

## ORIGINAL ARTICLE

# Protein kinase A participates in hyphal and appressorial development by targeting Efg1-mediated transcription of a Rab GTPase in *Setosphaeria turcica*

Yuwei Liu<sup>1,2,3</sup>  | Shen Shen<sup>1,2,3</sup> | Zhimin Hao<sup>1,2,3</sup> | Qing Wang<sup>2,3</sup> | Yumei Zhang<sup>2,3</sup> | Yulan Zhao<sup>2,3</sup> | Yameng Tong<sup>1,2,3</sup> | Fanli Zeng<sup>1,2,3</sup> | Jingao Dong<sup>1,3,4</sup>

<sup>1</sup>State Key Laboratory of North China Crop Improvement and Regulation, Baoding, China

<sup>2</sup>College of Life Sciences, Hebei Agricultural University, Baoding, China

<sup>3</sup>Key Laboratory of Hebei Province for Plant Physiology and Molecular Pathology, Baoding, China

<sup>4</sup>College of Plant Protection, Hebei Agricultural University, Baoding, China

## Correspondence

Zhimin Hao and Jingao Dong, State Key Laboratory of North China Crop Improvement and Regulation, Baoding, China.

Email: [haozhimin@hebau.edu.cn](mailto:haozhimin@hebau.edu.cn) and [shmdjg@hebau.edu.cn](mailto:shmdjg@hebau.edu.cn)

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## Abstract

The cyclic adenosine monophosphate (cAMP) signalling pathway plays an important role in the regulation of the development and pathogenicity of filamentous fungi. cAMP-dependent protein kinase A (PKA) is the conserved element downstream of cAMP, and its diverse mechanisms in multiple filamentous fungi are not well known yet. In the present study, gene knockout mutants of two catalytic subunits of PKA (PKA-C) in *Setosphaeria turcica* were created to illustrate the regulatory mechanisms of PKA-Cs on the development and pathogenicity of *S. turcica*. As a result, StPkaC2 was proved to be the main contributor of PKA activity in *S. turcica*. In addition, it was found that both StPkaC1 and StPkaC2 were necessary for conidiation and invasive growth, while only StPkaC2 played a negative role in the regulation of filamentous growth. We reveal that only StPkaC2 could interact with the transcription factor StEfg1, and it inhibited the transcription of *StRAB1*, a Rab GTPase homologue coding gene in *S. turcica*, whereas StPkaC1 could specifically interact with a transcriptional regulator StFlo8, which could rescue the transcriptional inhibition of StEfg1 on *StRAB1*. We also demonstrated that *StRAB1* could positively influence the biosynthesis of chitin in hyphae, thus changing the filamentous growth. Our findings clarify that StPkaC2 participates in chitin biosynthesis to modulate mycelium development by targeting the Efg1-mediated transcription of *StRAB1*, while StFlo8, interacting with StPkaC1, acts as a negative regulator during this process.

## KEYWORDS

chitin, filamentous growth, fungal pathogen, protein kinase A, *Setosphaeria turcica*

Yuwei Liu, Shen Shen, and Zhimin Hao contributed equally to the work.

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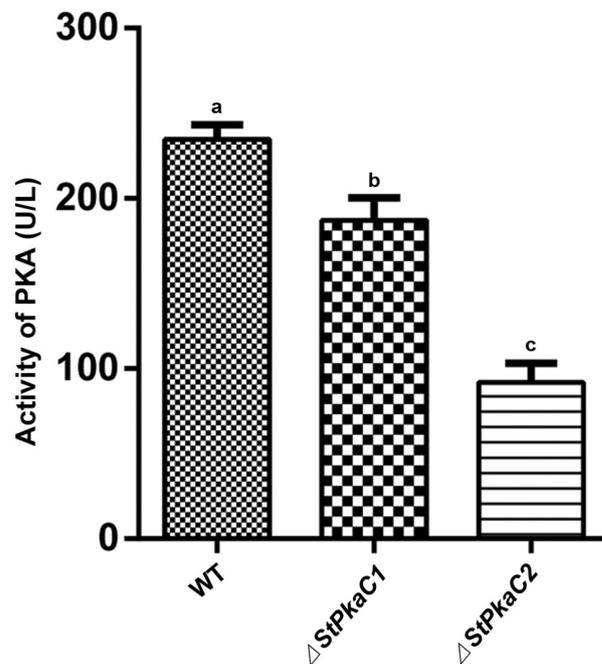
## 1 | INTRODUCTION

Northern corn leaf blight is a destructive foliar disease of cultivated maize caused by the fungal pathogen *Setosphaeria turcica* (Chang & Fan, 1986). The main infection strategy of *S. turcica* is mediated by a specialized cell, the appressorium, that develops from germinating conidia. Appressoria give rise to penetration pegs and directly penetrate the epidermal cell of maize (Kotze et al., 2019).

The cyclic adenosine monophosphate (cAMP) protein kinase A (PKA) cascade has emerged as a key player in the pathogenic development of *S. turcica* as well as other phytopathogenic fungi (Adachi & Hamer, 1998; Choi & Xu, 2010; García-Martínez et al., 2012; Hao et al., 2015; Ramesh et al., 2001; Ryder & Talbot, 2015; Williamson et al., 2007). The cAMP–PKA signalling pathway is well conserved (D'Souza & Heitman, 2001). As the main downstream effectors and best-defined targets of cAMP, the subunits of PKA are highly conserved in pathogenic fungi. PKA complexes are made up of two regulatory subunits (PKA-R) and two catalytic subunits (PKA-C). When two cAMP molecules bind with the PKA-R, the PKA-C is freed and activated (Schaap, 2011). Recent studies have found that hyphal growth, virulence, and pathogenicity are closely regulated by PKA in different plant-pathogenic fungi (Fuller & Rhodes, 2012; Lengeler et al., 2000). In *Magnaporthe oryzae*, PKA activities are essential for vegetative growth, appressorium development, and plant infection (Li et al., 2017; Selvaraj, Shen, et al., 2017). The *rpk1* mutants of *Colletotrichum lagenarium* show significant reduction in vegetative growth and conidiation, as well as defective penetration (Takano et al., 2001). In *Alternaria alternata*, PKA-C positively regulates vegetative growth and pathogenicity but negatively regulates conidial germination (Tsai et al., 2013; Xu et al., 2011), therefore these studies show that the PKA has roles in promoting growth and pathogenesis across fungi.

Chitin, a  $\beta$ -(1,4)-linked homopolymer of *N*-acetylglucosamine, plays a crucial structural role in maintaining the integrity and shape of fungal cells. In most fungi, chitin and cell wall synthesis occurs at sites of polarized growth and has an important effect on the hyphal morphogenesis (Bowman & Free, 2006). Chitin is synthesized by large families of chitin synthase. When chitin synthesis is disrupted, cell wall components are irregularly arranged, and fungal cells are deformed and osmotically unstable (Bago et al., 1996; Specht et al., 1996). The protein kinase C (PKC) and high osmolarity glycerol (HOG) mitogen-activated protein (MAP) kinase cascades and the  $\text{Ca}^{2+}$ /calcineurin pathway can regulate chitin synthase (*CHS*) gene expression and chitin synthesis PKC (Munro et al., 2007). *CHS* genes in pathogenic fungi such as *Magnaporthe grisea*, *Botrytis cinerea*, and *Fusarium oxysporum* are involved in the regulation of conidia formation, appressorium development, and the infection process (Cui et al., 2013; Kong et al., 2012; Martín-Urdíroz et al., 2008; Odenbach et al., 2009).

Our earlier studies demonstrated that appressorium-mediated infection is regulated by cAMP signalling pathways and identified two PKA-C subunits in *S. turcica*, namely *StPkaC1* and *StPkaC2*, which show different expression patterns in the development of *S. turcica* (Hao et al., 2015; Shen et al., 2013). In the current study,



**FIGURE 1** The protein kinase A (PKA) activity (U per litre of sample) in the wild-type strain (WT), knockout mutant of *StPkaC1* ( $\Delta$ StPkaC1), and knockout mutant of *StPkaC2* ( $\Delta$ StPkaC2). Error bars represent SE ( $n = 3$ ). Different letters above the bars indicate values are significantly different from each other (*t* test,  $p < 0.05$ ).

gene knockout mutants of *StPkaC1* and *StPkaC2* were produced to study their functional differentiation and the regulatory mechanisms of PKA-C involved in the development of the pathogen. The downstream transcriptional targets of *StPkaC1* and *StPkaC2* were identified, and one Rab-GTPase, namely *StRAB1*, was verified as the target of *StEfg1*, which is the downstream transcription factor of *StPkaC2*. *StPkaC1* was identified as a negative regulator of *StEfg1* together with the transcription regulator *StFlo8*. Taken together, our results demonstrate that *StPkaC2* is a regulator for chitin biosynthesis and mycelium development in *S. turcica*, and *StPkaC1* negatively regulates its downstream transcriptional targets. *StPkaC1*, *StPkaC2*, and their downstream transcriptional regulators constitute a balancer that maintains *StRAB1* at an appropriate expression level.

## 2 | RESULTS

### 2.1 | PKA activity in *S. turcica* is mainly dominated by *StPkaC2*

To investigate the roles of two PKA-C subunits in *S. turcica*, knockout mutants of *StPkaC1* ( $\Delta$ StPkaC1) and *StPkaC2* ( $\Delta$ StPkaC2) were obtained by homologous recombination, and the result was confirmed by locus-specific PCR and Southern blotting (Figure S1). The PKA activities of  $\Delta$ StPkaC1 and  $\Delta$ StPkaC2 were significantly lower than that of the wild type (WT), while  $\Delta$ StPkaC2 had the lowest PKA activity among all strains (Figure 1). These results show that *StPkaC2* is the main contributor of PKA activity in *S. turcica*.

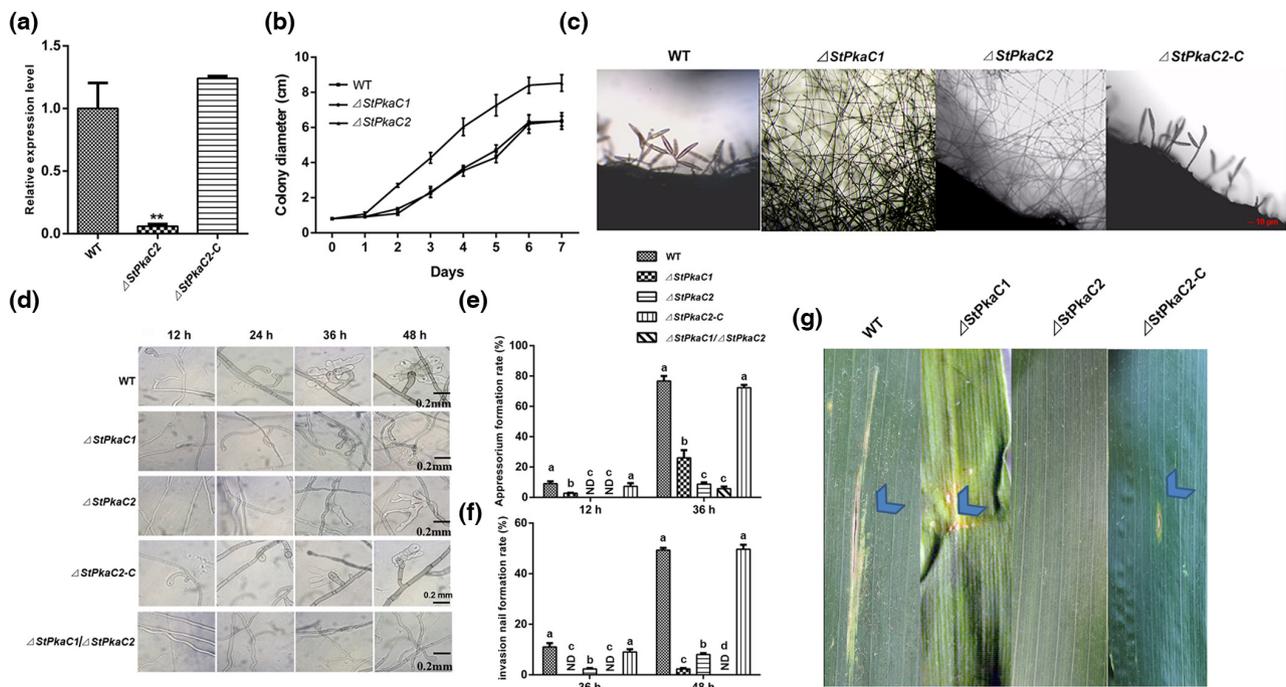
## 2.2 | StPkaC1 and StPkaC2 display different regulatory capacity in filamentous growth, but both are important for full pathogenicity

The morphogenesis of the WT and the mutants were observed to investigate the function of StPkaC1 and StPkaC2 in the growth and development of *S. turcica*. First, the *StPkaC2* deletion mutant was complemented with the *StPkaC2* gene, which was driven by the CaMV 35S promoter, to produce strain  $\Delta$ StPkaC2-C. We also attempted to generate a complemented control for  $\Delta$ StPkaC1, but we did not succeed for reasons that are not clear at this time. The expression level of *StPkaC2* in  $\Delta$ StPkaC2-C was slightly higher than that of the WT (Figure 2a). Compared with the WT and  $\Delta$ StPkaC1,  $\Delta$ StPkaC2 had the highest growth rate, while no difference was observed between the WT and  $\Delta$ StPkaC1 (Figure 2b). In addition, a comparative analysis of the mycelial morphology between the WT and the mutants revealed that the hyphae of the WT differentiated to produce short and straight conidiophores with five to six conidia, and those of  $\Delta$ StPkaC2-C produced two or three conidia, while  $\Delta$ StPkaC1 and  $\Delta$ StPkaC2 only developed slender and fluffy aerial hyphae without any conidia (Figure 2c), indicating that the deletion of *StPkaCs* blocked the conidiation. These results demonstrate that the two PKA-C subunits are involved in the regulation of the conidiation

in *S. turcica*, while there is an obvious functional difference between StPkaC1 and StPkaC2 in regulating filamentous growth.

The appressorium development and pathogenicity of different strains were studied. Hyphal suspensions of WT,  $\Delta$ StPkaC1,  $\Delta$ StPkaC2,  $\Delta$ StPkaC2-C, and  $\Delta$ StPkaC1/ $\Delta$ StPkaC2 were inoculated on an artificial cellophane surface to investigate their appressorium formation rates and penetration ability. At 12 h postinoculation (hpi), the hyphae of WT,  $\Delta$ StPkaC1, and  $\Delta$ StPkaC2-C formed appressoria. At 24 hpi, the appressoria formed by the hyphae of the WT began to penetrate the cellophane and form invasive hyphae. At 48 hpi, nearly all of the appressoria penetrated the cellophane. By contrast, the hyphae of  $\Delta$ StPkaC2 did not produce appressoria until after 24 hpi, and only a small fraction penetrated the cellophane and formed invasive hyphae at 48 hpi;  $\Delta$ StPkaC1/ $\Delta$ StPkaC2 did not form invasive hyphae at 48 hpi (Figure 2d), and even at 72 hpi there were still no invasive hyphae observed (data not shown). Appressorium formation rates of the WT and  $\Delta$ StPkaC2-C at 36 hpi exceeded 70%, and  $\Delta$ StPkaC1 was approximately 26%, whilst that of  $\Delta$ StPkaC2 was only 9% (Figure 2e). The penetration rates of WT and  $\Delta$ StPkaC2-C at 48 hpi were approximately 49%, whilst those of  $\Delta$ StPkaC1 and  $\Delta$ StPkaC2 were only 2% and 8%, respectively (Figure 2f).

Finally, hyphal suspensions of WT,  $\Delta$ StPkaC1,  $\Delta$ StPkaC2, and  $\Delta$ StPkaC2-C were sprayed onto susceptible maize seedlings (cv. OH43) to determine whether the two PKA-C subunits were involved



**FIGURE 2** *StPkaC2* is required for hyphal development and is essential for appressorium-mediated plant infection in *Setosphaeria turcica*. (a) Reverse transcription-quantitative PCR results of the relative expression of *StPkaC2* in the wild type (WT),  $\Delta$ StPkaC2, and  $\Delta$ StPkaC2-C (complemented) strains. Error bars represent SE ( $n = 3$ ), \*\* $p < 0.01$  (t test). (b) Statistical analysis of WT,  $\Delta$ StPkaC2, and  $\Delta$ StPkaC2-C colony diameter of growth of potato dextrose agar (PDA) at 25°C. Error bars represent the SE of three biological replicates. (c) Microscopic observation of WT and mutant conidial development grown on PDA for 7 days at 25°C. (d) Micrographs showing the formation of appressoria and penetration capacity of the WT,  $\Delta$ StPkaC1,  $\Delta$ StPkaC2,  $\Delta$ StPkaC2-C, and  $\Delta$ StPkaC1/ $\Delta$ StPkaC2 on cellophane. Error bars represent SE ( $n = 3$ ), different letters above bars indicate values that are significantly different from each other ( $p < 0.05$ , t test), ND indicates not determined. (e) Bar charts of the frequency of appressorium formation on cellophane at the indicated time points. (f) Bar charts of the frequency of cellophane penetration. (g) Maize leaf inoculated with mycelia showing the formation of blight lesions by the indicated strains. Blue arrows indicate disease lesions. ND, not determined.

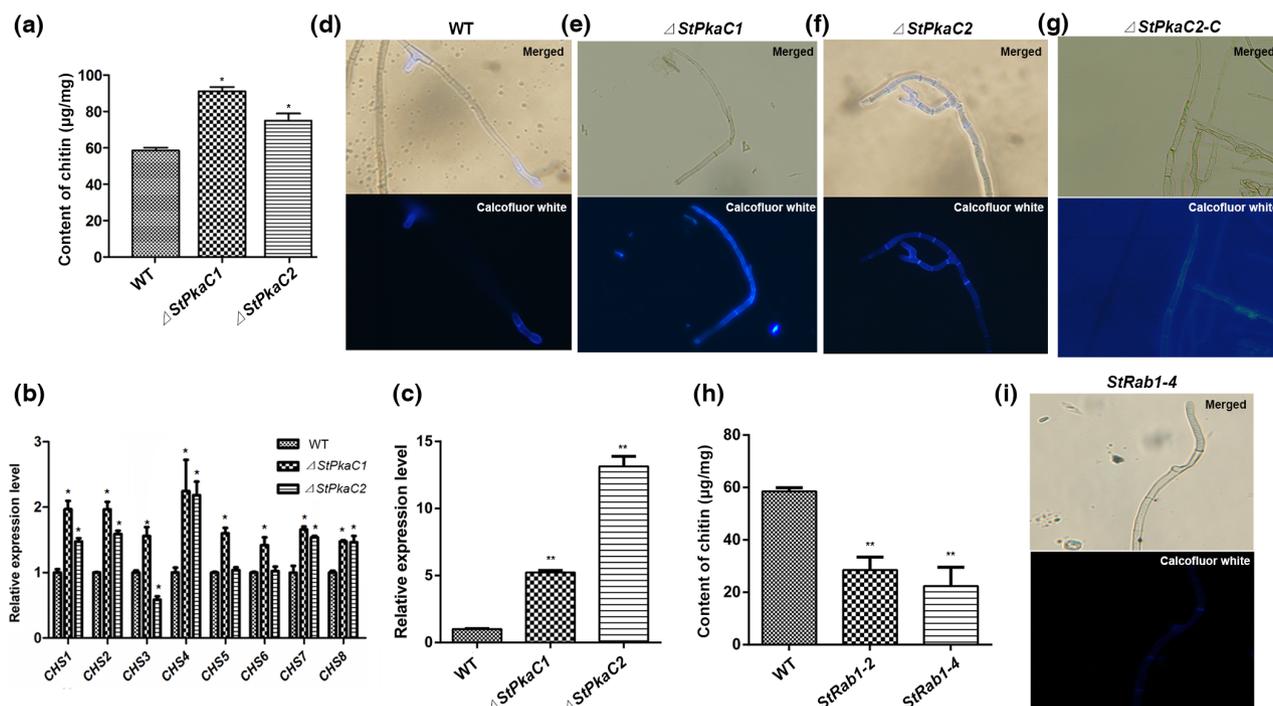
in the pathogenesis of *S. turcica*. Nine days after inoculation, leaves inoculated with the WT,  $\Delta StPkaC1$ , and  $\Delta StPkaC2$ -C showed numerous typical shuttle-like lesions that expanded over time. By contrast, leaves inoculated with  $\Delta StPkaC2$  showed no lesions (Figure 2g). The above results suggest that  $\Delta StPkaC2$  is defective in appressorium development and has completely lost its pathogenicity.

### 2.3 | PKA-C negatively regulates chitin synthesis and distribution mediated by *StRAB1*

Rapid tip elongation in filamentous fungi requires extensive membrane insertion and extension of rigid chitin-containing cell walls (Riquelme et al., 2018). Considering the impact of *StPkaC2* on the filamentous growth of *S. turcica*, we sought to determine the relationship between PKA and chitin biosynthesis. First, the content of chitin in  $\Delta StPkaC1$ ,  $\Delta StPkaC2$ , and the WT was assessed. The results showed that  $\Delta StPkaC1$  and  $\Delta StPkaC2$  contained significantly more chitin (90.1 and 72.3  $\mu\text{g}/\text{mg}$ , respectively) than the WT (59.7  $\mu\text{g}/\text{mg}$ ) (Figure 3a), indicating PKA-Cs negatively influence the content of chitin. However, the chitin content in  $\Delta StPkaC1$  was also significantly higher than that of the WT. Hence, the expression level of the genes encoding chitin synthase (CHS) were analysed by reverse transcription-quantitative PCR (RT-qPCR). The results showed that in the  $\Delta StPkaC1$  and  $\Delta StPkaC2$  mutant strains, the CHSs

were all up-regulated at the transcription level, except for *StCH3* which was down-regulated in  $\Delta StPkaC2$  (Figure 3b), and this result was in accordance with the chitin content. In addition, we found by RT-qPCR that one Rab-GTPase homologue, *StRAB1*, (JGI database ID 162018), which colocalizes with CHSs at the mycelial tip in *Neurospora crassa* (Sánchez-León et al., 2015), was up-regulated in  $\Delta StPkaC2$  by nearly 13-fold, and 5-fold in  $\Delta StPkaC1$  (Figure 3c). These results indicate that *StPkaC1* and *StPkaC2* have a negative transcription regulatory effect on these chitin biosynthesis-related genes, especially for *StRAB1*. The effect of *StPkaC2* on *StRAB1* was significantly stronger than that of *StPkaC1*, which was consistent with the effect on mycelial growth. These results prompted us to assess whether *StPkaC2* affects the distribution of chitin using calcofluor white (CFW) staining and microscopical observations. To our surprise, we found a significant difference in CFW fluorescence signals between WT,  $\Delta StPkaC1$ , and  $\Delta StPkaC2$ . The fluorescence signals in the WT were mainly manifested in one or two cells at the hyphal tips (Figure 3d), whereas the signals in  $\Delta StPkaC1$  (Figure 3e) and  $\Delta StPkaC2$  (Figure 3f) were spread to more cells than in the WT. The fluorescence signals in  $\Delta StPkaC2$  (Figure 3f) were enriched in the septa of hyphae. These results indicate that the two PKA-C subunits affect not only the synthesis of chitin in hyphae but also its distribution, and the two subunits have different effects on the distribution of chitin.

RNA interference (RNAi) mutants of *StRAB1* were generated to determine the relationship between *StRAB1* and the synthesis of chitin



**FIGURE 3** PKA-C-dependent chitin synthesis and *StPkaC2*-*StRAB1* pathway-mediated chitin distribution. (a) Quantification of the relative contents of chitin in the wild type (WT),  $\Delta StPkaC1$ , and  $\Delta StPkaC2$ . Error bars represent SE ( $n = 3$ ).  $*p < 0.05$ , *t* test. (b) Reverse transcription-quantitative PCR (RT-qPCR) analysis of the relative expression of chitin synthase genes in the WT,  $\Delta StPkaC1$ , and  $\Delta StPkaC2$ . Error bars represent SE ( $n = 3$ ).  $*p < 0.05$ , *t* test. (c) RT-qPCR analysis of the relative expression of *StRAB1* in the WT,  $\Delta StPkaC1$ , and  $\Delta StPkaC2$ . Error bars represent SE ( $n = 3$ ).  $**p < 0.01$ , *t* test. The chitin distribution observed using calcofluor white staining in the WT (d),  $\Delta StPkaC1$  (e),  $\Delta StPkaC2$  (f), and  $\Delta StPkaC2$ -C (g). (h) Quantification of the relative contents of chitin in the WT, *StRAB1-2*, and *StRAB1-4* RNAi strains. Error bars represent SE ( $n = 3$ ).  $**p < 0.01$ , *t* test. (i) The chitin distribution of *StRAB1-4* RNAi strain.

(Figure S2a). We found that *StRAB1* RNAi transformants grew slower than the WT strain, the conidiation and pathogenicity of them were impaired (Figure S2b,c), and their chitin content was lower than that of WT (Figure 3h). The CFW staining pattern was very weak, even at the septa of hyphae (Figure 3i), suggesting that chitin synthesis was inactive. These results reveal that StPkaC2 could influence the synthesis and distribution of chitin in the mycelium, which may be mediated by *StRAB1*.

## 2.4 | StPkaC2 specifically interacts with StEfg1, which could regulate the transcription of *StRAB1*

Transcription factors (TFs) are an important group of downstream transcriptional targets of PKA, and some TFs have been identified as the substrates of PKA, such as Efg1 and CreB (Bockmühl & Ernst, 2001; Singh et al., 2008). To clarify how StPkaC2 could affect the synthesis and distribution of chitin in the mycelium by mediating *StRAB1* expression, the downstream TF of StPkaC2 was analysed further. First, the *cis*-element of the *StRAB1* promoter was analysed and a binding motif (TATGCATA) of enhanced filamentous growth 1 (Efg1) was found at -256bp upstream of the transcription initiation site. Efg1 is a downstream TF of PKA and known as a morphogenetic regulator in *Candida albicans* (Kadosh, 2019). Using the BLASTP program, the homologue of Efg1 in *S. turcica* was identified (ID: 91357) and named StEfg1 (Figure S3a). Then, yeast two-hybrid (Y2H) and bimolecular fluorescence complementation (BiFC) assays were used to test whether StEfg1 was a downstream TF of StPkaC2. The Y2H and BiFC assays showed that StPkaC2 interacted with StEfg1 (Figure 4a,b), whereas StPkaC1 did not interact with it (data not shown). An electrophoretic mobility shift assay was used to verify the interaction between StEfg1 and the promoter of *StRAB1*. The results showed that the purified StEfg1 protein could specifically bind to the *StRAB1* promoter, and enhancing the concentration of the StEfg1 protein in the binding reaction enhanced the smear and shift bands (Figure 4c). We also performed dual-luciferase transient transcriptional activity assays to investigate whether StEfg1 could function as a transcriptional regulator of *StRAB1*. The results showed that  $\beta$ -glucuronidase (GUS) expression, which was driven by the promoter of *StRAB1*, was significantly suppressed by StEfg1 (Figure 4c). Combined with the expression level change of *StRAB1* in the WT and  $\Delta$ *StPkaC2*, these results illustrate that StEfg1 is the downstream TF of StPkaC2 and *StRAB1* is the target of StEfg1 in *S. turcica*.

## 2.5 | StPkaC1 interacts with StFlo8, which can rescue the expression of *StRAB1*

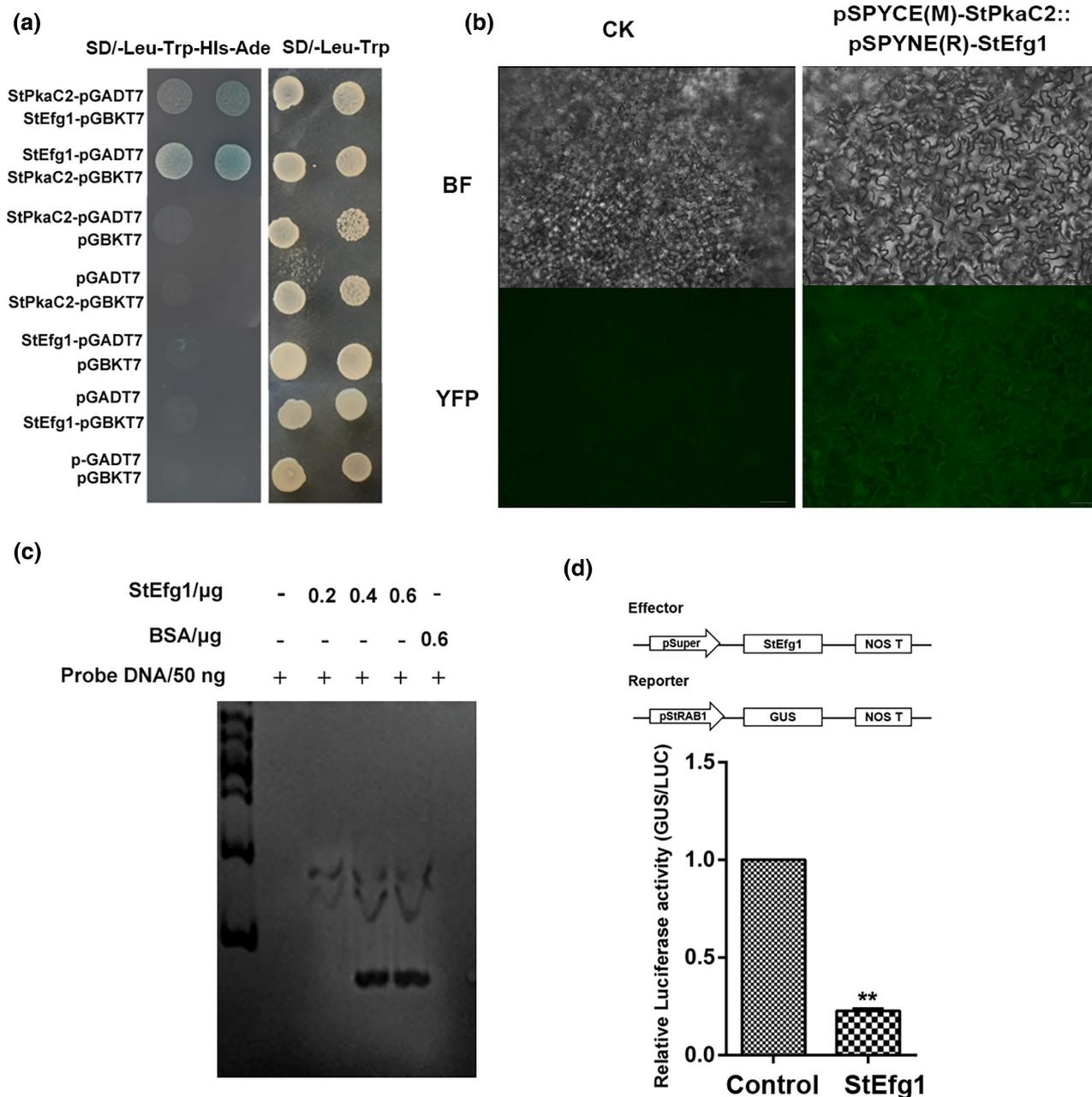
To explore how StPkaC1 affects the synthesis of chitin, the downstream transcriptional target of StPkaC1 was identified. In *C. albicans*, the transcriptional regulator Flo8 is a target of the cAMP/PKA pathway and is indispensable for filamentous growth (Cao et al., 2006). To examine whether Flo8 was a target of the cAMP/PKA pathway in *S. turcica*, the homologue of Flo8 was identified.

Using a BLASTP search, only one CaFlo8 homologue was found in *S. turcica* and we named it as StFlo8 (ID: 1411406) (Figure S3b). As a transcriptional regulator, StFlo8 contains only a LisH domain and does not have a DNA-binding domain. A Y2H assay was used to determine whether StPkaC1 and StPkaC2 interact with StFlo8. The results showed that StPkaC1 interacted with StFlo8 in vitro (Figure 5a), but StPkaC2 did not interact with it. We wondered if StFlo8 interacted with StEfg1, as in the case in *C. albicans*. We found that StFlo8 also interacted with StEfg1 in vitro (Figure 5b). These results suggest that StFlo8 and StEfg1 might function together in regulating hyphal development. To verify that the interaction of StFlo8 and StEfg1 was functionally related in *S. turcica*, StFlo8 protein was expressed in *Escherichia coli* BL21. The purified protein was added to the dual-luciferase transient transcriptional activity assay mentioned above. To our surprise, we found the expression level of *StRAB1* was up-regulated approximately 3-fold (Figure 5c). This result indicates that StFlo8 can rescue the inhibition of *StRAB1* expression by StEfg1.

## 3 | DISCUSSION

Previous research proved that the cAMP-PKA signalling pathway is essential for filamentation, and also revealed that its roles are diverse in different fungi. For example, in *Ustilago maydis* and *M. oryzae*, disruption of genes encoding PKA results in a constitutively filamentous growth phenotype (Dürrenberger et al., 1998; Mitchell & Dean, 1995), while in *Colletotrichum higginsianum* the deletion mutants of genes encoding PKA are significantly reduced in hyphal growth (Zhu et al., 2017). Although the function of cAMP signalling has been confirmed in filamentous fungi, the related mechanism is largely uncertain. Our data showed that the colony growth rate of  $\Delta$ *StPkaC2* was significantly higher than that of the WT, while the colony growth rate of *StPkaC1* was not. This result was similar to those reported for *U. maydis* (Dürrenberger et al., 1998), in which only one PKA-C subunit, *adr1*, negatively regulates hyphal development.

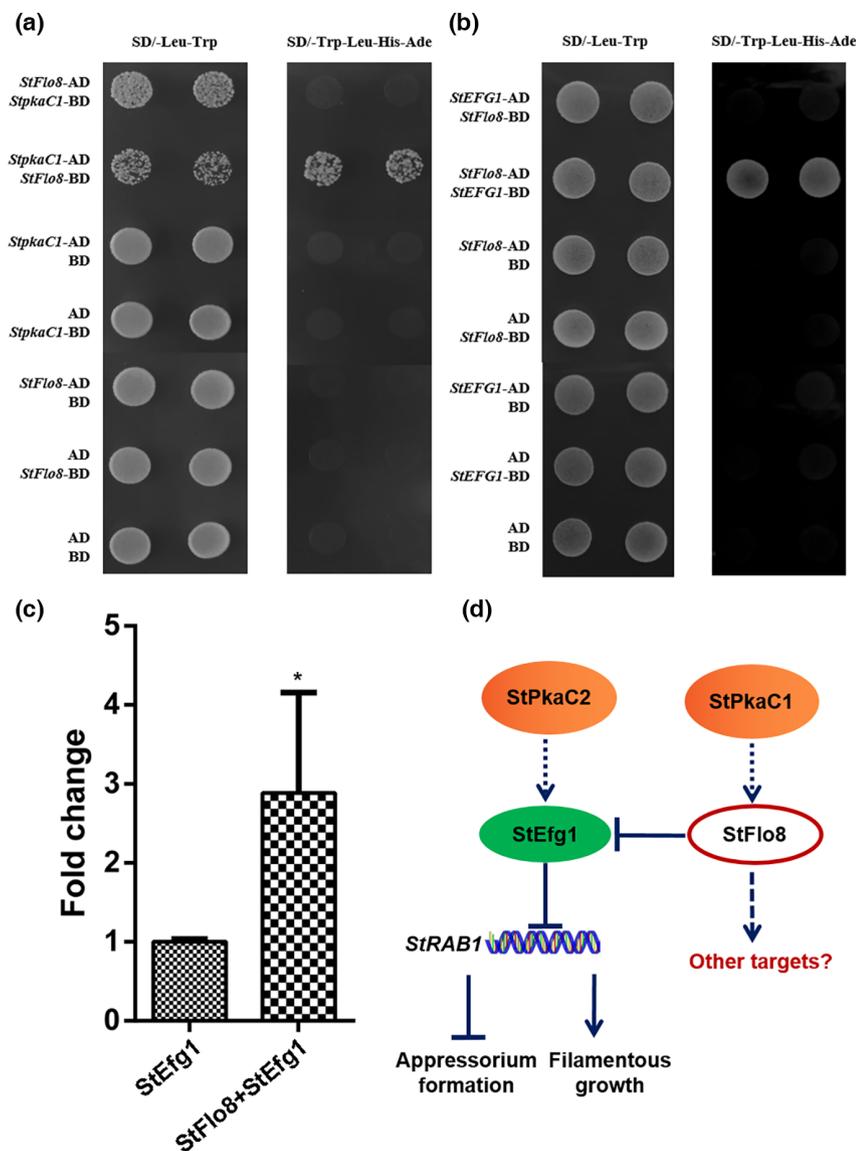
Chitin is a long linear polymer formed by *N*-acetylglucosamine linked by  $\beta$ -1,4-glycosidic bonds. Careful coordination of sufficient precursor and available CHSs at the plasma membrane determine the rate of chitin deposition on the cell wall and chitin chain length. Furthermore, the transport of chitin synthase needs to be executed through vesicles to its functional position, and this process is regulated by the small G protein Rab family (Riquelme et al., 2018). Research has indicated that chitin content can influence vegetative growth and sexual development in *N. crassa* (Fajardo-Somera et al., 2015), and proper maintenance of chitin is a crucial aspect of fungal development. In the present study, we found that the chitin content and the expression levels of most CHSs, especially *StCHS7* and *StCHS8*, were significantly higher in  $\Delta$ *StPkaC1* and  $\Delta$ *StPkaC2* than those of the WT. According to bioinformatics analysis, *StCHS7* belongs to class I and *StCHS8* belongs to class II of chitin synthases. In *N. crassa*, the green fluorescent protein (GFP) signal of transformants that expressed a fusion protein of the class I chitin



**FIGURE 4** StPkaC2-dependent regulation of *StRAB1* expression. (a) Yeast two-hybrid assay of transformants co-expressing different bait and prey plasmids carrying StPkaC2 and StEfg1, assayed on SD/-Leu-Trp-His-Ade selective medium. (b) Bimolecular fluorescence complementation assay. YFP signals were detected in the leaves of *Nicotiana benthamiana* co-expressing pSPYCE(M)-StPkaC2 and pSPYNE(R)-StEfg1. CK, empty vector; BF, bright field; YFP, yellow fluorescent protein. Scale bar = 100  $\mu$ m. (c) Electrophoretic mobility shift assay of the interaction of StEfg1 with the *StRAB1* promoter. BSA, bovine serum albumin. (d) Transactivation analysis in *N. benthamiana* showing that StEfg1 can suppress the expression of the  $\beta$ -glucuronidase (*GUS*) reporter gene when driven by the *StRAB1* promoter. *GUS* activity in the leaves co-transfected with the *GUS* reporter and empty effector vector (control) was set to 1. Error bars represent the SE of three biological replicates. \*\* $p < 0.01$ , t test.

synthase NcChs3 and GFP, was mainly concentrated at the tip of the hyphae, indicating that this type of chitin synthase may participate in the polar growth of fungi (Beth-Din & Yarden, 2000). The *Saccharomyces cerevisiae* class II chitin synthase ScChs2 participates in the formation of the primary membrane of yeast cells (Schmidt et al., 2002). In *Fusarium graminearum*, the absence of class II chitin synthase FgChs2 leads hyphal growth to slow down, and both the length of the spores and the number of membranes significantly decreases (Liu et al., 2016).

These results show that these two types of CHS have important regulatory effects on the filamentous growth and conidia development of fungi. Thus, we speculate that the deletion of *StPkaC2* leads to an increase in cell wall damage, which then triggers an increase in chitin synthesis and its altered distribution. Based on this, we wondered whether StPkaCs directly regulate the expression of these genes. Hence, the promoter of each *CHS* gene was further analysed, but no classical binding motif of PKA downstream TF was found in the promoters of



**FIGURE 5** StPkaC1 interacts with StFlo8 and rescues the expression of StRAB1. Yeast two-hybrid assay interaction of StPkaC1 with StFlo8 (a) and StFlo8 with StEfg1 (b). (c) Transactivation analysis in *Nicotiana benthamiana* leaves to analyse the effect of StFlo8 on the regulation of StRAB1 by StEfg1. StFlo8 rescued the expression of the GUS reporter gene which was driven by the StRAB1 promoter. Fold change in the GUS/LUC ratio is shown normalized to the ratio in StEfg1, set as 1. Error bars represent SE ( $n = 3$ ). \* $p < 0.05$ , t test. (d) Working model of the PKA-C regulatory network. StPkaC2 can phosphorylate StEfg1, which suppresses the expression of StRAB1 and then regulates the filamentous growth and appressorium formation of *Setosphaeria turcica*. In addition, StPkaC1 can phosphorylate StFlo8, which can inhibit the transcriptional activity of StEfg1. Solid lines with arrow or with T end represent positive or negative regulation, respectively; dotted line represents interaction of two proteins; dashed line represents unknown mechanism. AD, transcription activation domain; DB, DNA-binding domain.

StCHS1–StCHS8. More experiments are therefore needed to elucidate the molecular mechanism by which PKA affects CHS gene expression. Although  $\Delta$ StPkaC1 also showed the phenotype of increased chitin content and up-regulated CHS expression, it did not show the same filamentous growth-accelerating phenotype as  $\Delta$ StPkaC2. We speculate that the reason may be that the distribution of chitin leads to the difference in hyphal growth, in which StRAB1 may play an important role. The significantly different up-regulation levels of StRAB1 in the two PKA-C subunit mutants indicate that the functional differences between the two PKA-C subunits are most probably caused by the transcriptional regulation of StRAB1.

In most fungal species studied previously, the catalytic subunits of cAMP-PKA are usually encoded by two or more genes. In *S. cerevisiae*, three genes encode the PKA catalytic subunits, Tpk1p, 2p, and 3p. Studies have shown that a *tpk1 tpk2 tpk3* gene knockout mutant is not feasible, and these three genes share redundancy and different functions in vigour and pseudohyphae morphogenesis in *S. cerevisiae* (Pan & Heitman, 1999; Robertson & Fink, 1998; Toda et al., 1987). Many filamentous fungi, including *Aspergillus nidulans*, *B. cinerea*, and

*M. oryzae*, have shown differential roles between the different catalytic subunits concerning development and stress response, and these findings highlight the importance of studying these PKA subunits to fully understand the PKA signalling in filamentous fungi (Lee et al., 2003; Ni et al., 2005; Schumacher et al., 2008). For example, in *M. oryzae*, the single mutant of *cpkA* or *cpk2* shows no obvious or only slight growth defects but the *cpkA cpk2* double mutant shows a significant reduction in growth rate and rarely produces conidia (Selvaraj, Tham, et al., 2017). However, the molecular mechanisms by which different PKA catalytic subunits in the same species perform diverse functions have not been fully studied. In this report, we showed that PKA activity was dominated by StPkaC2 in *S. turcica* (Figure 1b). Hence, a comprehensive study of StPkaC2 functions could provide novel insights into the role of PKA in *S. turcica*. Compared with the WT, we found that  $\Delta$ StPkaC2 displayed a faster growth rate, a loss of pathogenicity, and could not produce conidia; moreover, the complemented mutant  $\Delta$ StPkaC2-C recovered pathogenicity and the ability to produce conidia (Figure 2a). These results suggest that the StPkaC2 plays a positive regulatory role in the formation of conidia and pathogenicity.

TFs are an important type of downstream transcriptional target of PKA. In model fungal species such as *S. cerevisiae*, *A. nidulans*, *N. crassa*, and *C. albicans*, some transcription factors have been reported as PKA downstream transcriptional targets, including Efg1, Flo8, and CerB, which provide valuable clues for studying the molecular mechanism of the action of PKA (Bockmühl et al., 2001; Du et al., 2018; Singh et al., 2008; Stoldt et al., 1997). The cAMP signalling pathway downstream TF Efg1 has recently been identified to be a major regulator that negatively regulates hyphal morphogenesis, and several targets of Efg1 related to hyphal development, such as UME6 and ACE2, have been identified in *C. albicans* (Saputo et al., 2014; Zeidler et al., 2009). In addition, the transcriptional regulator Flo8 has been proved to be essential for filamentous growth in *S. cerevisiae* and *C. albicans* (Cao et al., 2006; Liu et al., 1996). We therefore analysed the interaction between StPkaC1/2 and the homologues of Efg1 and Flo8 in *S. turcica*. We were surprised to find that the StEfg1 only physically interacted with StPkaC2, while StFlo8, which lacks a DNA-binding domain, only interacted with StPkaC1. We also found that StFlo8 interacted with StEfg1 and rescued the expression of *StRAB1*. Therefore, we preliminarily proposed the mechanism of action of StPkaC1 and StPkaC2 in regulating filamentous growth, that is, StPkaC2–StEfg1 negatively regulates *StRAB1*-mediated chitin synthesis, while StPkaC1–StFlo8 acts as a negative regulator of StEfg1, forming a balancer with StPkaC2 to maintain *StRAB1* at an appropriate expression level, which is an important regulator of filamentous growth (Figure 5d). In addition, although StFlo8 does not have a DNA-binding domain, it can interact with other TFs, such as StEfg1, to function as transcription regulators. In the future, we will conduct in-depth research on which TFs can be regulated by StPkaC1 through StFlo8.

The development, infection, and pathogenesis of fungi is a very complex regulatory network, and elucidating its regulatory mechanism and finding appropriate strategies to control *S. turcica* infection in maize is of great significance. In this study, we found that the two PKA-Cs influence mycelial development and pathogenicity in *S. turcica*. StPkaC2 is the main donor of active PKA, which can affect the development of *S. turcica* by regulating the expression of *StRAB1* through StEfg1. As a downstream transcriptional regulator of StPkaC1, StFlo8 can interact with StEfg1 to rescue the inhibition of *StRAB1*. However, the mechanism of *StRAB1* affecting hyphal growth at the molecular level is not clear. Therefore, more detailed information about its function needs to be elucidated in future studies.

## 4 | EXPERIMENTAL PROCEDURES

### 4.1 | Strains, plant materials, and culture conditions

The WT *S. turcica* strain 01–23 used in this study was deposited at the Mycotoxin and Molecular Plant Pathology Laboratory of Hebei Agricultural University. The knockout mutants ( $\Delta$ StPkaC1

and  $\Delta$ StPkaC2) were obtained by homologous recombination. The *StPkaC2* complementary mutant ( $\Delta$ StPkaC2-C) was driven by the CaMV 35S promoter and transformed into  $\Delta$ StPkaC2. All of the mutants were grown on potato dextrose agar (PDA; 2% glucose, 20% potato, 1.5% agar) at 25°C. Growth and storage methods for the *S. turcica* strains followed the standard procedures described previously. The maize inbred line OH43 was used as the susceptible host for *S. turcica* infection and grown in an artificial climate chamber under long-day light conditions (16 h light/8 h darkness).

### 4.2 | Identification of the $\Delta$ StPkaC1, $\Delta$ stPkaC2, and $\Delta$ StPkaC2-C mutants

Genomic DNA was isolated from the WT,  $\Delta$ StPkaC2, and  $\Delta$ StPkaC2-C mutants via the cetyltrimethylammonium bromide method. The specific primers were designed to verify the knockout mutants, and Southern blotting was performed to confirm the presence of *hph* in the genomic DNA of  $\Delta$ StPkaC2. The *php* probe was amplified using the primer HPH-F/HPH-R and the length of the probe was 584 bp. The probe was hybridized to the digoxigenin (DIG)-labelled *StPKA-c* genes using a DIG DNA labelling and detection kit (Roche Applied Science). The restriction enzymes *Hind*III and *Sma*I, sites for which were not present in the probe, were used to cut genomic DNA of the WT,  $\Delta$ StPkaC1, and  $\Delta$ StPkaC2. The specific primers of glufosinate ammonium (*bar*) Bar-F/R were designed to verify the *StPkaC2* complemented mutant  $\Delta$ StPkaC2-C. The primers are listed in Table S1.

### 4.3 | Generation of *StRAB1* RNAi mutant

The *StRAB1* RNAi constructs were described as we previously reported (Zeng et al., 2020). In brief, two different sites in *StRAB1* mRNA were selected to design the hairpin structure for *StRAB1* silencing and the strain 01–23 cDNA as the template. Sense and antisense sequences for target site 1 were amplified using the primer pairs *StRAB1*-RNAi-F1 and *StRAB1*-RNAi-R1; for target site 2, the primer pairs *StRAB1*-RNAi-F2 and *StRAB1*-RNAi-R2 were used. All primers used in this study are listed in Table S1. The constructs were transformed into protoplasts of strain 01–23 following the standard protocol described in our previous study (Zeng et al., 2020). The RNAi transformants were screened on PDA containing Basta (Coolaber) and confirmed by PCR detection of the *BAR* gene and RT-qPCR.

### 4.4 | Analysis of gene expression level

The total RNA of WT and transformant samples ( $\Delta$ StPkaC2 and  $\Delta$ StPkaC2-C) was extracted from mycelia inoculated on PDA in the dark at 25°C for 7 days by using TRIzol reagent and a total RNA extraction kit (BBI). First-strand cDNA was synthesized following the instructions of the PrimeScript Reverse Transcriptase Kit (TaKaRa).

The cDNAs of WT and the transformants were used as templates, and the specific primers of *StPkaC2*, *StPKS*, *St4HNR*, *StLAC1*, *StRAB1*, and *StSTK1* were used for amplification. qPCR was performed using TransStart Top Green qPCR SuperMix (TransGen) and an ABI StepOne Plus real-time PCR detection system (ABI). Three biological replicates were used to calculate the mean and standard deviation. The  $\Delta\Delta C_t$  method was used to calculate the relative gene expression levels (Livak & Schmittgen, 2001), which were normalized to the expression level of the *S. turcica* housekeeping gene 18S rRNA. The primers are listed in Table S1.

#### 4.5 | Measurements of PKA activity

The WT,  $\Delta$ StPkaC1 and  $\Delta$ StPkaC2 strains were cultured on PDA at 25°C in the dark for 7 days. Mycelia of each strain (1g) were taken for the measurement of PKA activity. The PKA activity was measured using a Human Protein Kinase, AMP Activated Alpha 1 (PRKAA1) ELISA kit (DLDEVELOP) according to the sandwich enzyme immunoassay method. The enzyme-substrate reaction was terminated by the addition of H<sub>2</sub>SO<sub>4</sub>, and the colour change was measured by spectrophotometry at 450 ± 10 nm. The PKA concentration of the three samples was calculated by comparing the absorbance of the sample with a standard curve. All experiments were repeated at least three times.

#### 4.6 | Phenotypic analyses

The WT and the transformants were inoculated on PDA in the dark at 25°C for 7 days and the colony morphologies were observed. The WT and the transformants were then cultured on PDA at 25°C in the dark, and the colony diameter were measured every 24 h for up to 7 days to analyse growth rates on solid medium. Growth rates were assessed on the basis of change in diameter. All experiments were performed three times, measuring 10 Petri dishes each.

#### 4.7 | Microscopic observation of hyphal morphology and determination of conidial yield

The mycelia of the WT and transformants were inoculated on PDA and incubated for 7 days at 25°C. Hyphae were randomly selected and placed on the surface of glass slides with 5 µl of distilled water, and their morphology was observed under a microscope (Eclipse E-200; Nikon). Mycelia that had been incubated for 10 days were collected with 5 ml of distilled water in individual Petri dishes. Hyphae of the same age were removed using two layers of gauze and then placed on the surface of a coverslip with 10 µl of distilled water. The number of conidia was counted using a microscope (Eclipse E-200; Nikon). All experiments were repeated three times and 10 Petri dishes were selected for each observation.

#### 4.8 | Appressorium formation and penetration assays

Vegetative hyphae of the WT and the transformants were harvested from 10-day-old PDA cultures and suspended in sterile water. The hyphal suspensions were then placed on an artificial cellophane surface and incubated at 25°C. The appressorium formation and penetration ability of the mycelia were observed under a microscope after 12, 24, 36 and 48 h.

#### 4.9 | Pathogenicity assay

Two-week-old maize seedlings (OH43) were used for the infection assay. The mycelia were evenly placed on the upper side of leaves. Inoculated plants were placed in a humidity chamber for 24 h at 25°C in the dark and then transferred to the growth chamber with a photoperiod of 12 h; illumination was provided by fluorescent lamps. Lesion formation of the top two leaves was examined 14 days after inoculation, and each infection assay was carried out with three maize seedlings.

#### 4.10 | Y2H assays

The bait constructs were generated by cloning *StPkaC2*, *StPkaC2*, *StEfg1*, and *StFlo8* full-length cDNAs into pGBKT7 and the prey constructs were obtained by cloning these cDNAs into pGADT7. All constructs were confirmed by sequence analysis. The corresponding vectors were then introduced to *S. cerevisiae* AH109 according to the instructions of the of BD library construction and screening kit (Clontech). The Trp+ and Leu+ transformants were isolated and further assayed for growing on SD-Trp-Leu-His-Ade medium. Yeast strains for positive and negative controls were obtained from the Y2H assay kit. The experiments were repeated three times.

#### 4.11 | BiFC assay

For BiFC assays, infiltration (10 mM MgCl<sub>2</sub>, 10 mM MES/KOH, pH 5.7) was done on the leaves of 3-week-old *Nicotiana benthamiana* plants with agrobacteria containing pSPYCE-StPkaC2::pSPYNE-StEFG1 and pSPYCE-StEFG1::pSPYNE-StPkaC2 at an optical density (OD<sub>600</sub>) of 0.8. Fluorescence was monitored approximately 48 h after infiltration using a Nikon fluorescence microscope with an YFP filter.

#### 4.12 | Electrophoretic mobility shift assay

An electrophoretic mobility shift assay was performed to assess the binding of StEfg1 to the *StRAB1* promoter using our previously reported method with some modifications (Zeng et al., 2020). A

specific 30 nucleotide-long promoter fragment of *StRAB1* containing the TATGCATA motif was synthesized as the probe for the assay. The full-length cDNA of the *StEfg1* gene was cloned and purified according to the manufacturer's protocol (GE Healthcare). Each binding reaction mixture (20  $\mu$ l final volume) contained 0.2, 0.4, and 0.6  $\mu$ g *StEfg1* or bovine serum albumen (control), 50 ng *StRAB1* promoter DNA, and binding buffer (50 mM Tris-HCl pH 7.8, 50 mM NaCl, 1 mM EDTA, 0.05% NP-40, 2.5% glycerol). Reaction mixtures were incubated at 30°C for 30 min and then loaded onto 1% agarose gels to separate free and bound DNA. The DNA on the gel was detected using ethidium bromide dye.

#### 4.13 | Dual luciferase transactivation assay

The dual luciferase transactivation analysis method using in this study was according to a previously reported method with some modifications (Xiao et al., 2018). A 443 bp fragment from the promoter region of *StRAB1* was inserted into the *EcoRI* and *BamHI* sites of pCAMV35S-GUS to generate the reporter plasmid pCAMV35S-GUS-*StRAB1*. The full-length coding sequence of the *StEfg1* gene was cloned and inserted into the *SmaI* and *SacI* sites of pSuper1300 to generate the effector plasmid pSuper1300-*StEfg1*. Tobacco leaves were injected with *Agrobacterium tumefaciens* GV3101, which were transformed with pSuper1300-*StEfg1* effector plasmid, pCAMV35S-GUS-*StRAB1* reporter plasmid, and 35S-Luc internal parameter plasmid. After 60 h of culture, the proteins were extracted from tobacco leaves with the same mass (0.16 g). GUS and luciferase (LUC) activity were detected by Power Wave XS2 (BioTek). Meanwhile, tobacco leaves injected with pSuper1300, pCAMV35S-GUS-*StRAB1* reporter plasmid, and 35S-LUC internal reference plasmid were used as the control group. The LUC activities were measured using the Luciferase Assay System (Promega) and the GUS activities were measured using 4-methylumbelliferyl- $\beta$ -D-glucuronide as a substrate. The GUS activities were normalized to the LUC activities in each infiltration. For this analysis, the ratio between GUS and LUC activities was measured three times.

#### 4.14 | Fluorescent staining of chitin

Six-day-old mycelia of WT and transformants were inoculated on slides with a thin layer of PDA medium and cultured in the dark for 3 days in an incubator at 25°C. The young mycelia were then removed from the slides, treated with 500  $\mu$ l of 10  $\mu$ g/ml CFW fluorescent dye, and placed in the incubator to stain for 5 min. The slides were then gently rinsed with sterile water for three times to wash off the dye. The morphology of the mycelia and distribution of chitin were observed with a fluorescence microscope under an excitation wavelength of 340 nm.

#### 4.15 | Analysis of chitin content in cell walls

Six-day-old mycelia were inoculated on PDA and cultured in the dark for 5 days in an incubator at 25°C. Vegetative hyphae of

the WT and the transformants were then harvested for further experiments. Chitin content was determined by measuring the amount of glucosamine released after the hydrolysis of cell walls. Quantitative lyophilized mycelium (1 g) was ground in liquid nitrogen and suspended in 5 ml of deionized water. After centrifugation for 10 min at 13,000  $\times$  g and 4°C, the precipitate was freeze-dried overnight. Exactly 1 ml of 6 M HCl was added to every 5 mg of the dry precipitate. After hydrolysis at 100°C for 4 h, the hydrolysate was adjusted to pH 7.0 with NaOH. Then, 1.25 volumes of solution A (1.25 M acetylacetone and added Na<sub>2</sub>CO<sub>3</sub> to achieve a final concentration of 4%) was added to the hydrolysate and heated in a boiling water bath at 100°C for 1 h. Ten volumes of ethanol, and 1.25 volumes of Roche's solution were added to the hydrolysate. The mixture was placed in a water bath at 60°C for 1 h and then centrifuged at 13,000  $\times$  g for 10 min. The absorbance of the supernatant was measured by spectrophotometry at 530 nm. Chitin content (mg glucosamine/mg dry weight fungal biomass) was calculated from a standard curve established by measuring the absorbance of known amounts of glucosamine hydrochloride.

#### AUTHOR CONTRIBUTIONS

D.J.G. and H.Z.M. conceived the project. L.Y.W., S.S., and H.Z.M. developed and managed the project. W.Q., Z.Y.M., Z.Y.L., T.Y.M., and Z.F.L. performed mutant creation and data analysis work. L.Y.W., Z.F.L., and H.Z.M. wrote the manuscript. All authors contributed substantially to revisions and approved of the final version.

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#### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

#### ORCID

Yuwei Liu  <https://orcid.org/0000-0002-9144-0014>

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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