., Leonard, K.J. and Leath, S.** (1989) Effects of temperature and light on virulence of *Exserohilum turcicum* on corn. *Phytopathology* **1989**, 631-635.
- Unterseer, S., Bauer, E., Haberer, G. et al.** (2014). A powerful tool for genome analysis in maize: development and evaluation of the high density 600k SNP genotyping array. *BMC Genomics* **15**, 823.
- Wang, P., Zhou, L., Jamieson, P. 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.
- Weems, J.D. and Bradley, C.A.** (2018) *Exserohilum turcicum* race population distribution in the North Central United States. *Plant Dis.* **102**, 292-299.
- Welz, H.G. and Geiger, H.H.** (2000) Genes for resistance to northern corn leaf blight in diverse maize populations. *Plant Breed.* **119**, 1-14.
- Yang, P., Herren, G., Krattinger, S.G. and Keller, B.** (2017) Large-scale maize seedling infection with *Exserohilum turcicum* in the greenhouse. *Bio-protocol* **7**, e2567.
- Yang, P., Praz, C., Li, B. et al.** (2019) Fungal resistance mediated by maize wall-associated kinase ZmWAK-RLK1 correlates with reduced benzoxazinoid content. *New Phytol.* **221**, 976-987.
- Yin, X.Y., Wang, Q.H., Yang, J.L., Jin, D.M., Wang, F., Wang, B., Zhang, J.R.** (2003) Fine mapping of the *Ht2* (*Helminthosporium turcicum* resistance 2) gene in maize. *Chin. Sci. Bull.* **48**, 165-169.
- Zhang, N., Pombo, M.A., Rosli, H.G. and Martin, G.B.** (2020) Tomato wall-associated kinase SlWak1 depends on Fls2/Fls3 to promote apoplastic immune responses to *Pseudomonas syringae*. *Plant Physiol.* **183**, 1869-1882.
- Zuo W.L., Chao Q., Zhang N. et al.** (2015) A maize wall-associated kinase confers quantitative resistance to head smut. *Nat. Genet.* **47**, 151-157.

Figure and table legends

Figure 1 Map-based cloning of *Ht2/Ht3*. (a) Genetic mapping of *Ht2*. The diseased lesions of parental lines RP5 and RP5Ht2 are given. (b) Genetic mapping of *Ht3*. The diseased lesions of parental lines RP6 and RP6Ht3 are given. The genetic position of the molecular markers is given based on the position in the B73 reference genome. Only critical markers are indicated, and important flanking markers are highlighted in red. The number of tested recombinants in each recombination type is represented. R, resistant; S, susceptible; Het, heterozygous. (c) Snapshot of the flanking segments genotyped using 600K SNP array. The fragment, which is defined by two flanking markers PZE-108092843 and PZE-108096469, expands 2.81 Mb with 675 SNP markers. (d) High-resolution mapping of *Ht2/Ht3*. (e) Construction of the physical map surrounding *Ht2/Ht3*. (f) Gene structure of *ZmWAK-RLK1*. SP, signal peptide; GUB_WAK, wall-associated receptor kinase galacturonan binding; WAK, wall-associated domain; TM, transmembrane domain; non-RD Ser/Thr kinase, non-arginine-aspartate serine/threonine kinase domain.

Figure 2 Mutations in *ZmWAK-RLK1* compromise the NCLB resistance. Three mutants ht2/ht3-k, ht2/ht3-n and ht2/ht3-q as well as respective sibling lines Ht2/Ht3-k-sib, Ht2/Ht3-n-sib and Ht2/Ht3-q-sib are in the RP6Ht3 background. (a) Graphic representation of EMS-induced mutants. (b) The diseased symptoms of the mutant and sibling lines at 17 dpi. (c) Percentage of the diseased leaf area at 11, 13, 15 and 17 dpi. Statistical analysis was conducted at each time-point using Tukey's honestly significant difference (HSD, $\alpha = 0.05$) test, and the plants used from each genotype were indicated. A complete randomization strategy was applied to test the parental and mutant lines, and three independent experiments were conducted with similar results. (d-f) Quantification of the field disease severity, separately inoculated with race 0 at Neupotz of Germany, race 1 at Homburg of France and race N at Quimperlé of France. Two to six replicates at each location were achieved. Statistical analysis was conducted using Tukey's HSD test, and rows tested for resistance were indicated.

Figure 3 Sequence variation of *ZmWAK-RLK1* and its flanking region. (a) Three-way comparison of scaffold sequences from maize cultivars B73, *Htn1*- and *Ht2/Ht3*-carrying genotypes. Sequences conserved between two cultivars are connected with shaded areas. Genes are indicated as black boxes. A pseudogene *ZmWAK-RLP1* is indicated with an asterisk. (b) Graphic representation of multiple alignment

of 32 *ZmWAK-RLK1* haplotypes. SNPs and Indels are indicated by black line and white blocks respectively. Haplotypes with the extension 'p' are pseudogenes which contain frameshift or in-frame stop codons. Hap-33p (Table S6), which contains a >500 bp insertion, is not indicated. The sequence encoding for the signal peptide is excluded for this analysis.

Table 1 Haplotype analysis of *ZmWAK-RLK1* in genotypes carrying the resistance genes *Ht2*, *Ht3* or *Htn1*.

Table 1 Haplotype analysis of *ZmWAK-RLK1* in genotypes carrying the resistance genes *Ht2*, *Ht3* or *Htn1*.

No.	Genotypes/accessions	Providers	Phenotype	Haplotype [†]
1	NN14 (<i>Ht2</i>)	AusPGRIS	R	<i>Ht2/Ht3</i> ^{1, 2}
2	Pa91Ht2 (Ames25373)	USDA-ARS	S	<i>Ht2/Ht3</i> ^{1, 2}
3	A619Ht2 (Ames25220)	USDA-ARS	R	<i>Ht2/Ht3</i> ^{1, 2}
4	A619Ht2	KWS [†]	R	<i>Ht2/Ht3</i> ^{1, 2, 3}
5	B37Ht2	KWS [†]	R	<i>Ht2/Ht3</i> ^{1, 2, 3}
6	RP5Ht2	KWS	R	<i>Ht2/Ht3</i> ^{1, 2, 3}
7	W22Htn1 (PI406128)	USDA-ARS	R	<i>Htn1</i> ²
8	B37Htn1 (PI406120)	USDA-ARS	R	<i>Htn1</i> ²
9	W22Htn1	KWS [†]	R	<i>Htn1</i> ^{2, 3}
10	B37Htn1	KWS [†]	R	<i>Htn1</i> ^{1, 2, 3}
11	RP1Htn1	KWS	R	<i>Htn1</i> ^{2, 3}
12	RP3Htn1	KWS	R	<i>Htn1</i> ^{2, 3}
13	RP4Htn1	KWS	R	<i>Htn1</i> ^{2, 3}
14	U940A Oh43Ht3	MGCSC	R	<i>Ht2/Ht3</i> ^{1, 2}
15	U940A Va26Ht3	MGCSC	R	<i>Ht2/Ht3</i> ²
16	U940A A619Ht3	MGCSC	R	<i>Ht2/Ht3</i> ²
17	U940A Pa91Ht3	MGCSC	S	<i>Ht2/Ht3</i> ²
18	Pa91Ht3 (Ames25374)	USDA-ARS	S	<i>Ht2/Ht3</i> ^{1, 2}
19	A619Ht3 (Ames25221)	USDA-ARS	R	<i>Ht2/Ht3</i> ^{1, 2}
20	A619Ht3	KWS [†]	R	<i>Ht2/Ht3</i> ^{1, 2, 3}
21	B37Ht3	KWS [†]	R	<i>Htn1</i> ^{1, 2, 3}
22	RP6Ht3	KWS	R	<i>Ht2/Ht3</i> ^{1, 2, 3}

R, Resistance; S, Susceptibility. The phenotypic data was based on the greenhouse seedling assay when infecting with Passau-1 (race 0). The *ZmWAK-RLK1* haplotype was revealed by Southern blot⁽¹⁾, re-sequencing *ZmWAK-RLK1* coding sequence⁽²⁾ and genotyping by 600K SNPs array⁽³⁾. [†]Genotypes

collected from USDA-ARS were multiplied by KWS SAAT SE & Co KGaA. [‡]The genetic resistance genes characterized from this study.

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