Plant Pathol. J. 33(6) : 597-601 (2017)
https://doi.org/10.5423/PPJ.OA.04.2017.0079
pISSN 1598-2254 eISSN 2093-9280

The Plant Pathology Journal
©The Korean Society of Plant Pathology

Note Open Access

Involvement of a Polyketide Synthetase ClPKS18 in the Regulation of Vegetative Growth, Melanin and Toxin Synthesis, and Virulence in *Curvularia lunata*

Jin-Xin Gao[1,2,3] and Jie Chen[1,2,3]*

[1]*School of Agriculture and Biology, Shanghai Jiaotong University, 800 Dongchuan Road, Shanghai 200240, China*
[2]*State Key Laboratory of Microbial Metabolism, Shanghai Jiaotong University, 800 Dongchuan Road, Shanghai 200240, China*
[3]*Ministry of Agriculture Key Laboratory of Urban Agriculture (South), Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai 200240, China*

(Received on April 11, 2017; Revised on May 22, 2017; Accepted on June 12, 2017)

The *clpks18* gene was first cloned and identified in *Curvularia lunata.* It contains 6571 base pairs (bp) and an 6276 bp open reading frame encoding 2091 amino acids. The ClPKS18 deletion mutant displayed an albino phenotype, and almost lost the ability to product 5-(hydroxymethyl) furan-2-carboxylate (M5HF2C) toxin, implying that *clpks18* gene in *C. lunata* is not only involved in 1,8-dihydroxynaphthalene melanin synthesis, but also relatively associated with M5HF2C toxin biosynthesis of the pathogen. The pathogenicity assays revealed that ΔClPKS18 was impaired in colonizing the maize leaves, which corresponds to the finding that ClPKS18 controls the production of melanin and M5HF2C in *C. lunata*. Results indicate that ClPKS18 plays a vital role in regulating pathogenicity of in *C. lunata*.

Keywords **:** melanin, PKS18, toxin

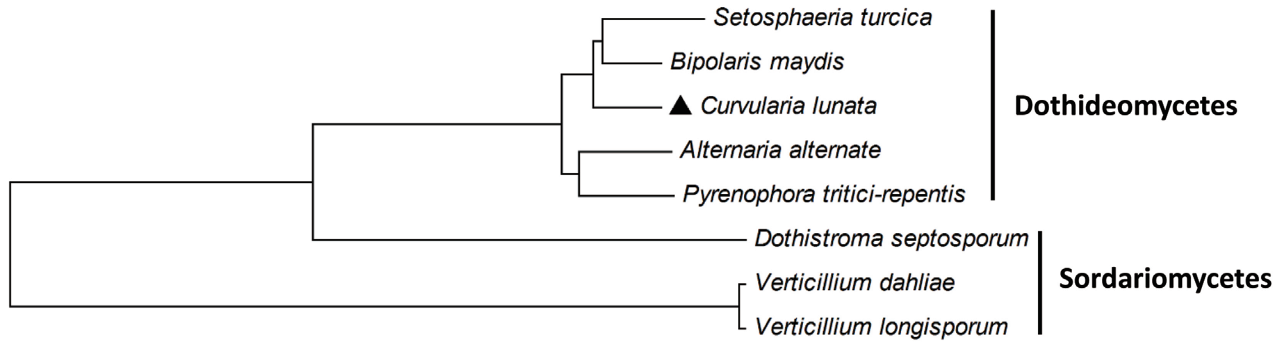
Handling Associate Editor **:** Jeon, Junhyun

Curvularia lunata is an important fungal pathogen that causes *Curvularia* leaf spot (CLS), which is one of the most widely distributed maize leaf diseases worldwide (Gao et al., 2015a; Liu et al., 2016). To uncover the pathogenicity mechanisms of CLS, most researches have focused on identifying virulence factors, such as melanin (Gao et al., 2012, 2015b), and toxin (Gao et al., 2014a; Liu et al., 2009). Fungal melanins, formed by the oxidative polymerisation of phenolic compounds, are essential for enhance the mechanical strength of the infection into the host plant epidermis and contribute to virulence in numerous types of plant diseases (Gao et al., 2015e). Previous studies have also indicated that the albino *C. lunata* strains present in wild-type (WT) strains are weakly virulent to maize leaves, supporting the conclusion that melanin is a crucial virulence factor for *C. lunata* in susceptible maize leaves (Yan et al., 2005). Researchers have successfully identified a non-host-specific toxin called methyl 5-(hydroxymethyl) furan-2-carboxylate (M5HF2C). The super-virulence capability of M5HF2C was also observed on some other plant species, such as *Capsicum annuum*, *Nicotiana tabacum, Oryzae sativa*, and (Liu et al., 2009). However, few genes involved in the production of melanin (Gao et al., 2015d) and toxin (Gao et al., 2015c) have been researched.

The PKS18 protein is involved in melanin synthesis in some pathogens, such as *Cochliobolus heterostrophus* (Eliahu et al., 2007). However, no research has revealed the exact correlation of PKS18 with melanin production and pathogenicity in *C. lunata*. This study first reports the sequence and characterization of *pks18* gene from *C. lunata*. The *Bipolaris maydis* PKS18 (accession number: AY495659) was used to query the *C. lunata* genome database (Dryad Digital Repository) for orthologs (Gao et al., 2014b). The open reading frame of *clpks18* comprises 6571 bp and an 6276 bp open reading frame encoding a 209-amino-acid protein. Phylogenetic analysis indicated that the ClPKS18 protein falls in a well-supported group of dothideomycete polyketide synthetase homologs (Fig. 1A). Alignment of ClPKS18 with *B. maydis* BmPKS18 and *Se-*

*Corresponding author.
Phone) +86-21-34206141, FAX) +86-21-34206141
E-mail) jiechen59@sjtu.edu.cn
ⓒ This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/4.0) which permits unrestricted noncommercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Articles can be freely viewed online at www.ppjonline.org.



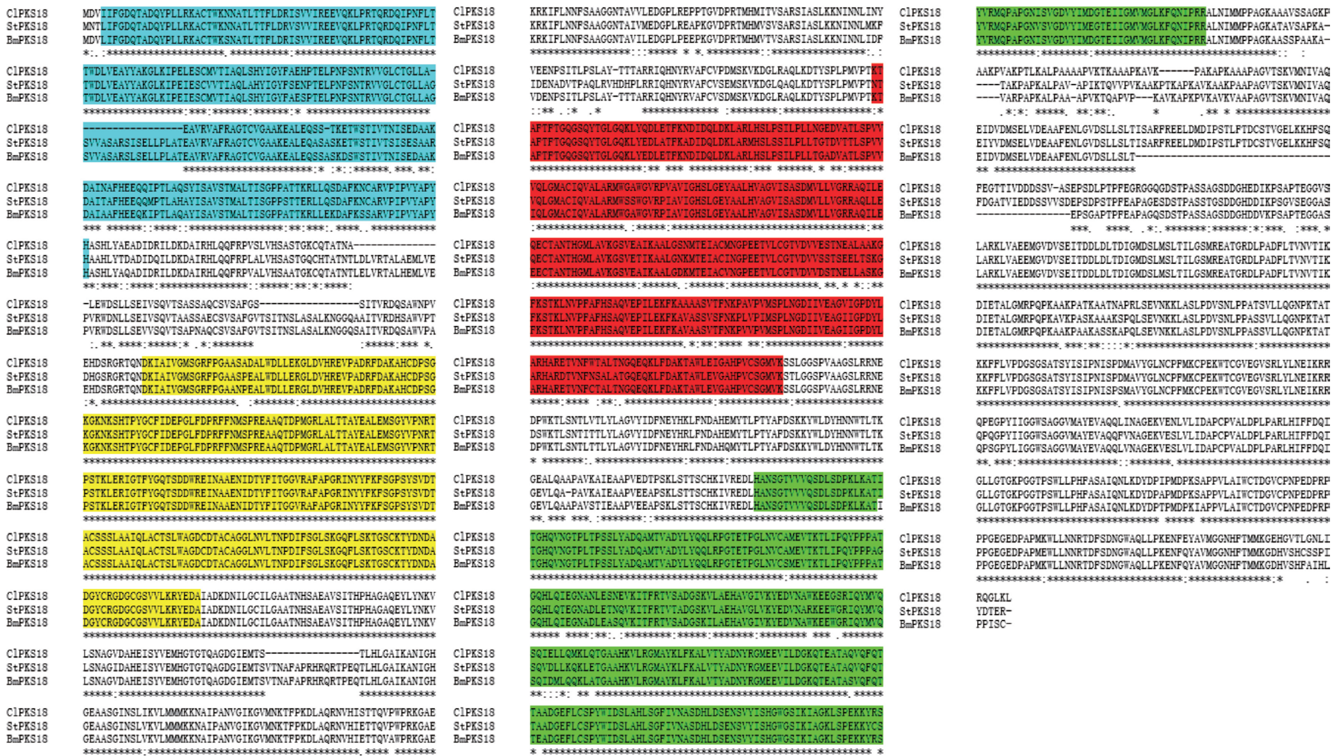


Fig. 1. *C. lunata* PKS18 is an ortholog of *Biolaris maydis* PKS18. (A) Phylogenetic analysis. PKS18 protein sequences were obtained from GenBank using *B. maydis* BmPKS18 as a query. (B) *C. lunata* ClPKS18, *B. maydis* BmPKS18 and *Setosphaeria turcica* StPKS18 were aligned using ClustalW. Conserved Acyl transferase domains are highlighted in blue, Beta-ketoacyl synthase domains are highlighted in yellow, malonyl CoA-acyl carrier protein transacylase domains are highlighted in red, dehydratase domains are highlighted in green, asterisks mark identical residues, colons mark conserved residues, and periods indicate semi-conserved residues.

GenBank Accession numbers

Dothideomycetes: *Alternaria alternate* AaPKS18: AFN68292; *Bipolaris maydis* BmPKS18: AAR90272; *Curvularia lunata* ClPKS18: MF114294; *Dothistroma septosporum* DsPKS18: EME39782; *Pyrenophora tritici-repentis* PtPKS18: XP_001933656; *Setosphaeria turcica* StPKS18: AEE68981.

Sordariomycetes: *Verticillium dahlia* VdPKS18: AGI15329; *Verticillium longisporum* VlPKS18: CRK15634.

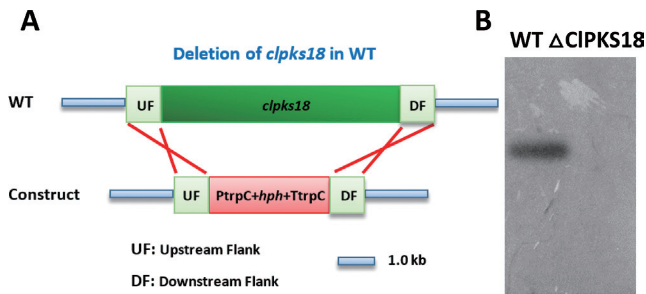


Fig. 2. Graphical representation of screening *clpks18* deletion mutant. (A) *clpks18* deletion strategy used by homologous recombination. *clpks18* and hygromycin resistance (*hph*) genes are represented by green and red boxes, respectively. (B) Southern blot to confirm *clpks18* was replaced using the 600 bp fragment of *clpks18* as probe. All primers used for gene deletion and confirmation are showed in Table 1.

tosphaeria turcica StPKS18 showed 88% identity and 99% positives, respectively (NCBI BlastP Align). Meanwhile, alignment of them identified highly conserved Acyl transferase, Beta-ketoacyl synthase, malonyl CoA-acyl carrier protein transacylase, and dehydratase domains, characteristic of fungal polyketide synthases (Fig. 1B).

Target gene deletion strategy was employed by replacing *clpks18* with a hygromycin resistance (*hph*) cassette to investigate the biological functions of ClPKS18 in *C. lunata* (Fig. 2A). The Southern hybridization pattern confirmed that homologous recombination occurs at the *clpks18* locus in ΔClPKS18 (Fig. 2B). The radial growth rates of the mutant and WT on the potato dextrose agar (PDA) medium were compared. ΔClPKS18 had a significantly slower mycelial growth rate than WT on the PDA medium. However, the conidiation of ΔClPKS18 was not evidently different from those of the WT (Table 2).

In *B. maydis*, BmPKS18 was functionally characterized to encode a polyketide synthetase that converts malonyl-CoA to 1,3,6,8-tetradroxynaphthalene (1,3,6,8-THN) in the DHN-melanin synthesis pathway (Eliahu et al., 2007). This research showed that the ClPKS18 deletion mutant of *C. lunata* displayed an albino phenotype, indicating that ClPKS18 is an orthologue of PKS18 from *B. maydis* (Fig. 3A). We detected the expression of the polyketide synthase gene (*pks18*), the transcription factor gene (*cmr1*), and three synthase genes (*brn1*, *brn2*, and *scd*) related to the

Table 1. Primer used for this study

Primers	Sequence 5' to 3'	Description	PCR (kb)[a]	Purpose
pks18-FL-F pks18-FL-R	ATGGATGTCATCATCTTCGG TTATAGCTTAAGGCCTTGCC	*clpks18* coding region, FP[b] *clpks18* coding region, RP[c]	6.571	[d]*clpks18* confirmation
pks18U-F pks18U-R	<u>ACGCGTCGAC</u>ACCCGCAGGATATATCA <u>GCTCTAGA</u>TTTAGCAAGGGTATAATT	flanking region upstream of *clpks18* FP flanking region upstream of *clpks18* RP	0.925	ΔClPKS18
pks18D-F pks18D-R	<u>GGGGTACC</u>GCATTTTGATTATTCATC <u>CGGAATTC</u>CCAGAGGCATGGGTCGGT	flanking region downstream of *clvelB* FP flanking region downstream of *clvelB* RP	0.943	
hyg-F hyg-R	CGACAGCGTCTCCGACCTGA CGCCCAAGCTGCATCATCGAA	*hph* coding region, FP *hph* coding region, RP	0.811	ΔClPKS18 confirmation
pks18-sb-F pks18-sb-R	AGATTGTCTCTCAGGTCACC AATTGATTCTGCCAGGCGCG	*clpks18* coding region, FP[b] *clpks18* coding region, RP[c]	0.600	
gapdh_2F gapdh_2R	TCGTCGCCGTAAACGACCCC CGCCCTTGAACTGGCCGTGT	*gapdh* coding region, FP *gapdh* coding region, RP	0.207	qRT-PCR
brn1-1F brn1-1R	TGGCCAGCCAGTAGACATTG ACCTTTCCGTTGACCCACTC	*brn1* coding region, FP *brn1* coding region, RP	0.075	
brn2-2F brn2-2R	AACAACGGCCGTATCATCCT AGCGTTGTAAAGAGCGTGGT	*brn2* coding region, FP *brn2* coding region, RP	0.078	
cmr1-1F cmr1-1R	GTTTGGACTGACTCGCTGGT TAGGATGATCGGCGGGAAGA	*cmr1* coding region, FP *cmr1* coding region, RP	0.118	
scd_1F scd_1R	CGGTCGTTCCTGGACAAGATG GTGTGCCTCCGATAAAGTGCTG	*scd* coding region, FP *scd* coding region, RP	0.121	
clt1-F clt1-R	GCACACACATACCCAAGACG AGTTGATGGGAATGTAGGCG	*clt-1* coding region, FP *clt-1* coding region, RP	0.150	

[a]PCR (kb) = PCR product length in kb; [b]FP = forward primer; [c]RP = reverse primer; [d]Δ = gene deletion. The underlined regions identify the added restriction sites.

Table 2. Phenotypic analysis of ClPKS18 mutant compared with wild-type (WT) isolate CX-3[a]

Strain	Growth rate (mm/24 h)[b]	Conidiation (log_{10} CFU/ml)[c]
WT	7.26 ± 0.19a	5.90 ± 0.09a
ΔClPKS18	6.13 ± 0.06cb	5.87 ± 0.11a

[a]Different letters in each data column indicate significant differences at $P = 0.05$.
[b]Diameter of hyphal radii.
[c]Conidial numbers (log transformation).

synthesis of DHN melanin in the mutant and WT to further confirm this observation (Gao et al., 2017). As expected, almost no expression of *pks18* was detected in ΔClPKS18. Besides, the expression of *scd* in ΔClPKS18 has a 70.92-fold decrease, and the expression of *brn1* and *brn2* have above 30-fold decrease in ΔClPKS18 compared to those in WT. As for *cmr1*, it also showed a downward trend, which is almost 0.36-fold decrease in ΔClPKS18 compared with WT (Fig. 3B). Overall, we conclude that ClPKS18 plays a positive regulation role in the synthesis of melanin.

The mutants were cultured in Fries 3 medium for 30 days to determine whether they retained the ability to produce the virulence-related toxin M5HF2C (Liu et al., 2009). As shown in Fig. 4, the mutant lost the ability to produce M5HF2C toxin. The expression of the M5HF2C biosynthesis related gene *clt-1* was analyzed by qRT-PCR to further confirm that ΔClPKS18 acts as a positive regulator of M5HF2C toxin production. Meanwhile, the expression of *brn1*, which is also responsible for M5HF2C biosynthesis (Liu et al., 2011), fell sharply in ΔClPKS18 (Fig. 3B). The experiment results indicate that ClPKS18 played a major role in the regulation of M5HF2C biosynthesis in *C. lunata*. We further assayed the infective ability of ΔClPKS18 on maize leaves because the deletion of *clpks18* compromised

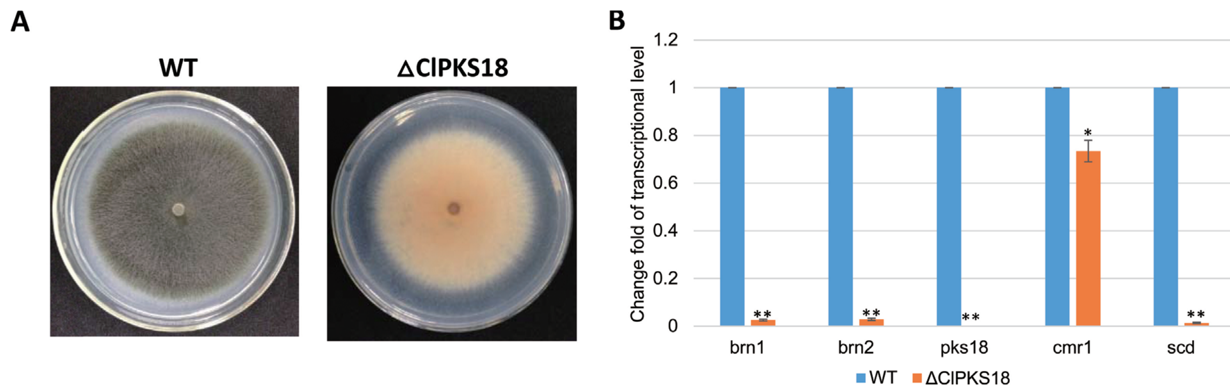


Fig. 3. ClPKS18 possively regulates the mycelial melanization of *C. lunata*. (A) Cultures of WT strain (CX-3), and *clpks18* deletion mutant (ΔClPKS18) grown on PDA plates. Note the white mycelia of ΔClPKS18 compared to WT. (B) qRT-PCR analyses of *pks18*, *cmr1*, *brn1*, *brn2*, and *scd*. Error bars are the standard deviation. A single asterisk indicates the $P < 0.05$ while double asterisks indicate the $P < 0.001$ in the *T*-test analysis. All primers used for qRT-PCR are showed in Table 1.

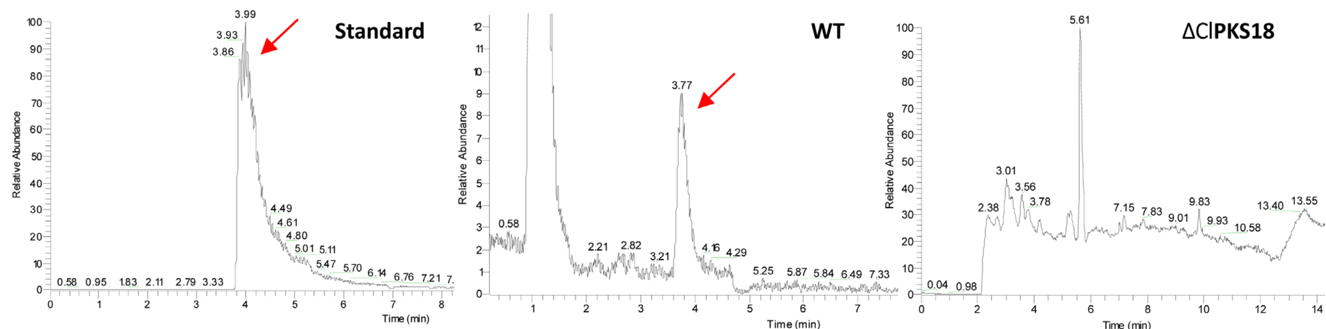


Fig. 4. ClPKS18 regulates the biosynthesis of M5HF2C toxin. HPLC-MS chromatograms of the methyl 5-(hydroxymethyl)-furan-2-carboxylate standard and toxins extracted from the WT strain (CX-3) and *clpks18* deletion mutant (ΔClPKS18).

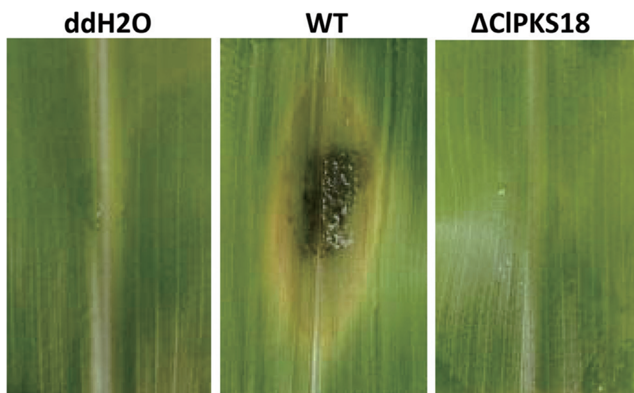


Fig. 5. Virulence of the WT (CX-3), and *clpks18* deletion mutant (ΔClPKS18) on maize leaves. ΔClPKS18 is impaired in the colonization of maize leaves. Detached leaves of HUANGZAO-4 were inoculated with conidial suspensions and incubated on two layers of filter papers moisturized with 10 mM 6-Benzyladenine (6-BA) in Petri dishes at 28°C for 96 h.

the ability of *C. lunata* to produce M5HF2C. Similarly, ΔClPKS18 lost the pathogenicity, and the lesion areas also cannot be found in maize leaves which treated with ΔClPKS18 (Fig. 5), indicating that ClPKS18 was essential to the complete virulence in *C. lunata*. We proposed that the *clpks18* gene may be one of the node genes controlling two separate metabolic pathways for melanin and toxin biosynthesis, respectively. In conclusion, this study would help us understand the synthesis mechanism of melanin and toxin in *C. lunata* and may provide target sites for designing a new agent to control *C. lunata* and a few similar fungi.

Acknowledgments

We would like to thank Zhe Li for critical reading of the manuscript. The work was supported by the National Science Foundation of China (31471734) and China Agriculture Research System (CARS-02).

References

Eliahu, N., Igbaria, A., Rose, M. S., Horwitz, B. A. and Lev, S. 2007. Melanin biosynthesis in the maize pathogen *Cochliobolus heterostrophus* depends on two mitogen-activated protein kinases, Chk1 and Mps1, and the transcription factor Cmr1. *Eukaryot. Cell* 6:421-429.

Gao, J. X., Gao, S. G., Li, Y. Q. and Chen, J. 2015a. Genome-wide prediction and analysis of the classical secreted proteins of *Curvularia lunata. J. Plant. Prot.* 42:869-876.

Gao, J. X., Jing, J. and Chen, J. 2015b. Elementary coordinated expression research on genes related to the synthesis of pathogenesis-related melanin and toxin in *Cochliobolus lunatus*. *J. SJTU Agr. Sci.* 33:53-58.

Gao, J. X., Jing, J., Liu, T. and Chen, J. 2015c. Identification of Clt-1-regulated proteins associated with the production of non-host-specific toxin and pathogenicity in *Cochliobolus lunatus. J. Phytopathol*. 163:33-41.

Gao, J. X., Jing, J., Liu, T., Yu, C. J., Li, Y. Q. and Chen, J. 2015d. Identification of proteins associated with the production of melanin and with pathogenicity in maize pathogen *Curvularia lunata*. *Australas. Plant Pathol.* 44:599-603.

Gao, J. X., Jing, J., Yu, C. J. and Chen, J. 2015e. Construction of a high-quality yeast two-hybrid library and its application in identification of interacting proteins with Brn1 in *Curvularia lunata*. *Plant Pathol. J.* 31:108-114.

Gao, J. X., Liu, T. and Chen, J. 2014a. Insertional mutagenesis and cloning of the gene required for the biosynthesis of the non-host-specific toxin in *Cochliobolus lunatus* that causes maize leaf spot. *Phytopathology* 104:332-339.

Gao, J. X., Yu, C. J., Wang, M., Sun, J. N., Li, Y. Q. and Chen, J. 2017. Involvement of a velvet protein ClVelB in the regulation of vegetative differentiation, oxidative stress response, secondary metabolism, and virulence in *Curvularia lunata*. *Sci. Rep.* 7:46054.

Gao, S. G., Li, Y. Q., Gao, J. X., Suo, Y. J., Fu, K. H., Li, Y. Y. and Chen, J. 2014b. Genome sequence and virulence variation-related transcriptome profiles of *Curvularia lunata*, an important maize pathogenic fungus. *BMC Genomics* 15:627.

Gao, S. G., Liu, T., Li, Y. Y., Wu, Q., Fu, K. H. and Chen, J. 2012. Understanding resistant germplasm-induced virulence variation through analysis of proteomics and suppression subtractive hybridization in a maize pathogen *Curvularia lunata*. *Proteomics* 12: 3524-3535.

Liu, T., Liu, L. X., Jiang, X., Huang, X. L. and Chen, J. 2009. A new furanoid toxin produced by *Curvularia lunata*, the causal agent of maize *Curvularia* leaf spot. *Can. J. Plant Pathol.* 31:22-27.

Liu, T., Wang, Y. Y., Ma, B. C., Hou, J. M., Jin, Y. Z., Zhang, Y. L., Ke, X. W., Tai, L. M., Zuo, Y. H. and Dey, K. 2016. Clg2p interacts with Clf and ClUrase to regulate appressorium formation, pathogenicity and conidial morphology in *Curvularia lunata*. *Sci. Rep.* 6:24047.

Liu, T., Xu, S. F., Liu, L. X., Zhou, F. H., Hou, J. M. and Chen, J. 2011. Cloning and characteristics of *Brn1* gene in *Curvularia lunata* causing leaf spot in maize. *Eur. J. Plant Pathol.* 131:211-219.

Yan, H., Chen, J., Gao, Z., Xia, S. and Zhang, R. 2005. The heredity and variation of the interaction between *Curvularia Lunata* and Plant. *J. Maize Sci.* 13:119-120.