Cytological and Molecular Characterization of *ZmWAK*-Mediated Head-Smut Resistance in Maize

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Head smut, caused by the fungal pathogen *Sporisorium reilianum*, poses a threat to maize production worldwide. *ZmWAK*, a cell wall-associated receptor kinase, confers quantitative resistance to head smut disease. Here, two near-isogenic lines (NILs), susceptible line Huangzao4 and its *ZmWAK*-converted resistant line Huangzao4R, were used to decipher the role of *ZmWAK* in head smut resistance. Cytological and molecular characterization in response to *S. reilianum* infection was compared between two NILs. Upon *S. reilianum* infection, the growth of pathogen hyphae was severely arrested in the *ZmWAK*-converted resistant line Huangzao4R, relative to its susceptible parental line Huangzao4. Infected cells exhibited apoptosis-like features in Huangzao4R and hyphae were sequestered within dead cells, whereas pathogen invasion caused autophagy in Huangzao4, which failed to prevent hyphal spreading. Integrated transcriptomic and metabolomic analysis indicated that *ZmWAK* functions as a hub in the trade-off between growth and defense, whereby *ZmWAK* promotes cell growth in the absence of the pathogen and switches to a defense response upon *S. reilianum* attack. These findings shed light on an elegant regulatory mechanism governed by *ZmWAK* in the trade-off between growth and head smut defense.

Head-smut disease, caused by *Sporisorium reilianum*, poses a devastating threat to maize production worldwide. Teliospores in soil or plant debris serve as the initial source of infection. Under favorable soil conditions, teliospores germinate into yeast-like haploid basidiospores to form sporidia. When compatible, haploid sporidia mate to form infectious dikaryotic hyphae (Martinez et al. 2002). Filamentous hyphae enter maize during seedling emergence and grow in a prolonged biotrophic phase, until invading the floral meristems, in which *S. reilianum* transforms into its destructive necrotrophic form (Martinez et al. 1999, 2002). Maize suffering from head smut displays diverse malformations such as stunted plants, loss of apical dominance, numerous tillers, phyllody in the inflorescences, and excessive ears, among others. The classic symptom is that tassels and ears are partly or completely replaced by large smut

galls. Differences in these morphologic deformities likely depend on the developmental stage of the inflorescence when *S. reilianum* colonizes maize seedlings (Ghareeb et al. 2011). When galls rupture, new spores are released and dispersed by wind and contaminate soil, thereby perpetuating the next life cycle.

Zea mays WAK (*ZmWAK*), a quantitative disease-resistance gene, confers resistance to head-smut disease (Zuo et al. 2015). *ZmWAK* contains a cytoplasmic serine/threonine kinase domain, a calcium-binding epidermal growth factor domain, and an extracellular galacturonan-binding domain; hence, *ZmWAK* belongs to the wall-associated kinase (WAK) family (Zuo et al. 2015). Genes of the WAK family have tended to expand among plant species during evolution (de Oliveira et al. 2014; He et al. 1999; Verica and He 2002; Zhang et al. 2005). Given the presence or absence of the conserved arginine (R) adjacent to the catalytic aspartate (D), WAK proteins can be divided into two evolutionarily related subfamilies, i.e., RD WAK and non-RD WAK, the latter of which is restricted to monocots (de Oliveira et al. 2014). Unlike the well-studied WAK proteins in *Arabidopsis*, *ZmWAK* is a typical monocot non-RD kinase (Zuo et al. 2015). Moreover, the novel non-RD WAK family member *ZmWAK-RLK1* (receptor-like kinase 1) of maize was identified based on its ability to improve resistance to northern corn leaf blight (Hurni et al. 2015). In *Arabidopsis*, WAK proteins serve as receptors for two kinds of ligands, namely, native pectin and its degradation products, oligogalacturonic acids (OGs) (Brutus et al. 2010; Decreux and Messiaen 2005; Kohorn et al. 2009). A previous review envisioned a model for the perception of ligands by WAK proteins (Kohorn 2016), that is, the recognition of native pectin activates the mitogen-activated kinase (MAPK) MPK3 and maintains cell expansion under normal growth conditions, whereas WAK proteins sense cell-wall damage, bind pectin fragments, and evoke MPK6-mediated stress signaling under stress (Kohorn 2016; Kohorn et al. 2012).

Programmed cell death (PCD) is a highly conserved and fundamental program that requires precise management of the cell death systems. Apoptosis-like PCD (AL-PCD) and autophagy constitute two forms of plant PCD that are governed by distinct molecular pathways and have remarkably distinct morphologic features (Dickman and Fluhr 2013). AL-PCD is an irreversible endogenous self-killing process that manifests distinct features, including cell shrinkage, membrane blebbing, nuclear condensation and fragmentation, and DNA laddering (Reape and McCabe 2008, 2010). The BAG (Bcl-2 athanogene) family, homolog to the mammalian BAG family, regulates AL-PCD in plants (Doukhanina et al. 2006). Autophagy is the

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process by which cellular components are degraded and recycled (Liu and Bassham 2012). Cellular cargo is packaged by a double-membrane structure, termed the autophagosome, and becomes destined for degradation in vacuoles. Autophagy requires collaboration among specific autophagy-related genes (Atgs). In plant-microbe interactions, PCD can benefit or harm the plant depending on the pathway by which host cells die (Dickman and Fluhr 2013). Infection with the necrotrophic pathogen *Sclerotinia sclerotiorum* triggers AL-PCD, which promotes disease (Dickman et al. 2001). Overexpression of the antiapoptotic BCL-2 family protein CED-9 from *Caenorhabditis elegans* was found to reduce cell death and increase resistance to *S. sclerotiorum* (Kim et al. 2008). The *S. sclerotiorum* oxalic acid-deficient nonpathogenic A2 mutant could generate autophagy in the host and restrict lesion expansion (Kabbage et al. 2013; Williams et al. 2011).

We previously found that *ZmWAK* confers head-smut resistance by arresting *S. reilianum* growth in the mesocotyl, a structure in which *ZmWAK* is highly expressed (Zuo et al. 2015). Despite progress made on defining the functions of WAK proteins, the mechanism underlying *ZmWAK*-mediated head-smut resistance remains to be fully understood. In the current study, we used two near-isogenic lines (NILs)—a susceptible line Huangzao4, which lacks a functional *ZmWAK*, and its *ZmWAK*-converted resistant line Huangzao4R—to decipher the cytological and molecular mechanisms underlying *ZmWAK*-dependent maize resistance to head-smut disease. Our findings highlighted that an intriguing switch between cell growth and AL-PCD was monitored by *ZmWAK*, which suggest that *ZmWAK* promote

cell growth under normal growth circumstances and activate AL-PCD to restrict hyphal growth, leading to subsequent head-smut resistance upon *S. reilianum* invasion.

RESULTS

ZmWAK-triggered hypersensitive response (HR)-like PCD restricts *S. reilianum* invasion in mesocotyls.

To gain a whole-plant view of *ZmWAK* function, seeds from two NILs were sowed in soil mixed with *S. reilianum* inoculum and were grown in an illumination incubator to mimic natural infection. Infected mesocotyls were collected from the two NILs at different time points after pathogen inoculation (Fig. 1A). At 7 days after sowing (DAS), mesocotyls of Huangzao4 appeared symptomless, whereas a handful of Huangzao4R mesocotyls showed small and restricted lesions, resembling HR-like cell death (Fig. 1A and B). By 14 DAS, Huangzao4 mesocotyls had shrunk and some of the cells had died, whereas Huangzao4R mesocotyls appeared essentially the same as observed at 7 DAS. At 21 DAS, Huangzao4 mesocotyls exhibited severe damage with brownish discoloration and necrosis, whereas Huangzao4R mesocotyls still had a few lesions. A key step to guarantee the pathogenic success of *S. reilianum* is its colonization of shoot apical meristems (SAMs) (Ghareeb et al. 2011), and thus, the presence or absence of *S. reilianum* in SAMs was investigated, via polymerase chain reaction (PCR) at 21 DAS (before the floral transition), with the *S. reilianum*-specific primer SRac. The infection rate was strikingly different between the two NILs; for Huangzao4, 45% $\pm$ 6% of SAMs were infected, which was significantly greater

Fig. 1. *ZmWAK* restricts *Sporisorium reilianum* from gaining access to shoot apical meristems (SAMs). **A**, Time course of symptom development of mesocotyls in response to *S. reilianum* colonization. **B**, Enlarged view of the dashed rectangle in **A** (7 days after sowing [DAS]) showing hypersensitive response-like programmed cell death of epidermal cells from Huangzao4R mesocotyls. **C**, Rate of *S. reilianum* invasion of SAMs in Huangzao4 and Huangzao4R. SAMs were collected at 21 DAS. Three independent experiments were conducted, n = number of plants investigated. The error bars denote standard error. Asterisks (**) indicate $P < 0.01$ (Student's *t* test).

than the $8\% \pm 3\%$ for Huangzao4R (Fig. 1C). The difference in mesocotyl morphological features and SAM invasion rates implied that hyphal growth status differed between these two NILs.

We used wheat germ agglutinin (WGA) staining to visualize the dynamic infection process under a fluorescence microscope. At 4 DAS, dikaryotic filamentous hyphae were observed on the bottom surface of mesocotyls (Supplementary Fig. S2). At 5 DAS, copious hyphae were present on the surface, which initiated penetration into epidermal cells. In this regard, there was no significant difference between the two NILs at the initial infection stage, and only at 7 DAS were there noticeable differences. Whereas *S. reilianum* hyphae expanded steadily in Huangzao4, they were impeded after early invasion of a few epidermal cells in Huangzao4R (Fig. 2A). We further adopted scanning electron microscopy to assess hyphal growth in detail in the two NILs. At 7 DAS, hyphae grew aggressively in Huangzao4, as shown in both transverse and longitudinal sections, whereas invading hyphae were sequestered in necrotic areas in Huangzao4R (Fig. 2B). Trypan blue was used to examine both cell viability and hyphal growth (Fig. 2C). In Huangzao4R, HR-like cell death occurred in the colonized areas, in which hyphae were confined to dead cells. In Huangzao4, however, hyphae spread extensively across living cells, implying ongoing interactions between pathogen and host. Calculation of *S. reilianum*-infected NILs at 7 DAS revealed that Huangzao4R had a significantly higher rate of HR-like cell death response than Huangzao4 (Fig. 2D). High expression of *ZmWAK* in the mesocotyl channel restricted hyphae access

to SAMs by inhibiting hyphal growth in Huangzao4R, but this did not occur in Huangzao4 because it lacks functional *ZmWAK* (Zuo et al. 2015).

Distinct cell death modes between the two NILs.

S. reilianum induced acute cell death within infected areas of Huangzao4R, in contrast to the latent and prolonged cell death observed in Huangzao4 (Fig. 1). Distinct cell fates between Huangzao4 and Huangzao4R prompted us to wonder whether different modes of PCD occurred in these two NILs. Transmission electron microscopy was performed on the two NILs at 7 DAS to seek ultrastructural evidence for the distinction between the PCD processes. In infected Huangzao4, cells underwent autophagy with typical double-membrane autophagic structures (Fig. 3A to C). Infected Huangzao4R, however, exhibited diagnostic features of AL-PCD, such as nuclear condensation and chromatin fragmentation, cell shrinkage, and plasma membrane blebbing (Fig. 3D to F). Large vacuoles were present within *S. reilianum* hyphae in the infected Huangzao4 but not in the infected Huangzao4R. Moreover, the infected Huangzao4R showed swollen mitochondrion and plasma membrane contraction from the cell wall, vacuole rupture, and peroxisome emergence (Supplementary Fig. S3). Both infected NILs were also stained with the fluorescent dye monodansylcadaverine (MDC), an autophagosome marker (Supplementary Fig. S4). MDC-marked autophagosomes were observed only in infected Huangzao4. These data indicated that Huangzao4 underwent autophagy while Huangzao4R underwent

Fig. 2. Hypersensitive response (HR)-induced cell death restricts *Sporisorium reilianum* growth. **A**, Confocal images of *S. reilianum* invasion of Huangzao4 and Huangzao4R. *S. reilianum* hyphae were stained with wheat germ agglutinin, and samples were taken 7 days after sowing and were assessed for green fluorescent protein fluorescence. Images were merged with differential interference contrast images. Scale bars = 1 mm. **B**, Scanning electron microscopy images of introgressed hyphae for both transverse and longitudinal sections. Red arrowheads denote *S. reilianum* hyphae. The white dashed line denotes the area of necrosis, in which hyphae were sequestered. Scale bars = 100 μ m. **C**, Trypan blue staining for both hyphae and cell viability. Red arrowheads denote *S. reilianum* hyphae. White dashed lines denote the areas of necrosis. Scale bars = 100 μ m. **D**, Calculation of HR-like cell death in the two near-isogenic lines. Mesocotyls were taken 7 days after sowing, samples were hand cut and were observed under a light microscope. Three independent experiments were carried out, and the mesocotyls with HR cell death is expressed as a percentage. n = number of plants investigated. The error bars denote standard error. Asterisks (**) indicate $P < 0.01$ (Student's *t* test).

AL-PCD upon *S. reilianum* infection, *ZmWAK* introgression leads to the activation of cellular AL-PCD machinery in Huangzao4R.

A transcriptional switch between growth and defense was regulated by *ZmWAK*.

Given the alternative cell-death strategy between two NILs, we were interested to discover the specific role that *ZmWAK* played in response to *S. reilianum* invasion at the transcriptional level (Table 1). Global transcript profiling was performed to investigate *ZmWAK*-associated transcriptional changes. Mesocotyls from the two NILs, with or without *S. reilianum* infection, were taken at 7 DAS. Differentially expressed genes (DEGs) between the two NILs or between treatments were defined as genes having a log₂-fold change of >1 and false discovery rate of <0.01, the DEGs were functionally annotated with MAPMAN BIN (barcode index number). The *ZmWAK*-associated response could be classified as a basal response (DEGs observed in non-infected mesocotyls) or defense response (DEGs between non-infected and infected mesocotyls). Supplementary Table S1 lists all the DEGs, and Figure 4 summarizes the observed transcriptional changes.

A comparison of noninfected mesocotyls between Huangzao4 and Huangzao4R allowed us to assess the *ZmWAK*-associated basal response (Supplementary Table S2). A total of 1,526 DEGs were identified as unique to Huangzao4R, of which 811 were up-regulated and 715 down-regulated. BIN enrichment analysis indicated significant over-representation of DEGs involved in cell-wall organization (cellulose synthase), cell wall-loosening agents (expansions and xyloglucan endotransglycosylase/hydrolase), and cell-wall arabinogalactans. Additionally, DEGs were also enriched in cell viability, i.e., cell cycle, cell division, cellular organization, and vesicle transport. Upregulated DEGs related to the cell cycle and cell-wall biosynthesis indicated that *ZmWAK*

promotes cell growth. Consistent with growth promotion, genes for sugar and fatty-acid biosynthesis were enriched in Huangzao4R. Genes involved in signal transduction (calcium signaling, MAPK cascade, G-protein signaling) were also up-regulated. MPK3 was up-regulated during the *ZmWAK*-associated basal response, which is similar to the *AtWAK*-associated growth response. On the other hand, the *ZmWAK*-associated basal response resulted in repression of genes involved in the biosynthesis of secondary metabolites (flavonoids, terpenoids, phenylpropanoids), production of reactive oxygen species (peroxidase, cytochrome P450), biotic stress response, and glycolysis. With regard to hormone signaling, *ZmWAK* promoted the expression of a set of genes that control auxin transport and response (*AUX*, *GH3*, *PIN*, and *SAUR*). Key genes involved in abscisic acid (ABA) biosynthesis (*NCED9*) and response (*ABI* and *HAI*) were up-regulated. Transcripts involved in the jasmonic acid (JA) (*JAZ*, *MYC2*) and ethylene (ET) biosynthesis (1-aminocyclopropane-1-carboxylate [ACC] synthase) and signaling pathways (*ERF/AP2*)

Table 1. RNAseq read counts and read mapping to the maize genome

Sample	All reads	Mapped to maize genome		Unique reads	
		Reads	Rate	Mapped	Rate
HZ4C-1	44,764,682	33,464,141	74.76%	28,586,092	63.86%
HZ4C-2	43,825,910	31,831,040	72.63%	26,236,353	59.86%
HZ4RC-1	42,011,266	32,608,605	77.62%	27,582,138	65.65%
HZ4RC-2	41,705,354	32,318,571	77.49%	26,945,178	64.61%
HZ4I-1	40,191,622	27,897,122	69.41%	23,647,272	58.84%
HZ4I-2	30,120,716	20,855,446	69.24%	16,406,790	54.47%
HZ4RI-1	37,247,598	28,711,029	77.08%	20,541,787	55.15%
HZ4RI-2	35,483,886	27,473,459	77.43%	20,575,789	57.99%

Fig. 3. Different cell death strategies between two near-isogenic lines in response to *Sporisorium reilianum* infection. **A, B, and C,** Transmission electron microscopy images of *S. reilianum*-infected Huangzao4. Black arrows indicate autolysosome/autophagosome-like structures. **D, E, and F,** *S. reilianum*-infected Huangzao4R. Dashed rectangles indicate nuclear condensation and chromatin fragmentation. Red arrows indicate plasma membrane blebbing. The black arrowhead indicates plasma membrane contraction from the cell wall. All samples were taken 7 days after sowing. H = hyphae; V = vacuole, N = nucleus, and PM = plasma membrane. Scale bars = 1 μm.

with 573 DEGs for Huangzao4. After eliminating 336 DEGs in common, the remaining 1671 DEGs, of which 797 were up-regulated and 874 down-regulated, were assigned to *ZmWAK*-associated defense response (Supplementary Table S3). Surprisingly, up to 886 DEGs showed opposite enrichment between the *ZmWAK*-associated basal and defense responses (Fig. 4B and 4C), implying a contrasting role for *ZmWAK* between two responses. It is also fascinating to notice that a total 179 transcripts for TFs were shown to participate *ZmWAK*-mediated defense response, including NAC, bZIP, bHLH, WRKY, MYB, GRAS, and other transcription family, indicating a strong reprogramme of downstream pathways. For instance, WRKY33, an important transcription factor for necrotrophic pathogens was over-represented in *ZmWAK*-mediated basal response while decreased after *S. reilianum* invasion (Zheng et al. 2006). In contrast to the *ZmWAK*-associated basal response, typical transcripts for biotic stress response, such as SA-induced pathogenesis-related proteins, RLKs, chitinase, glutathione *S* transferase, and production of reactive oxygen species, were highly up-regulated in the *ZmWAK*-associated defense response. DEGs that were repressed included those for cell-wall synthesis, cell organization, vesicle transport, and signal transduction (calcium signaling, MAPK cascade, G-protein signaling). However, in contrast to what has been reported for MPK6 in the *AtWAK*-associated defense response, MEK1, an upstream activator of MPK4, was up-regulated in the *ZmWAK*-associated defense response (Huang et al. 2000). We speculate that *ZmWAK* activated a new MAPK pathway in head-smut defense response.

With regard to metabolic pathways, genes related to biosynthesis of terpenoids, phenylpropanoids, and lignin were significantly up-regulated, whereas genes involved in biosynthesis of sucrose and fatty acids were down-regulated, suggesting a general induction of defense-related compounds. Additionally, upregulation of glycolysis, along with repression of the tricarboxylic acid (TCA) cycle, suggested that maize cells respond to infection by predominantly making use of glycolysis rather than the TCA cycle for energy provision. A large fraction of induced transcripts were also targeted to new BINs as present in protein degradation through the ubiquitination pathway, mitochondrial electron transport (NADH dehydrogenase, cytochrome *c* oxidase, apocytochrome b), ATPases, and ATP synthesis. Thus, upon *S. reilianum* infection, *ZmWAK* repressed mesocotyl growth and initiated a plant resistance response typical for defense against biotrophic fungi.

S. reilianum invasion also broke the balance of phytohormones. Genes for auxin transport and response (*AUX*, *GH3*, *PIN*, and *SAUR*), ABA biosynthesis (*NCED3* and *NCED9*) and response (*ABI* and *HAI*), JA biosynthesis (*AOS*) and signaling (*JAZ*), ET biosynthesis (*ACC* synthase), and ET-responsive transcription factors of the ERF/AP2 family were all down-regulated, whereas genes for SA (*PAL*) and GA biosynthesis (*GA3*) were up-regulated.

Arabidopsis AtBAG6 is a plant-specific calmodulin-binding protein and a cell-death regulator. Notably, BAG1 and BAG6—the two main plant apoptosis regulators—exhibited unique expression pattern in Huangzao4R. *BAG* genes were overexpressed in Huangzao4R but had undergone a drastic decrease upon *S. reilianum* infection, which was not observed in Huangzao4. Moreover, Ca²⁺ signaling/calmodulin proteins had expression patterns similar to those of BAG proteins, which were induced during the *ZmWAK*-associated basal response but repressed during the *ZmWAK*-associated defense response. Changes in mRNAs encoding BAG6 and Ca²⁺ signaling/calmodulin proteins hint at a potential cell-death control mechanism governed by *ZmWAK*. We hypothesize that *ZmWAK* regulates the activities of BAG proteins and calmodulin to maintain cells in an antideath state in noninfected plants, whereas

ZmWAK promotes apoptosis via downregulation of BAG proteins to release cells from the antideath state and activate intrinsic apoptotic signaling upon *S. reilianum* infection. But we failed to detect an expression change of *Atgs* upon *S. reilianum* infection in Huangzao4, perhaps because autophagy was weak in Huangzao4 at 7 DAS and the number of cells that had undergone autophagy was limited.

Metabolic switch is regulated by *ZmWAK*.

Transcriptomic analysis implicated the *ZmWAK*-associated defense response in the expression of numerous genes related to metabolism. Liquid chromatography–coupled mass spectrometry (LC-MS) enabled comprehensive and quantitative analysis of metabolite fluctuations in the two infected NILs and their noninfected controls. A metabolite was considered enriched or depleted when the intracellular concentration changed by more than 1.2-fold with *P* < 0.01. The relative changes for all metabolites are visualized in a Venn diagram and heatmap (Fig. 5).

In noninfected plants, *ZmWAK* introgression altered concentrations of 64 metabolites, including 39 that were increased and 25 that were decreased (Supplementary Table S5). In comparison with Huangzao4, Huangzao4R had elevated levels of nucleotides and fatty acids. In line with the transcriptome data, the content of certain phytohormones, like auxin, zeatin, ABA, and JA, were increased. The accumulation of phenylpropanoids might be a consequence of downregulated PAL for SA biosynthesis. Likewise, higher content of GA12, which lies at the initial branch point of GA biosynthesis, is possible, due to an outcome of repression of downstream GA biosynthesis. With regard to secondary metabolites, seven flavonoids were increased, whereas 13 were decreased.

The concentrations of 73 metabolites in Huangzao4R were altered in response to *S. reilianum* infection compared with 46 in Huangzao4. After filtering out 23 metabolites with a common trend of change between the two NILs, 50 altered metabolites (five metabolites changed in opposite trends between the two NILs) were unique to Huangzao4R (Supplementary Table S6). A striking number of amino acids were at lower concentrations in infected relative to noninfected Huangzao4R, whereas this was the case for only three amino acids in infected Huangzao4, implying a reduced carbon/nitrogen flux for amino acid biosynthesis in Huangzao4R after *S. reilianum* infection. Likewise, nucleotide and fatty-acid levels were lower in the infected than noninfected Huangzao4R. The concentrations of metabolites involved in the TCA cycle, including glutaric acid, aconitic acid, citrate, isocitrate, and malic acid, were remarkably lower in infected Huangzao4R as well. Glucose concentrations were generally much lower in the infected than noninfected Huangzao4R, whereas glucose 6-phosphate and fructose 1,6-bisphosphate concentrations were higher after infection. Changes in the concentrations of metabolic intermediates for both glycolysis and the TCA cycle are consistent with the previous transcriptomic data, i.e., that glycolysis rather than the TCA cycle replenishes cellular ATP in response to *S. reilianum* infection. For flavonoids, an increasing trend was generally observed. Analysis of phytohormones revealed a relatively low content for each of JA, ABA, and auxins after infection, whereas the SA level increased, which is consistent with our observation in transcriptomic analyses that the *ZmWAK*-associated defense response resulted in down-regulation of JA biosynthesis but promotion of the biotrophic defense response.

DISCUSSION

Prior to the floral transition and internode expansion, the SAM is located just above the mesocotyl, and this architectural

feature renders the mesocotyl a vital entryway for *S. reilianum* to access SAMs. Syringe infection of the SAM with a hyphae culture is an effective way to experimentally generate head smut in maize (Ghareeb et al. 2011), but this approach is time-consuming and bypasses the mesocotyl—the crucial tissue that determines head-smut resistance. Considering the pathogenic

mechanism of *S. reilianum*, the passage of this pathogen through the mesocotyl may be the decisive factor for head-smut pathogenesis. We suggest that inspection of mesocotyls at the seedling stage and assessment of the SAM invasion rate would be a time-saving means of discriminating susceptible from resistant varieties.

Fig. 5. Analysis of metabolites Huangzao4 and Huangzao4R with or without *Sporisorium reilianum* infection. **A**, Venn diagram illustrating the number of metabolites whose levels changed significantly in three pairs of samples, i.e., HZ4C and HZ4RC, HZ4I and HZ4C, and HZ4RI and HZ4RC (fold change >1.2, $P < 0.05$) and the overlapping metabolites among the three comparisons. **B**, Heatmap showing all metabolites whose levels increased or decreased significantly in at least one of the three comparisons. Fold changes were $\log_2$ -transformed, and the data represent the average from six biological replicates. HZ4C = noninfected Huangzao4, HZ4RC = noninfected Huangzao4R, HZ4I = infected Huangzao4, and HZ4RI = infected Huangzao4R.

In the current study, cytological analysis provided convincing evidence that cell death modes control the outcome of the interaction between a biotrophic fungus and host. Before the floral transition, *S. reilianum* propagates in a biotrophic mode, relying on living cells for nutrient acquisition. Thus, AL-PCD in Huangzao4R blocks pathogenesis, whereas the prosurvival process of autophagy in Huangzao4 facilitates fungal colonization. Growing research over the past decades have drawn a picture of the important role of BAG proteins in plant biotic and abiotic stress response, particularly in cell-death control, in which BAG6 is well identified and characterized. Upon over-expression of *AtBAG6* in yeast, the cells exhibited normal growth in glucose-containing medium but underwent apoptosis in galactose-containing medium (Kang et al. 2006). An *atbag6* mutant showed enhanced susceptibility to necrotrophic *Botrytis cinerea* (Doukhanina et al. 2006). Cleavage of *AtBAG6* by two aspartyl proteases resulted in autophagy and enhanced resistance to *B. cinerea* (Li et al. 2016). In our study, the *ZmBAG* genes are preinduced and undergo a drastic decrease upon *S. reilianum* infection, which expression pattern is unique in Huangzao4R. It is conceivable to infer that *ZmWAK* exerts its specific role in dictating the expression of *ZmBAG* genes and that downregulation of *ZmBAG* genes could prevent them from being cleaved or inducing autophagy.

The well-established *Arabidopsis* molecular model suggests that WAK proteins act at the front line of plant cell defense, where they sense and respond to changing environmental conditions. Recognition of pectin or OGs by WAK proteins provides a mechanism by which plant cells can switch between growth and the defense response. Consistent with previous studies, global transcript profiling along with LC-MS-based metabolite profiling of the two NILs we used provide compelling evidence of functional trade-offs in the *ZmWAK*-associated response to a changing environment. If *ZmWAK* acts similarly to *Arabidopsis* WAK (*AtWAK*), our findings

could help to define the mechanism of *ZmWAK*-mediated head-smut resistance (Fig. 6). It is quite possible that *ZmWAK* binds pectin to promote growth and depress the defense response in the absence of pathogen. Invading *S. reilianum* may secrete polygalacturonase to release OGs from the cell wall. Perception of OGs by *ZmWAK* thus downregulates cell growth and upregulates the SA-dependent defense response, thereby promoting AL-PCD to prevent *S. reilianum* from further invasion. This growth-defense trade-off requires coordination among multiple pathways, including signal transduction, transcription factors, hormone metabolism, production of reactive oxygen species, reallocation of primary and secondary metabolites, and mobilization of the PCD machinery. What is impressive is if such a broad spectrum of responses were modulated by one gene, *ZmWAK*. Knowing how *ZmWAK* signaling transduct downstream might broaden our understanding of the mechanisms.

Concerning resistance genes, plants have always had the dilemma of whether to promote growth or defense, as enhanced defense often has a yield penalty. *ZmWAK*, with its plasticity of adaption to environmental fluctuations, could promote growth in noninfected plants and switch to the defense response upon *S. reilianum* infection. This fine-tuning of the trade-off between growth and defense makes *ZmWAK* an ideal target for breeding programs. Although *ZmWAK* contributes to the normal growth of plants, the lack of *ZmWAK* in Huangzao4 does not substantively impact growth. We speculate that this is a consequence of the redundancy of WAK families in maize (Zuo et al. 2015). Indeed, transcriptome data revealed a relatively greater abundance of other WAK family members in Huangzao4 than Huangzao4R, although the absolute abundance of other WAK proteins was much less than that of *ZmWAK*. Functional compensation from other WAK family members may also explain the partial resistance observed for Huangzao4.

It has long been recognized that the complex crosstalks of hormones optimize plant response to diverse environmental cues and balance the resistance response and fitness penalty. SA and JA/ET have been shown to function antagonistically in the defense against specific pathogen with different lifestyles. SA is required for the regulation of biotrophic pathogen defense; whereas JA/ET are positive regulators of plant defense against necrotrophic pathogens (Glazebrook 2005). SA has also been reported to inhibit growth via repression of auxin signaling, and elevated auxin signaling mostly leads to enhanced susceptibility to biotrophic pathogens (Huot et al. 2014; Robert-Seilanian et al. 2011). It is noteworthy that the hormone homeostasis state was different between Huangzao4 and Huangzao4R under the noninfected condition. Huangzao4R showed higher expression of JA-, ET-, ABA-, and auxin-responsive genes while showing lower expression of GA and SA, as compared with Huangzao4. Intriguingly, *S. reilianum* infection disturbed the original balance of hormonal crosstalk in Huangzao4R, leading to the global alteration of hormone biosynthesis and the expression of related genes. *S. reilianum* growth restriction in Huangzao4R was accompanied by the accumulation of SA and activation of SA signaling marker gene PR proteins, suggesting that SA-dependent biotrophic pathogen resistance also exerts its role in mesocotyl defense against *S. reilianum*. Consistent with the negative crosstalk between SA and JA, JA content was markedly decreased and the expression of key genes for JA and ET biosynthesis and signaling were strongly down-regulated. In addition to the classic defense hormone, other hormone pathways have also been affected by *S. reilianum* infection. ABA and auxin content and signaling were decreased, whereas GA content and signaling increased. It is surprising to find that *ZmWAK* controls all of the key genes for these hormones, making it logical to infer that *ZmWAK*

Fig. 6. Mechanism of *ZmWAK*-mediated head-smut resistance.

stands at the hub of the intricate hormonal networks and modulates the entire hormone network, rather than merely one specific hormone-related pathway, to precisely respond to environmental changes. *ZmWAK* introgression changed the state of hormonal equilibrium in Huangzao4, in which growth-promoting auxin was overrepresented to benefit plant growth. Upon pathogen attack, defense hormone SA was increased to switch plants into a defense mode.

Given our findings for *ZmWAK*, we conclude that this gene functions as a hub in the growth and defense trade-off to optimize plant fitness. Still, the following five questions must be addressed. First, is *ZmWAK* a receptor for both pectin and OGs? Second, how does upstream signaling lead to a switch between basal and defense response pathways downstream? Third, how does *ZmWAK* alone orchestrate such a broad range of transcriptional and metabolic responses? Fourth, how does *ZmWAK* impact the abundance of BAG proteins? Finally, do any pathogen components participate in the *ZmWAK*-associated defense response? Our understanding of *ZmWAK*-associated head-smut resistance is rather rudimentary and numerous aspects await exploration.

MATERIALS AND METHODS

Plant growth and inoculation.

The F₁ hybrid was made between the donor line Ji1037 and recurrent parent Huangzao4, followed by continuous backcrossing to Huangzao4 to generate BC₉F₁ generation. The BC₉F₁ plant with the shortest *ZmWAK* donor segment (152 kb) was selfed twice to produce a pair of NILs, Huangzao4R (with *ZmWAK*) and Huangzao4 (without *ZmWAK*), identified by using a codominant marker MZAI (Supplementary Table S7). We further used a GoldenGate 6KSNP chip (Illumina) to examine the two NILs, and the result showed they have 99.65% identity in their genetic backgrounds.

The field infection study was performed in Gongzhuling, Jilin Province, China. Galls from susceptible lines were collected to prepare teliospores. After sowing, seeds were covered with contaminated soil containing 0.1% teliospores. Disease severity was scored at the filling stage when symptoms fully appeared.

Indoor infection was performed in an illumination incubator under a 16-h-light and 8-h-dark cycle at 28°C and 60% relative humidity. Seeds were soaked in 2% NaClO for 30 min and were rinsed three times with distilled water. Soil was autoclaved at 121°C for 20 min prior to sowing seeds. Sterilized seeds were sown in sterile soil (mock treatment) or soil containing 0.1% spores.

WGA staining.

WGA staining was carried out as described by Ghareeb et al. (2011). Bottom mesocotyls were harvested, were cut into thin sections, and were placed into 2-ml tubes. Samples were rinsed with distilled water once and were then soaked in 10% KOH overnight. Samples were then rinsed three times with distilled water and were incubated with WGA stain solution (10 mg of WGA per milliliter, 0.02% (vol/vol) Tween 20 in phosphate-buffered saline [PBS], pH 7.4) for 30 min under vacuum infiltration. After washing three times with PBS, samples were observed under an Olympus FV 1000 fluorescence microscope with excitation at 488 nm and emission at 500 to 540 nm.

Trypan blue staining.

Seedlings were collected 7 DAS. Removal of all leaves and roots yielded intact mesocotyls. Mesocotyls were then incubated at 100°C for 5 min in trypan blue staining solution, which consisted of two volumes ethanol plus one volume stock

solution (0.02 g of trypan blue, 10 g of phenol, 10 ml of glycerol, 10 ml of lactic acid, 10 ml of water). After further staining overnight, trypan blue was removed and was replaced with chloral hydrate several times, for bleaching before inspection. Samples were cut and were observed under a light microscope.

Analysis of SAM invasion rate.

SAMs were harvested from infected NILs at 21 DAS. Total RNA was extracted using the plant Total RNA kit (Tiangen), and first-strand cDNA was synthesized using the Easy Script first-strand cDNA synthesis kit (Transgen). The presence or absence of *S. reilianum* was assessed with PCR and the *S. reilianum*-specific primer SRac (sr11345). PCR cycling was as follows: 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, with a final 72°C for 10 min. PCR products were examined in 1% agarose gels, after staining with ethidium bromide.

Scanning electron microscopy.

Mesocotyl samples were fixed for >2 h in 2.5% glutaraldehyde in PBS at 0 to 4°C, were rinsed three times with the same buffer, and were immersed in 1.0% osmium tetroxide in PBS for 2 h. After rinsing twice with PBS, samples were dehydrated in a graded series of ethanol (30, 50, 70, 80, 90, and 100%), were subjected to critical-point drying, and were coated with gold. Scanning electron microscopy images of the patterned gold samples were acquired using a Hitachi S-3400N.

Transmission electron microscopy.

Mesocotyl samples were fixed for >2 h in 2.5% glutaraldehyde in PBS at 0 to 4°C, were rinsed three times in the same buffer, and were immersed in 1.0% osmium tetroxide in PBS for 2 h. After rinsing three times with PBS, samples were dehydrated in a graded series of acetone (30, 50, 70, 80, 90, and 100%). Samples were then embedded in epoxy resin. Ultrathin sections were cut with a Leica UC6 ultramicrotome and were then stained with uranyl acetate and lead citrate. Finally, samples were examined and were photographed using transmission electron microscopy (JEOLJEM-1230).

MDC staining.

Mesocotyls were stained with MDC as described by Kabbage et al. (2013). Seedlings were incubated for 30 min in 0.05 mM MDC in PBS. After washing three times with PBS, samples were observed with the Olympus FV 1000, using the UV channel.

RNA sequencing.

Infected and noninfected mesocotyls were taken 7 DAS with two replicates. Total RNA was extracted using TRIzol (Invitrogen) and were purified with the PureLink RNA mini kit (Invitrogen). RNA quality was assessed, using the Agilent RNA 6000 Pico kit, prior to preparation of the sequence library. A cDNA library for each sample was constructed using “mRNA Seq sample prep kit” (Illumina). Sequencing of each cDNA library was carried out on an Illumina HiSeq 2500 paired-end 100-bp (PE 100) system. Raw sequencing data were evaluated by FAST-QC. Total reads were mapped to maize B73 RefGen_V2 (Schnable et al. 2009), using Bowtie and TopHat software. Expression values were determined as fragments per kilobase exon per million mapped reads, using Cufflinks. DEGs between samples were defined as having a log₂-fold change >1 and false discovery rate <0.01. Functional annotation of DEGs was conducted with MAPMAN.BIN tool. The heatmap for DEGs was generated by Hemi (Deng et al. 2014).

Real-time PCR.

Infected and noninfected mesocotyls were taken 7 DAS. Total RNA was extracted and the first-strand cDNA was synthesized as described above. Quantitative PCR was performed on a Rotor-Gene Q 6000, using SYBR green (Takara). The conditions for PCR amplification were as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. The fold change in target gene expression was normalized to expression levels of tubulin and GAPDH, and relative expression was determined by the cycle threshold ($2^{-\Delta\Delta C_t}$) method. The mean and standard deviation were calculated from three biological repeats.

LC-MS-based metabolomics analysis.

Infected or noninfected mesocotyls (150 mg) were harvested 7 DAS, and six replicates were set up. Samples were frozen immediately in liquid nitrogen and were stored at -80°C until extraction. Mesocotyls were ground in liquid nitrogen and were mixed with 400 µl methanol and water (1:1). After 3 min of vigorous agitation, samples were centrifuged at 12,000 × g at 4°C for 10 min. The supernatant was transferred to a vial, and a 100-µl sample was subjected to ultraperformance liquid chromatography (UPLC)-coupled quantitative time-of-flight (Q-TOF) mass spectrometry.

The supernatant was separated with chromatography using an Agilent 1290 Infinity UHPLC system (Agilent Technologies). A Waters UPLC HSS T3 C18 column (1.8 µm 100 × 2.1 mm) was used for the reverse-phase separation. The mobile phase consisted of 0.1% formic acid (buffer A), and 0.1% formic acid in acetonitrile (buffer B). Gradient elution consisted of 2% B for 2 min, 2 to 95% B from 2 to 17 min, and 95% B from 17 to 19 min. The total run time was 25 min including a 6-min period to re-equilibrate at 2% B. The flow rate was 350 µl/min, and the injection volume was 3 µl.

An Agilent 6538 UHD and Accurate-Mass (Q-TOF) mass spectrometer was operated in positive ion mode and negative ion mode with a capillary voltage of 4 kV in positive ion mode and 3.5 kV in negative ion mode, drying gas flow of 11 liters per min, and a gas temperature of 350°C. The nebulizer pressure was set at 45 psi. The fragmentation voltage was set at 120 V and skimmer voltage at 60 V. All analyses were obtained by means of an automated calibrant delivery system using a dual-nebulizer ESI source that introduces a low flow (100 ml/min) of a reference solution (Agilent Technologies), which contains the internal reference masses at m/z 121.0509, 922.0098 in positive ion mode and m/z 112.9856, 1,033.9881 in negative ion mode to ensure mass accuracy and reproducibility. The mass range was set at m/z 100 to 1,100.

Raw data obtained in instrument-specific format (.d) were converted to common data format (.mzData) files using Agilent Mass Hunter qualitative analysis (B.03.01) software that eliminated isotope interferences. The program XCMS was used to extract raw data signals (peak identification and integration), correct retention time, and for automatic integration and deconvolution analysis (best mass spectrometry fragmentation) in R software platform. Data were collected in centroid mode, and metabolites were identified by matching m/z values in the METLIN database with mass error <5 ppm. The list of peaks (deprotonated m/z values) along with corresponding retention time, putative annotation, and peak intensity in a matrix were exported to Microsoft Excel for further data processing.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

MAPMAN.BIN tool: <http://www.gabipd.de/projects/MapMan>
 METLIN database: <https://metlin.scripps.edu/index.php>