Transgenic expression of a receptor-like kinases gene in corn to reduce aflatoxin contamination

Weiran Li & Charles P. Woloshuk, Department of Botany and Plant Pathology, Purdue University | Lilly Hall of Life Sciences • 915 W. State Street • West Lafayette, IN 47907-2054

PURDUE UNIVERSITY

Background

Aspergillus ear rot and aflatoxin contamination

In 2012, the entire state of Indiana experienced severe heat and drought conditions. Prior to corn harvest, Aspergillus ear rot was observed in fields throughout the state. *A. flavus* is the main causal agent of this disease. The aflatoxin contamination caused by Aspergillus ear rot can lead to extreme decreased grain quality.

Limited control of Aspergillus ear rot

Efforts to control Aspergillus ear rot through breeding, fungicides, tillage practices and biocontrol agents have not provided good solutions. Up to date, genetically modified (GMO) varieties were increasingly planted in the US, however, none of GMO traits is developed for ear rot resistance. Unsurprisingly, a 2013 survey indicated that 80% of Indiana corn producers would be interested in using corn hybrids with genetically modified mycotoxin resistance traits.

Receptor-like protein kinases

Considering that *Aspergillus flavus* is a necrotrophic phytopathogen, genetic engineering could aiming at introducing genes to enhance PAMP-triggered immunity (PTI) to fungal pathogens, in order to enable new methods of managing Aspergillus flavus infections.

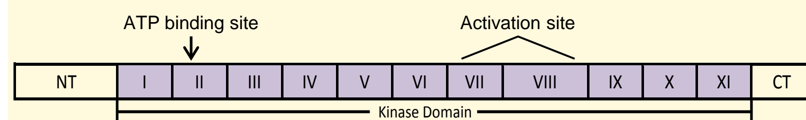


Figure 1. A schematic structure of receptor-like protein kinase. NT: N-terminal region; CT: C-terminal region; I~XI: 11 kinase subdomains

Receptor-like kinases (RLKs) in plants are a large superfamily of proteins that are structurally similar. Current studies have focused attention on plant receptor-like kinases as an important class of sentinels acting in plant defense responses. RLKs in *Arabidopsis* (BIK1) and (TPK1b) have been identified to have conserved *Botrytis cinerea* resistance function. Therefore, identification and expression of corn RLKs in corn is an optional strategy to manage Aspergillus ear rot disease and aflatoxin contamination.

Hypothesis & Objective

We hypothesize that expression of the regulatory gene (ZmPK1) in transgenic corn will reduce Aspergillus ear rot disease and aflatoxin contamination.

Objectives:

1, Identification of the receptor-like protein kinase (ZmPK) in corn

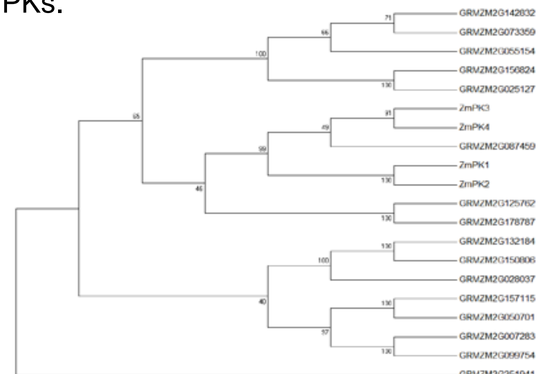
2, Generation of transgenic corn expressing ZmPK1a

3, Analyze ZmPK1a gene expression pattern in corn under pathogenic and wounding pressure.

Identification of ZmPK

Method: Sequence alignment was used to screen RLK homologs in corn genome. BIK1 and TPK1b sequences were used for BLAST in B73 RefGen_v3 database. Selected homolog genes were aligned by Clustal Omega and neighbor-joining phylogenetic tree was developed by Mega 6.

Result: Top rated 20 genes were selected from BLAST results (Figure 2). According to alignment score, the highest four genes were named as ZmPK1, ZmPK2, ZmPK3 and ZmPK4. All the four ZmPK genes have conserved 11 kinase subdomains and highly conserved activation region. In figure 3, the highlighted T (threonine) residues indicated the two important residues required by BIK1 and TPK1b phosphorylation activation, which are also conserved in ZmPKs.



BIK1	DFGLARDGPMGDLSYVSTRVMGTYGYAAPE
TPK1b	DFGLAKDGPTGDKSHVSTRVMGTYGYAAPE
ZmPK1a	DFGLAKDGPVGEKSHVSTRVMGTYGYAAPE
ZmPK1b	DFGLAKDGPVGEKSHVSTRVMGTYGYAAPE
ZmPK1c	DFGLAKDGPTGDKSHVSTRVMGTHGYAAPE
ZmPK1d	DFGLAKDGPTGDQSHVSTRVMGTYGYAAPE

Figure 2 (left). Rooted Neighbor-joining tree of 20 top rated RLK homologs in corn B73 genome.

Figure 3 (right). Conserved kinase activation sites in BIK1, TPK1b and ZmPK genes. Highlighted threonine residues are previous identified as required for BIK1 and TPK1b activation.

Generation of transgenic corn expressing ZmPK1a

Vector construction

Method: ZmPK1 was selected as the first candidate of corn RLK. This gene was amplified (noted as ZmPK1a afterwards) from corn B73 cDNA library by PCR. The construct carrying ZmPK1a gene (Figure 4) was cloned in the binary vector pTF101.1 for *Agrobacterium tumefaciens* mediated gene transfer. *A. tumefaciens* strain GV3101 was transformed with the binary vector by electroporation method.

Three putative constitutively activated ZmPK1a mutants (ZmPK1a-CA1, CA2 and CA3) were generated by substituting the two important T (threonine) residues to the polar charged E (glutamic acid), aiming at evaluating phosphorylation activity. A kinase dead mutant (ZmPK1a-IA) was generated by killing the ATP binding site (Figure 5). Site-directed mutagenesis was performed by overlap PCR base method. Activated and kinase dead mutants were cloned in same vector and *Agrobacterium* strain describe above.

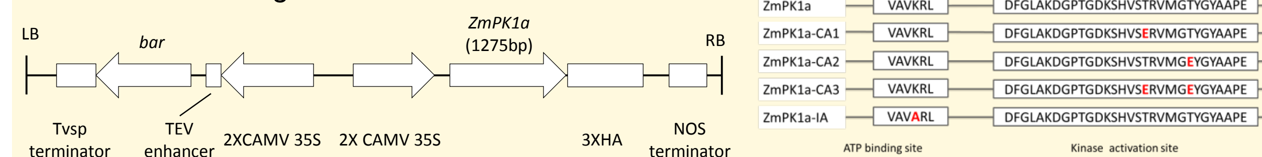


Figure 4. Construct maps used for ZmPK1a overexpression transgenic corn. Activated and kinase dead mutants of ZmPK1a used the same construct

Figure 5. Site-directed mutagenesis strategy for constitutively activated and kinase dead mutants. Red letters indicate the amino acid residues after substitutions

All transformed *A. tumefaciens* strains were infiltrated into *Nicotiana benthamiana* leaves for expression validation. Infiltrated leaves was collect after 48 hr incubation followed by protein extraction. Immunoprecipitation (IP) of total protein extraction was carried out by anti-HA beads.

Result: ZmPK1a and mutant proteins were expressed well in *N. benthamiana* (Figure 6). So ZmPK1a and CA1, CA2, CA3 transformed *A. tumefaciens* strains were sent to Burt Bluhm Lab, Arkansas for generation of transgenic corn lines。

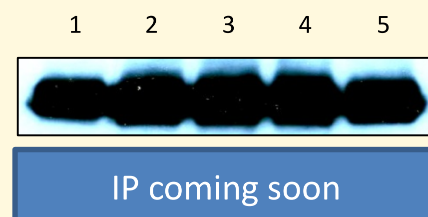


Figure 6. Western blot analysis of ZmPK1 in protein extracts from *N. benthamiana*. A) Total protein and B) IP protein. ZmPK1a was visualized with anti-HA antibodies. 1: wild-type ZmPK1a, 2: ZmPK1a-CA1, 3: ZmPK1a-CA2, 4: ZmPK1a-CA3, 5: ZmPK1a-IA

In vitro kinase activity assay

Method: IP ZmPK1 proteins were incubated with the substrate myelin basic (MBP) protein and radioactive-ATP. Proteins were separated by SDS-PAGE and visualized by autoradiography.

Result: constitutively activated mutants were found not activating the kinases, compared to wild type ZmPK1a (Figure 7). Especially, ZmPK1a-CA2 result in a large decrease of activity.

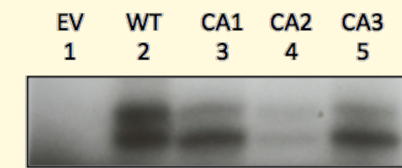


Figure 7. Protein kinase activity of ZmPK1 in protein extracts from *N. benthamiana*. Autoradiography was used to visualize transfer of radiolabel phosphate to MBP. 1: Empty vector, 2: wild-type ZmPK1a, 3: ZmPK1a-CA1, 4: ZmPK1a-CA2, 5: ZmPK1a-CA3

ZmPK expression under pathogenic and wounding pressure

Kernel inoculation

Method: For another research project, we have transcriptome (RNAseq) data from corn kernel inoculated with *A. flavus*. The data consist of seven biological replicate collected 0, 2, 3, 4, 5, and 6 days after wound inoculation B73 kernels. Expression of the 20 genes in figure 2 was processed by CLC Genome Workbench.

Result: most genes expression is not induced by *A. flavus*, including the ZmPK3 and ZmPK4. ZmPK1a expression decreased in the two days but increased afterwards. Expression of ZmPK2 steadily increased of the six-day (Figure 8).

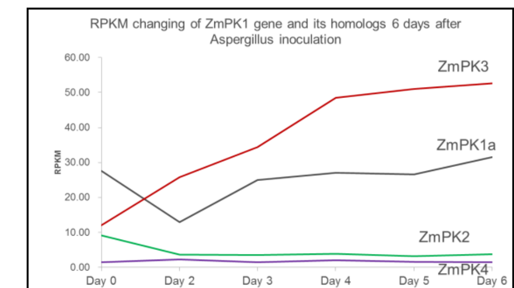


Figure 8. Expression (RPKM) of ZmPK in corn kernels inoculated with *A. flavus*.

Ear inoculation

Method: B73 ears 25 day after pollination were used. On each ear, all the kernels was treated by wounding, *A.flavus* inoculation or a control group. Kernels were collected 0, 12, 24, 72 hours after inoculation for qPCR protocol. Gene expression of ZmPK1 and ZmPK3 was examined and normalized by α-tubulin gene.

Result: Under inoculation treatment, ZmPK1a expression decreased in the first day and increased at the end. By wounding, ZmPK1a expression decreased at 12 hours, and then increase afterward (Figure 9A). ZmPK3 expression increased after 24 hours by wounding. In inoculation treatment, ZmPK3 start increasing later but expressed more compared with wounding treatment (Figure 9B).

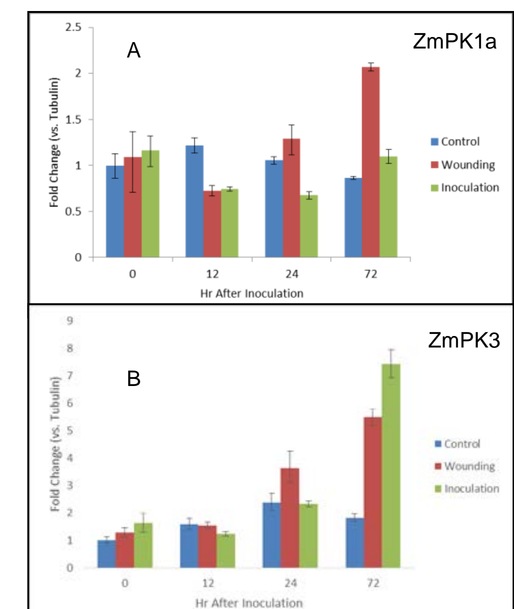


Figure 9. Relative expression of ZmPK1a (A) and ZmPK3 (B) after non-inoculation, wounding and Aspergillus inoculation.

Discussion

- According to the sequence alignment and RNA-seq result. Although a lot of genes and the translated proteins share high similarity as a kinase, but only ZmPK1a ZmPK3 responded to *A. flavus* infection. Although ZmPK1a is more identical to BIK1 and TPK1b in sequence, it is induced less than ZmPK3 by wounding or *A. flavus*. Comparing the upstream region of this two genes, a possible reason could be that ZmPK1a lacks strong promoter elements.

- The site direct mutations of ZmPK1a can mimic the kinase activity but fail to constitutively increase their kinase activity. We are questioning if the CA mutants can be induced by flagellin effectors stronger than the wild type ZmPK1a in *N. benthamiana*. leaf tissues.

- In order to verify the function of ZmPK1a, virus induced gene silencing of corn was strongly suggested for phenotype screening. In addition, Mu insertion corn lines which disrupt these genes also can be used for phenotyping.

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