

# The maize disease resistance gene *Htn1* against northern corn leaf blight encodes a wall-associated receptor-like kinase

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Northern corn leaf blight (NCLB) caused by the hemibiotrophic fungus *Exserohilum turcicum* is an important foliar disease of maize that is mainly controlled by growing resistant maize cultivars. The *Htn1* locus confers quantitative and partial NCLB resistance by delaying the onset of lesion formation. *Htn1* represents an important source of genetic resistance that was originally introduced from a Mexican landrace into modern maize breeding lines in the 1970s. Using a high-resolution map-based cloning approach, we delimited *Htn1* to a 131.7-kb physical interval on chromosome 8 that contained three candidate genes encoding two wall-associated receptor-like kinases (*ZmWAK-RLK1* and *ZmWAK-RLK2*) and one wall-associated receptor-like protein (*ZmWAK-RLP1*). TILLING (targeting induced local lesions in genomes) mutants in *ZmWAK-RLK1* were more susceptible to NCLB than wild-type plants, both in greenhouse experiments and in the field. *ZmWAK-RLK1* contains a nonarginine-aspartate (non-RD) kinase domain, typically found in plant innate immune receptors. Sequence comparison showed that the extracellular domain of *ZmWAK-RLK1* is highly diverse between different maize genotypes. Furthermore, an alternative splice variant resulting in a truncated protein was present at higher frequency in the susceptible parents of the mapping populations compared with in the resistant parents. Hence, the quantitative *Htn1* disease resistance in maize is encoded by an unusual innate immune receptor with an extracellular wall-associated kinase domain. These results further highlight the importance of this protein family in resistance to adapted pathogens.

wall-associated receptor-like kinase | quantitative disease resistance | pattern recognition receptor | *Exserohilum turcicum* | maize

Plants are constantly attacked by potential pathogenic microbes, specifically, viruses, bacteria, and fungi. Although lacking an adaptive immune system comparable to the one found in vertebrates, plants have evolved a plethora of strategies to fend off microbial pathogens. The first tier of defense is formed by plasma membrane-anchored pattern recognition receptors (PRRs). These receptor proteins monitor the extracellular space for the presence of microbial- or host-derived elicitors, also called pathogen-associated molecular patterns (PAMP) or danger-associated molecular patterns (DAMP), respectively. Perception of PAMPs and DAMPs triggers a signaling cascade that activates numerous defense responses, called PAMP-triggered immunity and DAMP-triggered immunity, respectively. PAMPs are often highly conserved microbial structures that are characteristic for entire pathogen classes (1, 2). Examples are the fungal cell wall component chitin or bacterial flagellin. Perception of highly conserved PAMPs therefore results in broad-spectrum resistance against whole groups of nonadapted microbes, also referred to as nonhost resistance. To avoid PAMP-triggered immunity and DAMP-triggered immunity, pathogens are equipped with specific effectors that are tailored to suppress the plant's immune response. These virulence effectors are injected into the host cytoplasm, where they can then, in turn, be recognized by cytoplasmic

receptor proteins, which results in effector-triggered immunity (ETI) (3). The intracellular recognition of effector molecules is mediated by structurally related proteins belonging to the nucleotide-binding site-leucine-rich repeat (NBS-LRR) family (4). ETI forms the second tier of the plant immune response. In contrast to nonhost resistance, the ETI response against adapted pathogens is much stronger and often results in the death of the infected cell through hypersensitive reaction. Hence, there is a strong selective pressure on the pathogen to avoid ETI by evolving new virulence effectors that escape recognition by NBS-LRR immune receptors. This coevolutionary arms race is the major reason for rapid breakdown of NBS-LRR-based disease resistance in crop plants, which often occurs within only a few years (5, 6).

Cell surface PRRs are encoded by receptor-like kinase (RLK) and receptor-like protein (RLP) genes. Both RLKs and RLPs contain an extracellular elicitor-binding domain and a transmembrane domain. In contrast to RLKs, RLPs lack a cytoplasmic serine/threonine kinase domain (7). The best-studied PRR is the leucine-rich repeat (LRR) receptor-like kinase flagellin-sensitive-2 (FLS2) of *Arabidopsis*, which binds an epitope of bacterial flagellin. On flagellin perception, this kinase initiates a signaling cascade that results in nonhost resistance (8). Most PRRs described until today contain such an extracellular LRR

## Significance

Northern corn leaf blight (NCLB) is one of the most devastating fungal diseases of maize. The *Htn1* disease resistance gene confers quantitative field resistance against most NCLB isolates. Here we show that *Htn1* encodes a putative wall-associated receptor-like kinase (RLK). RLKs act as important components of the first tier of the plant innate immune system by perceiving pathogen- or host-derived elicitors on the cell surface. RLKs are often associated with resistance to nonadapted pathogens and are a component of nonhost resistance. Our work demonstrates that the *Htn1*-RLK plays an important role in host resistance against adapted fungal pathogens.

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domain. Not all PRRs, however, are involved in nonhost resistance. The rice LRR-RLK *Xa21*, for example, confers race-specific resistance against the bacterial rice blast pathogen *Xanthomonas oryzae* pv. *oryzae* (9). The effector recognized by *Xa21* is still unknown. In addition, plants have evolved the ability to perceive endogenous molecules through PRRs that are produced during pathogen infection. For example, the *Arabidopsis* wall-associated kinase 1 (WAK1) binds cell wall-derived oligogalacturonides that are released during pathogen infection and serve as DAMPs (10). AtDORN1, an *Arabidopsis* lectin-RLK, binds plant-derived extracellular ATP likely produced during pathogen infection or wounding (11).

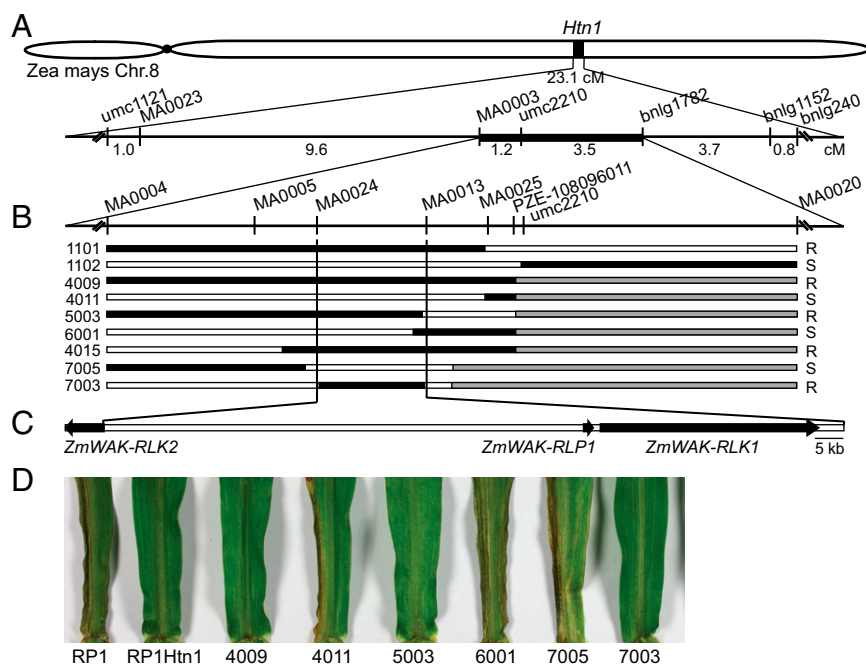
Maize (*Zea mays* L.) is the most widely grown crop in the world and represents an important source of food, feed, biofuel, and industrial products. Fungal diseases are a major threat to maize production and can result in severe crop losses. Northern corn leaf blight (NCLB) is the most devastating foliar disease of maize. It is caused by the hemibiotrophic ascomycete fungus *Exserohilum turcicum* (teleomorph *Setosphaeria turcica*) (12). The fungus spreads biotrophically during the initial infection process before switching to a necrotrophic lifestyle. Infections manifest as local lesions and necrosis, which lead to reduced photosynthetically active leaf area and yield losses. The disease occurs prevalently under conditions of high humidity and moderate temperatures and can be found in most regions where maize is grown (13–15). The *Htn1* locus confers quantitative and partial resistance against NCLB by delaying lesion formation. *Htn1* was originally introgressed into modern maize cultivars from the Mexican landrace Pepitilla in the 1970s (16). The *Htn1* resistance reaction is different from the other known major NCLB resistance genes *Ht1*, *Ht2*, and *Ht3*, which confer qualitative resistance, resulting in chlorotic-necrotic lesions. In contrast, *Htn1* leads to a delay of sporulation without chlorotic

lesions (17, 18). The dominant and race-specific *Htn1* gene is effective against the most prevalent NCLB races, but virulent isolates have been found (18). The strength of the *Htn1* resistance depends on environmental conditions and maize genotype (19). *Htn1* has been mapped to maize chromosome 8 ~10 cM distal to the NCLB resistance gene *Ht2* (20–23).

Here, we describe the map-based isolation of the *Htn1* gene, which encodes for a pattern recognition receptor with a putative extracellular, wall-associated domain.

## Results and Discussion

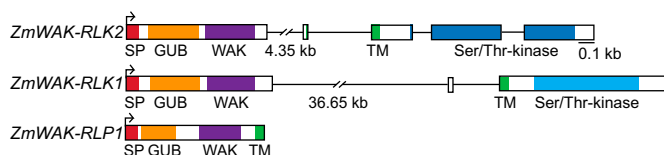
**High-Resolution Genetic Mapping of *Htn1*.** The *Htn1* locus from the Mexican landrace Pepitilla was introgressed into the NCLB susceptible maize line B37. B37Htn1 (17) served as the donor to develop three pairs of near isogenic lines for map-based isolation of *Htn1*. The three recurrent parents RP1, RP3, and RP4 were moderately to highly susceptible to NCLB in the field (*SI Appendix*, Tables S1 and S2). All three *Htn1* introgression lines, RP1Htn1, RP3Htn1, and RP4Htn1, were more resistant than their respective recurrent parent and only developed few and small lesions typical for the effect of *Htn1*. Using six microsatellite markers and two single nucleotide polymorphism (SNP) markers, *Htn1* was initially mapped at low resolution to a 23.1-cM interval on the short arm of chromosome 8 in a F2 population consisting of 528 progeny of a cross between the resistant parent RP1Htn1 and the susceptible parent RP1 (Fig. 1A and *SI Appendix*, Fig. S1). In parallel, we established a NCLB greenhouse seedling infection assay and phenotyped an additional 262 F2 progeny of the same cross. By including additional markers, we reduced the *Htn1* region to a 4.7-cM target interval flanked by SNP marker MA0003 and microsatellite marker bnlg1782. Microsatellite marker umc2210 cosegregated with *Htn1* (Fig. 1A).



**Fig. 1.** Map-based isolation of *Htn1*. (A) Initial mapping located *Htn1* to a 4.7-cM region on the long arm of maize chromosome 8. (B) High-resolution mapping delimited *Htn1* to a 131.7-kb physical interval flanked by SNP markers MA0024 and MA0013. Recombination breakpoints for critical recombinants are shown. Black segments represent the resistant RP1Htn1 genotype, and white segments the susceptible RP1 genotype. Gray segments indicate regions that were not tested with markers. Only critical markers around the recombination breakpoints are indicated for simplicity. Phenotypic scores (R = resistant, S = susceptible) are indicated on the right. (C) The *Htn1* target interval contains three predicted candidate genes: *ZmWAK-RLK2*, *ZmWAK-RLP1*, and *ZmWAK-RLK1*. (D) Leaves of critical recombinants and parents of the mapping population infected with *E. turcicum* in a seedling greenhouse assay. Infections can be seen as black sporulating lesions on the leaves.

For this flanking interval, a 1.3-Mb physical scaffold was established and sequenced, using a BAC library of the resistant parent RP4Htn1. Sequence conservation of the 1.3-Mb RP4Htn1 scaffold with the B73 reference genome sequence (24) was rather weak across the entire region, and gene content differed between the two lines (*SI Appendix, Figs. S2 and S3A*). The RP4Htn1 sequences were then used to saturate the target interval with SNP markers. To increase recombination events in the target region, heterozygous F2 progeny plants of the RP1 × RP1Htn1 population were backcrossed to RP1 and selfed. New recombinants were phenotyped in the field and in the greenhouse. This strategy allowed us to ultimately delimit *Htn1* to a 131.7-kb physical interval flanked by SNP markers MA0024 and MA0013 (Fig. 1 *B* and *D*). The region contained three putative candidate genes in the RP4Htn1 sequences: two wall-associated receptor-like kinases (*ZmWAK-RLK1* and *ZmWAK-RLK2*) and one wall-associated receptor-like protein that lacked a cytoplasmic kinase domain (*ZmWAK-RLP1*; Fig. 1*C*). Genes encoding WAK-RLKs and WAK-RLPs are often found as clusters in plant genomes (25). The left-flanking marker MA0024 was located in the 5' region of the *ZmWAK-RLK2* coding sequence, 296 bp downstream of the start codon. One critical recombinant, line 7003, had the susceptible allele for MA0024 but was clearly resistant in the seedling NCLB infection assay (Fig. 1 *C* and *D*), thereby excluding the sequence downstream of nucleotide 296 in *ZmWAK-RLK2* as a *Htn1* candidate region. We still considered *ZmWAK-RLK2* a candidate gene because we could not exclude that the recombination in line 7003 occurred in the promoter region or the first 296 bp of this gene. Recombinants 5003 and 6001 excluded the region to the right of *ZmWAK-RLK1* (Fig. 1 *C* and *D*), resulting in the three candidate genes mentioned earlier.

**Characterization of the Three Candidate Genes Found in the *Htn1* Target Interval.** All three candidate genes were expressed in line RP1Htn1 and have ORFs possibly encoding functional proteins. A conserved domain search revealed high structural similarity among the three predicted proteins. Their N-terminal, putatively extracellular domain contained a cysteine-rich galacturonan-binding domain (GUB-WAK\_bind; pfam13947), followed by a wall-associated receptor kinase domain (WAK\_assoc domain; pfam14380). These two cysteine-rich domains are characteristic for wall-associated kinases (26). Furthermore, the three proteins contained a predicted transmembrane domain (Fig. 2). The coding sequence of *ZmWAK-RLP1* consists of a single, 878-bp exon. The predicted protein lacks a cytoplasmic kinase domain, which classifies *ZmWAK-RLP1* as a receptor-like protein. Receptor-like proteins are thought to interact with other kinases for signaling (11). The 7,162-bp *ZmWAK-RLK2* gene has five exons and is located in reverse orientation compared with the other two genes (Fig. 1*C*). The genomic sequence of *ZmWAK-RLK1* is nearly 39 kb in size (Fig. 2). This massive increase in gene size is

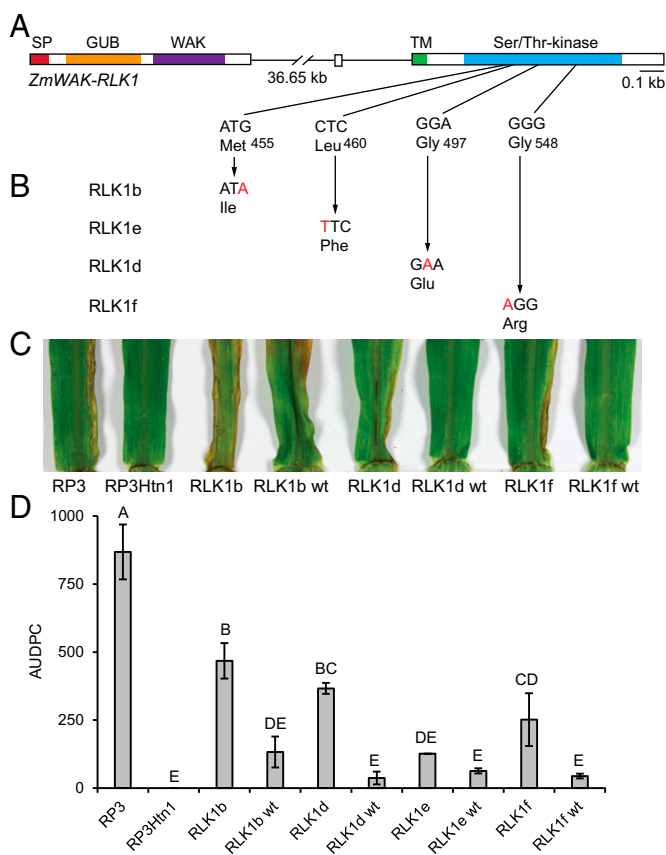


**Fig. 2.** Gene structure and protein domain prediction of the three candidate genes, *ZmWAK-RLK2*, *ZmWAK-RLK1*, and *ZmWAK-RLP1*, from the resistant *Htn1* locus. Predicted domains are in color. Exons are represented as rectangular bars, and introns as black lines between the exons. GUB (GUB-WAK), wall-associated receptor kinase galacturonan-binding; Ser/Thr-kinase, catalytic domain of the serine/threonine kinases and interleukin 1 receptor-associated kinases; SP, signal peptide; TM, transmembrane domain; WAK (WAK\_assoc), wall-associated receptor kinase C-terminal. Dark blue, RD-kinase; bright blue, non-RD kinase.

a result of the insertions of several transposons in the first intron (*SI Appendix, Fig. S3B*). cDNA amplification showed that this intron was correctly spliced, despite its large size. In addition to the extracellular and transmembrane domain, *ZmWAK-RLK1* and *ZmWAK-RLK2* contain a predicted cytoplasmic serine/threonine protein kinase domain at the C terminus. Receptor-like kinases can be further divided into RD and non-RD kinases, depending on the presence or absence of an arginine residue at the catalytic site of the kinase domain. *ZmWAK-RLK2* contains an RD kinase and *ZmWAK-RLK1* has a non-RD kinase domain (Fig. 2). Most receptor-like kinases involved in plant immunity identified so far belong to the non-RD kinases, whereas RD kinases are thought to play a role in other processes such as development (27). Interestingly, although the kinase domains of the two candidate proteins only showed 27% amino acid identity, their extracellular domains were highly similar (86% amino acid identity), indicating a recent gene conversion event. Despite having the same domain features, *ZmWAK-RLP1* only showed weak sequence conservation to the extracellular domains of the two *ZmWAK-RLKs*. Compared with dicotyledonous plants, the WAK gene family has considerably expanded in cereals. Although *Arabidopsis* contains 26 WAK and WAK-like genes, more than 100 WAK genes have been identified in the genomes of maize and rice (28, 29). A Southern blot analysis with probes derived from the conserved extracellular domains of *ZmWAK-RLK1* and *ZmWAK-RLK2* revealed that there is no additional gene with a similar wall-associated kinase domain in the genomes of RP1Htn1 and RP1 (*SI Appendix, Fig. S4*). Despite the large number of WAK genes in the maize genome, the extracellular domains of *ZmWAK-RLK1* and *ZmWAK-RLK2* are therefore unique.

On the basis of our mapping data and the protein structure, we considered the non-RD *ZmWAK-RLK1* as the best candidate gene for *Htn1*. A similar target interval for NCLB resistance on chromosome 8 has been identified in maize line PH99N. The PH99N NCLB interval contains a homolog of *ZmWAK-RLK1* with identical predicted cDNA sequence but different intron sequences (patent international publication number WO 2011163590 A1). However, no functional candidate gene validation is described in this patent application.

**Analysis of TILLING Mutants for Functional Identification of *Htn1*.** For functional validation of the three candidate genes, we developed a targeting induced local lesions in genomes (TILLING) population consisting of 10,000 plants of the resistant parent RP3Htn1. The TILLING population was screened with PCR primers targeting the transmembrane and kinase domains of *ZmWAK-RLK1* and *ZmWAK-RLK2* (*SI Appendix, Table S3*). Four mutant lines for *ZmWAK-RLK1* and three mutants for *ZmWAK-RLK2* were identified, all of them containing SNPs that resulted in amino acid substitutions (Fig. 3 *A* and *B* and *SI Appendix, Table S4*). Mutant lines, along with their respective segregating wild-type sister lines, were tested for resistance to NCLB in greenhouse infection tests. The TILLING parent RP3Htn1 was completely resistant. The three mutants in *ZmWAK-RLK2*, RLK2b, RLK2d, and RLK2e, showed no increase in susceptibility compared with RP3Htn1 and their corresponding wild-type segregants (*SI Appendix, Fig. S5*). In contrast, three of the four mutant lines in *ZmWAK-RLK1* (mutants RLK1b, RLK1d, and RLK1f) were more susceptible than line RP3Htn1 and the corresponding wild-type segregants (Fig. 3 *C* and *D*). One *ZmWAK-RLK1* mutant, RLK1e, showed wild-type levels of disease resistance (Fig. 3*D*). Identical results were obtained in field infection tests. Again, the *ZmWAK-RLK1* mutant lines RLK1b, RLK1d, and RLK1f were more susceptible than wild-type (*SI Appendix, Fig. S6*). These results demonstrate that the NCLB disease resistance in the TILLING parent RP3Htn1 is conferred by the wall-associated kinase gene *ZmWAK-RLK1*. The coding



**Fig. 3.** *ZmWAK-RLK1* mutant plants are more susceptible to NCLB than wild-type segregants. (A) Gene structure of *ZmWAK-RLK1*. (B) Nucleotide and resulting amino acid changes in the four *ZmWAK-RLK1* mutants. (C) Representative images of the second leaves of maize plants 21 d post-infection with *E. turcicum* in the greenhouse. Necrotic, sporulating NCLB areas are spreading from the leaf base toward the leaf apex along leaf borders in the susceptible control line RP3 and in three of the four *ZmWAK-RLK1* mutant lines (RLK1b, RLK1d, and RLK1f). The TILLING parent RP3Htn1 and the wild-type segregants (RLK1b wt, RLK1d wt, and RLK1f wt) are free of disease. (D) Area under the disease progress curve values of the four *ZmWAK-RLK1* mutants and wild-type. Values represent means of two to three biological replicates, and error bars represent SEs. Different letters on top of bars denote significant differences in area under the disease progress curve values (Student's *t* test,  $P < 0.05$ ).

sequence of *ZmWAK-RLK1* was identical in the three near-isogenic lines, RP1Htn1, RP3Htn1, and RP4Htn1, used for map-based cloning and in the two independent *Htn1* introgression lines B37Htn1 and W22Htn1 (16). Marker analysis showed that all *Htn1* introgression lines carry the same haplotype in the target interval (SI Appendix, Table S5). The NCLB resistance gene *Ht2* also maps on maize chromosome 8 to a similar interval to that of *Htn1* (16, 20–23). We amplified the *ZmWAK-RLK1* homolog from the *Ht2* introgression line B37Ht2. The gene is different from the one found in the *Htn1* near-isogenic lines, showing only 79% amino acid identity in the extracellular domain. In addition, the *E. turcicum* isolate used in this study is avirulent on B37Htn1 and B37Ht3, but virulent on B37, B37Ht1, and B37Ht2, therefore revealing a difference of B37Htn1 and B37Ht2 in resistance reaction to *E. turcicum*. Therefore, we conclude that the *ZmWAK-RLK1* gene identified in this study indeed represents *Htn1*.

**Comparison of *ZmWAK-RLK1* Sequence and Gene Expression Between RP1Htn1 and RP1.** Both the resistant line RP1Htn1 and the susceptible recurrent parent RP1 contain an expressed *ZmWAK-RLK1* allele.

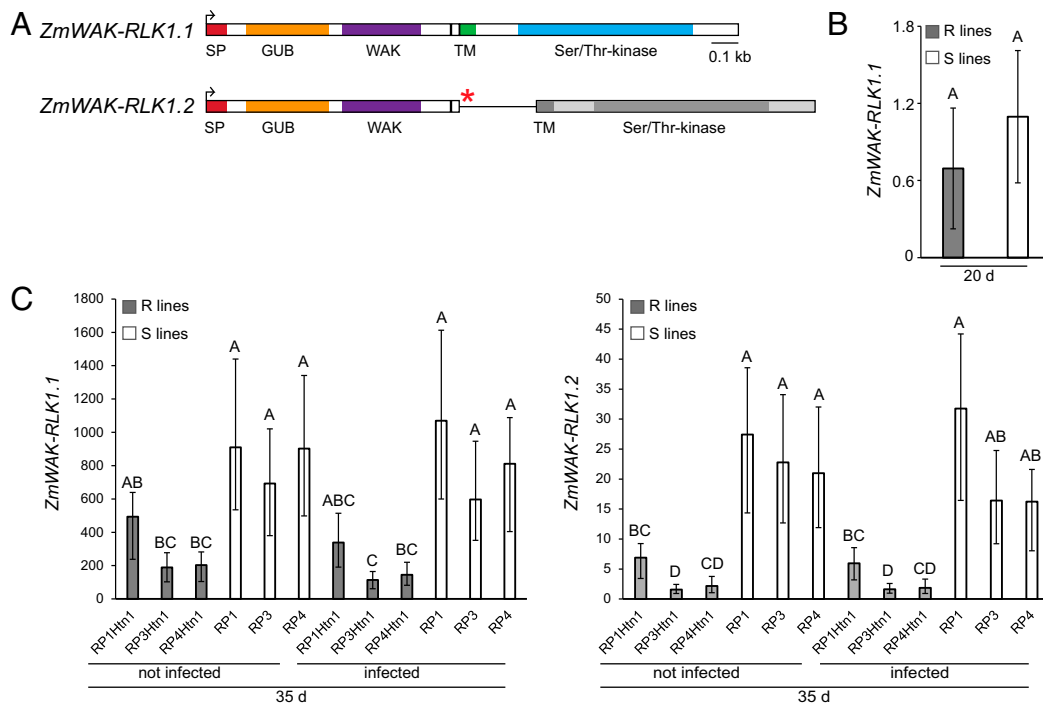
The predicted amino acid sequence of the extracellular WAK domain was highly divergent between the two proteins (SI Appendix, Figs. S3C and S7). The *ZmWAK-RLK1* protein version of the susceptible parent RP1 showed 46 amino acid changes, 19 amino acid deletions, and 2 amino acid insertions in the extracellular domain compared with in the resistant protein version of RP1Htn1, resulting in an amino acid identity of only 79%. The kinase domain of *ZmWAK-RLK1*, in contrast, was 98.5% identical between the two near-isogenic lines. In the B73 reference genome, multiple sequences showed homology to the *ZmWAK-RLK1* gene. However, none of the sequences comprised a complete gene with the same structure as *ZmWAK-RLK1* (SI Appendix, Fig. S3B). The closest homolog of *ZmWAK-RLK1* in maize variety B73 is gene *GRMZM2G164612*. Homology was restricted to the kinase domain, and the extracellular domain was only weakly conserved. The *ZmWAK-RLK1* ortholog in rice (LOC\_Os01g49614) showed 83% amino acid identity in the kinase domain but only 49% amino acid identity in the wall-associated kinase domain. These sequence comparisons indicate that the extracellular domain of *ZmWAK-RLK1* is highly diverse among different maize genotypes and is not conserved in closely related species such as rice.

Full-length cDNA amplification from the susceptible line RP1 revealed an alternative splice variant of *ZmWAK-RLK1* that retained the second intron (*ZmWAK-RLK1.2*; Fig. 4A). This results in a premature stop codon, which would lead to a truncated protein version that only contained the extracellular domain, not the transmembrane and kinase domains. Expression analysis of the correctly spliced *ZmWAK-RLK1.1* variant showed no difference in transcript level between susceptible RP1 and resistant RP1Htn1 parents in 20-d-old seedlings without infection (Fig. 4B). Surprisingly, *ZmWAK-RLK1.1* expression was 1.8–5.6 times higher in the three susceptible lines, RP1, RP3, and RP4, in both infected and uninfected 35-d-old seedlings compared with in the three *Htn1* introgression lines, RP1Htn1, RP3Htn1, and RP4Htn1 (Fig. 4C). Infection did not induce *ZmWAK-RLK1.1* expression in either of the parents (Fig. 4C). Only trace amounts of the alternatively spliced variant *ZmWAK-RLK1.2* were detectable in the resistant parents, RP1Htn1, RP3Htn1, and RP4Htn1 (Fig. 4C). In comparison, the susceptible parents, RP1, RP3, and RP4, showed a 4.0–14.4 times higher expression of *ZmWAK-RLK1.2* in both infected and uninfected 35-d-old seedlings (Fig. 4C). The correctly spliced *ZmWAK-RLK1.1* transcript variant was 57–120 times and 30–50 times higher expressed compared with the incorrectly spliced transcript *ZmWAK-RLK1.2* in the resistant and susceptible lines, respectively.

We therefore consider it unlikely that these low levels of alternative *ZmWAK-RLK1.2* transcript have an influence on the *Htn1* resistance phenotype. It is more plausible that the amino acid differences found in *ZmWAK-RLK1* between resistant and susceptible maize lines explain the difference in resistance phenotype.

## Conclusion

In this study, we identified a wall-associated receptor-like kinase as the *Htn1* NCLB disease resistance gene. Wall-associated kinases have been found to play a role in disease resistance (30, 31), phosphorus-starvation tolerance (32), and developmental processes such as root growth (33) and gametophyte development (34). A recent study reported that the maize quantitative head smut resistance gene *qHSR1* also encodes a wall-associated kinase. *Htn1* and *qHSR1* share structural homology, but their extracellular domains have no sequence identity detectable by BLAST (28). Hence, these two genes represent very diverse members of the same protein superfamily that are involved in quantitative resistance against different fungal pathogens. Wall-associated non-RD kinases might therefore represent a novel



**Fig. 4.** Expression analysis of the two *ZmWAK-RLK1* splice variants. (A) cDNA structure of the two splice variants *ZmWAK-RLK1.1* and *ZmWAK-RLK1.2*. Exons are represented as rectangular bars, and the retained intron two in *ZmWAK-RLK1.2* as a black line. Predicted protein domains are indicated in color. The red asterisk indicates the premature stop codon. (B and C) Relative *ZmWAK-RLK1* expression levels. (B) *ZmWAK-RLK1.1* in resistant lines (R lines; mix of line RP1Htn1 and three resistant recombinants) and susceptible lines (S lines; mix of RP1 and two susceptible recombinants) of 20-d-old seedlings and (C) *ZmWAK-RLK1.1* (Left) and *ZmWAK-RLK1.2* (Right) in resistant and susceptible mapping parents of 35-d-old seedlings, either noninfected or after *E. turcicum* infection. Mean (B) and untransformed mean (C) values are presented, and the 95% confidence intervals (back-transformed) are plotted. On top of the bars, different letters denote a significant difference in expression level (Tukey's honestly significant difference test,  $\alpha = 0.050$ ).

class of quantitative immune receptors in monocots. Wall-associated kinases are the only known proteins that can physically link the cell wall to the plasma membrane, which enables them to perceive changes to the cell wall structure (10, 35). WAKs can therefore serve as DAMP receptors that recognize changes to the cell wall during pathogen entry. It is therefore possible that *Htn1* perceives cell wall fragments generated during fungal penetration. In contrast, the high cultivar-specific diversity of the extracellular *ZmWAK-RLK1* domain could also indicate that this protein is involved in the perception of a microbial elicitor. These results provide the basis to study the molecular function of a novel pattern recognition receptor involved in plant immunity against an adapted fungal pathogen.

## Materials and Methods

**Plant Material.** Line B37Htn1 (17), carrying the *Htn1* locus from the Mexican landrace Pepitilla, served as donor for the *Htn1* introgression into three recurrent parents from the German maize breeding program of KWS SAAT SE, RP1, RP3, and RP4. Introgressions were enriched for the recurrent genome by backcrossing three times to the recurrent parent and by checking for the presence of the *Htn1* locus with flanking markers. For fine mapping, an  $F_2$ -population was generated from the cross RP1  $\times$  RP1Htn1. The following generations were obtained by selfing and were genotyped and phenotyped at each selfing step. The genotyping was done in a first step with simple sequence repeat markers, but during the process of selfing and recombinant selection, new SNP markers were established as Kompetitive Allele Specific PCR-Assays markers for the region. The phenotyping was done at different field sites with two replications. Recombinant plants for the *Htn1* region were selected and correlated with the phenotypic observations. The selection incorporated plants that had different segments of the target region, as well as heterozygous plants, to get new recombinants in the region. Each year, two generations of selfings lead to new recombinants. These new recombinants were phenotyped in the field and in the greenhouse. The results were then correlated with the genotypic data.

Maize lines B37, B37Htn (B37Htn1), B37Ht1, B37Ht2, B37Ht3, W22, and W22Htn1 were obtained from the Maize Genetics Cooperation Stock Center ([maizecoop.cropsci.uiuc.edu](http://maizecoop.cropsci.uiuc.edu/)) and from KWS SAAT SE.

**NCLB Field Infection Tests.** At each location, 20 kernels per row were sown for each genotype. The artificial inoculation was done with crushed infected leaf material, which was collected the year before at the same location. This method allowed for a comparable disease pressure from year to year at different locations. As a control, the near isogenic parents of the population were also planted at all locations. Infection rates were scored as described in *SI Appendix, Table S1*.

**Maize Infection with *E. turcicum* in the Greenhouse.** To generate an *E. turcicum* spore suspension for infection, a single spore was isolated from dried leaf material infected with NCLB. The dried leaf material was collected from infected maize plants in Passau in 2005. This isolated spore was propagated on potato dextrose agar (Difco, 213400) plates. The isolate was further propagated for infection tests by inoculating fresh plates with a small piece of mycelium with an inoculation needle. Plates were incubated at room temperature in the dark, upside down, for approximately 3 wk. Spores were harvested by pouring 0.1% Tween into the plates and removing spores by scraping, followed by pouring the spore suspension through a fine mesh. The spore solution was adjusted to a final concentration of  $4.5 \times 10^3$  spores/mL by counting spores in a Neubauer chamber.

Maize plants were grown each in Jiffy pots ( $\phi$  8 cm) filled with standard soil (Einheitserde Classic ED 73 + BIMS). Fifteen plants of the same maize line were placed in one tray. Plants were grown in the greenhouse at cycles of 16 h at 20 °C with light and 8 h at 18 °C in the dark and around 60% relative humidity. After the second leaf had fully emerged, the newly emerging leaves were cut and removed until the end of the experiment. Maize plants were infected at 19–21 d after sowing, and a second time 1 or 2 d after the first inoculation. At both infection points, a total of 100  $\mu$ L freshly made spore suspension ( $4.5 \times 10^4$  spores/mL) was distributed into the leaf sheath of the second leaf and into the stem whorl. Alternatively, first infection tests with recombinants were made by placing a small piece of dried leaf material infected with NCLB (field Passau 2005) on the base of the second leaf. Plastic

hoods were placed on top of each tray after the first infection to keep humidity very high. Disease symptoms were evaluated between 11 and 25 d after infection. For each evaluated day and plant line, disease severity was calculated as the percentage of infected plants. From this, we calculated the area under the disease progress curve by the trapezoidal method (36).

A greenhouse infection test with lines B37, B37Htn1, B37Ht1, B37Ht2, and B37Ht3 classified our single spore isolate as race 12 (37).

**Map-Based Cloning of *Htn1*.** Detailed descriptions of materials and methods for BAC library screening, marker development for genetic mapping and development, and screening of a TILLING mutant population are provided in *SI Appendix, Materials and Methods* and *SI Appendix, Tables S6 and S7*.

**Candidate Gene Characterization.** The proteins were analyzed with the National Center for Biotechnology Information conserved domain search tool ([www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi](http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi)). In all three proteins, a signal peptide was predicted with the SignalP 4.0 Server ([www.cbs.dtu.dk/services/SignalP/](http://www.cbs.dtu.dk/services/SignalP/)) at the aminoterminal end and a transmembrane domain with DAS ([www.sbc.su.se/~miklos/DAS](http://www.sbc.su.se/~miklos/DAS)), HMMTOP ([www.enzim.hu/hmmtop](http://www.enzim.hu/hmmtop)), and TMPred ([www.ch.embnet.org/software/TMPRED\\_form.html](http://www.ch.embnet.org/software/TMPRED_form.html)).

**Quantitative Real-Time PCR Analysis for Detection of *ZmWAK-RLK1* Expression.** Expression of *ZmWAK-RLK1* was quantified in a reverse transcription, quantitative real-time PCR (RT-qPCR) assay, using a CFX96 Real-Time System C1000TM Thermal cycler (Bio-Rad). Leaf material was harvested from 20-d-old plants just before infection and 15 d later with *E. turcicum*-infected plants, as well as noninfected plants. RNA extraction was performed as de-

scribed by Hurni et al. (38), and first-strand cDNA was synthesized from 0.5  $\mu$ g RNA, using 1/2 reaction of the iScript Advanced cDNA Kit (172-5038, Bio-Rad). RT-qPCR primers and master mixes used for the targets *ZmWAK-RLK1.1* and *ZmWAK-RLK1.2* and the reference gene *FPG5* are described in *SI Appendix, Table S8*. RT-qPCR was performed with 4  $\mu$ L of 10-fold-diluted cDNA for 20-d-old plants and with 4  $\mu$ L of 20-fold-diluted cDNA for 35-d-old plants in a total reaction volume of 10  $\mu$ L. Technical duplicates were made for 20-d-old plants, and technical triplicates for five biological replicates of 35-d-old plants. Thermocycling conditions were 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s, then 60 °C for 20 s (5 s for *FPG5*). Specificities of amplicons, RT-minus control check, and efficiency calculation were performed as described in Hurni et al. (38). To allow comparison of the expression levels between the two splice variants *ZmWAK-RLK1.1* and *ZmWAK-RLK1.2*, the RT-qPCR data were calibrated on the basis of plasmid DNA containing the *ZmWAK-RLK1.1* or *ZmWAK-RLK1.2* construct, respectively. Relative quantities were calculated and normalized to the reference genes *FPG5* revealing the calibrated normalized relative quantities (CNRQ) values, using the program qbase+ V 3.0 (Biogazelle).

Data analysis was performed using the statistical package JMP version 11.0 (SAS Institute). In Fig. 4B, Tukey's honestly significant difference test was done on  $\log_{10}$  transformed expression values. The untransformed means are given in columns, and back-transformed 95% confidence intervals are plotted.

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