CLASSIFICATION OF RECEPTOR-LIKE CYTOPLASMIC KINASES IN MAIZE AND FUNCTIONAL ANALYSIS OF ZMBLK1

by

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For my family and friends

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ABSTRACT

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Receptor-like cytoplasmic kinases (RLCKs) form a large family of proteins in plants. RLCKs have been found in different plant species, regulating plant immunity to different bacterial and fungal pathogens. Previous studies implicated *Arabidopsis botrytis induced kinase1 (BIK1)* and *tomato protein kinase 1b (TPK1b)* in plant resistance to *Pseudomonas syringae* and *Botrytis cinerea*. In this study, we classified 195 putative maize RLCKs into ten subfamilies. Based on the amino acid sequence similarity to BIK1 and TPK1b, a novel maize RLCK, *zea mays bik1-like kinase 1 (ZmBLK1)* was identified. Enzyme assays with cloned *ZmBLK1* revealed a functional kinase when expressed in planta. The recombinant protein located to the plasma membrane. Expression of *ZmBLK1* is highest in maize leaves compared to other structures at silking stage. Expression of the recombinant *ZmBLK1* significantly reduced the rate of lesion spread in maize leaves inoculated with the Goss's wilt pathogen. In maize kernels, expression of *ZmBLK1* increases during kernel maturation. Kernels from transgenic maize overexpressing *ZmBLK1* were not resistant to *Aspergillus flavus* or to aflatoxin contamination. In addition, mutations were made in *ZmBLK1* that were hypothesized to create a constitutively active kinase. However, resulting proteins had similar activity to the wild-type ZmBLK1 and transgenic plants showed similar responses to the Goss's wilt and Aspergillus ear rot pathogens. Overall, this research established the first

characterization of RLCKs in maize and described a potential contribution of ZmBLK1 to maize

immune responses.

CHAPTER 1.   BACKGROUND AND RESEARCH OBJECTIVES

1.1   Background

Protein kinase superfamily is one of the largest group of enzymes in eukaryotic organisms (Hanks et al., 1995). These enzymes catalyze the reversible transfer of γ-phosphate from adenosine triphosphate (ATP) to serine, threonine or tyrosine residues in the acceptor proteins. The protein domain is responsible for this phospho-transfer reaction, which is defined as the kinase domain, is highly conserved between kinases (Hanks et al., 1988; Stone et al., 1995). A kinase domain generally consists of 250 to 300 amino acid residues, which are divided into 11 subdomains (I to XI) (Stone et al., 1995; Hanks et al., 1988). In plants, a growing number of protein kinases are known and phylogenetic analyses classify these kinases into 9 groups with more than 80 families, which include the AGC group (consisting of PKA, PKG, and PKC families), the CAMK group (CAMK and CDPK families), the CK1 group (casein kinase 1), the CMGC group (CDK, MAPK, GSK-3, and CKII families), the STE group, the TK group (algae specific tyrosine kinase), the TKL (plant-specific tyrosine kinase-like kinase, and the "other" group (all other kinases without grouping) (Lehti-Shiu et al., 2012). Some families have central roles in metabolic processes that are conserved across eukaryotes. For example, AMP-activated protein kinases (AMPKs) maintain cellular energy homeostasis and cyclin-dependent kinases (CDKs) regulate cell cycle progression (Thelander et al., 2004; Hardie, 2011; Joubès et al., 2010). On the other hand, some of the kinase families display different level of plant specificity functions both phylogenetically and functionally. For instance, the calcium-dependent protein kinases (CDPKs) family and the receptor like kinase (RLK/Pelle) family have a high degree of expansion. Lehti-Shiu & Shiu (2012) suggest that these kinases are recent in the evolutionary history of plant genomes and that their numbers have

increased to fulfill a wide range of plant specific functions. The RLK family is dramatically larger compared to all the other plant kinase families (Lehti-Shiu et al., 2012; Stone et al., 1995). *Arabidopsis* has 610 RLKs and rice has 1100 RLKs, respectively in their genomes (Shiu et al., 2001b, 2004). The large number of RLKs suggest that they have a wide range of functions in plant, which is supported by a growing number of studies.

RLKs are reported to have essential or regulatory roles in development, disease resistance, hormone perception, and self-incompatibility (Shiu et al., 2001a). Generally, an RLK consists of an extracellular domain, a transmembrane region, and an intracellular kinase domain (Shiu et al., 2001a; Stone et al., 1995). However, a large group (25%) of kinases in the RLK family lack the extracellular domain. These kinases are subclassified as receptor-like cytoplasmic kinase (RLCK). In *Arabidopsis*, RLKs are classified into 44 subfamilies (Shiu et al., 2001a). These 44 include 33 containing 15 different types of extracellular domains and 11 lacking the extracellular domain (RCKLs) (Shiu et al., 2001a). In rice, which is the only other plant system that RLKs are phylogenetically categorized, 1100 RKLs are in 56 different types of extracellular domains and others sort into 23 RLCK subfamilies.

Among the RKLs with extracellular domain, the most abundant ones contain leucine-rich repeats (LRRs). There are 239 LRR-RLKs identified from the *Arabidopsis* RLKs (Shiu et al., 2001a). Some well-studied LRR-RLKs include BRASSINOSTEROID INSENSITIVE 1 (BRI1), FLAGELLIN SENSITIVE 2 (FLS2) and CLAVATA1 (CLV1) (Song et al., 1995; Friedrichsen et al., 2000; Clark et al., 1993; Gómez-Gómez et al., 2000). BRI1 is the receptor for brassinosteroid hormones (BRs), a series of small growth promoting molecules (Belkhadir et al., 2006). FLS2 binds to bacterial elicitor flagellin to initiate bacteria-induced immune responses (Gómez-Gómez et al., 2000). Another major group of RLKs with extracellular domains are the lectin-binding RLKs

(LecRLKs). This group is involved in recognition of extracellular carbohydrate signals, such as plant hormones, growth factors and pathogen effectors. *Arabidopsis* has 74 LecRLKs (Vaid et al., 2012) with roles in regulating plant development and stress response and pathogen defense. For instance, the *Arabidopsis* SGC is required for pollen development (Wan et al., 2008). Mutation of *SGC* leads to pollen deformation and collapse after anther stage 8. *LecRK-b2* and *AtLPK1* were reported to provide salt stress tolerance by regulating the ABA signaling pathways, and LecRK-VI.2 was found to be required for *Arabidopsis* pattern-triggered immunity (PTI) (Deng et al., 2009; Huang et al., 2013; Singh et al., 2013).

Shiu and Bleecker (2001b) first classified RLCKs in *Arabidopsis* by phylogenetic methods. Typically, RLCKs only contain a Ser/Thr-specific cytoplasmic kinase domain consisting of 11 kinase subdomains, a short N-terminal region and a short C terminal region (Shiu et al., 2001b). The relationships between RLCKs and RLKs in plants are analogous to animal nonreceptor tyrosine kinases (NRTKs) and receptor tyrosine kinases (RTKs) (Hubbard et al., 2000; Shiu et al., 2001a). In animals, a family of NRTKs are involved in signaling cascades regulated by RTKs (Hubbard et al., 2000). In plants, many RLCKs are localized to the plasma membrane by N-terminal myristoylation or palmitoylation and they can physically interact with RLKs that are also localized to the plasma membrane (Hohmann et al., 2017; Lin et al., 2013; Lu et al., 2010). These RLK/RLCK interaction-complexes regulate downstream pathways via transphosphorylation events to achieve the perception of a broad range of ligands such as plant hormones and molecular patterns. The biological impacts include responses in development and to abiotic and biotic stresses (Hohmann et al., 2017; Liang et al., 2018).

There are several good examples of RCLKs involved in plant growth and development. Brassinosteroids (BRs) are a group of steroid hormones regulating cell elongation, cell division,

senescence, vascular differentiation, reproduction and photomorphogenesis (Ye et al. 2011). In Arabidopsis, BRs are perceived by the LRR-RLK BRASSINOSTEROID INSENSITIVE 1 (BRI1) (He et al., 2000). In absence of BRs, the kinase activity of BRI1 is inhibited by the RLCK, BRI1 KINASE INHIBITOR 1 (BKI1) (Wang et al., 2006). When BRs bind to BRI1, BKI1 is dissociated, allowing the initiation of downstream cascade events (Jia et al., 2002; Nam et al., 2002). Other RLCKs are also involved in the downstream events, including CONSTITUTIVE DIFFERENTIAL GROWTH 1 (CDG1) and Brassinosteroid Signaling Kinases (BSKs) (Kim et al., 2011; Sreeramulu et al., 2013).

Another important RCLK involved in plant development is SHORT SUSPENSOR (SSP), which functions in a MAPK cascade regulating embryonic pattering. *SSP* transcripts produced in the sperm cell are transported into the egg cell and translated in the zygote and central cell (Bayer et al., 2009). Mutations in *SSP* suppresses zygote elongation and leads to excess stomata formation (Lukowitz et al., 2004; Bergmann et al., 2004). Genetic analyses revealed that SSP functions upstream of a cascade that includes the MAPKK kinase YODA (YDA), the MAPK kinase MKK4/MKK5, the MAP kinase MPK3/MPK6 and the transcription factor GROUNDED (GRD) (Lukowitz et al., 2004; Bayer et al., 2009). However, it has not been reported whether SSP directly interacts with YDA in these cascade events.

Besides controlling plant development, RLCKs have a role in plant responses to environmental (abiotic) changes. In *Arabidopsis*, an RLCK, CALMODULIN-BINDING RECEPTOR-LIKE CYTOPLASMIC KINASE 1 (CRCK1) is induced under cold, salt, H_2O_2, and ABA stresses (Yang et al., 2004). Upon binding to calmodulin, the kinase activity of CRCK1 increases and is thought to transduce a stress signal through a downstream cascade that is poorly understood. A similar gene in soybean (*Glycine soja*), CALMODULIN-BINDING RECEPTOR-

LIKE KINASE (GsCBRLK) enhances tolerance to salt and ABA stresses when overexpressed in *Arabidopsis* (Yang et al., 2010). Other less understood RLCKs included *OsRLCK253* from rice and *GsRLCK* from soybean, which enhanced salt and drought tolerance when overexpressed in *Arabidopsis* (Giri et al., 2011; Osakabe et al., 2010).

Our best understanding of the role RLCKs have in plant responses to pathogens is from studies on *botrytis-induced kinase 1* (*BIK1*) in *Arabidopsis*. The loss of *BIK1* function leads to increased susceptibility to *Botrytis cinerea* but increased resistance to *P. syringae* (Veronese, 2006). BIK1 interacts with two LRR-RLKs, FLAGELLIN-SENSING 2 (FLS2) and BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1), under conditions without a pathogen effector, such as bacterial flagellin. FLS2 and BAK1 form a complex which directly phosphorylates BIK1 when flagellin is present. The phosphorylated BIK1 then dissociates with FLS2 to activate downstream signaling cascades (Lu et al., 2010; Zhang et al., 2010). The resulting responses include calcium burst, ROS burst, stomatal closure, activation of hormone pathways (salicylic acid, ethylene and jasmonate). Research also indicates that BIK1 is essential for these responses (Laluk et al., 2011; Li et al., 2014; Liu et al., 2013).

In tomato, *the tomato protein kinase 1b* (*TPK1b*) encodes an RLCK protein homologous to BIK1 (AbuQamar et al., 2008). TPK1b and BIK1 are also comparable in terms of their functions in plant immunity. Interference of *TPK1b* transcription leads to altered responses in the ethylene pathway and increased susceptibility to *B. cinerea* and tobacco hornworm *Manduca sexta*. Expression of *TPK1b* can restore the *Botrytis* resistance phenotype of *Arabidopsis bik1* mutants. Further investigations indicate that TPK1b and PEPR1/2 ORTHOLOG RECEPTOR-LIKE KINASE1 (PORK1) form a complex like FLS2-BIK1 in Arabidopsis and transduce immunity signaling through phosphorylation events in response to systemin and wounding (Xu et al., 2018).

Another RLCK in tomato, PTO-INTERACTING 1 (PTI1) was found to be essential for the ROS accumulation in response to the bacterial peptides flg22 and flgII-28 and for *P. syringae* pv. *tomato* resistance (Schwizer et al., 2017). The receptors of the two peptides flg22 and flgII-28 are FLS2 and FLS3, respectively. Thus, it is highly possible that PTI1 may directly interact with FLS2 and FLS3 to contribute to their regulations.

Finally, several rice RLCKs are associated with pathogen responses. Genetic evidence indicates that OsRLCK176 functions downstream of the rice LysM receptor-like kinase OsCERK1 during signaling induced by peptidoglycan (PGN) and chitins (Ao et al., 2014). OsRLCK185 also directly interacts with and is phosphorylated by OsCERK1 (Yamaguchi et al., 2013). Both OsRLCK176 and OsRLCK185 dissociate from OsCERK1 after chitin treatment, suggesting a similar regulation mechanism as the FLS2-BIK1 complex in Arabidopsis. Moreover, four rice RLCKs, OsRLCK57, OsRLCK107, OsRLCK118 and OsRLCK176 are required for XA21-mediated immunity to *Xanthomonas. oryzae* pv. oryzae (Zhou et al., 2016).

1.2   Research Objectives

The idea of the studies described in this thesis came from the question of whether maize contains a homologue of *BIK1 and TPK1b* from *Arabidopsis* and tomato, respectively. Our initial hypothesis was that the maize gene functions in resistance to diseases and aflatoxin production by *Aspergillus flavus*. Very little is known about RLKs and RLCKs in maize. The first described RLK in maize is ZmPK1, which appears to have an extracellular lectin-binding domain (Walker et al., 1990). The only described RLCK in maize is ZmPti1a, which is involved in pollen activity (Walker et al., 1990). In this dissertation, I identified the RLKs and RLCKs in maize and selected a RLCK candidate as a putative homologue of *BIK1 and TPK1*, which was named <u>*Zea mays BIK1*</u>

Like Kinase 1 (*ZmBLK1*). To characterize *ZmBLK1* and test my initial hypothesis, transgenic maize lines were obtained that constitutively expressed *ZmBLK1*. In the following chapters, three objectives were addressed:

- Objective 1: Characterize the maize RLCKs and identify *ZmBLK1*.

- Objective 2: Determine if ZmBLK1 contributes to maize resistance to Goss's wilt disease.

- Objective 3: Determine if ZmBLK1 impacts resistance to kernel disease and aflatoxin

  contamination *by A. flavus*.

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CHAPTER 2.   A MAIZE BIK1-LIKE RECEPTOR-LIKE CYTOPLASMIC KINASE CONTRIBUTES TO DISEASE RESISTANCE

2.1   Abstract

Receptor-like cytoplasmic kinases (RLCKs) form a large subfamily of proteins in plants. RLCKs have been found in different plant species, regulating plant immunity to different bacterial and fungal pathogens. Previous studies implicated *arabidopsis botrytis induced kinase1 (BIK1)* and *tomato protein kinase 1b (TPK1b)* in plant resistance to *Pseudomonas syringae* and *Botrytis cinerea*. In this study, a novel maize RLCK, *zea mays bik1-like kinase 1 (ZmBLK1)*, was identified based sequence similarity. We demonstrated that ZmBLK1 displays protein kinase activity *in vitro*. The localization of ZmBLK1 to the plasma membrane implies that the protein may interact with other membrane proteins early in the immune response pathway. Constitutive expression of *ZmBLK1* in transgenic maize increased resistance to the Goss's wilt disease caused by the bacterial pathogen *Clavibacter michiganensis* subsp. *nebraskensis* (CMN). Furthermore, expression of *ZmBLK1* was induced in non-transgenic maize by CMN infection 12 h after inoculation. These findings support our hypothesis that *ZmBLK1* contributes to plant resistance to bacterial pathogens likely by modulating events early after infection.

2.2   Introduction

Receptor-like kinases (RLKs) belong to a large superfamily of plant proteins, with 610 members in *Arabidopsis* and 1100 members in rice (Shiu et al. 2004). In many plant species, RLKs participate in various signal transduction pathways underlying development, defense against pathogens, and self-incompatibility (Stein et al. 1991; Torii 2000; Becraft 2002; Walker and Zhang 1990). Generally, RLK proteins contain a ligand-binding extracellular domain, a transmembrane

region, and a C-terminal kinase domain (Shiu and Bleecker 2001). In the RLK superfamily, plants evolved a distinct subfamily called receptor-like cytoplasmic kinase (RLCK), which lacks the extracellular domain (Shiu et al. 2004). RLCKs have a serine/threonine protein kinase domain, including 11 highly conserved kinase subdomains (I to XI), a short N-terminal region and a short C-terminal region (AbuQamar et al. 2008; Hanks and Hunter 1995). In *Arabidopsis*, there are 147 RLCKs, and rice has 379 members (Vij et al. 2008). RLCKs are predicted to be localized to the plasma membrane through a post-translational N-terminal myristoylation. The myristic acid, which is attached to the N-terminal Gly residue, facilitates the anchoring of the proteins to membranes (Podell and Gribskov 2004; Maurer-Stroh et al. 2002). This plasma membrane localization enhances interaction between RLCKs and other membrane proteins. Frequently, RLCKs associate with RLKs to function in transphosphorylation events (Yamaguchi et al. 2013b; Lin et al. 2015).

RLCKs are involved in plant immune responses in several plant species, including *Arabidopsis*, tomato and rice (Veronese 2006; AbuQamar et al. 2008; Ao et al. 2014; Vij et al. 2008). A well-established model in *Arabidopsis* describes the interaction between the RLCK BIK1 with two leucine-rich repeat-receptor kinases, FLS2 and BAK1 (Lu et al. 2010; Chinchilla et al. 2007). The interaction between BIK1, FLS2 and BAK1 happens during the pathogen- or microbe-associated molecular patterns (PAMPs/ MAMPs) triggered immunity (PTI) (Jones and Dangl 2006). In response to the elicitor flagellin, FLS2 binds to flagellin. FLS2 and BAK1 then form a protein complex in which the activated BAK1 phosphorylates BIK1, which in turn phosphorylates the FLS2/BAK1 complex. The phosphorylated FLS2/BAK1 complex further phosphorylates BIK1. These transphosphorylation events are suggested to be required for the flagellin-induced immune signaling (Lu et al. 2010; Chinchilla et al. 2007). BIK1 also directly interacts with pattern-

recognition receptor PEPR1, NADPH Oxidase RbohD, EF-Tu receptor EFR and LysM receptor kinase CERK1 to mediate ethylene-induced immunity, reactive oxygen species (ROS) burst, bacterial elongation factor-Tu, and chitin-induced immunity, respectively (Liu et al. 2013; Li et al. 2014; Zhang et al. 2010). In addition, BIK1 was found to increase *Arabidopsis* susceptibility to aphids through suppression of the aphid resistance and senescence-promoting gene PAD4 (Lei et al. 2014). These studies provide evidence for a central role of BIK1 in plant innate immune responses.

In other plant species, RLCKs have been reported to have similar regulatory activities. *Tomato Protein Kinase 1b* (*TPK1b*) encodes an RLCK that is localized to the plasma membrane and required for tomato resistance against the fungal pathogen *B. cinerea* and the chewing insect tobacco hornworm (AbuQamar et al. 2008). In rice, *OsRLCK185* encodes an RLCK that is directly phosphorylated by a lysine motif-containing PAMP-receptor OsCERK1. Suppression of *OsRLCK185* expression resulted in reduced MAP kinase activation and reduced expression of chitin-induced genes *PBZ1* and *PAL1* (Yamaguchi et al. 2013a; Wang et al. 2017). In addition, the OsRLCK185/ OsCERK1 interaction is suppressed by Xoo1488, an effector produced by *Xanthomonas oryzae*, causing bacterial blight in rice, suggesting an essential role for the OsRLCK185/ OsCERK1 complex during PTI responses. Similarly, OsRLCK176/OsCERK1 interaction is required for chitin-induced ROS production (Ao et al. 2014). These previous studies indicate the significant function of RLCKs in plant immunity responses. Furthermore, based on protein structure and phylogenetic analyses of RLCKs in *Arabidopsis* and rice, most immunity-related RLCKs belong to the RLCK VII subfamily (Shiu et al. 2004; Rao et al. 2018). Therefore, one can hypothesize that RLCK-VII members, which are highly conserved in terms of their protein structures, are also conserved in functions across dicot and monocot species.

In the maize genome, the RLCK subfamily has not been classified. Few studies have focused on individual maize RLCKs and their functions in mediating disease resistance. The objectives of this study were to identify putative RLCKs in maize and to study the function of *ZmBLK1* (Zm00001d034662), identified by a screen for maize orthologs of *Arabidopsis* BIK1 and tomato TPK1b. We describe the features of ZmBLK1 and provide evidence for its role in disease resistance through gain of function lines that constitutively express *ZmBLK1*.

2.3   Materials and Methods

2.3.1   Identification of maize RLCKs and ZmBLK1

Maize RLCKs were identified by comparing maize kinases with *Arabidopsis* RLCK subfamily members in a phylogenetic analysis. All maize kinases were selected in maize B73 RefGen_v4 databases (Jiao et al. 2017) with the hmmersearch tool (https://www.ebi.ac.uk/Tools/hmmer/search/hmmsearch). The hidden Markov models (HMMs) profile of eukaryotic protein kinases (PF00069) was used for the search. The 610 reported *Arabidopsis* RLKs were retrieved from TAIR database (Berardini et al. 2015, Shiu and Bleecker 2001). All the maize kinases and *Arabidopsis* RLKs were aligned using ClustalW (Larkin et al. 2007). The kinase domains of these sequences were used to generate a phylogenetic tree with the neighbor-joining method with 500 bootstrap replicates (Saitou and Nei 1987). Maize sequences in the same cluster as known *Arabidopsis* RLCKs were classified into maize RLCK subfamilies. The maize BIK1-like RLCKs were identified by BLASTp analysis in the B73 RefGen_v4 translations database with the amino acid sequences of BIK1 (NP_181496.1) and TPK1b (NP_181496.1). Annotation information was retrieved from Phytozome v12.1 (https://phytozome.jgi.doe.gov/pz/portal.html). Sequence alignments were obtained with Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and visualized with the Mega 7 tool (Kumar

et al. 2016; Sievers et al. 2011). Maize gene Zm00001d034662 was assigned with the name *ZmBLK1*.

2.3.2   Nucleic acid purification, cDNA synthesis and qRT-PCR protocols.

DNA was extracted from maize leaves by standard CTAB method (Minsavage et al. 1994). Total RNA was extracted with Trizol (Invitrogen) following the manufactures protocols and then purified with an RNeasy Mini Kit (Qiagen). For kernels, total RNA was extracted from kernel samples by a phenol-chloroform method. Kernels were ground with mortar and pestle in liquid nitrogen and approximate 3 g of fine powder was mixed with 10 ml of Tris-saturated phenol (pH 4.3) and 10 ml of 1 M Tris-HCl (pH 8.0). The extracts were centrifuged at $10,000 \times$ g for 10 min at 4°C and the supernatant was then extracted with an equal volume of chloroform: phenol (1:1), followed by an extraction with equal volume of choloroform: isoamyl alcohol (24:1). RNA was precipitated overnight at -20°C and pelleted by centrifugation. The pellet was dissolved in DEPC-treated water and precipitated again by ethanol. After centrifugation the RNA pellet was washed with 70% ethanol and dissolved in DEPC-treated water. Total RNA was then purified with an RNeasy Mini Kit (Qiagen)

cDNA was synthesized with SuperScript™ III reverse transcriptase (Invitrogen) according to standard protocols (Reese et al. 2011). For qRT-PCR, a reaction mixture containing 7.5 µl of SYBR Green Supermix (Bio-rad), 1 µl of each primer (10 µM), 2 µl of cDNA template, and 3.5 µl of nuclease-free water. The reaction cycling consisted of 3 min at 95 °C, 40 cycles of 5 s at 95 °C and 30 s at 57 °C. The ΔΔCt method was used to calculate relative gene expression with α-tubulin gene as the internal normalizer. *ZmBLK1* specific primers were qZmBLK1F2 (5'-CGAGCCTCTTCAGCTTCTATG-3') and qZmBLK1R2 (5'-TGTGGCTGCCTTGAGATTATT-

3') and the α-tubulin gene-specific primers were AlphaTUBF (5'-CACTGATGTTGCTGTCCTGC-3') and AlphaTUBR (5'-CGCTGTTGGTGATTTCGG-3').

2.3.3 Construction of ZmBLK1 vectors.

Full-length *ZmBLK1* coding region was amplified by PCR with cDNA generated from RNA purified from B73 maize leaves. PCR primers were ZmBLK1F (5'AAAGGCGCGCCTATGGGGAACTGCTGGG-3', *Asc*I site underlined) and ZmBLK1R (5'-CGCCCCGGGACGAGAATGGGCCAATGG-3', *Xma*I site underlined). The reaction product was cloned into the *Asc*I and *Xma*I sites of the binary vector pTF101HA. The resulting construct (pTFZBLK1) contained a T-DNA cassette consisting *ZmBLK1* coding sequence with a triple HA tag (5'-TACCCATACGATGTTCCTGACTATGCGGGCTATCCCTATGACGTCCCGGCCTATGCAGGATCCTATCCATATGACGTTCCAGATTACGCTGCT-3') driven by the cauliflower mosaic virus 35S promoter. The cassette also contained the *bar* gene as glufosinate-ammonium resistance for callus selection (Schröder et al. 1994; Rajasekaran et al. 2017). A green fluorescent protein (GFP) -tagged ZmBLK1 vector (pTFZBLK1G) was also constructed. The green fluorescent protein (GFP) was amplified from pCAMBIA99 (Mang et al. 2009) by PCR with primers GFPF (5'-GCGTACGTACTAGCTAGCTTTGTATAGTTC-3', *Sna*BI site underlined) and GFPR (5'-GGTCCCGGGTCAGGCGGATCGGTAGATCTGACTAGTAAAG-3', *Xma*I site underlined). The PCR product was cloned into pTFZBLK1 and into pTF101HA replacing the HA tag in both vectors. The resulting vectors were named pTFZBLK1G and pTFGFP, respectively. All vectors were transformed into *Agrobacterium tumefaciens* strain GV3101 (Sheludko et al. 2006).

2.3.4 Transformation of maize with *ZmBLK1*

Binary vector pTFZBLK1 was used to transform maize Hi-II plants by the Plant Transformation Facility at Iowa State University (Ames, IA USA). Transformed callus tissue was sent to the University of Arkansas (Fayetteville, AR USA), and after plantlet formation, sent to Purdue University (West Lafayette, IN USA). The transgenic plantlets were grown in ground-beds (16 h of light daily) at the Purdue Lilly Greenhouse Facility. Transgenic plants were identified by their resistance to glufosinate-ammonium herbicide. At the fourth-leaf stage a solution (500 mg/L) of the herbicide was applied on leaves as described by Rajasekaran et al. (2017). Expression of the ZmBLK1 was determined by Western analysis. Total proteins were isolated from herbicide-resistant plants with extraction buffer (50 mM HEPES, 50 mM EGTA, 25 mM NaF, 1 mM Na_3VO_4, 50 mM β-glycerophosphate, 100 mM NaCl, 1 mM PMSF, 2 mM DTT, 20% glycerol, 1x proteinase inhibitor cocktail (Sigma)) and separated by 12% SDS-PAGE. Blots were probed with an anti-HA antibody as described (Wood et al. 2006).

Out of the 4 transgenic events, two regenerated lines ZMBLK1 lines (ZMBLK1-1 and ZMBLK1-3) were identified. The T0 transgenic plants were crossed to B73, and the BC1 transgenic plants were backcrossed to B73. BC2 transgenic plants were used for the Goss's wilt assay. The non-transgenic control plants (WT1-1) were generated from non-transgenic Hi-II plants by the same transformation and crossing procedures.

Location of the insertion into the maize genome was determined by Wideseq.Genomic DNA was purified from ZMBLK1-1 and ZMBLK1-3 leaf tissues by the CTAB method as described (Minsavage et al. 1994). DNA (2 μg) was digested with fragmentase (NEB), run on 2% agarose electrophoresis gels and the 500-1500 bp fragments were gel-purified. The recovered fragments were ligated with a 5' terminal adapter

GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGCT and a 3'
terminal adapter 5'-phosphate-AGCAGCCCGGG-amino C7-3'. Subsequently, two rounds of
PCR were conducted with the ligation products to enrich the T-DNA and genome junction region.
The first PCR primer set was AP1-out: 5'-GTAATACGACTCACTATAGGGCACGCGTGGTC-
3' and pTF-out: 5'-ACTGGCCGTCGTTTTACAACGTCGTGACTG-3'. The second PCR primer
set was: AP1-nest: 5'-TGGTCGACGGCCCGGGCTGC-3' and pTF-nest: 5'-
AAACCCTGGCGTTACCCAACTTAATCGCCTT-3'. After the second PCR step, the products
were run on 2% agarose electrophoresis gels, the 500-1500 bp products were purified and used for
Wideseq high throughput sequencing at Purdue Genomics Core Facility. Sequence reads that
covered the T-DNA and maize genome junctions were manually identified by searching the
pTFZBLK1 left border sequence against all the sequencing readings with Notepad++ software.
The selected reads contained the left border sequences of pTFZBLK1 and a flanking maize genome
sequence. The maize sequences were used to search the maize genome by the BLASTn analysis
of maize genome database (MaizeGDB). For the transgenic lines ZMBLK1-1 and ZMBLK1-3, 24
out of 82146 reads and 47 out of 62830 reads, respectively, were identified containing the T-DNA
and genome junction sequence. The analysis indicated that the T-DNA cassette was inserted in the
same position at chromosomes 4 (36,635,505) in both transgenic lines.

2.3.5   Analysis of ZmBLK1 kinase activity.

Agrobacterium tumefaciens strain GV3101 carrying pTFZBLK1 was grown at 25°C in LB
medium supplemented with 25 mg/ml rifampicin and 100 mg/mL spectinomycin. After 2 days,
the cultures were centrifuged and the cells washed twice with the infiltration media (10 mM MES,
10 mM $MgCL_2$ and 100 μM acetosyringone). Bacterial suspensions were then maintained at room
temperature for 3 h. Leaf-infiltrations were applied to the abaxial surface of fully expanded

Nicotiana benthamiana leaves with a 1-ml disposable syringe. After 2 days of incubation under constant illumination at 25 °C, infiltrated-leaves were ground in liquid nitrogen and suspended in cold immunoprecipitation buffer (50 mM HEPES, 50 mM EGTA, 25 mM NaF, 1 mM Na_3VO_4, 50 mM β-glycerophosphate, 100 mM NaCl, 1mM PMSF, 2 mM DTT, 20% glycerol, 1x proteinase inhibitor cocktail (Sigma-Aldrich)). Monoclonal anti-HA-agarose (Sigma) was used to immunoprecipitate the ZmBLK1-HA protein. The protein was washed three times with the immunoprecipitation buffer and twice with kinase reaction buffer (50 mM Tris, pH 7.5, 20 mM $MnCl_2$, 2 mM EGTA, and 2 mM DTT). The protein was then tested for kinase activity in a 50 μl reaction containing 25 μg of myelin basic protein (MBP) substrate, 200 mM of ATP, and 1 μCi [γ-^{32}P] ATP. After 30 min, the reaction mixture was boiled for 5 min and the proteins were separated by 8% SDS–PAGE. ^{32}P-labeled products were visualized by autoradiography.

2.3.6 Analysis of Goss's wilt disease

C. michiganensis subsp. *nebraskensis* (CMN) was cultured on NBY (Nutrient Broth Yeast extract) agar medium at room temperature. After 4 days of growth, bacteria were harvested from the plates with sterile water and the concentration adjusted to OD_{640} = 0.3 (10^8 CFU/ml). Transgenic maize ZMBLK1-1 and ZMBLK1-3 and the non-transgenic line (WT1-1) were grown in 4-inch pots in greenhouse. At the V2 stage, leaf tissue was analyzed by Western blots to verify ZmBLK1 expression. Subsequently, the third leaf of ten V4-stage plants were cut at the tip and inoculated with CMN by submerging the cut end into the inoculum for 5 seconds. Lesion length on each inoculated leaf was measured every 24 hr. After 8 days, bacteria within the inoculated leaves were measured by a modified 6x6 drop plate method (Mbofung et al. 2015; Chen et al. 2003). From each leaf, a 15 cm segment, measured from the inoculation site, was collected. The leaves were flattened and scanned. The area of each leave was analyzed using ImageJ software.

The tissue was surface sterilized in 10% bleach for 30 s, and immediately washed three times with sterile distilled water. The tissue was placed in a sterile tube containing 15 ml of phosphate-buffered saline (PBS) buffer, placed in a sonication bath for 7 min and then vortexed for 15 s. The resulting extract was serially diluted and 10-µl drops of each dilution were transferred to two replicate plates of NBY agar media. The plates were incubated in the dark at room temperature and the bacterial colonies were counted after 4 days. The number of CFU/mm^2 leaf tissue was calculated.

2.3.7   Subcellular localization of ZmBLK1.

In vivo localization of ZmBLK1 was examined in both intact tissues and protoplasts. *A. tumefaciens* carrying pTFPK1G was infiltrated into 3-week old *Nicotiana benthamiana* plants on the 4th or 5th leaves as described (Goodin et al. 2002). Microscope observation was carried out 2 days after infiltration. Prior (30 min) to the observation, *N. benthamiana* leaves that received *Agrobacterium* infiltration were infiltrated with 50 µM 4´,6-diamidino-2´-phenylindole, dihydrochloride (DAPI) and 1 µg/ml FM 4-64.

Protoplasts were isolated from infiltrated *N. benthamiana* leaf tissues as described with slight modifications (Ibrahim et al. 2012). Briefly, whole infiltrated leaves were cut into about 1 cm^2 large pieces and digested in an enzyme mixture containing 2% cellulysin (Calbiochem), 0.1% pectolyaseY-23 (Seishin Pharmaceutical), and 400 mM mannitol for 90 min at 30°C with gentle shaking. Protoplasts were collected by centrifugation at 50 x g for 2 min and then resuspended in mannitol/CaCl$_2$ solution (400 mM mannitol and 70 mM CaCl$_2$). Protoplast suspensions were layered onto 20% sucrose solution and centrifuged at 50 x g for 10 min. Protoplasts were collected and washed with mannitol/CaCl$_2$ solution twice, pelleted, and resuspended in 4 ml mannitol/MgCl$_2$ solution (400 mM mannitol, 15 mM MgCl$_2$, and 5 mM MES, pH 5.7).

Water-mounts prepared from the lower epidermis of infiltrated leaves were examined with a Nikon A1R confocal laser scanning microscope system and protoplasts were examined with a Zeiss LSM 880 upright confocal system. For both systems, blue fluorescence of DAPI-stained nuclei was detected in 455 nm channel. Green fluorescence of the ZmBLK1::GFP fusion protein was detected in 488 nm channel. Red fluorescence of FM4-64-stained plasma membrane was detected in 560 nm channel.

2.4   Results

2.4.1   Identification of maize RLCKs and *ZmBLK1*.

Of the 1,512 protein kinases identified in maize, 195 clustered with the *Arabidopsis* RLCKs (Figure 2.1). Ten of the *Arabidopsis* RLCK subfamilies (I, II, IV, V, VI, VII, VIII, IX, X and XI) were identified in maize. No maize RLCKs clustered with subfamily III of *Arabidopsis*. Also, three *Arabidopsis* RLCK IX members (AT3G21450, AT5G65500 and AT3G26700) failed to cluster with the other RLCK IX members in our phylogenetic analysis.

ZmBLK1 was identified as a putative maize ortholog of the *BIK1* and *TPK1b*. BIK1 (At2g39660) and TPK1b (NP_001234409.2) amino acid sequences were used for BLASTp search in the maize genome. Among the output sequences, five proteins shared the highest sequence identity and phylogenetic relationship with both BIK1 and TPK1b (Table 2.1, Figure 2.2). These five proteins were designated as ZmBLK1 (Zm00001d034662), ZmBLK2 (Zm00001d012958), ZmBLK3 (Zm00001d011066), ZmBLK4 (Zm00001d011779) and ZmBLK5 (Zm00001d028613). In the phylogenetic tree, these five proteins were all in the same clade and branched out from BIK1. ZmBLK1 and ZmBLK2 are the two proteins branched from TPK1b. ZmBLK1 and ZmBLK2 were therefore selected as candidates for functional analyses. An RNAseq expression data set generated from R3-stage maize kernels, 6 days post inoculation with *Aspergillus flavus,* indicated that

ZmBLK1 was higher in the non-inoculated control (RPKM 27.68) and the inoculated kernels (RPKM 33.82) compared to the ZmBLK2 (RPKM 6.69 and 4.05, respectively) (C. P. Woloshuk, unpublished). Thus, we hypothesized that *ZmBLK1* is the functional RLCK ortholog of *BIK1* and *TPK1b* in maize.

2.4.2   ZmBLK1 is a putative RLCK.

ZmBLK1 is a single copy gene located in chromosome 1, encoding a 419 amino acid protein with an estimated molecular weight of 46.02 kDa. Phylogenetic analysis clustered ZmBLK1 with the *Arabidopsis* RLCK VII subfamily. The amino acid sequence of ZmBLK1 shows all features of an RLCK. ZmBLK1 has a protein kinase domain (residues 76 to 361), containing all 11 conserved protein kinase subdomains I to XI (Figure 2.3) (Hanks and Hunter 1995; Angermayr and Bandlow 2002). The subdomain II contains a protein kinase ATP-binding region signature (residues 82 to 114). In subdomain VI, there is a serine/threonine protein kinases active-site signature. In subdomain VII and VIII, ZmBLK1 has a conserved sequence between the DFG and APE motif like BIK1 and TPK1b (Figure 2.3) (Laluk et al. 2011; AbuQamar et al. 2008; Taylor and Radzio-Andzelm 1994).

The conserved structure has been reported as the activation segment of a protein kinase (Johnson et al. 1996; Taylor and Radzio-Andzelm 1994). To determine kinase activity of ZmBLK1, an *in vitro* assay was performed with transiently expressed ZmBLK1 protein in *N. benthamiana* leaf tissues. The immune-purified ZmBLK1 displayed autophosphorylation and phosphorylation of the myelin basic protein (MBP), which is a commercially available artificial substrate for *in vitro* kinase assays, indicating that ZmBLK1 is an active kinase (Figure 2.4). Expression of *ZmBLK1* in various maize tissues was determined by qRT-PCR. Expression in leaves was over 7-fold higher than in silks, husks, and roots, (Figure 2.5A). These results are consistent with the

expression data available in the MaizeGDB database (Stelpflug et al. 2016). *ZmBLK1* expression was detectable in all stages of kernel development (Figure 2.5B). The expression increases from the R2 stage (blister) to a maximum at the R5 stage (dent).

2.4.3   ZmBLK1 is located to the plasma membrane.

Although no trans-membrane motif was identified in ZmBLK1, it does have an N-myristoylation motif at the N terminus. N-myristoylated proteins are often targeted to membrane for binding (Resh 1999; Maurer-Stroh et al. 2002; De Vries et al. 2006). To assess the subcellular localization of ZmBLK1, ZmBLK1::GFP fusion protein was constructed and transiently expressed in epidermal cells of *N. benthamiana* leaves. The abaxial epidermal tissues were examined by confocal laser scanning microscopy. FM4-64 is a red fluorescence marker of plasma membranes (Figure 2.6D) and DAPI is a blue fluorescence marker that stains nuclei (Figure 2.6B). In *N. benthamiana* epidermal cells, the green fluorescence signal of ZmBLK1::GFP was observed along the plasma membrane (Figure 2.6C) and the FM4-64 signal overlapped with ZmBLK1::GFP but not the DAPI stained nuclei (Figure 2.6E). Localization was also investigated with protoplasts isolated from infiltrated leaves of *N. benthamiana*. In the protoplasts, the green fluorescence of ZmBLK1::GFP fusion protein overlapped with the red fluorescence of FM4-64 (Figures 2.6F, G, H, I). These observations support our prediction that ZmBLK1 is localized to the plasma membrane.

2.4.4   Constitutive expression of *ZmPK1 increases resistance to* Goss's wilt disease.

When susceptible maize leaves were inoculated with the Goss's wilt pathogen *C. michiganensis* subsp. *nebraskensis* (CMN), a lesion formed at the inoculation site at the leaf tip and rapidly spread to the base. Two days post inoculation with CMN, symptoms (necrosis and water soaking) were visible at the inoculation site on the two transgenics ZMBLK1-1 and

ZMBLK1-3 and the non-transformed plants. Over the next 7 days, lesion development on the ZMBLK1-1 and ZMBLK1-3 was significantly slower (P<0.05) than the non-transgenic plants (Figure 2.7). The rate of lesion spread on the non-transgenic line was 23.96 mm/day ($\pm$ 1.18). For ZMBLK1-1 and ZMBLK1-3, the rate of spread was 9.48 mm/day ($\pm$ 1.37) and 10.99 mm/day ($\pm$1.26), respectively (Figure 2.7). At 8 DPI, the number of bacteria in the inoculated leaves varied greatly. However, the number in the transgenic lines were significantly less (ZMBLK1-1, P=0.0026; ZMBLK1-3, P<0.001) than the non-transgenic line. CMN growth in the infected leaves of ZMBLK1-1 and ZMBLK1-3 were 3.5 x 10^4 CFU/mm^2 of leaf ($\pm$ 3.4 x 10^5) and 3.8 x 10^4 CFU/ mm^2 of leaf ($\pm$ 3.8 x 10^4) respectively, which were lower compared to the non-transgenic plants 4.9 x 10^6 CFU/ mm^2 of leaf ($\pm$ 3.9 x 10^5). These results indicate that constitutive expression of *ZmBLK1* provides resistance to Goss's wilt in maize seedlings.

Transcript levels of *ZmBLK1*, measured by qRT-PCR, indicated that *ZmBLK1* was not induced until 12 h post inoculation in non-transgenic leaves (Figure 2.8A). At 3 and 6 days post inoculation, *ZmBLK1* expression was at the basal pre-inoculation level (data not shown). In the transgenic lines, the *ZmBLK1* expression level was as high as 150 times that in the non-transgenic plants at 0, 4, and 8 h after inoculation (Figures 2.9A, B, C). Expression in the transgenic plants decreased with time (Figures 2.8B, C), and expression at 12 h post inoculation was similar to the non-transgenic plants (Figure 2.9D).

2.5   Discussion

Previous studies have shown that RLCKs are involved in PAMP-triggered immunity in various plants. Based on the well-studied *Arabidopsis BIK1* and tomato *TPK1b*, we identified five RLCK genes (*ZmBLK1* to *ZmBLK5*) in the maize genome. Although no previous reports about the

function of these putative RLCKs were revealed, the Phytozome v12.1 database annotates ZmBLK1, ZmBLK2 and ZmBLK5 as cytoplasmic protein tyrosine kinases. ZmBLK3 and ZmBLK4 were annotated as chloroplastic-related protein kinase APK1A.

We compared 1,512 protein kinases in maize B73 with the 610 reported *Arabidopsis* RLKs and identified a large number (195) of putative maize RLCKs. These maize RLCKs also clustered with ten of the eleven *Arabidopsis* subfamilies. Subfamily III cluster was not identified, which is similar to the rice RLCKs (Shiu et al. 2004). The *Arabidopsis* RLCK subfamily III contains ZRK3 (AT3G57720), which is required for recognition of the *Pseudomonas syringae* type III effector HopF2a (Seto et al. 2017), and RKS1 (AT3G57710), which is associated with broad-spectrum resistance to *Xanthomonas campestris* (Huard-Chauveau et al. 2013).

In *Arabidopsis*, 46 RLCKs, including BIK1, have been classified into the RLCK-VII subfamily (Lin et al. 2015; Shiu et al. 2004). As in *Arabidopsis*, RLCK VII is also the largest RLCK subfamily in maize. In our phylogenetic analysis, which included BIK1 and TPK1b, none of the 20 top maize RLCK candidates tightly clustered with BIK1, while ZmBLK1 and ZmBLK2 were most closely associated with TPK1b. ZmBLK1 and ZmBLK2 are also the most closely related to the rice RLCK, OsRLCK118 (83% identity with ZmBLK1 and 82% with ZmBLK2), which is necessary for *Xanthomonas* leaf blight resistance (Zhou et al. 2016). Both of ZmBLK1 and ZmBLK2 are predicted to belong in the RLCK-VII family and we can speculate that they play roles in regulating maize disease resistance. ZmBLK1 is on chromosome 1 and ZmBLK2 is on chromosome 5. They share 90.74% identity in amino acid sequence. There is high possibility that ZmBLK1 and ZmBLK2 have some functional redundancy. As in rice, maize RLCK VII subfamily proteins may also have overlapping functions (Shiu et al. 2004; Zhou et al. 2016).

In our study, ZmBLK1 exhibited kinase activities *in vitro*. Most protein kinases in the eukaryotic kingdoms have 11 conserved kinase catalytic subdomains (Hanks et al. 1988; Hanks and Hunter 1995). The kinase activation region (from DGP through APE) in subdomain VII and VIII is required as a structural component for the catalytic ability (Johnson et al. 1996). In ZmBLK1, two threonine residues Thr-246 and Thr-251, which are conserved among ZmBLK1, BIK1 and TPK1b, are in the kinase activation region. These two threonine residues were identified to have essential roles for kinase activity in BIK1 and TPK1b. Substitutions of any of these two residues lead to loss of kinase activity and *B. cinerea* resistance (AbuQamar et al. 2008; Laluk et al. 2011). These results suggest that Thr-246 and Thr-251 may also be required phosphorylatable residues in ZmBLK1.

We confirmed that ZmBLK1 is localized on the plasma membrane and the N-terminal myristate likely facilitates the localization as described by Resh (1999). Like BIK1, the localization of ZmBLK1 suggests that ZmBLK1 may directly interact with other RLKs or RLCKs. Wei Song et al. (2015) performed bioinformatics analysis of the entire maize genome and classified a series of maize immune related LRR-containing kinases, including a maize homolog of FLS2. FLS2 recognizes flagellin and activates signaling cascades by binding to BIK1 (Gómez-Gómez and Boller 2000; Zhang et al. 2010). Any signaling cascade that ZmBLK1 might regulate remains unknown.

BIK1 has been shown to be involved in plant development. The *bik1* mutant has wrinkled leaf surfaces with serrated leaf margins (Veronese 2006). During the reproductive stages, stems of *bik1* mutant are weak and small seedpods are produced. It is unknown whether *ZmBLK1* is involved in plant development. We found that overexpression of *ZmBLK1* in maize did not reveal altered morphology or development. A maize line (UFMu-00071), containing a Mu insertion in

the 5'-UTR of *ZmBLK1,* has been identified (McCarty and Meeley 2009). However, this line was not available for our study. We also found that the expression of ZmBLK1 increased in the whole kernel tissues during maize seed development. The results are consistent with expression profile data available in MaizeGDB database (Sekhon, 2011). In a microarray analysis carried out by Liu et al. (2011), genes encoding starch metabolic enzymes and storage proteins were most commonly upregulated during seed development, but expression pattern of protein kinases were variable. For example, some cyclin-dependent kinases involved in cell division were highly expressed after pollination but downregulated after blister stage (Liu, 2011). Some putative protein kinases involved in abscisic acid signaling was upregulated during seed development (Liu, 2011). No previous studies report that RLCKs are essential for plant seed development. However, it is still possible that *ZmBLK1* is involved in plant hormone pathways regulating seed development and its expression level may change along with the hormone changes in seeds.

Genetic data reveal that *BIK1* is a regulator of resistance to bacterial and fungal pathogens (Veronese 2006; Laluk et al. 2011). Knockout of *BIK1* leads to decreased resistance to *B. cinerea* and type III-secretion mutants of *P. syringae* pv *tomato.* (Lu et al. 2010). In tomato, *TPK1b* RNAi plants showed no impact on resistance to *P. syringae* (AbuQamar et al. 2008). Veronese et al (2006) showed that *BIK1* expression in *Arabidopsis* leaves increases about 6.5 fold after *B. cinerea* inoculation, and expression remains high for at least until 72 h post inoculation. AbuQamar et al. (2008) reported that TPK1b is induced within 12 h in inoculated tomato leaves with *B. cinerea* or *P. syringae.* In maize, we observed an increase in *ZmBLK1* expression 12 h after CMN inoculation and the expression decrease to the basal level by 72 hr. Considering that the *ZmBLK1* overexpressed lines provided resistance to Goss's wilt, it suggests that ZmBLK1 is involved in the bacterial resistance pathways.

In conclusion, this report classifies the maize receptor-like cytoplasmic kinase protein family including 195 members which can be further classified into ten subfamilies. One maize RLCK, *ZmBLK1*, is the maize ortholog of tomato *TPK1b*. The protein is localized on the plasma membrane and phosphorylates its substrate. Overexpression of *ZmBLK1* in maize increases resistance to Goss's wilt disease. We propose that *ZmBLK1* may regulate the signaling transduction in maize immune responses. Further studies are needed to determine how *ZmBLK1* and other receptor-like cytoplasmic kinases are involved in the signaling pathways.

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Table 2. 1 Putative receptor-like cytoplasmic kinases identified in maize.

Protein Name	MaizeGDB ID	Sequence Identity (%)	
		TPK1b	BIK1
ZmBLK1	Zm00001d034662	66.43	63.93
ZmBLK2	Zm00001d012958	71.93	66.30
ZmBLK3	Zm00001d011066	66.02	63.24
ZmBLK4	Zm00001d011779	69.41	58.48
ZmBLK5	Zm00001d028613	68.21	56.75

[a] Maize protein kinase were identified by BLASTp analysis maize genome database (MaizeGDB) with TPK1b and BIK1 sequences.
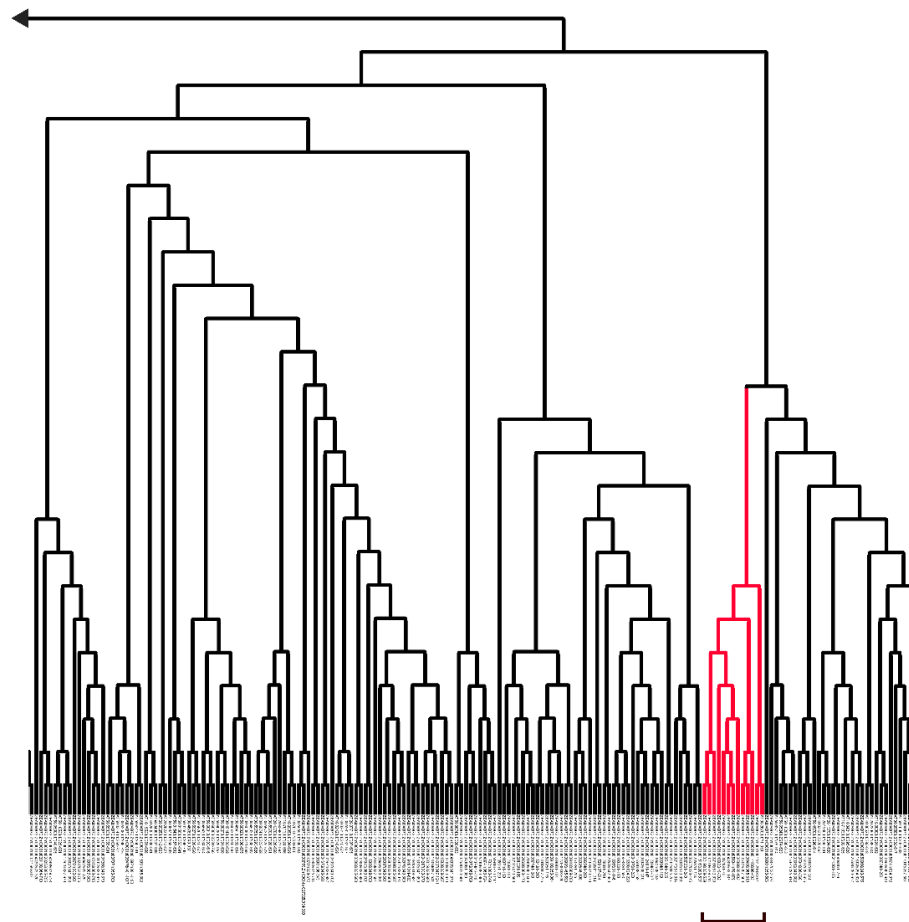
Figure 2. 1 Phylogeny of RLK proteins from Arabidopsis and kinase proteins from maize. The neighbor-joining tree was generated from the alignment of the amino acid sequences of the kinase domain. The red clades indicate RLCK clusters. The subfamily numbers are shown on the right of the phylogenetic tree.

Figure 2.1 continued
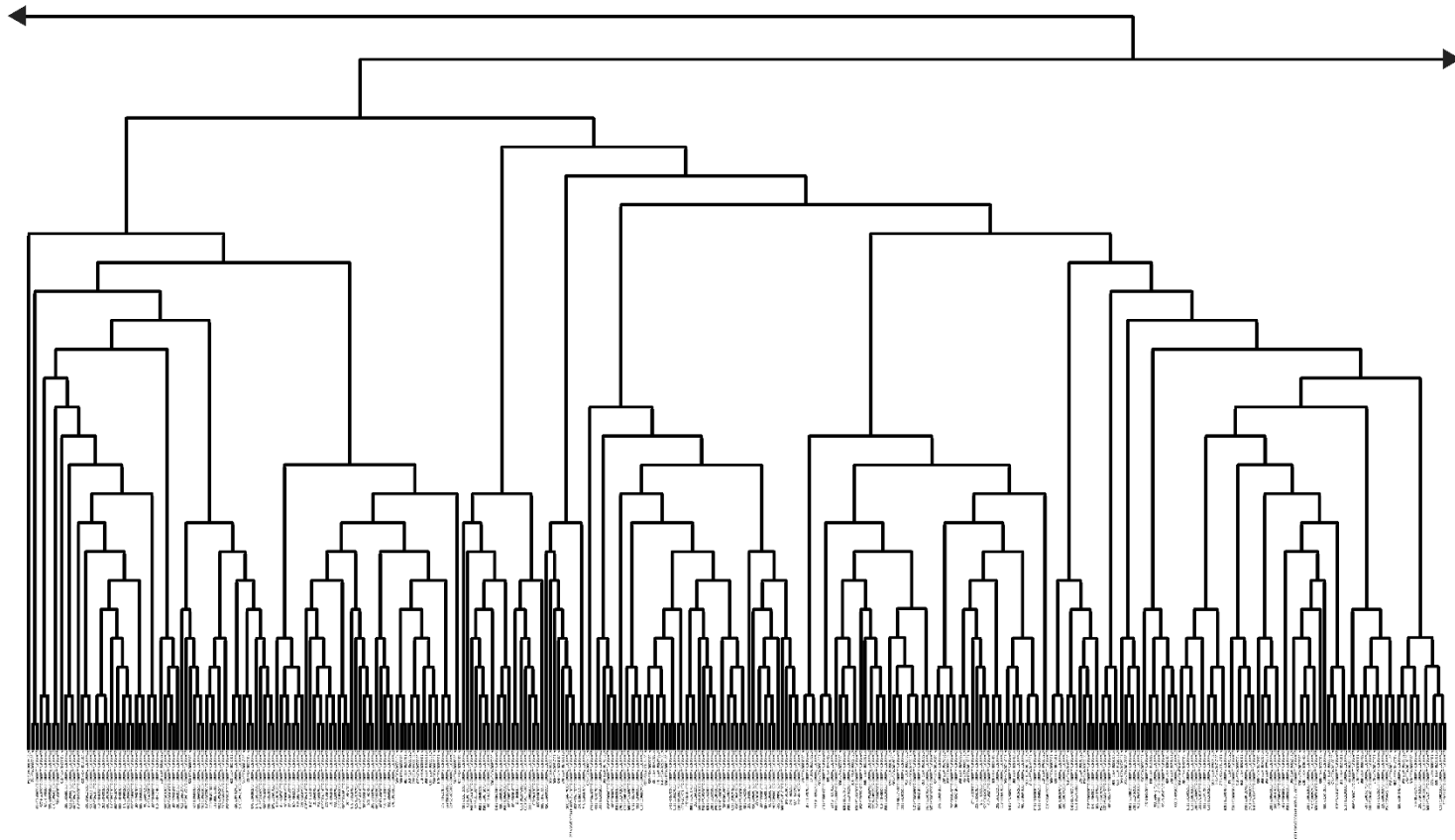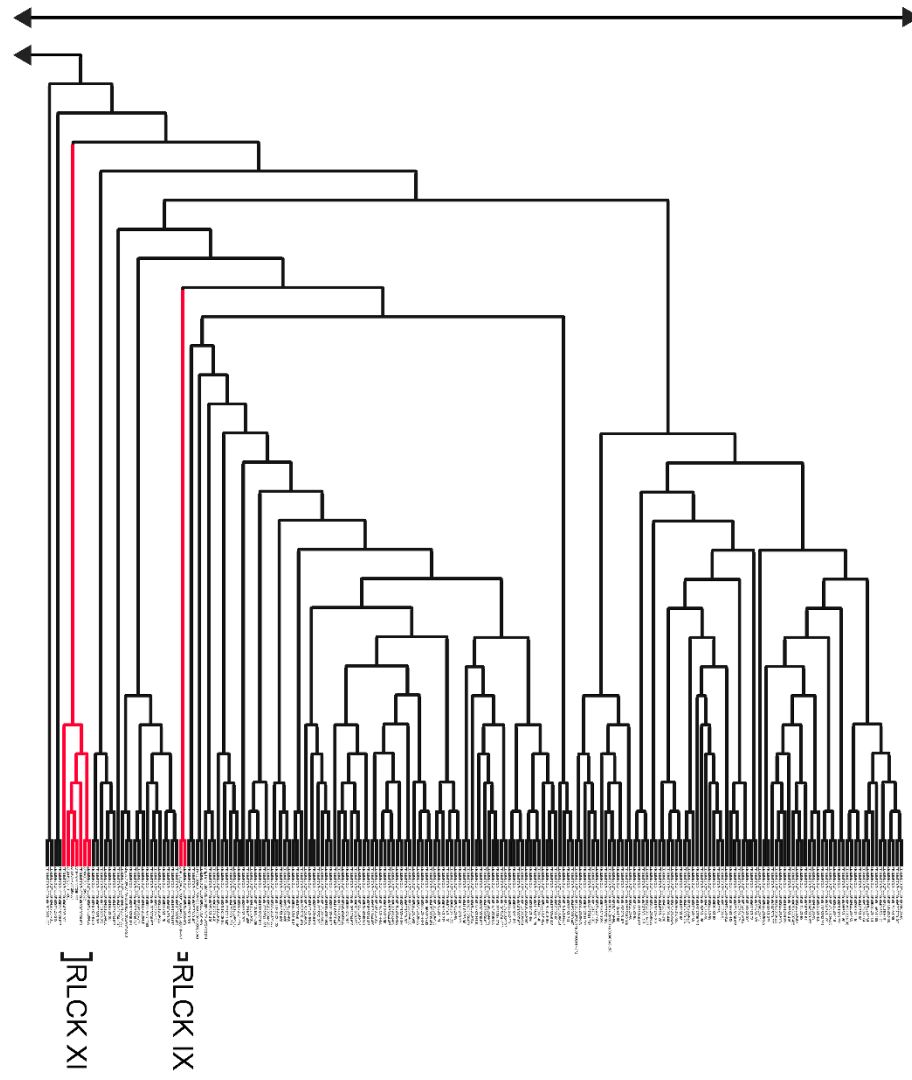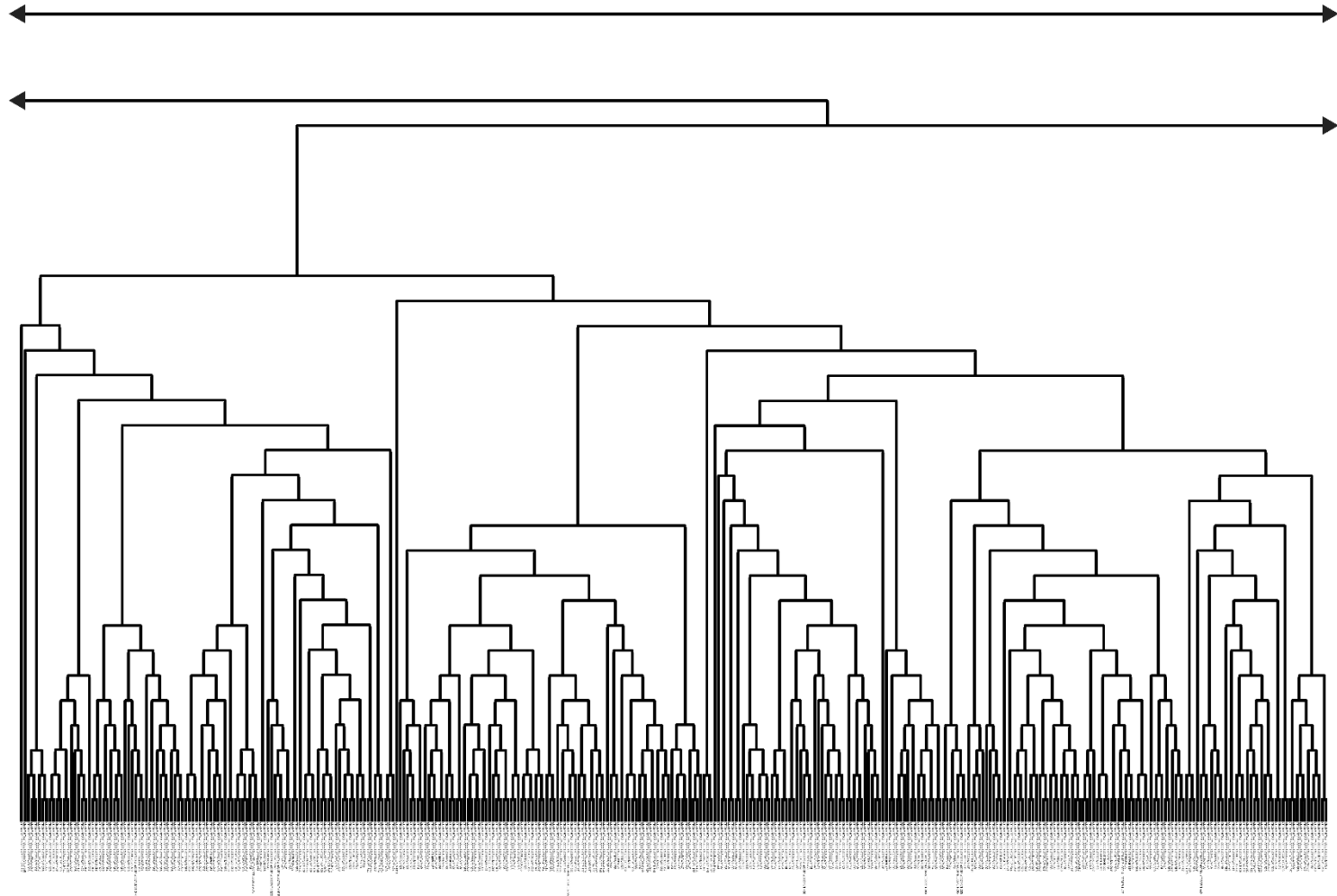
Figure 2.1 continued

Figure 2.1 continued

Figure 2.1 continued

RLCK V

RLCK II

RLCK VIII

Figure 2.1 continued

Figure 2.1 continued

RLCK X

RLCK IV

Figure 2.1 continued

RLCK VII
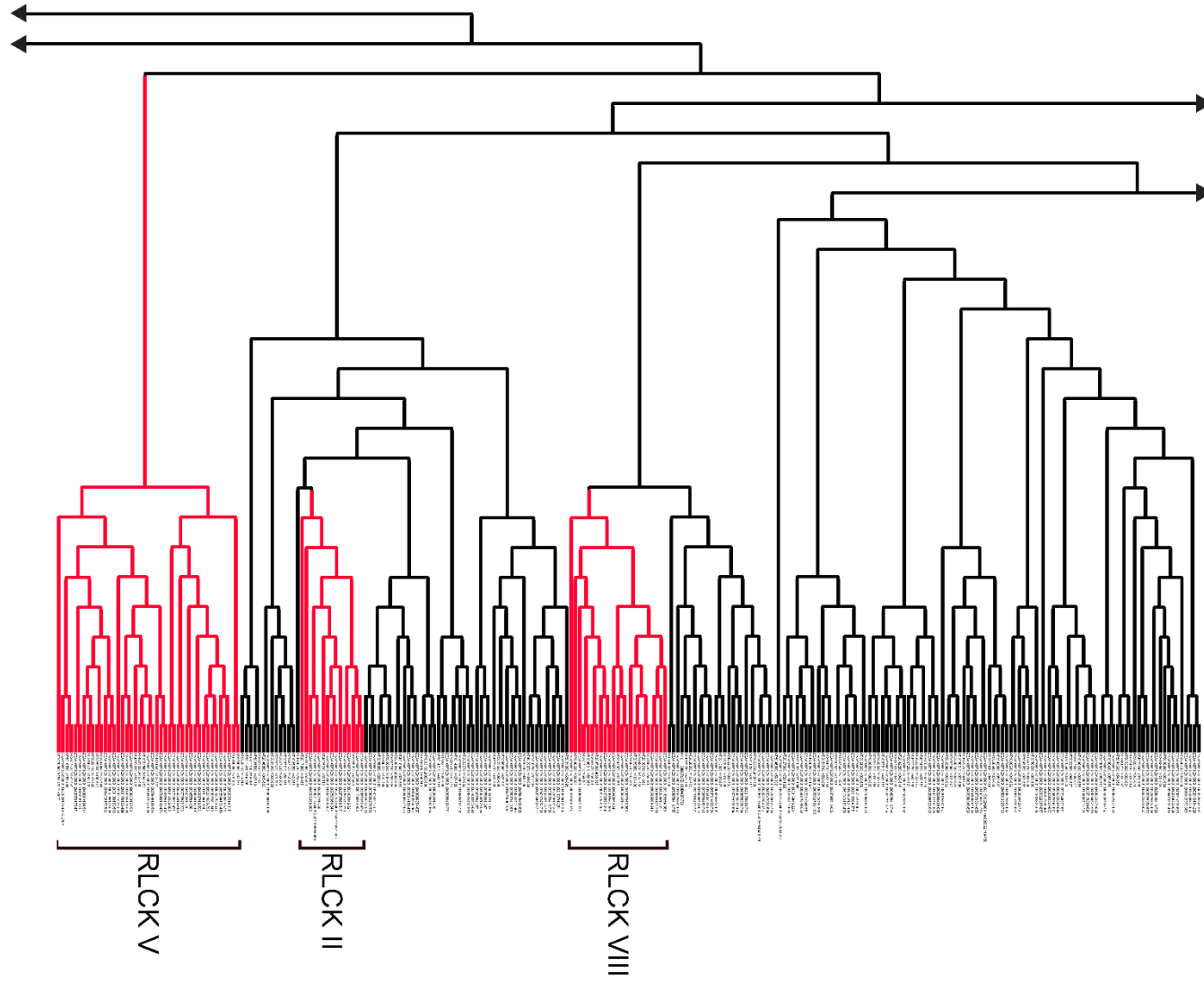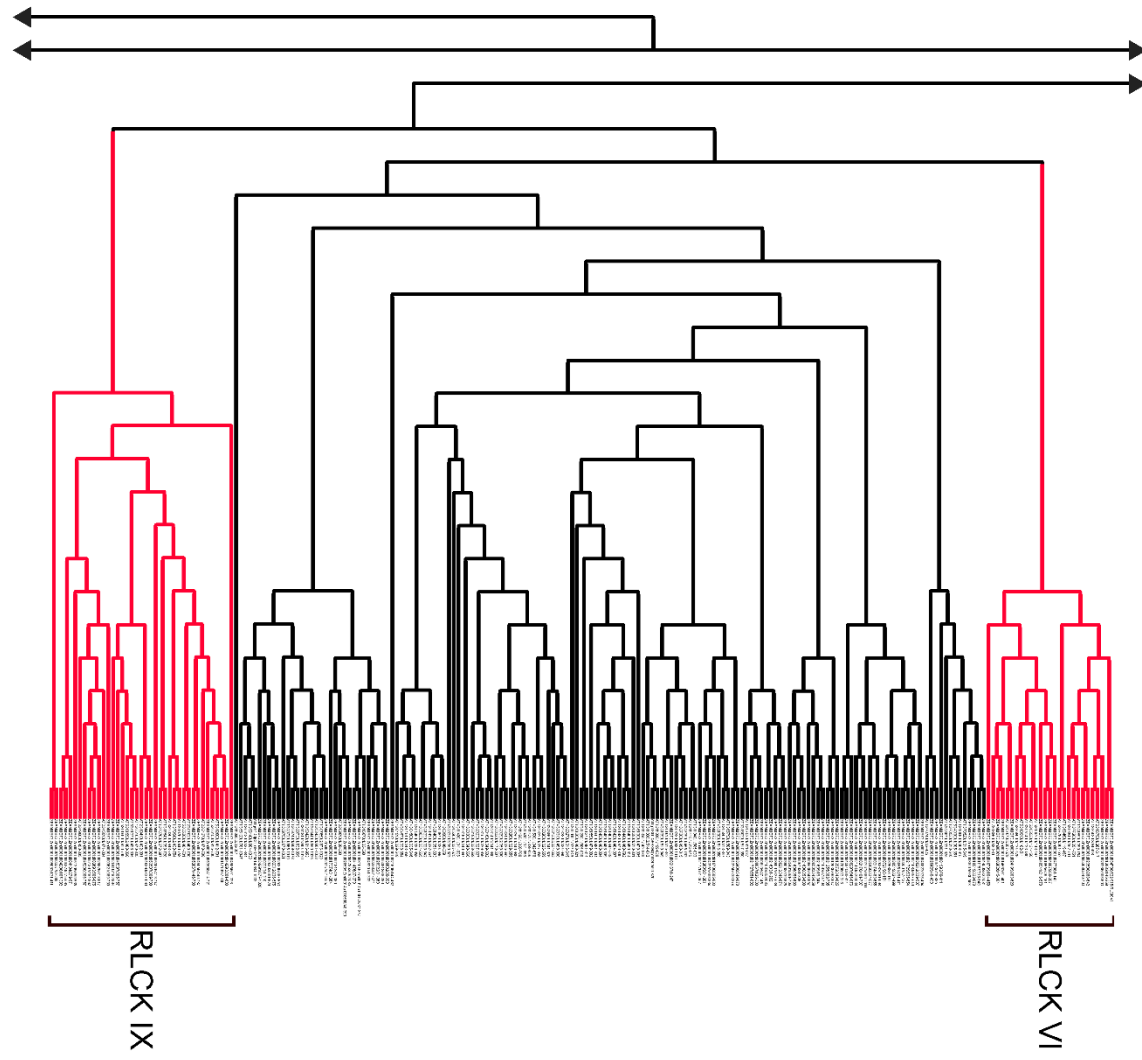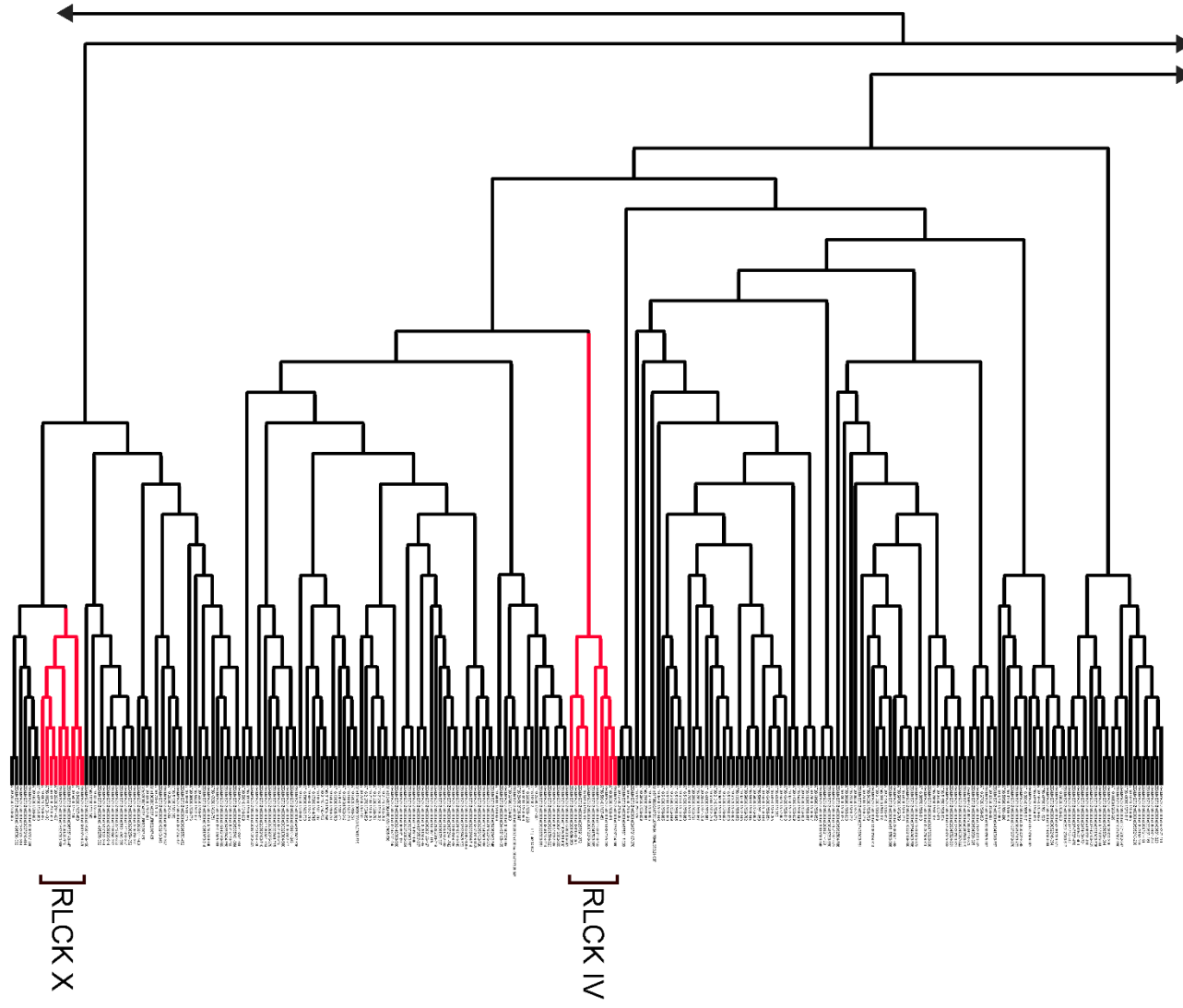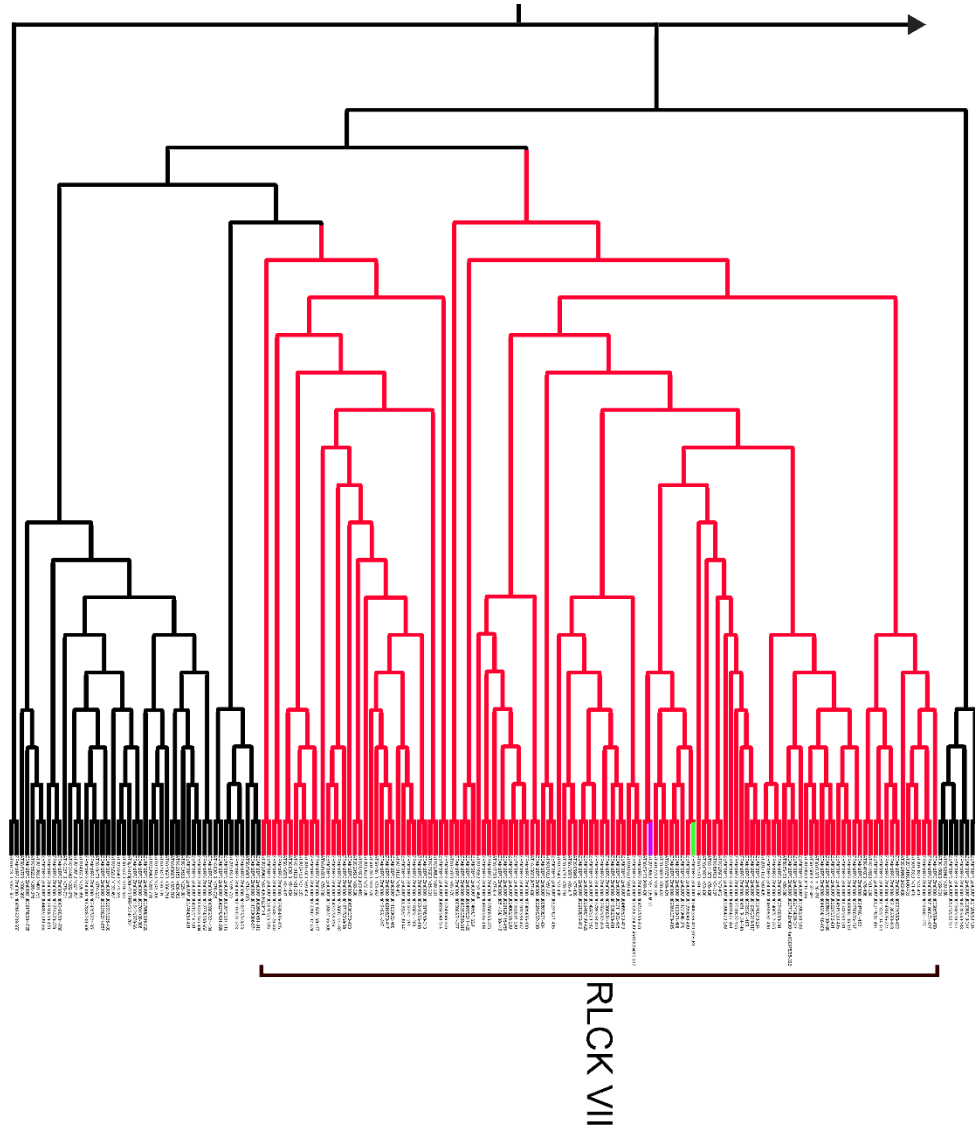
Figure 2. 2 Rooted UPGMA tree of 20 top RLCK homologs in maize B73 genome, TPK1b and BIK1. Sequences were aligned with Clustal Omega and the tree was developed with Mega 7.0. Numbers at the branches indicate the percentage of bootstrapping values with 1000 replicates. Maize PTI protein was used as the outgroup.

```
                     I                        II                      III                        IV
         ────────────────────        ───────────────        ───────────────────        ──────────────────
         1                                                                                                   100
ZmBLK1   FRPDSVLGEGGFGSVYKGWIDENTLSPCRPGTGIAVAVKRLNHEGLQGHREWLAEVNYLGQFCHPNLVKLIGYCLEDEHRLLVYEFMPRGNMENHLFR
TPK1b    FRPDSVLGEGGFGSVFKGWIDENTFAATKPGTGVIIAVKRLNQEGFQGHREWLAEVNYLGQFSHPHLVKLIGYCLEDEHRLLVYEFMPRGSLENHLFR
BIK1     FRPDSVIGEGGFGCVFKGWLDESTLTPTKPGTGLVIAVKKLNQEGFQGHREWLTEINYLGQLSHPNLVKLIGYCLEDEHRLLVYEFMQKGSLENHLFR


                   V                       VI                   VII                        VII
         ───────────────────        ───────────────        ─────────        ───────────────────       I
         101                                                                                I                    200
ZmBLK1   RGSYFQPLSWNLRMKVALGAAKGLAYLHSAEAKVIYRDFKTSNILLDTDYSAKLSDFGLAK DGPVGEKSHVSTRVMGTYGYAAPEYLSTGHLTAKSDI
TPK1b    RGSYFQPLSWKLRLKVALGAAKGLAFLHSAETKVIYRDFKTSNILLDSNYTAKLSDFGLAK DGPTGDKSHVSTRVMGTYGYAAPEYMATGHLTSKSDV
BIK1     RGAYFKPLPWFLRVNVALDAAKGLAFLHSDPVKVIYRDIKASNILLDADYNAKLSDFGLAR DGPMGDLSYVSTRVMGTYGYAAPEYMSSGHLNARSDV


                   IX                       X                                    X
         ───────────────────        ───────────────        ───────────────────────────
         201                                                                                                    286
ZmBLK1   YSFGVVLLEMLSGRRAIDKNRPQGEHNLVEWARPYLTHKRKIFRILDTRLEGQYNLNGAQTIAALALECLSYEAKMRPTMDAVVAILEEL
TPK1b    YSFGVVLLEMLSGRRAIDKNRPSGEHNLVEWAKPYLGNKRKVFRVLDTRLEGQYSMEVASKVANLALRCLSKDPRFRPSMSDIVKELEQL
BIK1     YSFGVLLLEILSGKRAIDHNRPAKEENLVDWARPYLTSKRKVLLIVDNRLDTQYLPEEAVRMASVAVQCLSFEPKSRPTMDQVVRALQQL
```

Figure 2. 3 Amino acid sequence alignment of ZmBLK1, BIK1 and TPK1b. Bars indicate the 11 kinase subdomains (I-XI). Red frame indicates the conserved activation regions.
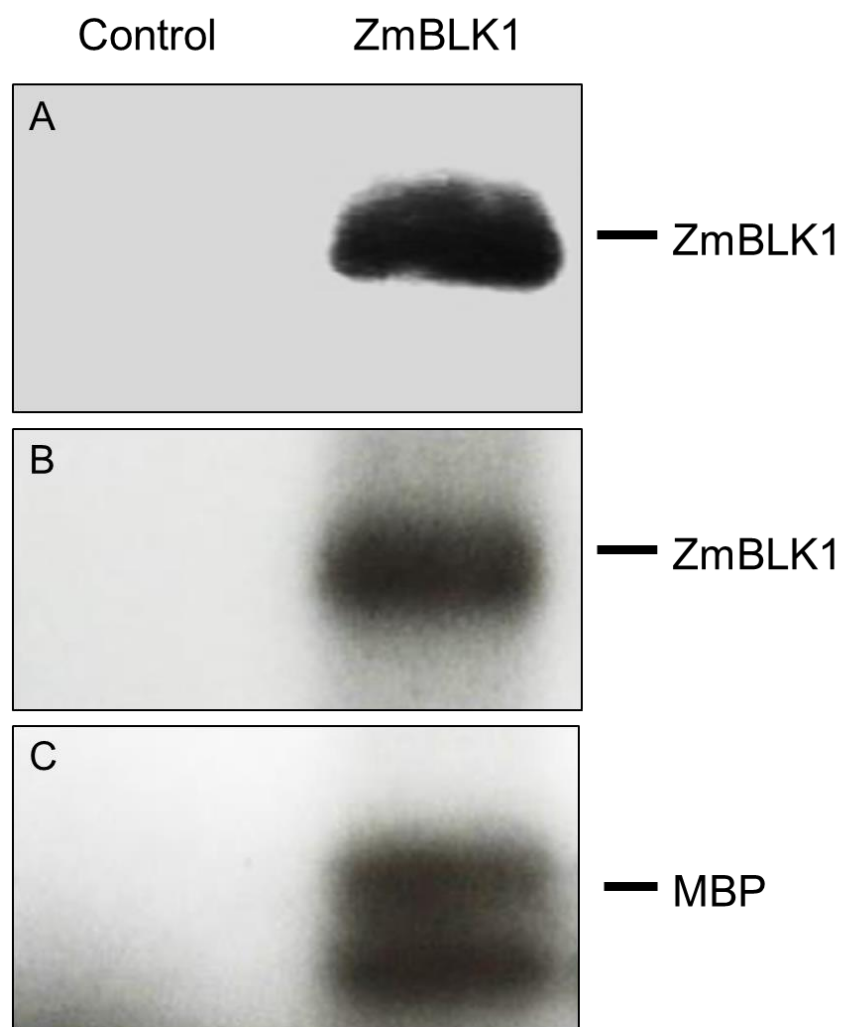
Figure 2. 4 Kinase activity of ZmBLK1. Protein was extracted from *N. benthamiana* leaves transiently expressing *ZmBLK1,* and ZmBLK1 protein was immuno-purified. (A) Western blot of ZmBLK1 probed with anti-HA antibody. Autoradiographs of SDS-PAGE gels, showing (B) autophosphorylation of ZmBLK1 and (C) phosphorylation of MBP substrate. Control lanes contain protein extracts from non-treated *N. benthamiana* leaf tissues.
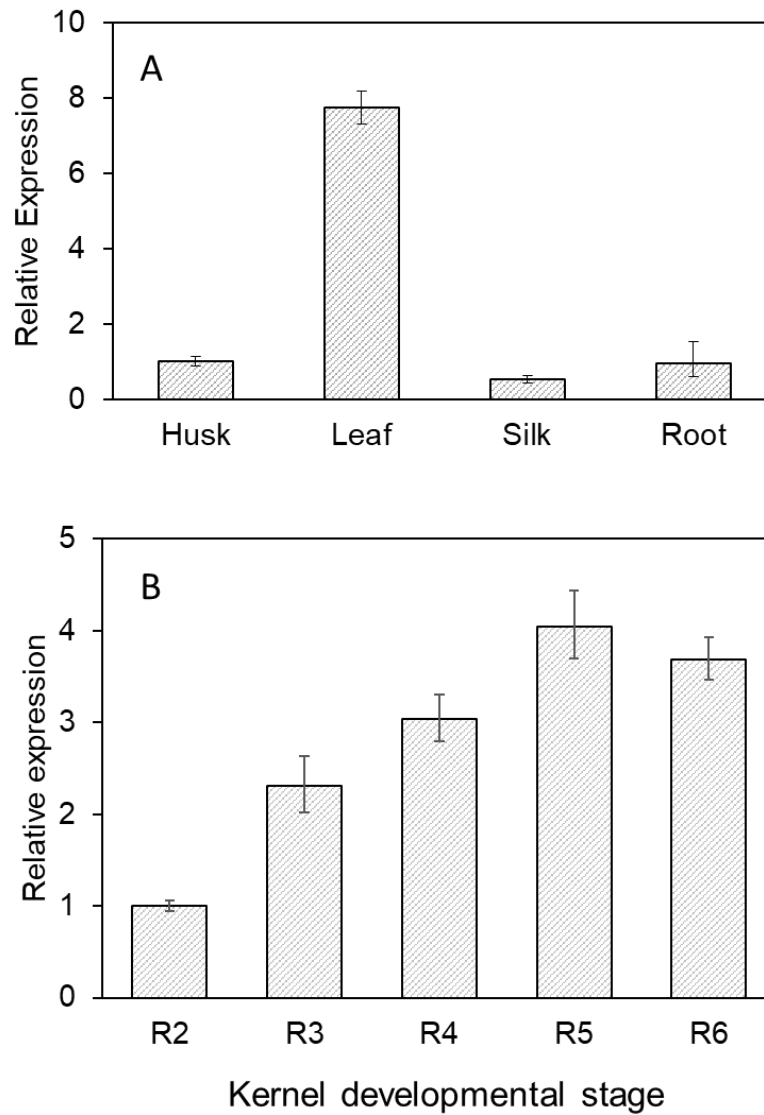
Figure 2. 5 Relative expression of *ZmBLK1* in different maize tissues. RNA was isolated from (A): leaves, silks, husks and roots tissue at the silk (R1) stage of development and (B): kernels at the blister (R2) milk (R3) dough (R4) dent (R5) and maturity (R6) stages of development. Expression was measured by qPCR, normalized to α-tubulin, and calculated relative to expression in (A): husk tissue or (B): blister kernel tissue. The relative expression of each gene was calculated as 2ΔΔCt. Bars indicate standard deviations of three technical replicates. The analysis was repeated on at least two biological replicates with similar results.
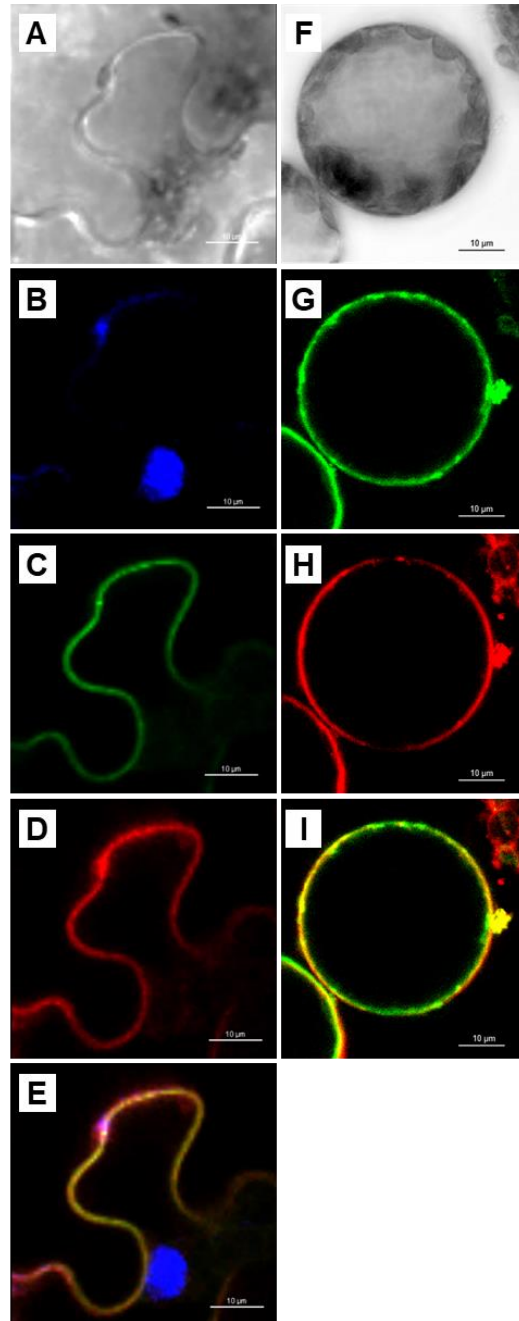
Figure 2. 6 Subcellular localization of ZmBLK1 in epidermal cells of *N. benthamiana* leaves (A through E) and protoplast (F through I). Image (A) and (F) were taken under transmitted light. Image (B) was taken under blue fluorescence (455 nm) to detect DAPI-stained nucleus. Image (C) and (G) were taken under green fluorescence (488nm) to detect ZmBLK1::GFP fusion protein. Image (D) and (H) were taken under red fluorescence (560 nm) to detect FM4-64-stained plasma membrane. Image (E) is a merge of 455 nm (blue), 488nm (green) and 560 nm (red) channels. Image (I) is a merge of 488nm (green) and 560 nm (red) channels.
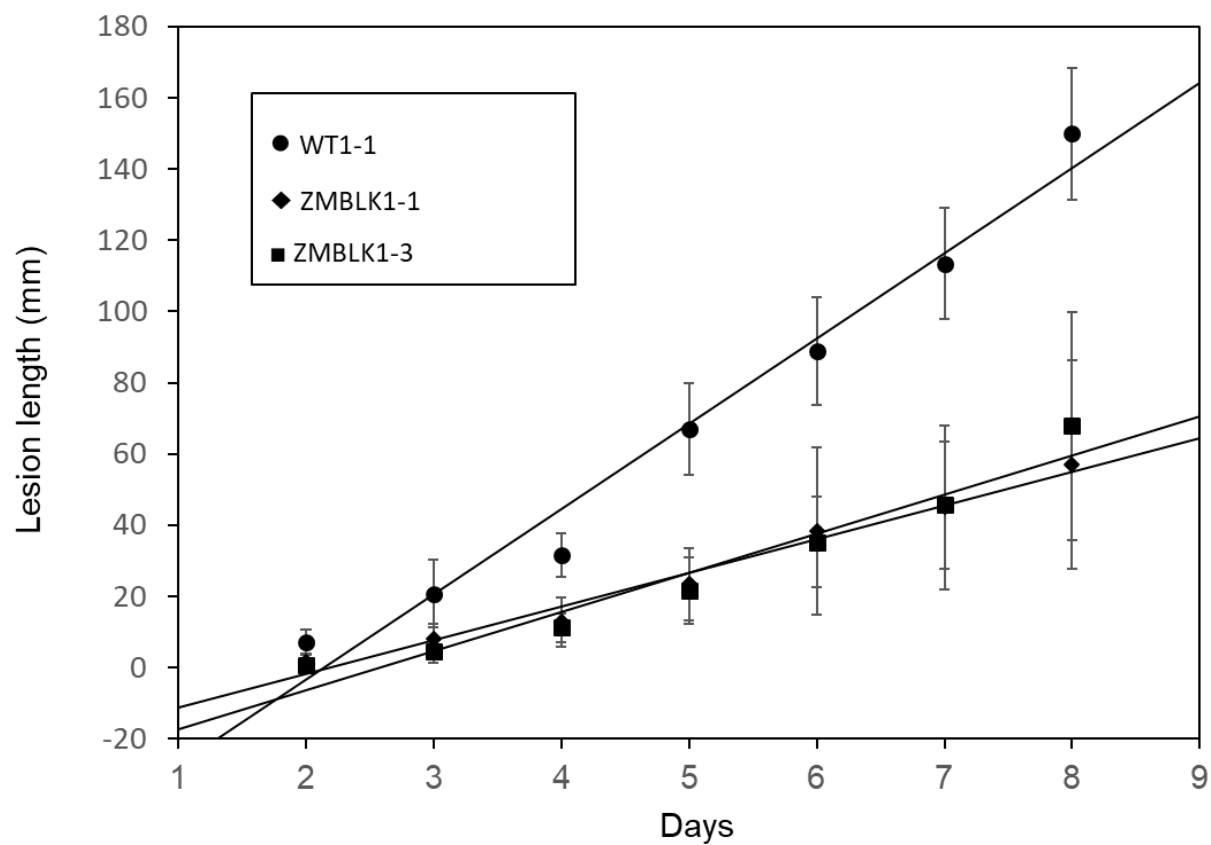
Figure 2. 7 Disease development of Goss's wilt lesion on non-transgenic (WT-1), ZMBLK1-1 and ZMBLK1-3 plants. Lesion length was measure from the inoculation point. Data are the mean from 10 plants and the bars represent the standard error.
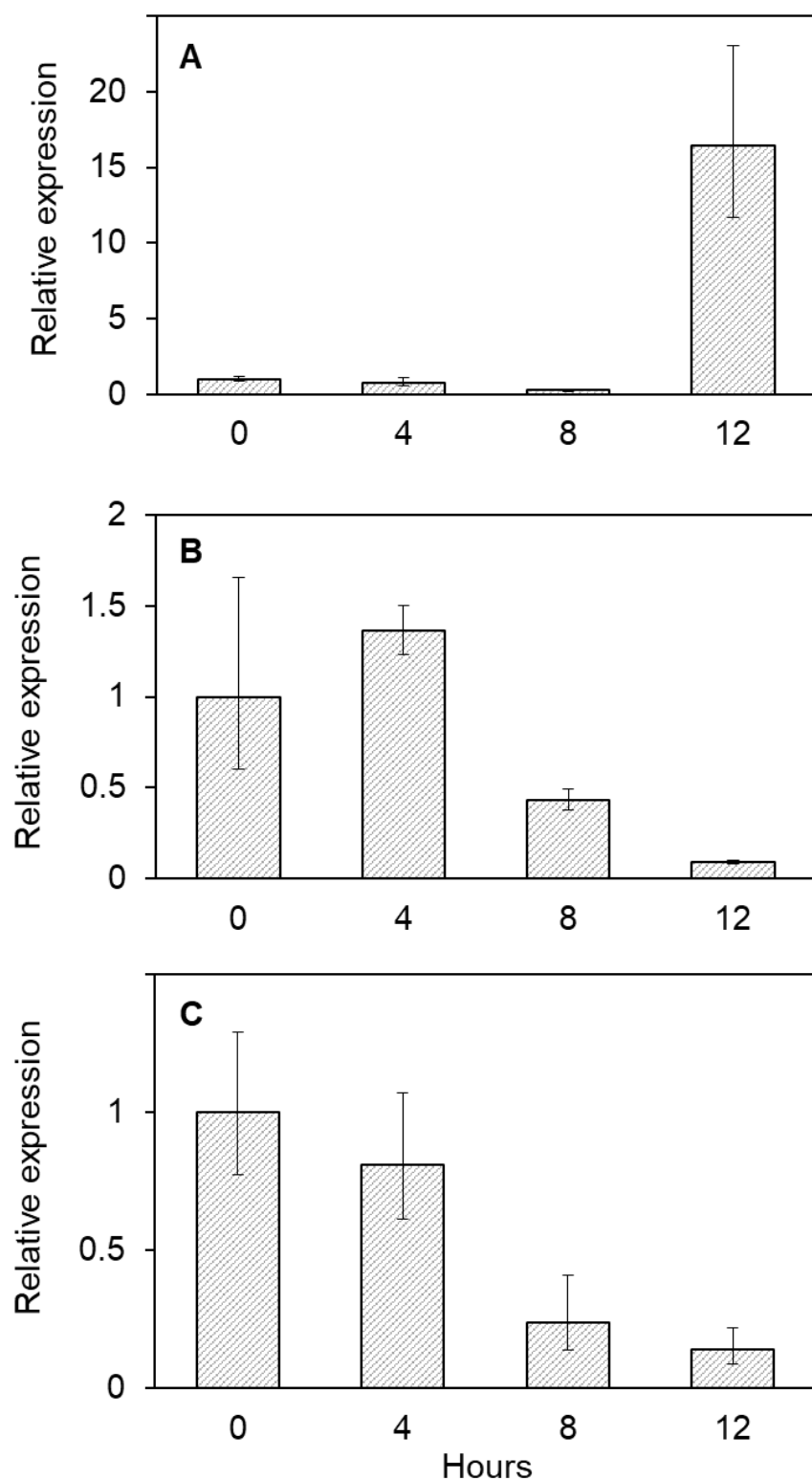
Figure 2. 8 *ZmBLK1* expression level in WT1-1 (A), ZMBLK1-1 (B) and ZMBLK1-3 (C) plants after CMN inoculation. Expression was measured by qPCR, normalized to α-tubulin, and calculated relative to expression at time zero. The relative expression of ZmBLK1 was calculated as 2ΔΔCt. Bars indicate standard deviations of three technical replicates.

Figure 2. 9 *ZmBLK1* expression level in plants. Expression was measured in WT1-1, ZMBLK1-1 and ZMBLK1-3 plants 0 h (A), 4 h (B), 8 h (C) and 12 h (D) after CMN inoculation by qRT-PCR. Data were normalized to α-tubulin, and expression calculated relative to expression in the non-transgenic plant (WT1-1). The relative expression of *ZmBLK1* was calculated as $2^{\Delta\Delta Ct}$. Bars indicate standard deviations of three technical replicates.

CHAPTER 3.   OVEREXPRESSING ZMBLK1 DOES NOT AFFECT RESISTANCE TO AFLATOXIN CONTAMINATION IN MAIZE KERNELS

3.1   Abstract

The Aspergillus ear rot caused by Aspergillus flavus usually results in aflatoxin accumulation in the maize kernels. Variety of efforts have been made to control the disease and the correlated aflatoxin contamination. Previous studies revealed that the maize RLCK, zea mays bik1-like kinase 1 (ZmBLK1) contributes to resistance against Goss's wilt disease. In this study, ZmBLK1 and three mutated ZmBLK1 genes were respectively overexpressed in maize to investigate if they confer resistance against Aspergillus ear rot and aflatoxin contamination. We demonstrated that substitution of the two threonine residues with negatively charged residues in the activation segment of ZmBLK1 does not affect its activity. Overexpressing the wild type or the mutated ZmBLK1 protein did not altered the resistance against A.flavus infection and aflatoxin amount in maize kernels. Furthermore, maize overexpressing the mutated ZmBLK1 proteins had similar resistance level against Goss's wilt as the ZmBLK1 overexpressing maize. These results suggest that ZmBLK1 does not contribute to maize resistance to Aspergillus ear rot and aflatoxin contamination.

3.2   Introduction

The *Aspergillus* ear rot is one of the most significant fungal diseases of maize. The major pathogen of this disease is *Aspergillus flavus* Link: Fries (teleomorph stage: *Petromyces flavus*) which infects maize ear through wounds or silks. The air-borne conidia can infect the silk and

grow down through the silk channel to the kernels, and they can also infect through wounds created by ear-feeding insects, such as corn earworm (Helicoverpa zea), (Dowd, 2003; Ni et al., 2011; Magbanua et al., 2013; Smart et al., 1990; Mideros et al., 2012; Horner et al., 2009). Abiotic stresses, such as drought and heat, weaken the plant, increase susceptibility, and often leads to silk cuts (Payne et al., 1986; Kebede et al., 2012; Chen et al., 2004; Ayerst, 1969; Odvody et al., 2007).

Aspergillus ear rot disease often results in aflatoxin contamination, which dramatically lowers the grain quality (Warburton et al., 2014; Woloshuk et al., 2013). Aflatoxins are polyketide secondary metabolites which are highly carcinogenic to humans and livestock. Liver is the main target of aflatoxin and consumption can lead to acute toxicity, cancer, and immune suppression (Peers et al., 1973; Qian et al., 1994; Peers et al., 1976; Svoboda et al., 1966; Clifford et al., 1967). The Group 1 carcinogen assignment by the International Agency for Research on Cancer (IARC) has resulted in worldwide regulations on aflatoxin-contaminated products. The US Food and Drug Administration (FDA) established an series of action level regulations, which include a maximum of 20 ppb aflatoxins in maize for interstate commerce (Codifer et al., 1976). As a result of these regulations, an outbreak of *Aspergillus* ear rot disease can lead to a huge economic impact.

A variety of efforts have been undertaken to control *Aspergillus* ear rot and aflatoxin contaminations such as biocontrol, host induced gene silencing and especially resistance breeding (Tubajika et al., 2001; Jiang et al., 2011; Cary et al., 2011; Payne et al., 1986; Paul et al., 2003). Since the disease and aflatoxin accumulation is largely associated with abiotic stresses, breeding efforts have focused on drought tolerance in maize varieties (Kebede et al., 2012; Tubajika et al., 2001). Maize germplasm lines with drought tolerance, such as P31G70, PI474210 and PI504146, had significantly lower aflatoxin accumulation when inoculated with *A. flavus* (Tubajika et al., 2001; Kebede et al., 2012; Clements et al., 2004). Molecular evidence also indicates the

relationship between aflatoxin contamination and stress responses of plants. Proteome studies identified a series of stress-related proteins and the higher production of a specific 14-kDa trypsin inhibitor protein in aflatoxin-resistant genotypes compared to susceptible genotypes (Chen et al., 2007, 2002). In an effort to identify potential aflatoxin resistance, Robert et al. (2016) selected high resistant inbred lines from West and Central Africa and obtained hybrid lines with different resistance levels. Chiuraise et al. (2015) successfully stacked mycotoxin resistance traits from South Africa inbred lines by crossing. In addition, QTL mapping and Genome-Wide association mapping studies have identified several QTLs and genes associated with resistance to aflatoxin accumulation (Luo et al., 2009; Warburton et al., 2015; Betrán et al., 2013; Yin et al., 2014). The candidate genes include a group of heat shock proteins (HSPs), late embryogenesis abundant proteins (LEAs), individual glycine rich RNA binding protein2, ribosomal protein L30 and exonuclease endonuclease phosphatase (Fountaina et al., 2015; Warburton et al., 2015). However, these studies have not determined any resistance mechanism in the maize and *A. flavus* interaction.

Ogunolab et al. (2017) characterized maize lipoxygenase (LOX) genes that are highly associated with reduce aflatoxin contamination in maize. Six WRKY transcription factors in maize were also found to respond to *A. flavus* infection (Guo et al., 2014). These results suggest several candidate genes and metabolic pathways that need further analyses, including ROS recycling, SA mediated pathways, ethylene mediated pathways and MAPK pathways. Based on the current studies on maize and other model plants, Fountaina et al (2015) presented a hypothetical model to describe the maize and *A. flavus* interaction. The model suggests that the necrotrophic nature of *A. flavus* leads to PTI responses in maize. The molecular mechanisms associated with PTI have been best studied in *Arabidopsis*, including the importance of receptor-like kinases (RLKs) and receptor-like cytoplasmic kinases (RLCKs). The RLCK BIK1 in *Arabidopsis* plays an essential

role in regulating PTI responses to the necrotrophic fungus *Botrytis cinerea*. Additional studies indicate that phosphomimic mutation of BIK1 can be created by substitution of the threonine amino acid (Thr-242) in the kinase activation region with negatively charged residues. This phosphomimic mutation was found to enhance plant resistance to *B. cinerea* (Laluk et al., 2011).

In a previous study, we compared the protein kinases in maize to the 610 RLKs in Arabidopsis and identified 195 putative RLCKs (Chapter 2). We also identified a putative ortholog of BIK1, which we named ZmBLK1. We hypothesized that ZmBLK1 is involved in disease resistance and found that overexpression of ZmBLK1 in transgenic maize reduced the severity of Goss's Wilt disease. In this current study, the impact of ZmBLK1 expression on *Aspergillus* ear rot and aflatoxin contamination was investigated. We also tested the hypothesis that overexpression of putative ZmBLK1 phosphomimic mutations further increase maize resistance.

3.3   Method

3.3.1   Site-direct mutagenesis, vector construction and maize transformation

Full-length *ZmBLK1* coding region was amplified by PCR with cDNA generated from RNA purified from B73 maize leaves. PCR primers were ZmBLK1F and ZmBLK1R (Table 3.1). The reaction product was cloned into the *Asc*I and *Xma*I sites of the binary vector pTF101HA. The resulting construct (pTFZBLK1) contained a T-DNA cassette consisting *ZmBLK1* coding sequence with a triple HA tag (5'-TACCCATACGATGTTCCTGACTATGCGGGCTATCCCTATGACG TCCCGGCCTATGCAGGATCCTATCCATATGACGTTCCAGATTACGCTGCT-3') driven by the cauliflower mosaic virus 35S promoter. The cassette also contained the *bar* gene as glufosinate-ammonium resistance for selection (Schröder et al. 1994; Rajasekaran et al. 2017).

To obtain phosphomimic mutations of ZmBLK1 proteins, site-directed mutagenesis based on overlapping PCR was carried out to convert threonine residues at positions 246 and 251 in ZmBLK1 to glutamic acid. The primers used for PCR are in Table 3.1. The overlapping PCR was carried out as described with pTFZBLK1 as template DNA (Landt et al., 1990). Briefly as an example, to createTo create ZmBLK1^{T246E}, a PCR fragment was amplified with primers BLK1LF1 and 246LR (Table 3.1), containing the desired point mutation. A second PCR fragment was amplified with primer 246RF and BLK1RR2. Subsequently, the two PCR products were annealed and extended by 14 PCR cycles. The full-length mutated gene was amplified by a final PCR with primer BLK1LF1 and BLK1RR2. ZmBLK1^{T251E} and ZmBLK1^{T246E,T251E} were created by the same strategy with primers 251LR, 251RF and 246251LR, 246251RF, respectively (Table 3.1). The mutated genes were individually cloned into the binary vector pTF101HA, resulting constructs pTFZBLK2, pTFZBLK3 and pTFZBLK4 that contained ZmBLK1^{T246E}-HA, ZmBLK1^{T251E}-HA and ZmBLK1^{T246E,T251E}-HA coding sequences, respectively.

3.3.2   Maize transformation

The pTFZBLK1 construct and the three mutation constructs pTFZBLK2, pTFZBLK3 and pTFZBLK4 were used to obtain transformed maize plants. Hi-II maize plants were transformed by the Plant Transformation Facility at Iowa State University (Ames, IA USA). Transformed callus tissue was sent to the University of Arkansas (Fayetteville, AR USA), and after plantlet formation, sent to Purdue University (West Lafayette, IN USA). The transgenic plantlets were grown in ground-beds (16 h of light daily) at the Purdue Lilly Greenhouse Facility. Transgenic plants were identified by their resistance to glufosinate-ammonium herbicide. At the fourth-leaf stage a solution (500 mg/L) of the herbicide was applied on leaves as described by Rajasekaran et al.

(2017). Expression of the ZmBLK1 was determined by Western analysis. Total proteins were isolated from herbicide-resistant plants with extraction buffer (50 mM HEPES, 50 mM EGTA, 25 mM NaF, 1 mM Na_3VO_4, 50 mM β-glycerophosphate, 100 mM NaCl, 1 mM PMSF, 2 mM DTT, 20% glycerol, 1x proteinase inhibitor cocktail (Sigma)) and separated by 12% SDS-PAGE. After electroblot transfer, blots were probed with an anti-HA antibody as previously described (Wood et al. 2006).

From the four constructs (*ZmBLK, ZmBLK1^{T246E}, ZmBLK1^{T251E}, ZmBLK1^{T246E,T251E}),* a total of 4, 10, 9 and 9 transgenic events were obtained, respectively. From these events, two transgenic lines were identified expressing *ZmBLK* (Line ZMBLK1-1 and ZMBLK1-3), *ZmBLK1^{T246E}* (Line ZMBLK2-1and ZMBLK2-2), *ZmBLK1^{T251E}* (Line ZMBLK3-1 and ZMBLK3-1), and *ZmBLK1^{T246E,T251E}* (Line ZMBLK4-1and ZMBLK4-4). The T0 transgenic plants were crossed to B73, and the BC1 transgenic plants were backcrossed to B73. To generate homozygous plants, BC2 transgenic plants were selfed three times, twice under greenhouse conditions (Lilly Plant Growth Facility) and once in field (Agronomy Center for Research and Education). The homozygous plants of F3 generation were used for kernel and leave disease assays and the non-transgenic segregation lines from the F2 generation were used as controls. Transgenic plants were identified by resistance to glufosinate-ammonium treatment and by PCR. Genome DNA was extracted from plant leaf tissues as described by Xin et al (Xin et al., 2003). The HA-tag specific PCR primers used for the screen were ZmBLKGTF and ZmBLKGTR (Table 3.1).

3.3.3   Kinase activity assay

Total proteins were extracted from 300 mg of kernel samples as described for Western analysis. Monoclonal anti-HA agarose (Sigma) were used to immunoprecipitate the HA-tagged

proteins. The precipitated proteins were washed three times with the immunoprecipitation buffer and twice with kinase reaction buffer (50 mM Tris, pH 7.5, 20 mM $MnCl_2$, 2 mM EGTA, and 2 mM DTT). Prior to the kinase assay, 200 µg of myelin basic protein (MBP) was pre-treated with 50 unit of calf intestinal alkaline phosphatase (CIP) in CIP buffer (100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl2, 1 mM DTT) for 1 h at 37 °C. This dephosphorylation reaction was stopped by adding 5 mM EDTA followed by incubating 30 min at 65 °C. The kinase assay with the immuno-precipitation beads was in a 100 µl reaction volume in reaction buffer (50mM Tris-HCl, 10mM MgCl2, 200 mM of ATP, 2mM DTT) and 20 µg dephosphorylated MBP. After incubation for 30 min at room temperature, the reaction mixture was boiled for 5 min at 95 °C. Phosphorylated MBP was visualized by Western analysis with 15% SDS–PAGE gels and anti- phosphoserine/Thr antibodies.

3.3.4   RNA extraction, cDNA synthesis and qRT-PCR

Total RNA was extracted from kernel samples by a phenol-chloroform method. Kernels were ground with pestle and mortar in liquid nitrogen and approximate 3 g of fine powder was mixed with 10 ml of Tris-saturated phenol (pH 4.3) and 10 ml of 1 M Tris-HCl (pH 8.0). The extracts were centrifuged at $10,000 \times g$ for 10 min at 4°C. The supernatant was then extracted with equal volume of chloroform: phenol (1:1), followed by extraction with equal volume of chloroform: isoamyl alcohol (24:1). RNA was precipitated overnight at -20°C after adding 2 volume of ethanol and pelleted by centrifugation at $10,000 \times g$ for 30 min at 4°C. The pellet was dissolved in 400 µl DEPC treated water and precipitated again by adding 40 µl of sodium acetate and 800 ul of ethanol.

After centrifugation at $12,000 \times g$ for 30 min at 4°C, the RNA pellet was washed with 70% ethanol and dissolved in DEPC treated water.

cDNA was synthesized with SuperScript™ III reverse transcriptase (Invitrogen) according to standard protocols (Reese et al. 2011). For qRT-PCR, a reaction mixture containing 7.5 µl of SYBR Green Supermix (Bio-rad), 1 µl of each primer (10 µM), 2 µl of cDNA template, and 3.5 µl of nuclease-free water. The reaction cycling consisted of 3 min at 95 °C, 40 cycles of 5 s at 95 °C and 30 s at 57 °C. The ΔΔCt method was used to calculate relative gene expression with α-tubulin gene as the internal normalizer. *ZmBLK1* specific primers were qZmBLK1F2 and qZmBLK1R2 (Table 3.1) and the α-tubulin gene-specific primers were AlphaTUBF and AlphaTUBR (Table 3.1).

3.3.5 *Aspergillus flavus* inoculation on ears and kernels

Ears of transgenic lines were harvested from field at R3 stage. For the ear assay, three whole ears of each transgenic line were inoculated with an *A. flavus* conidial suspension (10^7 conidia/ml) by pin bars as described (King et al., 1982). The pin bars were surface sterilized by bleach and then washed three times before dipping into the *A. flavus* conidial suspension. The dipped pin bar is used to penetrate the kernels on the ears. The kernel screening assay was as described by Cary et al (2011). Briefly, 30 random individual kernels from each transgenic line were removed from each transgenic line and placed in foil cups (10 kernels per cup). Each kernel was wounded with an 18-gauge needle and inoculated with 10 µl of an *A. flavus* conidial suspension (10^5 conidia/ml) at the wounding site. Inoculated ears or kernels were incubated in covered plastic boxes. *Aspergillus* infection and colonization on ears and kernels were photographed each day after inoculation. Five days after inoculation, inoculated kernels were removed from the ears or foil cups and kept in -80 °C for further analysis.

3.3.6 Aflatoxin analysis

Kernels samples of ear or kernel assay were ground in a coffee grinder (Hamilton Beach, Southern Pines, NC). From each sample, 0.5 g of the ground kernel was extracted by shaking overnight in 2 ml of chloroform: methanol (1: 1) solution. Extracts were centrifuged and filtered by Whatman 1001055 filter papers (GE Healthcare Life SciencesBoston, MA). Aflatoxins were analyzed by thin layer chromatography (TLC) as described by Narendrakumar and Dhandapani (2011). Briefly, 10 µl of the extracted samples were spotted on the silica gel 60 F_{254} plates (Merck KGaA, Darmstadt, Germany. TLC plates were developed in chloroform: acetone: water (88:12:1), photographed under UV, and analyzed with ImageJ software (https://imagej.nih.gov/ij/index.html). Quantification was obtained from standard curse from a serial of aflatoxin standards (10 µg, 20 µg, 50 µg and 100 µg) spotted on each TLC plate.

3.3.7 Goss's wilt analysis

C. michiganensis subsp. *nebraskensis* (CMN) was cultured on NBY (Nutrient Broth Yeast extract) agar medium at room temperature. After 4 days of growth, bacteria were harvested from the plates with sterile water and the concentration adjusted to OD_{640} = 0.3 (10^8 CFU/ml). Transgenic maize ZMBLK1-1 and ZMBLK1-3 and the non-transgenic line (WT1-1) were grown in 4-inch pots in greenhouse. At the V2 stage, leaf tissue was analyzed by Western blots to verify ZmBLK1 expression. Subsequently, the third leaf of ten V4-stage plants were cut at the tip and inoculated with CMN by submerging the cut end into the inoculum for 5 seconds. Lesion length on each inoculated leaf was measured every 24 hr.

3.4   Result

3.4.1   Substitution of threonine-246 and threonine-251 to glutamic acid did not activated ZmBLK1

To determine if ZmBLK1 can be constitutively activated by modifying the threonine residues in its kinase activation domain, two threonine residues (246 and 251) were individually or both substituted with Glu residues. This modification was hypothesized to mimic the negative charge conferred by the phosphorylated threonine residue and keep the kinase in an activated configuration. We extracted non-mutant and mutant proteins from the 8 selected transgenic lines and conducted the in vivo kinase assays. According to the Western blots, the wild type ZmBLK1 has kinase activity without any modifications. The three mutations, ZmBLK1^{T246E}, ZmBLK1^{T251E}, ZmBLK1^{T246E,T251E} did not qualitatively activated the kinases from the wild type level. However, ZmBLK1^{T246E,T251E} had slightly higher MBP phosphorylation activity compared to ZmBLK1, and ZmBLK1^{T251E} had slightly lower MBP phosphorylation activity compared to ZmBLK1 (Figure 3.1A). We also extracted these four proteins from transgenic maize leaves and obtained similar results (Figure 3.1B).

3.4.2   *ZmBLK1* does not confer resistance to *A. flavus* and aflatoxin contamination in maize kernel

To determine if overexpression of *ZmBLK1* or any of the phosphomimic mutants in maize can confer resistance to *A. flavus* and associated aflatoxin contamination, we inoculated *A. flavus* on transgenic maize kernels. The transgenic lines showed different levels of protein expression in kernel tissues (Figure 3.2), however, at 5 days after *A. flavus* inoculation, transgenic lines overexpressing ZmBLK1 or the three phosphomimic mutants did not show any inhibited aflatoxin

accumulation based on two individual ear experiments and one kernel experiment (Table 3.2). *A. flavus* sporulation began on the transgenic and non-transgenic kernels 3 days post inoculation, and no altered disease development was found between transgenic and non-transgenic kernels within 5 days post inoculation (Figure 3.3). Also, in three separate experiments transcript level of *ZmBLK1* was measured after *A. flavus* inoculation to B73 maize kernels at the R3 stage (Table 3.3). No difference in *ZmBLK1* expression was found 12 hours post inoculation when compared to expression in the mock-inoculated controls. Similar results were obtained at 24 hours post inoculation in two of the experiments and reduced *ZmBLK1* expression in one experiment. Based on these results, it appears that *ZmBLK1* is not induced during colonization of *A. flavus*.

3.4.3 Overexpression of *ZmBLK1* phospho-mimic mutants does not increase resistance to Goss's Wilt.

Goss's wilt assay was also carried out on transgenic maize overexpressing *ZmBLK1^{T246E}*, *ZmBLK1^{T251E}* or *ZmBLK1^{T246E,T251E}*. The results showed that overexpression of *ZmBLK1* or the phospho-mimic mutants in maize all resulted in resistance to Goss's wilt. However, compared to the *ZmBLK1* overexpressing lines, none of the phosphomimic mutant overexpressing lines had lower disease development rate within 8 days post inoculation (Figure 3.4). Therefore, overexpression of *ZmBLK1^{T246E}*, *ZmBLK1^{T251E}* or *ZmBLK1^{T246E,T251E}* does not increase Goss's wilt resistance in maize compared to the *ZmBLK1* overexpression maize.

3.5 Discussion

In this study, we found that substitution of threonine-246 and threonine-251 to glutamic acid did not impact the kinase activity of ZmBLK1. These two threonine residues locate in the

activation segment which is highly conserved among serine/threonine protein kinases (Nolen et al., 2004; Hanks, 1991). The activation segment consists of an activation loop (the catalytic center) and a following P+1 loop (the substrate binding site). The first threonine residue locates in the activation loop and the second threonine residue locates in the P+1 loop. Previous studies of different protein kinases reveal that the threonine residues in the activation segment are important for their functions and constitutive activated mutation can be created by substituting these residues with negatively charge residues like glutamic acid and aspartic acid. For instance, substitution of the two conserved threonine residues with glutamic acid in the *Arabidopsis* MKK2 and rice OsMKK6 can activate their MAP kinase activity (Teige et al., 2004; Kumar et al., 2013). In BIK1, substitution of the second threonine residue to aspartic acid (BIK1^{T242D}) also increase its kinase activity from a basal level to a higher level (Laluk et al., 2011). In our study, the wild type ZmBLK1 protein had kinase activity in maize leaves and kernels without any stimulus, and the substitution of any or both of the reserved threonine residues did not change ZmBLK1 to a further activated status. This result may indicate that the activity of ZmBLK1 is already at an activated level when it is expressed in maize tissues. Therefore, the constitutive mutations of ZmBLK1 did not have further activity compared to ZmBLK1.

The kinase activities of the ZmBLK1 and mutations may also explain the phenotype observed in the disease assays. We found that overexpressing *ZmBLK1* or any of the *ZmBLK1* mutations in maize led to similar resistance against Goss's wilt disease. This may be the result of the similar kinase activity of ZmBLK1 and the three mutants. In *Arabidopsis*, overexpressing the phospho-mimic mutant *BIK1^{T242D}* enhances the resistance to *B. cinerea* compared to wild type *Arabidopsis* plants, but *B. cinerea* susceptibility difference between *BIK1* and *BIK1^{T242D}* overexpressing *Arabidopsis* was not reported (Laluk et al., 2011). However, overexpression of

BIK1 can activate the *Arabidopsis* NADPH Oxidase RbohD and enhance bacterial resistance (Li et al., 2014). In addition, overexpression of a rice RLCK, *OsRLCK278*, in *Arabidopsis* leads to resistance against *P. syringae* (Dubouzet et al., 2011). In another study, overexpressing a pepper (*Capsicum annuum*) RLCK gene *CaPIK1* in *Arabidopsis* leads to resistance against *P. syringae* pv. *tomato*. These results suggest that other than modulating the activity of an RLCK, overexpressing the wild type kinase can also impact the plant's disease resistance. In addition, according to the wester blots of the four kinase assays carried out in this study, the ZmBLK1^{T251D} always displayed the weakest MBP phosphorylation. This may account for the slightly higher rate of Goss's wilt lesion development on ZmBLK1^{T251D} overexpressing lines (13.57 (11.16-15.52) mm/day) compared to the ZmBLK1 overexpressing lines (9.44 (7.94-10.93) mm/day), while the lesion rate in ZmBLK1^{T246D} and ZmBLK1^{T246D,T251D} lines were not significantly different from the ZmBLK1 lines.

In this study, we also investigated the function of *ZmBLK1* in maize kernels. However, expression level of *ZmBLK1* did not increase in maize kernel tissues 12 and 24 after *A. flavus* inoculation. In addition, we did not observe any increase of aflatoxin and *A. flavus* resistance in *ZmBLK1* or any of the *ZmBLK1* mutant overexpression line. To date, molecular evidence of *A. flavus* and aflatoxin resistance in maize kernel is limited. Fountaina et al. (2015) described the hypothetical pathways involved in the maize-*A. flavus* interaction based on related *Arabidopsis* studies. However, most of the discovered maize genes which have resistance function against the *A. flavus* infection and aflatoxin contamination belong to two major groups. One group is the stress tolerance proteins like heat shock proteins (HSPs) and late embryogenesis abundant proteins (LEAs) (Chen et al., 2007, 2002; Luo et al., 2009). Another group is the antifungal proteins, such as β-1,3-glucanase, chitinases, and globulins (Moore et al., 2007; Ji et al., 2000; Chen et al., 2007;

Luo et al., 2009). The early immune response genes, like RLKs, MAPKs or CDPKs, have not yet been proofed to provide *A. flavus* and aflatoxin resistance. In this study, we also found that the putative PTI related gene *ZmBLK1* did not display any *A. flavus* resistance functions. Thus, these results suggest that *ZmBLK1* may not be an essential component for the immune responses to *A. flavus* and resistance to aflatoxin contamination in maize kernels.

In conclusion, this study reveals that substitution of threonine-246, threonine-251 or both residues with glutamic acid does not alter the kinase activity of ZmBLK1. Overexpressing these mutants of ZmBLK1 in maize does not confer a higher resistance to Goss's wilt compared to overexpressing ZmBLK1. In addition, overexpression of ZmBLK1 and the modified proteins does not impact maize susceptibility to *A. flavus* and the associated aflatoxin contamination.

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Table 3.1 Primers used in chapter 3.

Primer name	Sequence (5' to 3')
ZmBLK1F	AAAGGCGCGCCTATGGGGAACTGCTGGG
ZmBLK1R	CGCCCCGGGACGAGAATGGGCCAATGG
BLK1LF1	CGAATTGGGCCCGACGTCGCAT
BLK1RR2	GACACTATAGAATACTCAAGCTATGCATCC
246LR	CCATATGTTCCCATAACTCTCTCGGACACATGGCT
251LR	CTCAGGAGCTGCATAACCATATTCTCCCATAACTCTTGT
246251LR	CCATATTCTCCCATAACTCTCTCGGACACATGGCT
246RF	GAGTTATGGGAACATATGGTTATGCAGC
251RF	TATGGTTATGCAGCTCCTGAGTATCTTTC
246251RF	GAGTTATGGGAGAATATGGTTATGCAGC
ZmBLKGTF	GGTTATGCAGCTCCTGAGTATC
ZmBLKGTR	GCATAGTCAGGAACATCGTATGG
qZmBLK1F2	CGAGCCTCTTCAGCTTCTATG
qZmBLK1R2	TGTGGCTGCCTTGAGATTATT
AlphaTUBF	CACTGATGTTGCTGTCCTGC
AlphaTUBR	CGCTGTTGGTGATTTCGG

Table 3.2 Aflatoxin levels in kernels from maize overexpressing various *ZmBLK1* constructs.

Overexpressed gene	Aflatoxin (µg of AFB1/g of kernel)[a]	
	Transgenic	Non-transgenic
ZmBLK1	11.1-80.1	0-78.8
ZmBLK1[T246E]	6.6-43.2	16-165.0
ZmBLK1[T251E]	7.9-208.9	31.7-189.4
ZmBLK1[T246E,T251E]	5.9-82.5	23.7-102.5

[a]Values are range of AFB1 5 days after inoculation with *A. flavus* from three infection experiments.

Table 3.3 *ZmBLK1* expression in maize kernel inoculated with A. flavus.

Experiment[a]	ZmBLK1 expression[b]	
	12 Hr	24 Hr
1	1.02 (0.99-1.05)	0.53 (0.50-0.56)
2	0.96 (0.73-1.26)	1.00 (0.81-1.22)
3	0.82 (0.67-1.00)	0.94 (0.84-1.07)

[a]Expression analysis was carried out in three independent experiments.
[c]Expression was measured by qPCR, normalized to α-tubulin, and calculated relative to expression in control group inoculated with 0.05% triton X-100 solution. The relative expression of each gene was calculated as $2\Delta\Delta Ct$. The range of expression, in parentheses, for each gene = $(2^{\Delta\Delta Ct-s}–2^{\Delta\Delta Ct+s})$, where s = the standard deviation of the $\Delta\Delta Ct$ value
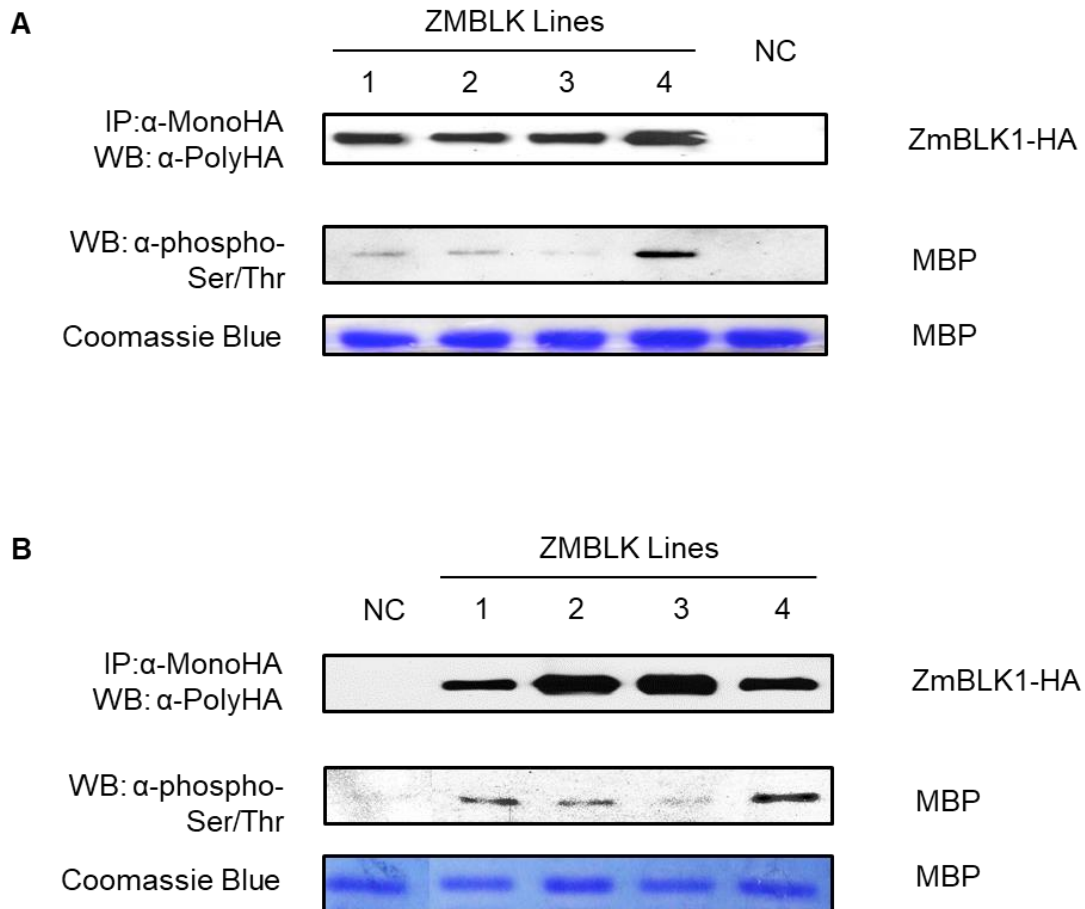
Figure 3. 1 Kinase activity of ZmBLK1 proteins extracted from transgenic maize kernels (A) and leaves (B). ZmBLK1 protein was immuno-purified with monoclonal anti-HA agarose beads (IP:α-MonoHA). Western blot of ZmBLK1 proteins were probed with polyclonal anti-HA antibody (WB: α-PolyHA). Phosphorylation of MBP substrate was detected by phosphoserine/Thr-specific antibody (WB: α-phospho- Ser/Thr). Coomassie blue staining shows equal loading of MBP. Control lanes contain protein extracts from non-transgenic maize kernels. NC: Nontransgenic control, 1: ZmBLK1, 2: ZmBLK1T246E, 3: ZmBLK1T251E, 4: ZmBLK1T246E,T251E. Experiments carried out in two independent transgenic lines showed similar results.
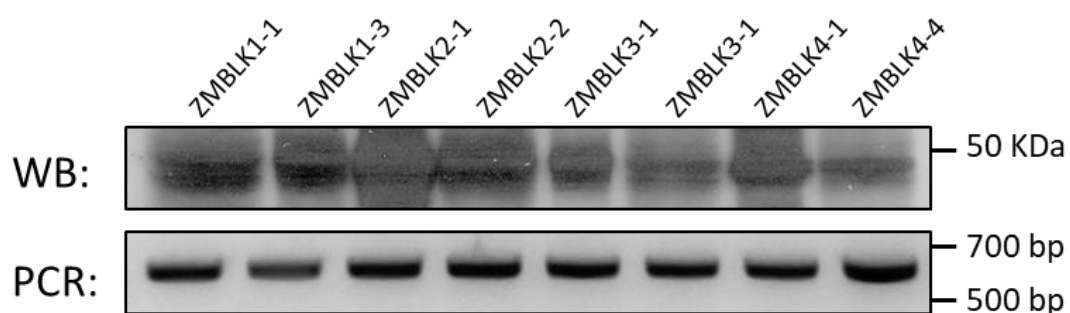
Figure 3. 2 Protein expression and genotype of transgenic maize lines used for disease assays. Protein was extracted from 300 mg transgenic maize kernels and ZmBLK1 proteins were probed with monoclonal anti-HA antibody. PCR was carried out using genome DNA extracted from transgenic maize leaves and ZmBLK1-HA specific primers. ZMBLK1-1 and ZMBLK1-3 express ZmBLK1, ZMBLK2-1 and ZMBLK2-2 express ZmBLK1T246E, ZMBLK3-1 and ZMBLK3-1 express ZmBLK1T251E, ZMBLK4-1 and ZMBLK4-4 express ZmBLK1T246E,T251E.
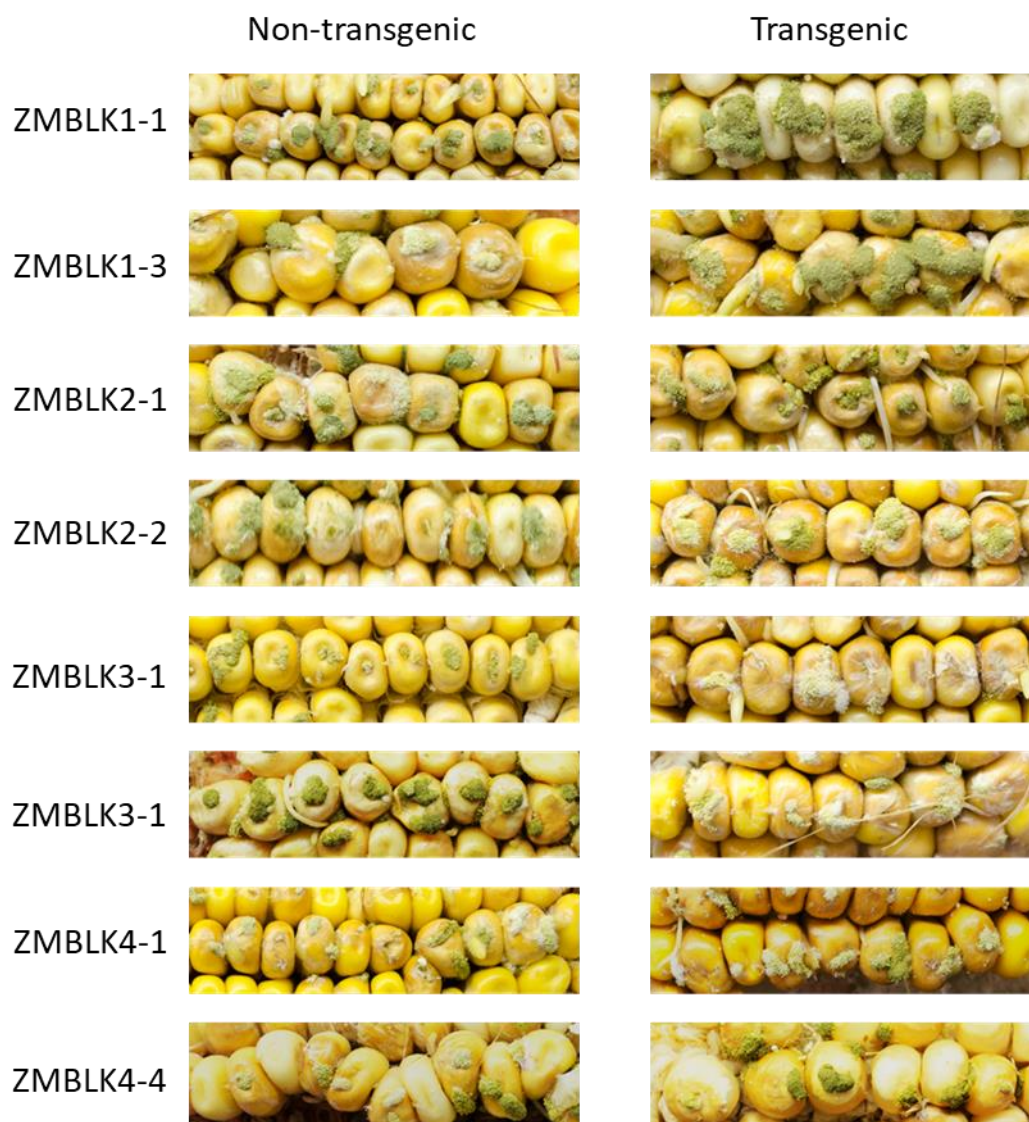
Figure 3. 3 A.flavus colonization on transgenic and non-transgenic kernels 5 days post inoculation. ZMBLK1-1 and ZMBLK1-3 express ZmBLK1, ZMBLK2-1 and ZMBLK2-2 express ZmBLK1T246E, ZMBLK3-1 and ZMBLK3-1 express ZmBLK1T251E, ZMBLK4-1 and ZMBLK4-4 express ZmBLK1T246E,T251E.
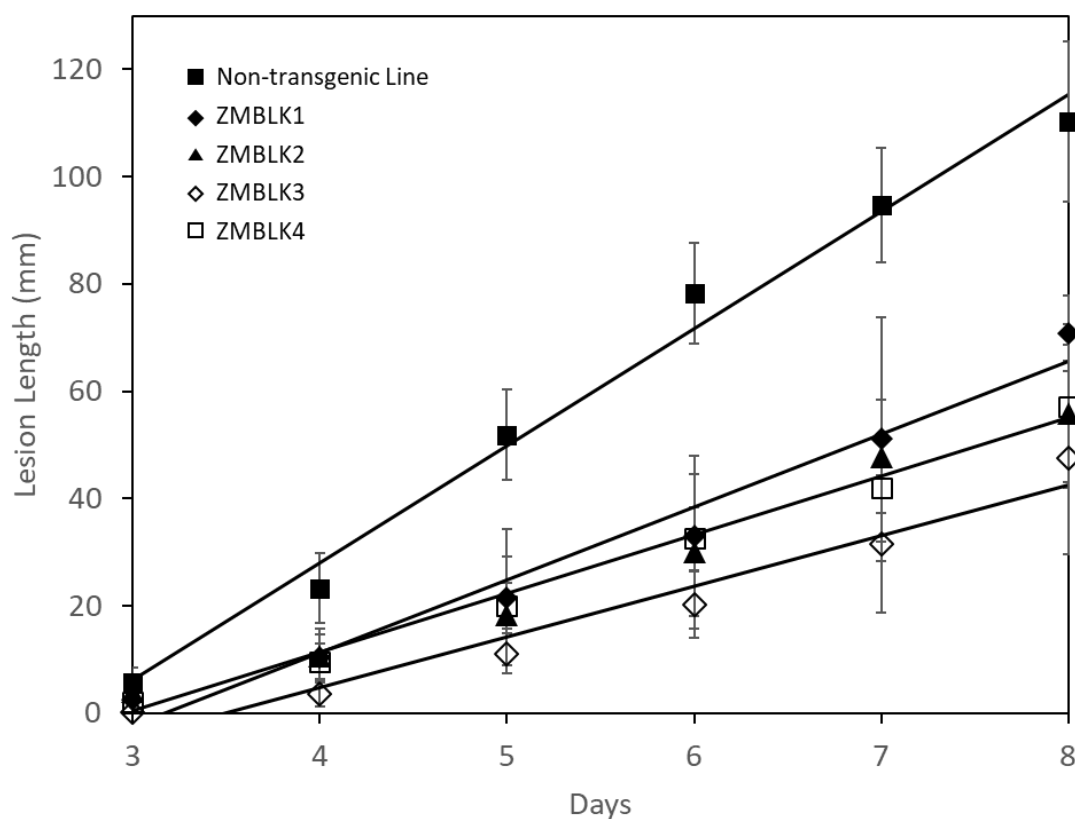
Figure 3. 4 Disease development of Goss's wilt lesion on non-transgenic and overexpression plants. Lesion length was measure from the inoculation point. Data are the mean from 10 overexpression plants (5 from each line) and the bars represent the standard errors. The rates of lesion development are: Non-transgenic: 21.87 (20.34-23.40) mm/day, ZMBLK1: 9.44 (7.94-10.93) mm/day, ZMBLK2: 10.95 (9.38-12.50) mm/day, ZMBLK3: 13.57 (11.16-15.52) mm/day, ZMBLK4: 11.02 (9.79-12.24) mm/day. The range in parentheses indicates the 95% confidence limits.