Fungal resistance mediated by maize wall-associated kinase ZmWAK-RLK1 is associated with the reduction of secondary metabolite benzoxazinoids

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26 **Summary**

27 - Wall associated kinases (WAKs) have recently been identified as major components of fungal
28 and bacterial disease resistance in several cereal crop species. However, the molecular
29 mechanisms of WAK-mediated resistance remain largely unknown.
30 - Here, we investigated the function of the maize gene *ZmWAK-RLK1* (*Htn1*) that confers
31 quantitative resistance to northern corn leaf blight (NCLB) caused by the hemibiotrophic fungal
32 pathogen *Exserohilum turcicum*.
33 - ZmWAK-RLK1 was found to localize to the plasma membrane and its presence resulted in a
34 modification of the infection process by reducing pathogen penetration into host tissues. A
35 transcriptome analysis of near-isogenic lines (NILs) differing for *ZmWAK-RLK1* revealed that a
36 large number of genes were differentially expressed in the presence of *ZmWAK-RLK1*, including
37 several genes involved in the biosynthesis of the secondary metabolites benzoxazinoids (BXDs).
38 The contents of BXDs such as DIMBOA-Glc, DIMBOA, HMBOA-Glc and DIM_2BOA-Glc were
39 significantly lower in the NILs with *ZmWAK-RLK1*. Maize mutants that were affected in overall
40 BXDs biosynthesis showed increased resistance to *E. turcicum* infection. Furthermore, DIM_2BOA-
41 Glc concentration was significantly elevated in *ZmWAK-RLK1* mutants with compromised NCLB
42 resistance. In agreement with this observation of higher DIM_2BOA-Glc level in NCLB
43 susceptibility, a mutant in the *bx13* gene which affects biosynthesis of DIM_2BOA-Glc showed
44 NCLB resistance.
45 - We conclude that *Htn1*-mediated NCLB resistance is associated with a reduction of BXD
46 secondary metabolites. These findings suggest a link between WAK-mediated quantitative
47 disease resistance and changes in biochemical fluxes starting with indole-3-glycerol phosphate.

48 **Key words**: Wall-associated kinase, receptor-like kinase, benzoxazinoids (BXDs), maize disease
49 resistance, *Htn1*

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51

52 **Introduction**

53 Plants have evolved multiple layers of defense against infection by pathogenic microbes (Jones &
54 Dangl, 2006; Krattinger & Keller, 2016). The primary defense is based on the extracellular perception
55 of pathogen-derived or host damage-derived signatures (PAMPs/DAMPs) by plasma membrane-
56 localized receptors. These signatures can be highly conserved and characteristic for entire pathogen
57 classes as in the case of the bacterial flagellin that is perceived by the leucine-rich repeat receptor
58 kinase (LRR-RK) FLS2, which results in basal and broad-spectrum resistance against most bacteria
59 (Dardick *et al.*, 2012; Macho & Zipfel, 2014). Other receptor kinases only confer resistance to certain
60 races of a particular pathogen (Hu *et al.*, 2017). Receptor kinases have different types of extracellular
61 domains, including leucine-rich repeats, lysine motifs, lectin motifs or epidermal growth factor like
62 extracellular domains (Gomez-Gomez & Boller, 2000; Dardick *et al.*, 2012; Macho & Zipfel, 2014). The
63 wall-associated kinases (WAKs) contain a cell wall-associated galacturonan-binding domain
64 (Kanneganti & Gupta, 2008). In grasses, there is emerging evidence that WAKs are important players
65 in fungal and bacterial disease resistance. The WAK genes *ZmWAK* (*qHSR1*), *ZmWAK-RLK1* (*Htn1*) and
66 *OsWAK* (*Xa4*) confer disease resistance against maize head smut, maize northern corn leaf blight
67 (NCLB) and rice bacterial blight, respectively (Hurni *et al.*, 2015; Zuo *et al.*, 2015; Hu *et al.*, 2017).
68 *WAK*-mediated resistance involves strengthening of the cell wall intensity by enhancing cellulose
69 biosynthesis and the biosynthesis of phytoalexin (Hu *et al.*, 2017), oxidative burst (Delteil *et al.*,
70 2016), and defense gene expression (Zuo *et al.*, 2015). Interestingly, there is also one case described
71 where the wheat WAK encoded by the *Snn1* gene acts as a susceptibility factor. It has been shown
72 that Snn1 perceives the SnTox1 toxin encoded by the fungal pathogen *Parastagonospora nodorum*,
73 which triggers cell death and allows the necrotrophic *P. nodorum pathogen* to proliferate on wheat
74 (Shi *et al.*, 2016). In dicots, the Arabidopsis AtWAK1 was found to physically associate with and to
75 recognize cell wall-derived oligogalactouronides, which result from polysaccharide degradation
76 (Decreux *et al.*, 2006; Brutus *et al.*, 2010).

77 Indole-3-glycerol phosphate (IGP) is an important metabolite and serves as a branch point compound
78 in the Trp-independent biosynthesis of the plant auxin indole-3-acetic acid (IAA) and the biosynthesis
79 of defense-related benzoxazinoids (BXDs) (Frey *et al.*, 2000; Di *et al.*, 2016). Benzoxazinoids (BXDs)
80 are a class of secondary metabolites found in maize and other cereal species that contain the 2-
81 hydroxy-2H-1,4-benzoxazin-3(4H)-one skeleton (Niemeyer, 2009; Wouters *et al.*, 2016). The
82 biosynthesis of BXDs is mostly under developmental control (Kohler *et al.*, 2015). The first step of
83 BXDs synthesis is based on the formation of indole derived from indole-3-glycerol phosphate (IGP),
84 converted by indole-glycerolphosphate lyase BX1 (Frey *et al.*, 2000). In addition to BX1, its homolog
85 IGL can convert IGP into free indole (Frey *et al.*, 2000; Niemeyer, 2009). BXD metabolism largely
86 depends on the content of indole and *Igl* can contribute up to 10% of total BXDs levels (Frey *et al.*,

87 2000). After several successive steps of oxidation and methylation, predominantly inactive glucosides
88 are produced and stored as vacuolar BXDs (*e.g*. DIMBOA-Glc). Upon biotic stress they are hydrolyzed
89 to the respective toxic active hydroxamic acids (e.g. DIMBOA) (Niemeyer, 2009; Wouters *et al.*,
90 2016). These compounds are known to be involved in defense against aphids, phloem-feeding
91 herbivores and other pests (Niemeyer, 2009), for instance the European corn borer *Ostrinia nubilalis*
92 and the cereal aphid *Rhopalosiphum padi* (Houseman *et al.*, 1992; Ahmad *et al.*, 2011). However,
93 certain herbivores can circumvent benzoxazinoid toxicity and use BXDs as foraging cues (Robert *et*
94 *al.*, 2012; Kohler *et al.*, 2015; Wouters *et al.*, 2016).

95 While the role of BXDs in insect resistance is quite well studied, the role of these compounds in
96 fungal disease resistance remains unclear. Several field studies proposed a relationship between
97 active benzoxazinoid hydroxamic acid DIMBOA and the disease resistance to maize stalk rot, maize
98 NCLB and wheat stem rust (Elnaghy & Pekka, 1962; Long *et al.*, 1978; Kostandi *et al.*, 1981). Induction
99 of DIMBOA accumulation possibly associated to the increased sheath blight disease caused by
100 *Rhizoctonia solani* (Song *et al.*, 2011). However, other studies found no effect of BXDs on fungal
101 disease resistance, including maize stalk rot, southern corn leaf blight, maize anthracnose, corn smut
102 and head blight (Niemeyer, 2009). Recently, *E. turcicum,* the causal agent of NCLB, has been shown
103 to elicit apoplastic BXDs accumulation at the early infection stages (Ahmad *et al.*, 2011), which
104 suggested a link between BXDs and *E. turcicum* infection. This study suggested an inhibition of
105 penetration success however did not show whether BXDs contribute to increased disease resistance
106 or susceptibility.

107 We recently isolated by map-based cloning the maize *Htn1* gene that encodes a putative wall-
108 associated receptor-like kinase (Hurni *et al.*, 2015). Unlike the dominant gene *Ht1* that causes
109 hypersensitive response-like chlorotic lesions (Welz & Geiger, 2000), *Htn1* confers quantitative and
110 partial resistance against NCLB by delaying lesion formation and sporulation (Hurni *et al.*, 2015). In
111 this study, we investigated the molecular basis of quantitative NCLB resistance conferred by *ZmWAK-*
112 *RLK1* (*Htn1*). We provide evidence that NCLB resistance caused by *ZmWAK-RLK1* is associated with a
113 reduction of secondary metabolite benzoxazinoids, a biochemical pathway of IGP metabolism.

114 **Materials and Methods**

115 **Plant material and growth conditions**

116 Nineteen maize inbred lines were used in the study, including: (1) historical cultivars B37 and w22,
117 and the NILs B37Htn1 and w22Htn1 that contain the NCLB resistance gene *Htn1* (Table S1), which
118 were originally developed via crossing the donor line “Pepitilla” (a Mexican landrace) and the
119 recurrent parental lines by Mr. Raymundo and colleagues from University of Illinois (Raymundo *et al.*,

120 1981); (2) Breeding line RP3 and its NIL RP3Htn1 carrying *Htn1* (KWS, Einbeck, Germany); (3) three
121 pairs of mutants RLK1b (1365', G to A, Met to Ile), RLK1d (1490', C to T, Leu to Phe) and RLK1f (1642',
122 G to A, Gly to Arg, susceptible/compromised resistance), and their corresponding sister lines RLK1b-
123 wt, RLK1d-wt, and RLK1f-wt (resistant, carrying functional *Htn1*), which were produced by EMS-
124 mutagenesis in RP3Htn1 (Hurni *et al.*, 2015); (4) three maize mutants (bx1, bx2 and bx6) and parental
125 line w22 (referred w22-wt, in order to make difference with the line w22, provided by KWS), which
126 were kindly provided by Prof. Georg Jander (Cornell University, Ithaca, US); (5) the NILs Bx13NIL-B73
127 that contains a functional *Bx13* allele and Bx13NIL-Oh43 that contains non-functional *Bx13* allele
128 compromising the synthesis of DIM_2BOA-Glc (Handrick *et al.*, 2016).

129 **NCLB infection tests in the greenhouse**

130 Testing for NCLB resistance using *E. turcicum* isolate Passau-1 was performed as previously described
131 with minor modification (Yang *et al.*, 2017). Two or three maize seeds were sown in a Jiffy pot (ø
132 8cm), and fifteen pots were placed in one tray. Seedling plants were grown in a greenhouse (16 h at
133 20°C in the day, 8 h at 18°C in the night and approximately 60% relative humidity). After the second
134 leaves had fully emerged, the later emerging leaves were cut and removed until the end of each
135 experiment. Single spore inoculation and culture on PDA medium plate, harvest and quantification of
136 progeny spores were performed as described (Yang *et al.*, 2017). Instead of infection by dropping 80
137 µl spore suspension into the leaf sheath of the second leaf twice, here maize seedlings were infected
138 once by spray (sprayer: ø 28mm, Semadeni, Ostermundigen, Switzerland). Each 4 trays (ca. 60-80
139 seedlings) were sprayed with 4 ml of spore suspension (4.5×10^4 spores/ml). A very high humidity
140 micro-condition was produced by placing plastic hoods on top of each tray after infection. Each plant
141 was scored for disease symptoms between 11 and 25 days and the severity was evaluated by
142 calculating the area under the disease progress curve (AUDPC) or by quantifying the diseased leaf
143 area of the inoculated second leaves (PrimDLA) (Yang *et al.*, 2017). About 15 seedling plants were
144 scored for each genotype in each experiment.

145 **Test for insect performance in the greenhouse**

146 *Spodoptera littoralis* and *Diabrotica balteata* eggs were kindly provided by Ted Turlings (University of
147 Neuchâtel, CHE) and Oliver Kindler (Syngenta Crop Protection, Stein), respectively. For insect feeding
148 assay, maize plants were sown in 1 L pots and grown under greenhouse condition (350 umol.m-2.s-1
149 light, 14 h day, 55% relative humidity, 26±2°C). Plants with four fully developed leaves were used for
150 the experiments. Three pre-weighed second-instar larvae of *S. littoralis* or *D. balteata* were added to
151 w22 and w22Htn1 plants. Control plants remained uninfested. Modified PET bottles were added on
152 all individual plants as previously described (Erb *et al.*, 2011). After 8 days, all larvae were collected

153 and weighed again to calculate their relative weight gain. Larval survival rate was calculated based on
154 the proportion of larvae recovered per pot.

155 **Vector construction, subcellular localization and western blotting**

156 The coding sequence of *ZmWAK-RLK1* was amplified using a cDNA clone as template, which was
157 initially amplified in NCLB resistance line RP1Htn1 (Hurni *et al.*, 2015). The primers used for this
158 construct are given (Table S2). The PCR fragment was introduced into the Gateway donor vector
159 pDONR207 using the Gateway® BP Clonase® II Enzyme mix (Thermo Fisher Scientific, Wilmington,
160 USA). The generated entry vector carrying the target *ZmWAK-RLK1* sequence was inserted by
161 recombination in the destination vector pUBC-GFP-DEST, to produce an in-frame *ZmWAK-RLK1* + c'-
162 eGFP fusion protein construct driven by the Arabidopsis ubiquitin-10 (UBQ10) gene promotor
163 (Grefen *et al.*, 2010). The *UBQ10:: ZmWAK-RLK1-c'-eGFP* construct together with a reference plasmid
164 PIP2A-mCherry (contains *35S::PIP2A_c'_RFP* construct, which is localized to the plasma membrane)
165 (Nelson *et al.*, 2007) were mixed with nanograde gold particles and co-bombarded into onion
166 epidermal cells, which were subsequently incubated at 20°C in the dark for 2-3 days until being ready
167 for observation using a Zeiss LSM 880 confocal microscope (CARL ZEISS, Jena, Germany) by following
168 the standard instructions. Plasmolysis was induced by adding a 0.8 M mannitol solution.

169 The sameplasmids were transformed into Agrobacterium GV3101 and co-infiltrated into 4-week-old
170 *N. benthamiana* leaves, which were ready for observation 2 days post infiltration. The
171 *Agrobacterium*-infiltrated tobacco leaves were harvested for the extraction of total proteins using
172 the lysis buffer (150 mM NaCl, 50 mM Tris-HCl at pH 7.5, 5 mM EDTA at pH 8.0, 0.1% Triton X-100,
173 0.2% NP-40) plus freshly added PMSF (phenylmethylsulphonyl fluoride, 10 mM). The GFP-tagged
174 protein was checked by western blotting using anti-GFP antibody (1:2000, Roche, 11814460001).

175 Maize protoplasts were isolated from the second leaves of maize seedling plants. After seed sowing,
176 these maize plants were planted 10-14 days under dark condition. Transformation of plasmid
177 constructs was following standard methods (Yoo *et al.*, 2007), and the protoplasts were incubated for
178 24-48 hours under dark condition until being ready for observation using the confocal microscope.

179 **Analysis of *E. turcicum* infection**

180 The second leaves of 21-day seedling plants were harvested and cut into 2 × 2 cm^2 leaf segments,
181 which were placed and incubated on phytoagar plates. A spore suspension (4.5 × 10^4 spores/ml) was
182 painted on the leaf surface using swabs. The petri dishes carrying samples were sealed using
183 PARAFILM and incubated 24 hours at room temperature until harvest.

184 Trypan blue staining was conducted as previously described (Chung *et al.*, 2010). The infected
185 segments at 1 dpi were incubated overnight in an acetic acid: ethanol (1:3, v/v) solution, and then in

186 a mixed solution of acetic acid : ethanol : glycerol (1:5:1, v/v/v) for 4 hours. The samples were stained
187 overnight in 0.01% (w/v) trypan blue lactophenol solution, and then washed once using ddH$_2$O and
188 stored in 60% glycerol ready for use. Specimens were placed on slides and examined under the ZEISS
189 Axio Imager 2 microscope system (CARL ZEISS, Jena, Germany) with normal light, by magnifying 10 or
190 20 times. In general, more than 50 spores were counted in each replicate of each sample, and at
191 least three specimens were checked. The numbers of germinated spores, germ tubes, appressoria
192 and successful penetrations (hyphae inside of cell or between cell walls) were counted. Three
193 independent experiments were performed.

194 **RNA extraction, RNA sequencing and data analysis**

195 The second leaves of seedling plants were harvested with four biological replicates at 0, 9-hpi, 3-dpi
196 and 10-dpi, which corresponded to before inoculation, the germination/penetration, biotrophic
197 growth and necrotrophic growth, respectively (Jennings & Ullstrup, 1957; Hilu & Hooker, 1964).
198 Forty-eight samples (4 genotypes, 4 time points, 3 biological replicates) were subjected for total RNA
199 extraction using SV Total RNA Isolation Kits (Promega, Dübendorf, Switzerland). 1 µl of total RNA was
200 checked by Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) to
201 estimate the RNA concentration. Meanwhile, 15 plants in each genotype were evaluated for the
202 AUDPC value to control if the infection worked.

203 The quantity and quality in RNA for RNA sequencing were determined using Qubit® 1.0 Fluorometer
204 (Thermo Fisher Scientific, Wilmington, USA) and Bioanalyzer 2100 (Agilent, Waldbronn, Germany).
205 The TruSeq Stranded mRNA Sample Prep Kit (Illumina, Inc., Hayward, USA) was used for library
206 preparation. 1 µg of total RNA per sample was ribosome depleted and then subjected for
207 synthesizing double-strand cDNA. Each cDNA sample was fragmented, end-repaired, polyadenylated
208 and then ligated with TruSeq adaptor that contains the index for multiplexing. The cDNA fragments
209 containing TruSeq adapters at the both ends were enriched with PCR reaction. The enriched libraries
210 were quantified and qualified, and then normalized to 10nM. The TruSeq SR Cluster Kit v4 cBot
211 (Illumina, Inc., Hayward, USA) was used for cluster generation using 8 pM of pooled normalized
212 libraries. Sequencing was performed on the Illumina HiSeq2500 at single end 125 bp using the TruSeq
213 SBS Kit v4 (Illumina, Inc., Hayward, USA).

214 The maize reference genome Zea_mays.AGPv3.27 and the corresponding annotation were
215 downloaded (http://www.maizegdb.org/). The RNA sequencing reads were mapped on the reference
216 genome with STAR (Dobin *et al.*, 2013) allowing one mismatch per 100 bp and no multimappers with
217 the following command: STAR -outFilterMultiMapNmax 1 - outFilterMismatchNoverLmax 0.01 -
218 alignIntronMax 10000. Read counts were determined from the mapping files with featureCounts
219 1.4.6 (Liao *et al.*, 2014) using standard parameters with the command "featureCounts bam -a gtf".

220 Statistical analyses were done with the R package edgeR and genes were tested for differential
221 expression with pairwise comparisons and tagwise estimation of dispersion (Robinson *et al.*, 2010). A
222 gene was considered to be expressed when at least 10 reads were mapped on it and a gene was
223 considered to be differentially expressed with $log_2FC \geq |2|$ and FDR < 0.01. First, pairwise
224 comparisons were performed between NILs with/without *Htn1* for each genotype and each time
225 point separately. The results were then compared between time points and then between the two
226 genotypes. The Gene Ontology analysis for DEGs was conducted by using online software agriGO (Du
227 *et al.*, 2010). The significant terms were colored if adjusted $p \leq 0.05$.

228 **RT-qPCR assay**

229 1µg total RNA was subjected for first strand cDNA synthesis using the iScript Advanced cDNA kit (172-
230 5038, Rio-Rad). 1:20 diluted cDNA was applied for quantifying expression using a Real-Time System
231 C1000TM Thermal cycler (96 or 384 wells, Bio-Rad). The expression of targets was normalized by the
232 reference genes *FPGS* and *Actin* as described (Balmer *et al.*, 2013; Hurni *et al.*, 2015). The primers for
233 expression analysis are shown (Table S2).

234 **Benzoxazinoids extraction and measurement**

235 60 - 100 mg leaves (without veins) of the seedling plants were harvested and frozen immediately in
236 liquid nitrogen, grinded and extraction buffer was added (1 mg sample + 10 µl extraction buffer). The
237 samples were mixed thoroughly and centrifuged at 13,000 rpm at 4˚C. The supernatant was
238 transferred into a new tube and centrifuged once more under the same condition to remove leaf
239 particles. The supernatant was collected for BXD measurements.

240 Benzoxazinoid contents were analyzed by an Acquity UPLC equipment (Waters) coupled to a UV
241 detector and a mass spectrometer (Waters) (Meihls *et al.*, 2013). An Acquity BEH C18 column
242 (Waters) was used. The temperatures of the autosampler and column were 15 °C and 40 °C,
243 respectively. The mobile phase consisted of 99% water, 1% acetonitrile, and 0.1% formic acid (A) and
244 acetonitrile and 0.1% Formic acid (B). Flow rate was set to 0.4 ml min^{-1} with 3% A and 97% B followed
245 by column reconditioning. The injection volume was 5 µl. The extracted trace at 275 nm was used for
246 benzoxazinoids quantification. The following extracted ion chromatograms were used for
247 quantification with a mass window of ±0.01 D: mass-to-charge ratio (m/z) for DIMBOA (retention
248 time [RT] 5.62 min) and DIMBOA-Glc (RT 5.64 min), m/z for HDMBOA-Glc (RT 8.19 min), m/z for
249 HMBOA-Glc (RT 5.34 min), m/z for DIM_2BOA-Glc (RT 5.825 min) and HDM_2BOA-Glc (RT 8.32 min).
250 Benzoxazinoids absolute concentrations were determined by external calibration curves obtained
251 from purified DIMBOA-Glc, DIMBOA and HDMBOA-Glc standards. TRIMBOA-Glc and HDM_2BOA-Glc
252 are below than the detection limit and not shown.

253 **Results**

254 ***ZmWAK-RLK1* encodes a plasma membrane localized protein**

255 To determine the subcellular localization of the ZmWAK-RLK1 protein, we generated a fusion
256 construct consisting of a full-length coding sequence fused to the sequence of an enhanced green
257 fluorescence protein at the C terminus (Grefen *et al.*, 2010). The ZmWAK-RLK1 fusion protein
258 localized to the plasma membrane before and after plasmolysis when transiently expressed in onion
259 epidermal cells (Fig. 1A-B). Secondly, infiltration into leaves of *Nicotiana benthamiana* indicated the
260 localization of ZmWAK-RLK1 to the plasma membrane two days after infiltration (Fig. 1C). A fusion
261 protein of ZmWAK-RLK1 and GFP was detected by western blot analysis (Fig. S1). Thirdly, we
262 transiently expressed the same gene fusion in maize protoplasts. The encoded protein was found to
263 be localized to the plasma membrane 36 hours after transformation (Fig. 1D). Thus, these data
264 indicate that ZmWAK-RLK1 is a plasma membrane-localized protein.

265 ***ZmWAK-RLK1* reduces fungal penetration rate**

266 Spores of the hemibiotrophic fungus *E. turcicum* penetrate the maize epidermis mostly between 6-18
267 hours after inoculation (hpi) (Jennings & Ullstrup, 1957; Hilu & Hooker, 1964). To investigate if
268 *ZmWAK-RLK1* changes the outcome of fungal penetration attempts, we investigated the infection
269 process at one day post inoculation (dpi) using trypan blue staining (Fig. 2A and Fig. S2A-C). The
270 numbers of successful penetration events were evaluated in three EMS-induced *ZmWAK-RLK1* loss-
271 of-function mutant lines (RLK1b, RLK1d and RLK1f) and their corresponding sister lines that were
272 generated in the near isogenic line (NIL) RP3Htn1 (Table S1) (Hurni *et al.*, 2015). No significant
273 differences in the establishment of germ tubes and appressoria were observed in mutants and sister
274 lines (Fig. S2D-E). In contrast, the number of successful penetration events was significantly lower if
275 *ZmWAK-RLK1* was functional compared to loss-of-function mutants at 1 dpi (Fig. 2B). In order to
276 compare the penetration ratio at different days post inoculation, we counted the penetration events
277 at 1 dpi and 3 dpi in genotype B37 and NIL B37Htn1. The rate of successful penetration significantly
278 decreased at 3 dpi vs 1 dpi (Fig. 3C). This indicates that *ZmWAK-RLK1* plays role in reduction of
279 pathogen penetration into host tissues, in agreement with the partial resistance/delayed
280 susceptibility.

281 **Transcriptome and metabolism analysis identifies alterations of the benzoxazinoids (BXDs)**
282 **biosynthesis pathway in the presence of *ZmWAK-RLK1***

283 The surface-localized RLKs act as crucial components in plant immune signaling (Zipfel *et al.*, 2017). In
284 order to decipher the transcriptional regulation network specifically influenced by *ZmWAK-RLK1*, we
285 performed a transcriptome analysis by RNA sequencing in two pairs of near isogenic lines, w22 and

286 w22Htn1 as well as B37 and B37Htn1. NCLB development was significantly reduced in the presence
287 of *ZmWAK-RLK1* in both NILs (Fig. S3A-C). Leaf samples were collected at 0 and 9 hpi (penetration
288 stage) as well as 3 dpi (biotrophic growth) and 10 dpi (necrotrophic growth) (Jennings & Ullstrup,
289 1957; Hilu & Hooker, 1964). Forty-eight samples were sequenced and 1.159 billion reads were
290 obtained (Table S3). More than 820 million reads were uniquely mapped with an average of 17.08
291 million reads per sample (70.7% of total reads) (Table S3). A total of 15,345 genes were expressed
292 and they were used for further analysis. By conducting a multidimensional scaling analysis using
293 expression normalized by reads per kilobase per million mapped reads (RPKM) using edgeR, the
294 biological replicates for the same genotype-timepoint combinations mostly grouped together,
295 demonstrating high similarity of replicates (Fig. S4A-C). A high number of differentially expressed
296 genes (DEGs) was detected in B37Htn1/B37 compared to w22Htn1/w22 (Fig. S5A). To identify DEGs
297 associated with *ZmWAK-RLK1* and to rule out genetic background effects*,* only genes that were
298 differentially expressed in both NIL pairs were further considered.

299 Two-hundred and fifteen common DEGs were identified across all time points (Table S4). 132 and 83
300 genes were induced and repressed, respectively, in NILs with *ZmWAK-RLK1* compared to the parental
301 lines without *ZmWAK-RLK1* (Fig. S5B-C). An overrepresentation analysis using agriGO revealed an
302 enrichment of Gene Ontology (GO) terms associated with defense response (e.g. GO:0009814) and
303 metabolic/biosynthetic process (e.g. GO:0006725) (Fig. S6). Twenty-nine DEGs were differently
304 expressed at all time points including timepoint 0 (Fig. S5C). Using the annotation information of the
305 best hit rice homologs, four genes were annotated as hypersensitive induced response protein
306 (GRMZM2G157869) and receptor-like kinase proteins (GRMZM2G433684, GRMZM2G165387,
307 GRMZM2G436455) that might indicate an association with disease resistance. We then considered
308 all the 215 DEGs that have available annotation information in maize. Several DEGs were found and
309 belonged to known pathways that are associated with disease resistance, including biosynthesis of
310 the defense hormones jasmonic acid (JA) and ethylene as well as lignin and cell wall biosynthesis
311 (Table S4). Interestingly, we found five DEGs that are part of the BXDs biosynthesis pathway. This
312 finding was surprising because these secondary metabolites have been mainly described to increase
313 plant resistance against insects (Niemeyer, 2009; Wouters *et al.*, 2016). The five genes *Bx2*, *Igl-like*,
314 *Bx6*, *Bx11* and *Bx14* showed differential expression in at least one time point (Table S4).

315 To analyze if the presence of *ZmWAK-RLK1* is associated with lower BXD content, BXDs were
316 quantified in the second leaves of w22Htn1 and w22 before and after infection (Fig. 3A-F). The
317 content of the four BXDs DIMBOA-Glc, DIMBOA, HMBOA-Glc and DIM_2BOA-Glc was significantly
318 lower in w22Htn1 compared to w22 at all time points (Fig. 3B-E), which indicated a constitutive
319 reduction of BXD accumulation in the presence of *ZmWAK-RLK1*. Furthermore, we determined by RT-
320 qPCR the transcriptional levels of *ZmWAK-RLK1* and specifically the genes in the BXDs biosynthesis

321 pathway before and after pathogen inoculation (Fig. S7A-P). Both the expression of *ZmWAK-RLK1*
322 and of the BXD genes *Bx1*, *Bx6* and *Bx13* were lower in w22Htn1 (Fig. S7B, S7H and S7N).

323 To test if the lower BXD content in w22Htn1 impaired resistance against herbivores, we evaluated
324 maize lines w22 and w22Htn1 upon infections with leaf-feeding *Spodoptera littoralis* and root-
325 feeding *Diabrotica balteata* (Fig. S7). Although the aboveground biomass of w22Htn1 plants was
326 lower than of w22 plants, food was not restricted for *S. littoralis* larvae. No difference in the growth
327 of the two generalist herbivores was noted.

328 Taken together, our data indicate a reduced content of BXDs in the presence of *ZmWAK-RLK1*.

329 **Mutations in BXDs biosynthesis genes increase NCLB resistance**

330 To further analyze a possible positive or negative role of BXD biosynthesis genes in NCLB resistance,
331 mutants in the three genes *Bx1*, *Bx2* and *Bx6* in the w22 genetic background (Vollbrecht *et al.*, 2010)
332 were tested for disease development after inoculation with *E. turcicum* (Fig. 4A). These mutants
333 showed strong reduction in several BXDs compounds (Fig. S9). For example, HMBOA-Glc became
334 nearly undetectable in mutants and DIMBOA was reduced by more than 80%. Interestingly, by
335 quantifying disease severity using AUDPC, all three mutants showed an increase in NCLB resistance in
336 five-week old plants if compared to susceptible genotype w22 (Fig. 4A-B). This confirmed a negative
337 association of BXDs content and NCLB disease resistance. Furthermore, we checked the *ZmWAK-*
338 *RLK1* expression at 10 dpi (Fig. 4C). No significant difference was detected in the *bx* mutants if
339 compared to the wild-type.

340 **Compromising the synthesis of DIM$_2$BOA-Glc increases NCLB resistance**

341 In addition, we tested for NCLB resistance in Bx13NIL-B73 that contains a wild type functional *Bx13*
342 allele and Bx13NIL-Oh43 that contains a non-functional *bx13* allele. This mutation results in a frame
343 shift mutation in *Bx13* that specifically compromised the synthesis of DIM$_2$BOA-Glc and its
344 downstream compound HDM$_2$BOA-Glc (TRIMBOA-Glc was below the detection limit), without
345 modified contents of BXD compounds upstream in the biosynthetic (Handrick *et al.*, 2016).
346 Interestingly, in the *bx13* mutant, we found an increase in NCLB resistance during the early infection
347 phase (Fig. 5).

348 **Mutations in *ZmWAK-RLK1* are associated with the induction of the secondary metabolite**
349 **DIM$_2$BOA-Glc**

350 To further analyze the role of different BXD biosynthesis genes as well as the metabolites of this
351 pathway in NCLB resistance, EMS-induced mutants of *ZmWAK-RLK1* and their sister lines in RP3
352 background were used (Hurni *et al.*, 2015). We quantified the content of major BXD compounds and

353 the transcript levels of several BXD genes in mutants which lost the resistance caused by *ZmWAK-*
354 *RLK1*. The content of DIM_2BOA-Glc in mutants relative to sister lines was significantly higher (Fig. 6A),
355 while we didn't observe consistent pattern on contents of other BXD compounds (Fig. S10). The
356 induction of DIM_2BOA-Glc content associated with higher *Igl* transcript level (Fig. 6B), while no
357 obvious difference in the transcriptional levels of *ZmWAK-RLK1* and *Bx1* was detected in mutants and
358 sister lines (Fig. S11A-B). *Bx6*, *Bx7* and *Bx13* are key genes of the BXDs pathway to produce DIM_2BOA-
359 Glc, and these genes were slightly but not significantly upregulated when *ZmWAK-RLK1* is present
360 (Fig. S11C-E). This phenomenon can be explained by a feedback regulation, which has been proposed
361 in BXD metabolism pathways (Ahmad *et al.*, 2011). We conclude that in RP3 genetic background,
362 *ZmWAK-RLK1* is associated with the reduction of secondary metabolite DIM_2BOA-Glc and the
363 reduction of the expression levels of *Igl*.

364 Taken together, our results indicated that *ZmWAK-RLK1* underlying quantitative NCLB disease
365 resistance is associated to a decrease of the biosynthesis of secondary metabolite BXDs (*e.g.*
366 DIM_2BOA-Glc), which are involved in biochemical pathways starting with indole-3-glycerol
367 phosphate.

368 **Discussion**

369 In this study, we investigated the functional basis of quantitative resistance to northern corn leaf
370 blight of maize mediated by the wall-associated kinase ZmWAK-RLK1 encoded by the *Htn1* gene
371 (Hurni *et al.*, 2015). Our work here provides evidence that *ZmWAK-RLK1* is associated with the
372 reduction of BXDs. BXDs have been found in many cereal species such as maize and wheat, which are
373 important food crops worldwide (Niemeyer, 2009).

374 Two earlier studies suggested a positive association of BXDs compound DIMBOA and resistance
375 against *E. turcicum* spore germination and penetration (Couture *et al.*, 1971; Ahmad *et al.*, 2011).
376 Infections by either *E. turcicum* or *B. maydis* resulted in the elevation of BXDs (e.g. HDMBOA-Glc)
377 (Ahmad *et al.*, 2011; Oikawa *et al.*, 2004). In contrast, our results revealed no significant difference of
378 the content of HDMBOA-Glc (0 and 3 dpi), but the reduction of several BXD compounds (DIMBOA,
379 DIMBOA-Glc, DIM_2BOA-Glc) in five week old plants of genotypes w22 and w22Htn1. An increase of
380 northern corn leaf blight resistance was observed in BXD-deficient mutants. The former study was
381 conducted on 8 days old seedlings (Ahmad *et al.*, 2011), and the discrepancy might be due to the
382 difference in BXD content between the two developmental stages (Kohler *et al.*, 2015). Although
383 BXDs were significantly lower in w22 relative to w22Htn1, we did not detect any difference on the
384 performance and biomass removal for the insect pests *S. littoralis* and *D. balteata*. This is surprising
385 given the described role of BXDs in resistance to these two herbivores (Niemeyer, 2009; Wouters *et*
386 *al.*, 2016). This might be explained by the presence of an *Htn1*-independent resistance factor which

387 compensates for the lower BXD content. Alternatively, the herbivores might induce BXDs synthesis to
388 similar levels.

389 Furthermore, a *Bx13* knock-out mutant that blockss the synthesis of DIM$_2$BOA-Glc and/or HDM$_2$BOA-
390 Glc has been shown to result in 50% more progeny of corn leaf aphids *Rhopalosiphum maidis*, but
391 showed no difference on the weight of chew feeding insects *S. littoralis*, *S. exigua* and *D. balteata* in
392 feeding experiments (Handrick *et al.*, 2016). That suggests a functional specificity of DIM$_2$BOA-Glc
393 that interplays with negative effects on aphid growth only. Our resulted confirmed that mutations in
394 *ZmWAK-RLK1* result in elevation of DIM$_2$BOA-Glc content. Compromising the synthesis of DIM$_2$BOA-
395 Glc in Bx13NIL-Oh43 resulted in elevated NCLB resistance. The resistance caused by *ZmWAK-RLK1* is
396 likely achieved via mediating a reduction of DIM$_2$BOA-Glc. Thus, the accumulative datasets suggest
397 the functional divergence of BXD compounds (*e.g.* DIM$_2$BOA-Glc) in interaction with aphids,
398 herbivores and fungal pathogens.

399 The biosynthesis pathway of BXDs starts with the formation of indole by conversion of indole-3-
400 glycerol phosphate (Niemeyer, 2009). IGP and indole are secondary metabolites found cross
401 kingdoms, and they can also stimulate the synthesis of auxin (*e.g.* the phytohormone indole-3-acetic
402 acid, IAA) via tryptophan dependent and independent pathways (Woodward & Bartel, 2005). A
403 former study found no difference of IAA content in the bx1 mutant compared to wild type (Maag *et*
404 *al.*, 2016), suggesting that termination of the BXD pathway does not necessarily result in an increase
405 of auxin biosynthesis. However, we cannot exclude at this stage that *Htn1* contributes to the
406 modulation of the metabolic flux from IGP and/or indole into the BXD and IAA biosynthesis
407 pathways. Thus, the reduced flux into the BXD pathway in the presence of *Htn1* could be
408 accompanied by an increased flux into the auxin biosynthesis pathway, resulting in metabolic
409 changes contributing to resistance.

410 The transcriptome data also revealed DEGs in several additional pathways related to immune
411 responses. The presence of *Htn1*modifies the biosynthesis pathways of the defense hormones
412 jasmonic acid and ethylene, lignin synthesis, cell wall strength and other receptor like kinases. For
413 instance, >16 fold up-regulation of *OPR2* (9 hours post inoculation) and *LOX3* (before inoculation)
414 were observed in w22Htn1 and B37Htn1 relative to the parental lines. Both genes are involved in the
415 biosynthetic pathway of the phytohormone jasmonic acid that plays a central role in regulating
416 resistance against hemibiotrophic and necrotrophic diseases (Glazebrook, 2005). JA treatment can
417 induce the accumulation of BXD compounds (Oikawa *et al.*, 2001; Oikawa *et al.*, 2002). At this stage it
418 remains unclear if there is a link between JAs, WAKs and BXDs metabolism. The gene *Caffeoyl-CoA O-*
419 *methyltransferase 2* (*ZmCCoAOMT2*) is a key functional gene in lignin metabolic pathway and up-
420 regulated (>5 fold) when *ZmWAK-RLK1* is present before pathogen inoculation. Recently, this gene

421 was shown to be associated with resistance against multiple foliage diseases including NCLB (Yang *et*
422 *al.*, 2017), suggesting a link between *ZmWAK-RLK1*, lignin and NCLB resistance. Since about 50% of
423 DEGs showed differential expression even before pathogen infection, this suggests a constitutive
424 *ZmWAK-RLK1*-mediated immune response that might be expressed independently of the recognition
425 of pathogen components (Jones & Dangl, 2006). Based on these data, we conclude that a number of
426 metabolic pathways are modified in the presence of *Htn1* and it is possible that the observed
427 resistance is the consequence of additive action of multiple biochemical changes in the plant.The
428 transcriptome analysis revealed large number of DEGs in B37Htn1/B37 compared to w22Htn1/w22,
429 but only a proportion of DEGs were shared. There implicated either *Htn1*-mediated resistance via
430 different mechanisms in diverse genetic backgrounds, or most likely the presence of *Htn1*-unrelated
431 un-specific DEGs caused by differences in genomic segments that are present in NILs.

432 In contrast to the Arabidopsis *WAK* gene family that consists of only five members, *WAK* genes in
433 monocots belong to large families. For instance, in rice >100 members were found (Zhang *et al.*,
434 2005; Kanneganti & Gupta, 2008). A number of WAK genes in monocots have been shown to be
435 associated to several functional aspects, e.g. biotic diseases (Li *et al.*, 2009; Hurni *et al.*, 2015; Zuo *et*
436 *al.*, 2015; Shi *et al.*, 2016; Hu *et al.*, 2017), tolerance to phosphorus deficiency (Hufnagel *et al.*, 2014),
437 root growth (Kaur *et al.*, 2013) as well as gametophyte development (Wang *et al.*, 2012). WAKs are
438 the only known proteins that physically link the cell wall to the plasma membrane (Brutus *et al.*,
439 2010; Kohorn & Kohorn, 2012). They have been shown to be often associated with either of these
440 two cell compartments (Brutus *et al.*, 2010; Wang *et al.*, 2012). ZmWAK-RLK1 is localized to the
441 plasma membrane, very similar to the maize WAK protein ZmWAK/*qHSR1* that confers quantitative
442 head smut disease resistance (Zuo *et al.*, 2015). ZmWAK-RLK1 can reduce pathogen penetration into
443 host tissues and our transcriptome data revealed different expression levels of several cell wall
444 related genes, suggesting a positive effect possibly in stabilization of the cell wall. This role has been
445 demonstrated for the rice *OsWAK/Xa4* gene conferring quantitative rice blight resistance by
446 strengthening the cell wall (Hu *et al.*, 2017). In contrast, the wheat WAK gene *TaWAK*/*Snn1* is
447 hijacked by the necrotrophic effector SnTox1 that triggers programmed cell death allowing the
448 pathogen to feed and grow on dead tissue (Shi *et al.*, 2016). Furthermore, these data show that
449 elicitors recognized by WAKs can both be cell wall derived degraded polysaccharides (e.g. OGs) or
450 pathogenic short peptides (SnTox1) (Brutus *et al.*, 2010; Shi *et al.*, 2016). Thus, although there is
451 increasing evidence for a complex nature and functional divergence of WAKs in perception of types
452 of ligands and in their role of interacting with biotic diseases, understanding the functional basis of
453 WAKs is of interest to explore novel anti-fungal strategies relevant for a series of important crop
454 plants such maize, rice and wheat.

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465 **Contributions**

466 P.Y., M.O., S.G.K., M.E. and B.K. designed research; P.Y., C.P., B.L., J.S., C.R., B. Kessel, D.S. and L.L.
467 performed research; P.Y., C.P., B.L., C.R., S.G.K., M.E. and B.K. analyzed data; and P.Y., S.G.K., M.E.,
468 and B.K. wrote the paper.

469

470 **References**

471 **Ahmad S, Veyrat N, Gordon-Weeks R, Zhang YH, Martin J, Smart L, Glauser G, Erb M, Flors V, Frey
472 M, Ton J. 2011.** Benzoxazinoid metabolites regulate innate immunity against aphids and
473 fungi in maize. *Plant Physiology* **157**(1): 317-327.
474 **Balmer D, de Papajewski DV, Planchamp C, Glauser G, Mauch-Mani B. 2013.** Induced resistance in
475 maize is based on organ-specific defence responses. *Plant Journal* **74**(2): 213-225.
476 **Brutus A, Sicilia F, Macone A, Cervone F, De Lorenzo G. 2010.** A domain swap approach reveals a
477 role of the plant wall-associated kinase 1 (WAK1) as a receptor of oligogalacturonides.
478 *Proceedings of the National Academy of Sciences of the United States of America* **107**(20):
479 9452-9457.
480 **Ceballos H, Gracen VE.** 1989. A dominant inhibitor gene inhibits the expression of *Ht2* against
481 *Exserohilum turcicum* race 2 in corn inbred lines related to B14. *Plant Breeding* **102**: 35-44.
482 **Chung CL, Jamann T, Longfellow J, Nelson R. 2010.** Characterization and fine-mapping of a resistance
483 locus for northern leaf blight in maize bin 8.06. *Theoretical and Applied Genetics* **121**(2): 205-
484 227.
485 **Couture RM, Routley DG, Dunn GM. 1971.** Role of cyclic hydroxamic acids in monogenic resistance
486 of maize to *Helminthosporium turcicum*. *Physiological Plant Pathology* **1**(4): 515-521.
487 **Dardick C, Schwessinger B, Ronald P. 2012.** Non-arginine-aspartate (non-RD) kinases are associated
488 with innate immune receptors that recognize conserved microbial signatures. *Current
489 Opinion in Plant Biology* **15**(4): 358-366.
490 **Decreux A, Thomas A, Spies B, Brasseur R, Van Cutsem P, Messiaen J. 2006.** In vitro characterization
491 of the homogalacturonan-binding domain of the wall-associated kinase WAK1 using site-
492 directed mutagenesis. *Phytochemistry* **67**(11): 1068-1079.
493 **Di DW, Zhang C, Luo P, An CW, Guo GQ. 2016.** The biosynthesis of auxin: how many paths truly lead
494 to IAA? *Plant Growth Regulation* **78**(3): 275-285.
495 **Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR.
496 2013.** STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* **29**(1): 15-21.

497 **Du Z, Zhou X, Ling Y, Zhang ZH, Su Z. 2010.** agriGO: a GO analysis toolkit for the agricultural
498 community. *Nucleic Acids Research* **38**: W64-W70.
499 **Elnaghy M, Pekka L. 1962.** The role of 4-o-glueosyl-2,4-dihydroxy-7-methoxy-l,4-benzoxazin-3-one
500 resistance of wheat to stem rust. *Pysiologia Plantarum* **15**: 764-771.
501 **Erb M, Robert CAM, Hibbard BE, Turlings TCJ. 2011.** Sequence of arrival determines plant-mediated
502 interactions between herbivores. *Journal of Ecology* **99**(1): 7-15.
503 **Frey M, Stettner C, Pare PW, Schmelz EA, Tumlinson JH, Gierl A. 2000.** An herbivore elicitor
504 activates the gene for indole emission in maize. *Proceedings of the National Academy of*
505 *Sciences of the United States of America* **97**(26): 14801-14806.
506 **Glazebrook J. 2005.** Contrasting mechanisms of defense against biotrophic and necrotrophic
507 pathogens. *Annual review of phytopathology* **43**: 205-227.
508 **Gomez-Gomez L, Boller T. 2000.** FLS2: An LRR receptor-like kinase involved in the perception of the
509 bacterial elicitor flagellin in Arabidopsis. *Molecular Cell* **5**(6): 1003-1011.
510 **Grefen C, Donald N, Hashimoto K, Kudla J, Schumacher K, Blatt MR. 2010.** A ubiquitin-10 promoter-
511 based vector set for fluorescent protein tagging facilitates temporal stability and native
512 protein distribution in transient and stable expression studies. *Plant Journal* **64**(2): 355-365.
513 **Handrick V, Robert CAM, Ahern KR, Zhou SQ, Machado RAR, Maag D, Glauser G, Fernandez-Penny**
514 **FE, Chandran JN, Rodgers-Melnik E, Schneider B, Buckler ES, Boland W, Gershenzon J,**
515 **Jander G, Erb M, Kollner TG. 2016.** Biosynthesis of 8-o-methylated benzoxazinoid defense
516 compounds in maize. *Plant Cell* **28**(7): 1682-1700.
517 **Hilu HM, Hooker AL. 1964.** localized infection by *helminthosporium turcicum* on cron leaves.
518 *Phytopathology* **55**: 189-192.
519 **Houseman JG, Campos F, Thie NMR, Philogène BJR, Atkinson J, Morand P, Arnason JT. 1992.** Effect
520 of the maize-derived compounds DIMBOA and MBOA on growth and digestive processes of
521 European corn borer (Lepidoptera: *Pyralidae*) *Journal of Economic Entomology* **85**(3): 6.
522 **Hu KM, Cao JB, Zhang J, Xia F, Ke YG, Zhang HT, Xie WY, Liu HB, Cui Y, Cao YL, Sun XL, Xiao JH, Li XH,**
523 **Zhang QL, Wang SP. 2017.** Improvement of multiple agronomic traits by a disease resistance
524 gene via cell wall reinforcement. *Nature Plants* **3**(3): 17009.
525 **Hufnagel B, de Sousa SM, Assis L, Guimaraes CT, Leiser W, Azevedo GC, Negri B, Larson BG, Shaff**
526 **JE, Pastina MM, Barros BA, Weltzien E, Frederick H, Rattunde W, Viana JH, Clark RT, Falcao**
527 **A, Gazaffi R, Garcia AAF, Schaffert RE, Kochian LV, Magalhaes JV. 2014.** Duplicate and
528 conquer: multiple homologs of *PHOSPHORUS-STARVATION TOLERANCE1* enhance
529 phosphorus acquisition and sorghum performance on Low-phosphorus soils. *Plant Physiology*
530 **166**(2): 659-677.
531 **Hurni S, Scheuermann D, Krattinger SG, Kessel B, Wicker T, Herren G, Fitze MN, Breen J, Presterl T,**
532 **Ouzunova M, Keller B. 2015.** The maize disease resistance gene *Htn1* against northern corn
533 leaf blight encodes a wall-associated receptor-like kinase. *Proceedings of the National*
534 *Academy of Sciences of the United States of America* **112**(28): 8780-8785.
535 **Jennings PR, Ullstrup AJ. 1957.** A histological study of three *Helminthosporium* leaf blights of corn.
536 *Phytopathology* **47**: 707-714.
537 **Jones JDG, Dangl JL. 2006.** The plant immune system. *Nature* **444**(7117): 323-329.
538 **Kanneganti V, Gupta AK. 2008.** Wall associated kinases from plants - an overview. *Physiol Mol Biol*
539 *Plants* **14**(1-2): 109-118.
540 **Kaur R, Singh K, Singh J. 2013.** A root-specific wall-associated kinase gene, *HvWAK1*, regulates root
541 growth and is highly divergent in barley and other cereals. *Functional & Integrative Genomics*
542 **13**(2): 167-177.
543 **Kohler A, Maag D, Veyrat N, Glauser G, Wolfender JL, Turlings TCJ, Erb M. 2015.** Within-plant
544 distribution of 1,4-benzoxazin-3-ones contributes to herbivore niche differentiation in maize.
545 *Plant Cell and Environment* **38**(6): 1081-1093.
546 **Kohorn BD, Kohorn SL. 2012.** The cell wall-associated kinases,WAKs, as pectin receptors. *Front Plant*
547 *Sci* **3**: 88.
548 **Kostandi SF, Koraiem YS, Kamara A, Omar MA. 1981.** Effect of phenols in host-pathogen: interaction
549 of maize (*Zea mays* L.). *Cephalosporium maydis* system. *Agrochimica* **25**(3-4): 367-375.

550 **Krattinger SG, Keller B. 2016.** Molecular genetics and evolution of disease resistance in cereals. *New Phytologist* **212**(2): 320-332.
552 **Li H, Zhou SY, Zhao WS, Su SC, Peng YL. 2009.** A novel wall-associated receptor-like protein kinase gene, *OsWAK1*, plays important roles in rice blast disease resistance. *Plant Molecular Biology* **69**(3): 337-346.
555 **Liao Y, Smyth GK, Shi W. 2014.** featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* **30**(7): 923-930.
557 **Long BJ, Dunn GM, Routley DG. 1978.** Relationship of hydroxamate concentration in maize and field reaction to *Helminthosporium turcicum*. *Crop Sci* **18**(4): 573-575.
559 **Maag D, Kohler A, Robert CAM, Frey M, Wolfender JL, Turlings TCJ, Glauser G, Erb M. 2016.** Highly localized and persistent induction of Bx1-dependent herbivore resistance factors in maize. *Plant Journal* **88**(6): 976-991.
562 **Macho AP, Zipfel C. 2014.** Plant PRRs and the activation of innate immune signaling. *Molecular Cell* **54**(2): 263-272.
564 **Meihls LN, Handrick V, Glauser G, Barbier H, Kaur H, Haribal MM, Lipka AE, Gershenzon J, Buckler ES, Erb M, Kollner TG, Jander G. 2013.** Natural variation in maize aphid resistance is associated with 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside methyltransferase activity. *Plant Cell* **25**(6): 2341-2355.
568 **Nelson BK, Cai X, Nebenfuhr A. 2007.** A multicolored set of in vivo organelle markers for co-localization studies in Arabidopsis and other plants. *Plant Journal* **51**(6): 1126-1136.
570 **Niemeyer HM. 2009.** Hydroxamic acids derived from 2-hydroxy-2H-1,4-benzoxazin-3(4H)-one: key defense chemicals of cereals. *Journal of Agricultural and Food Chemistry* **57**(5): 1677-1696.
572 **Oikawa A, Ishihara A, Hasegawa M, Kodama O, Iwamura H. 2001.** Induced accumulation of 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one glucoside (HDMBOA-Glc) in maize leaves. *Phytochemistry* **56**(7): 669-675.
575 **Oikawa A, Ishihara A, Iwamura H. 2002.** Induction of HDMBOA-Glc accumulation and DIMBOA-Glc 4-O-methyltransferase by jasmonic acid in poaceous plants. *Phytochemistry* **61**(3): 331-337.
577 **Oikawa A, Ishihara A, Tanaka C, Mori N, Tsuda M, Iwamura H. 2004.** Accumulation of HDMBOA-Glc is induced by biotic stresses prior to the release of MBOA in maize leaves. *Phytochemistry* 65(22): 2995-3001.
580 **Robert CAM, Veyrat N, Glauser G, Marti G, Doyen GR, Villard N, Gaillard MDP, Kollner TG, Giron D, Body M, Babst BA, Ferrieri RA, Turlings TCJ, Erb M. 2012.** A specialist root herbivore exploits defensive metabolites to locate nutritious tissues. *Ecology Letters* **15**(1): 55-64.
583 **Robinson MD, McCarthy DJ, Smyth GK. 2010.** edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**(1): 139-140.
585 **Shi G, Zhang Z, Friesen TL, Raats D, Fahima T, Brueggeman RS, Lu S, Trick HN, Liu Z, Chao W, Frenkel Z, Xu SS, Rasmussen JB, Faris JD. 2016.** The hijacking of a receptor kinase-driven pathway by a wheat fungal pathogen leads to disease. *Science Advances* **2**(10): e1600822.
588 **Song YY, Cao M, Xie LJ, Liang XT, Zeng RS, Su YJ, Huang JH, Wang RL, Luo SM. 2011.** Induction of DIMBOA accumulation and systemic defense responses as a mechanism of enhanced resistance of mycorrhizal corn (*Zea mays* L.) to sheath blight. *Mycorrhiza* **21**(8): 721-731.
591 **Vollbrecht E, Duvick J, Schares JP, Ahern KR, Deewatthanawong P, Xu L, Conrad LJ, Kikuchi K, Kubinec TA, Hall BD, Weeks R, Unger-Wallace E, Muszynski M, Brendel VP, Brutnell TP. 2010.** Genome-wide distribution of transposed dissociation elements in maize. *Plant Cell* **22**(6): 1667-1685.
595 **Wang N, Huang HJ, Ren ST, Li JJ, Sun Y, Sun DY, Zhang SQ. 2012.** The rice wall-associated receptor-like kinase gene *OsDEES1* plays a role in female gametophyte development. *Plant Physiology* **160**(2): 696-707.
598 **Welz HG, Geiger HH. 2000.** Genes for resistance to northern corn leaf blight in diverse maize populations. *Plant Breeding* **119**(1): 1-14.
600 **Woodward AW, Bartel B. 2005.** Auxin: regulation, action, and interaction. *Annals of Botany* **95**(5): 707-735.

602 **Wouters FC, Blanchette B, Gershenzon J, Vassao DG. 2016.** Plant defense and herbivore counter-
603 defense: benzoxazinoids and insect herbivores. *Phytochemistry Reviews* **15**(6): 1127-
604 1151.**Yang P, Herren G, Krattinger SG, Keller B. 2017.** Large-scale maize seedling infection
605 with *Exserohilum turcicum* in the greenhouse. *Bio-protocol* **7**(19): e2567.
606 **Yang Q, He Y, Kabahuma M, Chaya T, Kelly A, Borrego E, Bian Y, El Kasmi F, Yang L, Teixeira P,**
607 **Kolkman J, Nelson R, Kolomiets M, J LD, Wisser R, Caplan J, Li X, Lauter N, Balint-Kurti P.**
608 **2017.** A gene encoding maize caffeoyl-CoA O-methyltransferase confers quantitative
609 resistance to multiple pathogens. *Nature Genetics* **49**(9): 1364-1372.
610 **Yoo SD, Cho YH, Sheen J. 2007.** Arabidopsis mesophyll protoplasts: a versatile cell system for
611 transient gene expression analysis. *Nature Protocols* **2**(7): 1565-1572.
612 **Zhang SB, Chen C, Li L, Meng L, Singh J, Jiang N, Deng XW, He ZH, Lemaux PG. 2005.** Evolutionary
613 expansion, gene structure, and expression of the rice wall-associated kinase gene family.
614 *Plant Physiology* **139**(3): 1107-1124.
615 **Zipfel C, Oldroyd GED. 2017.** Plant signalling in symbiosis and immunity. *Nature* **543**(7645): 328-336.
616 **Zuo WL, Chao Q, Zhang N, Ye JR, Tan GQ, Li BL, Xing YX, Zhang BQ, Liu HJ, Fengler KA, Zhao J, Zhao**
617 **XR, Chen YS, Lai JS, Yan JB, Xu ML. 2015.** A maize wall-associated kinase confers quantitative
618 resistance to head smut. *Nature Genetics* **47**(2): 151-157.

619

620 **Figure legends**

621 **Fig. 1 ZmWAK-RLK1 localizes to the plasma membrane**. (A-B) Fluorescent signals in onion epidermal
622 cells after transient expression of ZmWAK-RKL1-eGFP and the positive control PIP2A-mCherry that is
623 known to localize to the plasma membrane (Kammerloher et al. 1994; Nelson et al. 2007). Signals are
624 shown before (A) and after (B) plasmolysis with 0.8 M mannitol. (C) Fluorescent signals in *N.*
625 *benthamiana* leaves two days after infiltration. Scale bars, 50µm.

626 **Fig. 2 *ZmWAK-RLK1* reduced the fungal penetration rates**. (A) Hyphae detected inside of host tissues
627 at 1 dpi. In the left and right panels the focus is on the epidermis and hyphae, respectively. The red
628 arrows indicate the hyphae inside the host tissue. Scale bars, 100µm. (B) Rate of successful
629 penetration events at 1 dpi. RLK1b, RLK1d and RLK1f are *ZmWAK-RLK1* mutants with compromised
630 NCLB resistance that were produced in RP3Htn1 (Hurni et al. 2015), while RLK1b-wt, RLK1d-wt and
631 RLK1f-wt are the corresponding sister lines, respectively. Red bar, *Htn1* resistance allele; black bar,
632 susceptible allele. (C) Rate of successful penetration events at 3 dpi in B37 and B37Htn1. Statistics
633 was conducted using Student's t test, based on three independent experiments. The asterisks
634 represent a significant difference of $^{**}p<0.01$ or $^{*}p<0.05$. Error bars indicate ± standard error.

635 **Fig. 3 Content of BXDs in w22 and w22Htn1**. (A) The proposed biosynthesis pathway of BXD
636 secondary metabolites. The genes that catalyze each step of enzymatic reactions are given. The
637 contents of BXD compounds DIMBOA-Glc (B), DIMBOA (C), HMBOA-Glc (D), DIM$_2$BOA-Glc (E) and
638 HDMBOA-Glc (F) were determined before inoculation, at 3 dpi and 10 dpi. TRIMBOA-Glc and
639 HDM$_2$BOA-Glc are below than the detection limit and not shown. The statistics were conducted using
640 Tukey's HSD (P = 0.05) in eight biological replicates. ns: no significance (p = 0.05). Error bars are ± SE.

641 **Fig. 4 Mutations in BXDs biosynthesis genes increased resistance to NCLB disease at the seedling**
642 **stage in the w22 genetic background**. (A) Visual symptoms and (B) quantified NCLB disease severity
643 in *bx* mutants in w22 background. (C) Expression of *ZmWAK-RLK1* at 10 dpi. Statistical analysis was
644 conducted using Student's t test. The asterisks represent a significant difference of $^{**}p<0.01$ or
645 $^{*}p<0.05$. The ns stands for no significance. Error bars are ± SE.

646 **Fig. 5 A knockout mutation of the *Bx13* gene increases NCLB resistance**. Bx13NIL-B73 contains a
647 wild type functional *Bx13* allele and Bx13NIL-Oh43 contains a non-functional *Bx13* allele, which
648 results in the elimination of DIM_2BOA-Glc (Hardick et al. 2016). Statistics was conducted using
649 Student's t test (n=45). PrimDLA, the primary diseased leaf area of the inoculated leaves. The
650 asterisks represent a significant difference of $^{**}p<0.01$ or $^{*}p<0.05$. Error bars are ± SE.

651 **Fig. 6 Mutations in *ZmWAK-RLK1* result in elevated DIM_2BOA-Glc content that is associated with *Igl***
652 **expression**. (A) Content of DIM_2BOA-Glc in mutants and corresponding sister lines before infection at
653 21 days after sowing (n=8). (B) Expression analysis of *Igl* (n=5). Statistics was conducted using
654 Student's t test. The asterisks represent a significant difference of $^{**}p<0.01$ or $^{*}p<0.05$. Error bars are
655 ± SE.

656

657 **Supporting information**

658 **Table S1** Summary of the genotypes with and without Htn1

659 **Table S2** Setup of RT-qPCR assays for target genes

660 **Table S3** Statistics of RNA-seq reads sequenced and mapped

661 **Table S4** The logFC and annotation of 215 differently expressed genes

662 **Fig. S1** Western blotting analysis of the fusion protein of ZmWAK-LRK1 plus GFP. Total protein was
663 subjected for western blotting using anti-GFP-antibody.

664 **Fig. S3** Quantitative analysis of the infection steps of E. turcicum in maize seedlings at 1 dpi. (A)
665 Micrograph of a germinated spore and appressorium. (B) Penetration peg. (C) Hyphae in epidermal
666 cells. (D) Number of germ tubes per spore. (E) Number of appressoria per spore. The numbers of
667 counted spores in each experiment are given. Statistics was conducted using Student's t test, based
668 on the results of three independent experiments. ns: no significance (p = 0.05). Scale bars, 100µm.

669 **Fig. S3** Disease phenotype of NILs with and without Htn1. (A) Disease symptoms of the second leaves
670 at 16 dpi. (B) Rate of infected plants. (C) Area under the disease progress curve (AUDPC). *AUDPC in

671 this panel was calculated as described in Hurni et al. 2015, based on calculating the sum of the rate
672 of infected plants (%).

673 **Fig. S4** Multidimensional scaling (MDS) analysis in RNAseq datasets using normalized expression by
674 edgeR (Robinson et al., 2010). Each symbol indicates one genotype-timepoint combined sample. (A)
675 MDS plot of two pairs of NILs and the corresponding parental lines. (B) MDS plot of B37 and
676 B37Htn1. (C) MDS plot of w22 and w22Htn1.

677 **Fig. S5** Transcriptome analysis in NILs with and without Htn1 revealed a set of DEGs. (A) Number of
678 DEGs in the two Htn1 NILs compared to the parental lines without Htn1. (B) Clustering of 215 DEGs
679 shared in comparisons of both NILs w22Htn1/w22 and B37Htn1/B37. These genes were differentially
680 expressed in both NILs in at least one of timepoints. The colors of the heat map correspond to logFC
681 (fold change). (C) Venn diagram representing the number of shared DEGs at the different time points.

682 **Fig. 6** Gene Ontology analysis for 215 DEGs. The Gene Ontology analysis was conducted by using
683 online software agriGO (Du et al. 2010). The significant terms were colored if adjusted p $\leqslant$ 0.05.

684 **Fig. S7** Expression levels of genes in Htn1-NILs and the corresponding parental lines. The expression
685 of genes (A) ZmWAK-RLK1, (B) Bx1, (C) Igl, (D) Bx2, (E) Bx3, (F) Bx4, (G) Bx5, (H) Bx6, (I) Bx7, (J) Bx8,
686 (K) Bx9, (L) Bx10/11, (M) Bx12, (N) Bx13, (O) Glu1 and (P) Glu2 is shown. The colors indicate
687 timepoints before and after infection. The statistics were conducted using Tukey's HSD (P = 0.05) in
688 four biological replicates. Error bars are ± SE.

689 **Fig. S8** Infection of S. littoralis and D. balteata in w22 and w22Htn1. (A) Performance of S. littoralis,
690 (B) performance of D. balteata, (C) leaf biomass after S. littoralis inoculation, (D) leaf biomass after D.
691 balteata inoculation, (E) root biomass after S. littoralis inoculation, (F) root biomass after D. balteata
692 inoculation. The statistics were conducted using Sigma Plot 13 (P = 0.05). Error bars are ± SE.

693 **Fig. S9** Content of BXD compounds DIMBOA-Glc (B), DIMBOA (C), HMBOA-Glc (D), and DIM2BOA-Glc
694 (E), HDMBOA-Glc (F) in second leaves at 10 dpi. HDM2BOA-Glc is below the detection limit and not
695 shown. w22-wt is provided by Prof. Georg Jander and represented the parental material for
696 obtaining the bx mutants. The statistics were conducted using Student's t test (P = 0.05) in eight
697 biological replicates. The asterisks represent a significant difference of **p<0.01 or *p<0.05. ns: no
698 significance (p = 0.05). Error bars are ± SE.

699 **Fig. S10** Content of BXD compounds DIMBOA-Glc (B), DIMBOA (C), HMBOA-Glc (D), and HDMBOA-Glc
700 (E) in second leaves of Htn1 NILs and mutants at 21 days after sowing. HDM2BOA-Glc is below than
701 the detection limit and not shown. The statistics were conducted using Student's t test (P = 0.05) in

702 eight biological replicates. The asterisks represent a significant difference of **p<0.01 or *p<0.05.
703 The ns stands for no significance. Error bars are ± SE.

704 **Fig. S11** Expression levels of genes ZmWAK-RLK1 (A), Bx1 (B), Bx6 (C), Bx7 (D) and Bx13 (E) in second
705 leaves of Htn1 NILs and mutants at 21 days after sowing (n=5). The statistics were conducted using
706 Student's t test (P = 0.05) in five biological replicates. The asterisks represent a significant difference
707 of **p<0.01 or *p<0.05. ns: no significance (p = 0.05). Error bars are ± SE.