Journal of Microbiology (2015) Vol. 53, No. 4, pp. 236–242
DOI 10.1007/s12275-015-4639-x

Trichoderma reesei Sch9 and Yak1 regulate vegetative growth, conidiation, and stress response and induced cellulase production

Xinxing Lv[†], Weixin Zhang[†], Guanjun Chen, and Weifeng Liu[*]

No.27 Shanda South Road, State Key Laboratory of Microbial Technology, School of Life Science, Shandong University, Jinan 250100, Shandong, P. R. China

(Received Nov 4, 2014 / Revised Dec 17, 2014 / Accepted Dec 22, 2014)

Protein kinases are key players in controlling many basic cellular processes in almost all the organisms via mediating signal transduction processes. In the present study, we characterized the cellulolytic *Trichoderma reesei* orthologs of *Saccharomyces cerevisiae* Sch9 and Yak1 by sequence alignment and functional analysis. The *T. reesei Trsch9*Δ and *Tryak1*Δ mutant strains displayed a decreased growth rate on different carbon sources and produced less conidia. The absence of these two kinases also resulted in different but abnormal polarized apical growth as well as sensitivity to various stresses. In addition, disruption of the genes *Trsch9* or *Tryak1* resulted in perturbation of cell wall integrity. Interestingly, while the induced production of cellulases was slightly compromised in the *Trsch9*Δ strain, the extracellular production of cellulases was significantly improved in the absence of Yak1. The results indicate that TrSch9 and TrYak1 play an important role in filamentous growth, stress response and induced production of cellulases in *T. reesei*.

Keywords: protein kinase, vegetative growth, conidiation, stress response, induced cellulase production

Introduction

All organisms have evolved sophisticated mechanisms to deal with the extreme environmental changes including nutrient shifts and stresses by reprogramming their metabolism and gene expression to achieve proper growth, proliferation, and development. Protein phosphorylation represents one such important regulatory mechanism to control many cellular processes. About 30% of proteins of the proteome are affected by protein phosphorylation in *Saccharomyces cerevisiae* (Ptacek *et al.*, 2005). Many protein kinases have thus been reported to be involved in regulating cellular processes as diverse as differentiation, survival and cell cycle progression.

In eukaryotes, PKA (protein kinase A) and TOR (target of rapamycin) are two major growth-promoting kinases acting by stimulation of protein synthesis, inhibition of stress responses and regulation of other cellular processes in response to nutrient availability (Thevelein and de Winde, 1999; Santangelo, 2006). The pathways controlled by PKA and TOR display diverse ways of cross-talk sharing several common targets. Yak1 and Sch9 are two such targets (Urban *et al.*, 2007; Lee *et al.*, 2011). Yak1 is an evolutionarily conserved serine/threonine protein kinase belonging to the DYRK family (dual specificity Yak1-related kinase). It was initially identified as an antagonizer of cell proliferation which is under the negative control of PKA (Garrett *et al.*, 1991; Lee *et al.*, 2008), and null mutations in *yak1* could suppress loss of function of the cAMP-PKA pathway (Garrett *et al.*, 1991). Yak1 can therefore be phosphorylated by the catalytic PKA subunit Tpk1 (Budovskaya *et al.*, 2005). It has been later established that Yak1 functions as a bridge between PKA and stress-responsive transcription factors, Hsf1 and Msn2/4 to regulate stress response (Lee *et al.*, 2008). Moreover, it has been reported that Yak1 is involved in the initiation and maintenance of hyphal growth in *Candida albicans* (Goyard *et al.*, 2008). Similar to *S. cerevisiae* Yak1, involvement of Yak1 orthologue in stress response in *Penicillium marrneffei* has been also reported (Suwunnakorn *et al.*, 2014).

Unlike Yak1, Sch9 is one of the AGC (protein kinase A, G, or C) kinases which is structurally related to the catalytic subunits of PKA. It was initially isolated as a high-copy suppressor of the growth defect resulting from loss of the *TPK* genes (Toda *et al.*, 1988). Sch9 is later identified as a major target of TOR complex 1 (TORC1) and can be directly phosphorylated by TORC1 to regulate ribosome biogenesis, translation initiation, and entry into G0 phase (Urban *et al.*, 2007). Despite their similar physiological roles in response to nutrient availability, it has become clear that PKA and Sch9 also oppositely regulate given target genes including those involved in stress responses and post-diauxic shift (PDS) (Toda *et al.*, 1988; Roosen *et al.*, 2005; Pascual-Ahuir and Proft, 2007). Moreover, loss of *sch9* in the *S. cerevisiae* expressing the HSF allele HSF (1-583) could be capable of restoring its high-temperature growth by regulating Hsp90 chaperone complex activity (Morano and Thiele, 1999). All these results thus demonstrate that Sch9 is a key player in integrating nutrient signals with various other cellular processes.

Trichoderma reesei (teleomorph *Hypocrea jecorina*) is one of the most prolific cellulase producers in industry and an organism to look into regulated responses to environmental cues (Lynd *et al.*, 2002, 2005). Despite recent advances in the understanding of the induced production of hydrolytic enzymes in *T. reesei*, less is known about how different carbon sources are sensed and inducing signals are transduced.

[†]These authors contributed equally to this work.
*For correspondence. E-mail: weifliu@sdu.edu.cn; Tel.: +86-531-88364324; Fax: +86-531-88565610

Although it has been reported that adenylatecyclase (ACY1) and PKA influenced the regulation of vegetative growth and the transcript abundance of cellulase genes for the light responsiveness (Schuster *et al.*, 2012), and that deletions of *schA* and *yakA* in *Aspergillus nidulans* abolished the induced transcription of endoglucanase genes *eglA* and *eglB* (Brown *et al.*, 2013), functional characterization of such important protein kinases has been lacking in the model cellulolytic fungus *T. reesei*. Moreover, due to the adoption of different life styles, it is anticipated that differences may exist between filamentous fungi and yeast. Here, we report the identification and characterization of *T. reesei* Sch9 and Yak1 (TrSch9 and TrYak1). The physiological role of these kinases is investigated with their gene locus disrupted. Collectively, we demonstrate that TrSch9 and TrYak1 play an important role in filamentous growth, stress response and induced production of cellulases.

Material and Methods

Strains, medium and culture conditions

The wild type strain *Trichoderma reesei* TU-6 (ATCC MYA-256) was uridine auxotrophy (*pyr4*Δ) and was maintained on malt extract agar containing 10 mM uridine when necessary (Gruber *et al.*, 1990b). Strains were grown in 1-L Erlenmeyer flasks on a rotary shaker (200 rpm) at 30°C in the medium as described by Mandels and Andreotti (1978). Carbon sources were added at a final concentration of 10 g/L. *Escherichia coli* DH5α was used for plasmid construction and cloning.

In order to analyze the production of celluases, *T. reesei* strains were precultured on glycerol for 36 h and grown for another 12 h in the same fresh medium. Mycelia were harvested by filtration and washed twice with medium with no carbon source. Equal amounts of mycelia were transferred to a fresh medium containing Avicel (1% w/v) without peptone, and incubation was continued for the indicated time periods.

Sequence analysis

Amino acid sequences of Sch9 and Yak1 from *T. reesei* and other relevant species were obtained from NCBI database. Amino acid sequence alignment was performed using Clustal (Larkin *et al.*, 2007). Phylogenetic analysis was performed with MEGA5.0 (Tamura *et al.*, 2011) using neighbor-join-

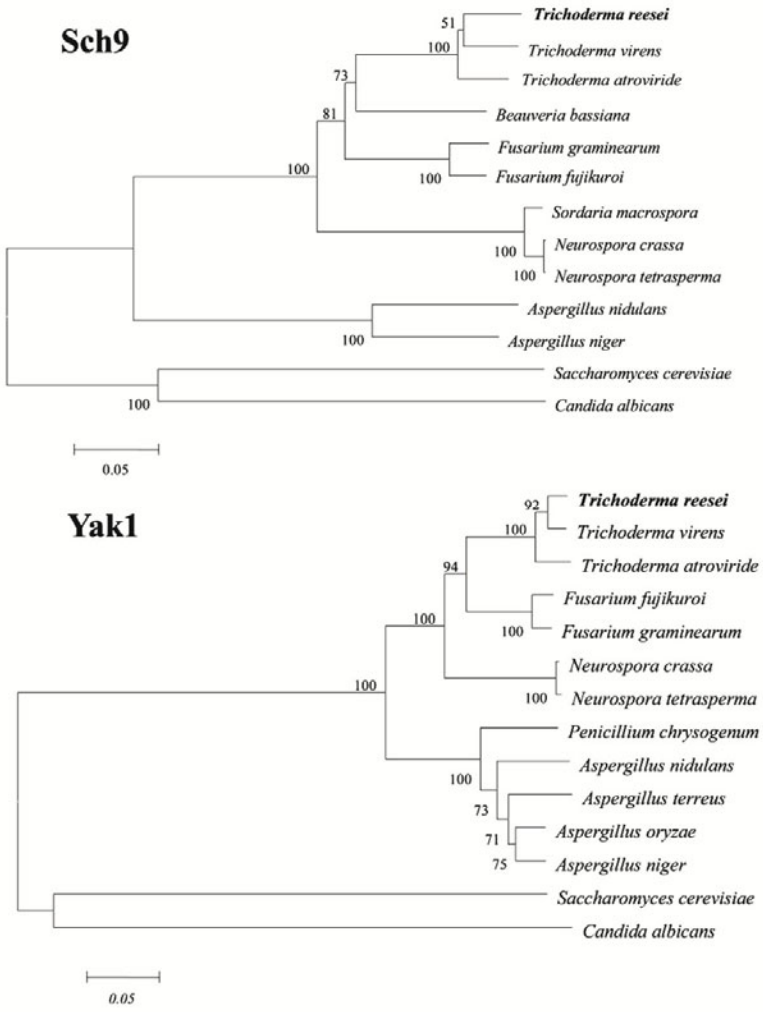

Fig. 1. Phylogenetic analysis of TrSch9 and TrYak1 and their homologues from other filamentous ascomycete fungi. Sch9 and Yak1 from *T. reesei* were represented in bold. Amino acid sequence alignment was performed using ClustalX. Phylogenetic analysis was performed with MEGA4.0 using neighbor-joining method with 1,000 bootstraps.

ing method with 1,000 bootstraps.

Disruption of the *sch9* and *yak1* genes in *T. reesei*

For disruption of *Trsch9* and *Tryak1*, the 2.7-kb *pyr4* gene fragment was amplified from pFG1 (Gruber *et al.*, 1990a), digested with *Spe*I and *Pme*I and was ligated into the pMD^{TM} 19-T vector to obtain pMD19-T*pyr4*. The two ~2.3-kb fragments upstream of ATG codon and downstream of the stop codon of the *sch9* and *yak1* gene separately were amplified from *T. reesei* chromosomal DNA, and were ligated into the corresponding sites in pMD19-T*pyr4* to yield the disruption vectors pMD*sch9pyr4* and pMD*yak1pyr4*. After linearization by *Bam*HI and *Ssp*I respectively, PMD*sch9pyr4* and pMD*yak1pyr4* were used to transform *T. reesei* TU6 essentially as described by Penttilä (1987). Transformants were selected on minimal medium for uridine prototroph.

Microscopy

The mycelia morphology was visualized under bright-field, differential interference contrast (DIC) microscopy (Olympus BX51) after the conidia were cultivated in a 16-well microplate with 1 ml of minimal medium and grown for 16 h at 30°C. For conidiophore observation, strains were grown on malt extra agar (Sigma-Aldrich) solid medium plates at 30°C for 3 days, and then the conidia were stained with lactophenol cotton blue according to the methods described by Parija *et al.* (2003). Microscopic images were captured on Nikon Eclipse 80i light microscope, and images were captured and processed with the NIS-ELEMENTS AR software.

For analysis of sporulation, the growing strains of TU6, *Trsch9*Δ, and *Tryak1*Δ were inoculated on plates with malt extract agar (Sigma-Aldrich) for 5 days. For analysis of the integrity of the cell wall, TU6, *Trsch9*Δ, and *Tryak1*Δ strains were inoculated on plates with minimal medium with 1% (w/v) glucose plus stressing agents of 0–50 mg/L calcofluor white (CFW) or 0–500 mg/L congo red (CR) for 4 days at 30°C. For analysis of the sensitivity of mutant strains to different stresses, growing strains of TU6, *Trsch9*Δ, and *Tryak1*Δ were inoculated on plates with minimal medium with 1% glucose plus 0–600 mM NaCl or 0–50 nM H_2O_2 for 3 days at 30°C. The effect of CFW, CR, temperature or NaCl on growth of *T. reesei* was evaluated by statistical analysis of mycelial diameter change according to an equation as follows: *Diameter change* (%) = $(D_{treated} - D_{untreated}) / D_{untreated} \times 100\%$, where D stands for mycelial diameter.

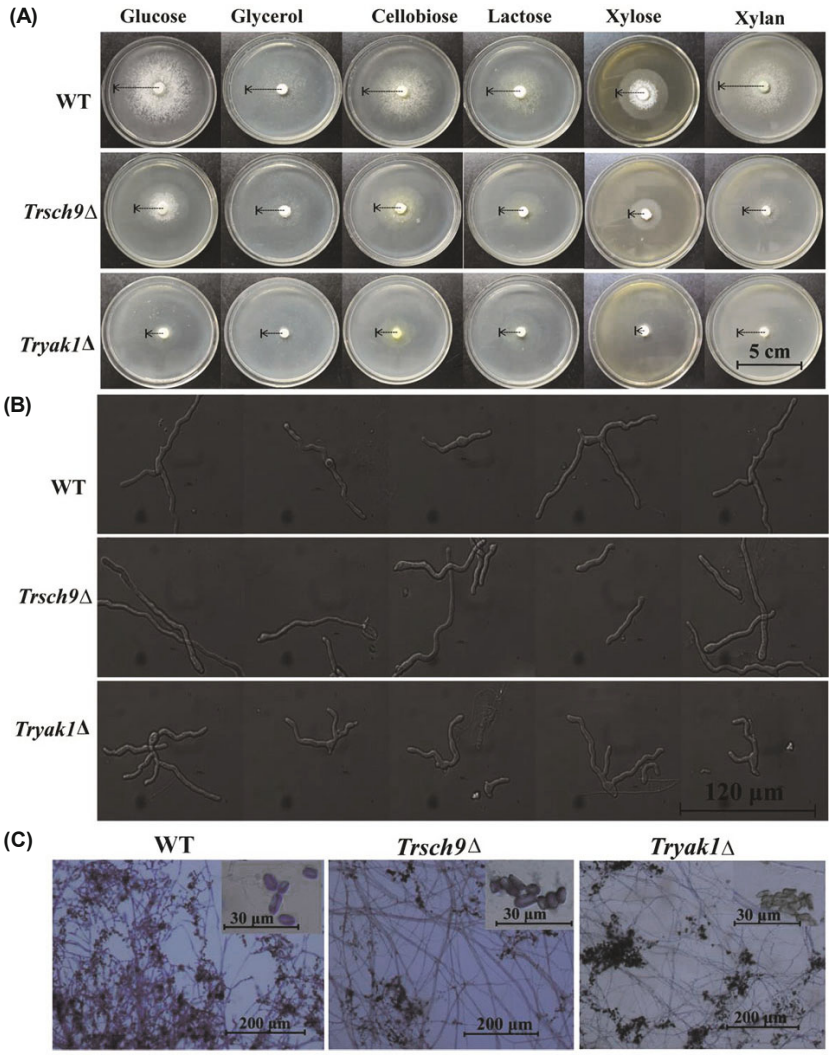

Fig. 2. The effect of the individual absence of TrSch9 and TrYak1 on the vegetative growth, hyphal polarity and conidiation of *T. reesei*. (A) Growth of WT, *Trsch9*Δ and *Tryak1*Δ strains on plates with various carbon sources as indicated at a final concentration of 1% (w/v) at 30°C for 4 days. The arrowed lines represented mycelial radius. (B) Microscopic analysis of the hyphal mophology of TU6, *Trsch9*Δ, and *Tryak1*Δ cultured on MM liquid medium for 16 h. Panels from left to right represent five independent observations for each strain. (C) Microscopic analysis of the effect of the individual absence of TrSch9 and TrYak1 on the conidiation of *T. reesei*. Micrographs were taken after staining of mycelia with lacto-phenol cotton blue. Inset graph indicated conidial morphology for each strain.

Phenotypic analysis

For assay of growth under different conditions, about a diameter of 1-cm agar with growing strains were inoculated on plates with minimal medium with different carbon sources. The diameters of final radial growth were determined after 4 days of growth.

Cellulase and protein analysis

For analysis of cellulase activity, 200 µl of reaction mixtures containing 50 µl of culture supernatant and 50 µl of the respective substrates, in 100 µl 50 mM sodium acetate (pH 5.0) were established. The cellobiohydrolase activity with 5 mM p-nitrophenol-D-cellobioside (pNPC) (Sigma-Aldrich) as substrate plus 0.1% δ-gluconolactone and β-glucosidase activity with 5 mM p-nitrophenyl-β-D-glucopyranoside (pNPG) (Sigma-Aldrich) as substrate were measured after incubation for 30 min at 45°C. CMCases activity with 1% sodium carboxymethyl cellulose (CMC-Na, Sigma-Aldrich) as substrate was determined after incubation at 50°C for 30 min. Total secreted proteins were determined using the method of Bradford protein assay with BSA as the standard (Bradford, 1976).

Results and Discussion

Identification of *T. reesei* TrSch9 and TrYak1

The protein kinases Sch9 and Yak1 are known to be functionally important in regulating cell growth and stress responses in *S. cerevisiae* (Hartley *et al.*, 1994). To investigate the physiological roles of these protein kinases in cellulolytic *T. reesei*, we searched the genome of *T. reesei* for homologs of the *S. cerevisiae* protein kinases Sch9 (NP_012075) and Yak1

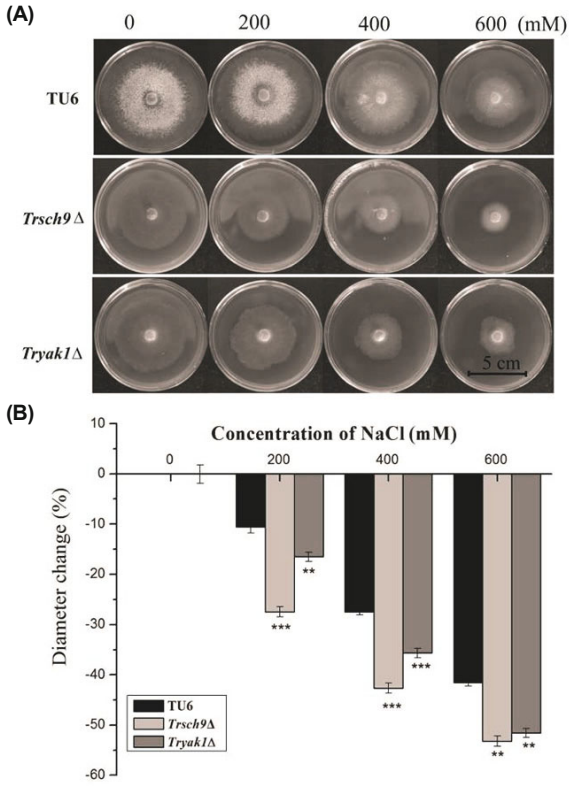

Fig. 3. The effect of the individual absence of TrSch9 and TrYak1 on response to salt stress in *T. reesei*. (A) Growth of WT and mutant strains on plates containing minimal medium with increasing concentrations of NaCl at 30°C for 60 h and (B) statistical analysis of mycelial diameter change for each strain. D stands for the diameter of mycelial colony. The differences of growth between the WT and mutants were found to be statistically relevant by Student's *t*-test ; two asterisks, *P* is less than 0.01; three asterisks, *P* is less than 0.001.

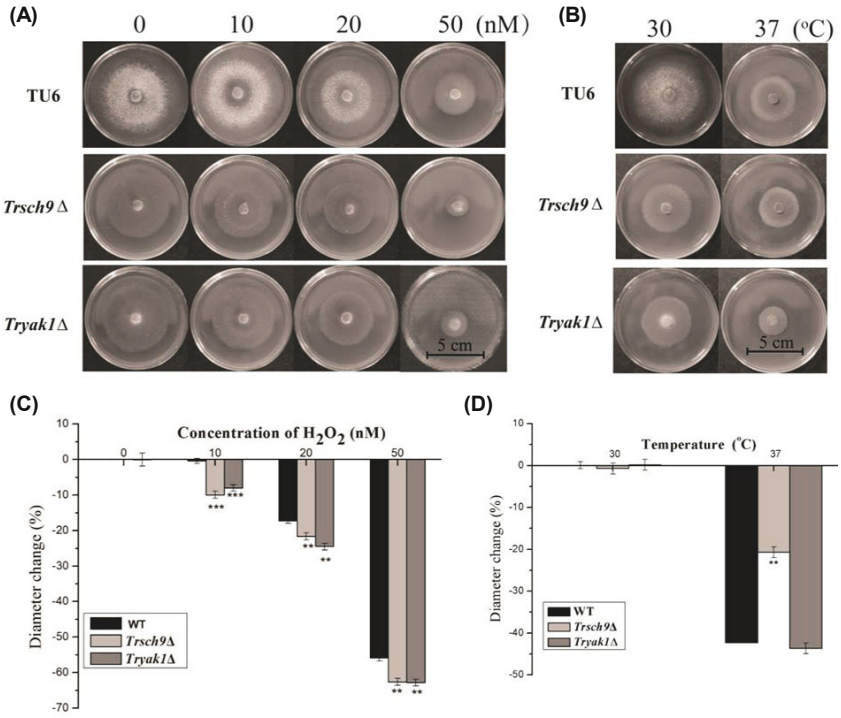

Fig. 4. The effect of the individual absence of TrSch9 and TrYak1 on response to oxidative stress and thermal-tolerance in *T. reesei*. (A) Growth of WT and mutant strains on plate containing minimal medium with increasing concentrations of H_2O_2 at 30°C for 60 h. (B) Growth of WT and mutant strains on plates containing minimal medium at 30°C and 37°C for 60 h respectively. (C) and (D), statistical analysis of mycelial diameter change for each strain respectively. D stands for the diameter of mycelial colony. The differences of growth between the WT and mutants were found to be statistically relevant by Student's *t*-test ; two asterisks, *P* is less than 0.01; three asterisks, *P* is less than 0.001.

(NP_012394). Two putative protein kinase genes were identified: *Trsch9* (Tr_119614) and *Tryak1* (Tr_44330). The *Trsch9* gene comprises a 4306-bp open reading frame, which is interrupted with three introns and encodes a protein of 528 amino acids. The *Tryak1* gene comprises a 2,902-bp open reading frame, which is interrupted with five introns encoding a protein of 836 amino acids. Comparisons of the amino acid sequences of TrSch9 and TrYak1 with those from *S. cerevisiae* revealed that they shared relatively high sequence identity (46% and 36%). Furthermore, phylogenic analysis revealed that orthologs of TrSch9 and TrYak1 are widely distributed among filamentous ascomycete fungi (Fig. 1).

TrSch9 and TrYak1 are involved in regulation of vegetative growth, hyphal polarity, and conidiation in *T. reesei*

For detailed analysis of the physiological functions of TrSch9 and TrYak1, we constructed *T. reesei* mutants that lacked the coding sequences of *Trsch9* or *Tryak1* via targeted gene replacement. Inoculation of the wild-type (WT, TU-6) and mutant strains on plates containing different carbon sources followed by incubation at 30°C for four days showed that both the mutants exhibited dramatically reduced colony size on all of the carbon sources investigated, thereby indicating that the hyphal extension rate greatly decreased compared with the parental strain (Fig. 2A). Loss of *Tryak1* led to a more significant reduction in the growth rate compared with the loss of *Trsch9*. Investigations of growth on the same carbon sources in liquid cultures also indicated decreased biomass formation (data not shown). Moreover, the mutants were microscopically examined to assess the effects on hyphal morphogenesis. The absence of *Trsch9* and *Tryak1* resulted in abnormal hyphal polarity in liquid cultures compared with the WT strain (Fig. 2B). Loss of *Trsch9* reduced hyphal branching, whereas the lack of *Tryak1* appeared to facilitate the branching of hyphae, which was in accordance with the observation that *yak1* contributes to the initiation

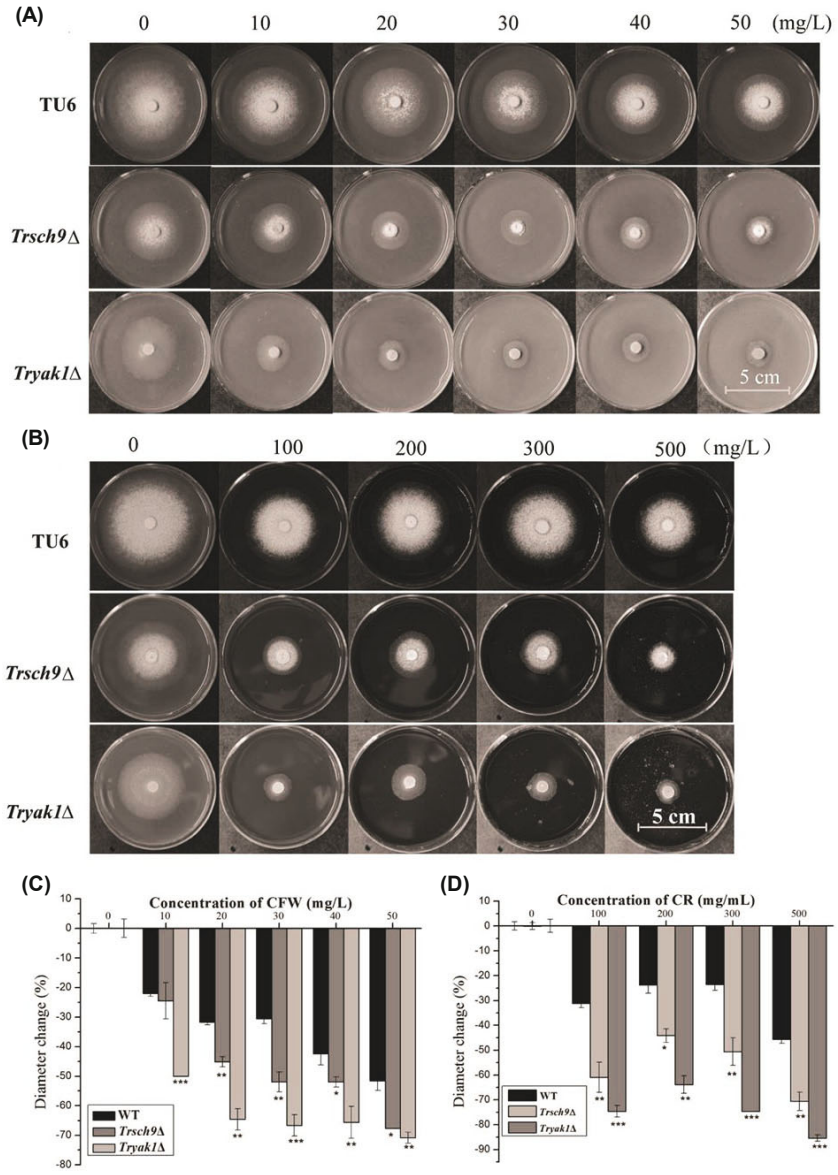

Fig. 5. The effect of the individual absence of TrSch9 and TrYak1 on cell wall integrity of *T. reesei* cells. (A) and (B) growth of WT and mutant strains on plate containing minimal medium with 0–50 mg/L calcofluor white (CFW) and 0–500 mg/L congo red (CR) at 30°C for 60 h respectively. (C) and (D) statistical analysis of mycelial diameter change for each strain treated by CFW and CR respectively. D stands for the diameter of mycelial colony. The differences of growth between the WT and mutants were found to be statistically relevant by Student's *t*-test ; one asterisk, *P* is less than 0.05; two asterisks , *P* is less than 0.01; three asterisks, *P* is less than 0.001.

and maintenance of hyphal growth in *Candida albicans* (Goyard *et al.*, 2008). Moreover, when the conidiation of the WT and mutant strains were evaluated on malt extract agar, both mutants exhibited compromised conidiation with fewer conidiophore structures compared with the WT strain (Fig. 2C). However, no changes in the conidial morphology were detected (Fig. 2C).

TrSch9 and TrYak1 are involved in regulating stress responses

It has been well established that Sch9 and Yak1 contribute to the adaptation of yeast cells to various stress conditions. In order to examine the functions of these two kinases in osmostress adaptation, we compared the growth of the WT and mutant *T. reesei* strains on plates containing various concentrations of NaCl (Fig. 3A). The deletion of *Trsch9* resulted in slightly more pronounced growth defects under salt stress, whereas *Tryak1*Δ mutants exhibited only moderate sensitivity to NaCl (Fig. 3B). *S. cerevisiae sch9* mutants have been characterized as hyper-resistant to oxidative stress in the stationary phase, whereas exponentially growing cells are sensitive (Fabrizio *et al.*, 2001). Therefore, we included H_2O_2 treatment in the phenotypic analysis of the kinase mutants. As shown in Fig. 4C, *Trsch9*Δ and *Tryak1*Δ mutants were both moderately more sensitive to oxidative stress than the WT strain.

It has been reported that overexpression of in *S. cerevisiae sch9* resulted in sensitivity to heat shock, whereas the inactivation of *sch9* suppressed the temperature-sensitive growth caused by Hsp90 mutant (Morano and Thiele, 1999). Therefore, we analyzed the thermotolerance of the kinase mutants (Fig. 4B and D). In contrast to the WT and *Tryak1*Δ mutant strains, *Trsch9*Δ mutant exhibited significantly pronounced thermotolerance. Taken together, these results demonstrate that both TrSch9 and TrYak1 kinases are important determinants of osmotic and oxidative stress adaptation in *T. reesei*, and TrSch9 may also be involved in regulating *hsp* chaperone complex signal transduction, as found in *S. cerevisiae.*

TrSch9 and TrYak1 contribute in maintaining cell wall integrity

To determine whether the stress response defects exhibited by the mutant strains were due to perturbations of the cell wall, the integrity of the cell wall was analyzed based on the sensitivity of the WT and mutant strains to calcofluor white (CFW) and Congo red (CR) (Ram and Klis, 2006). When increasing concentrations of CFW and CR were added to the plates, the growth rates of *Trsch9*Δ and *Tryak1*Δ mutants were dramatically inhibited compared with the WT strain, even at lower concentrations (Fig. 5). In particular, *Tryak1*Δ mutant was more sensitive to CFW and CR than *Trsch9*Δ. These results suggest that TrSch9 and TrYak1 are involved in the maintenance of cell wall integrity, which may further contribute to stress resistance.

TrSch9 and TrYak1 differentially regulate the induced production of cellulases

T. reesei is well established as an efficient cellulase producer. Previous studies have shown that the protein kinases SchA and YakA are important for the production of cellulases in *Aspergillus nidulans* (Brown *et al.*, 2013). Thus, we analyzed whether the absence of TrSch9 and TrYak1 affected the induced production of cellulases in *T. reesei*. In contrast to the phenotypes obtained with *schA* and *yakA* deletions in *A. niudulans*, the induced production of cellulases was not completely abolished in *Trsch9*Δ mutant and only a slight

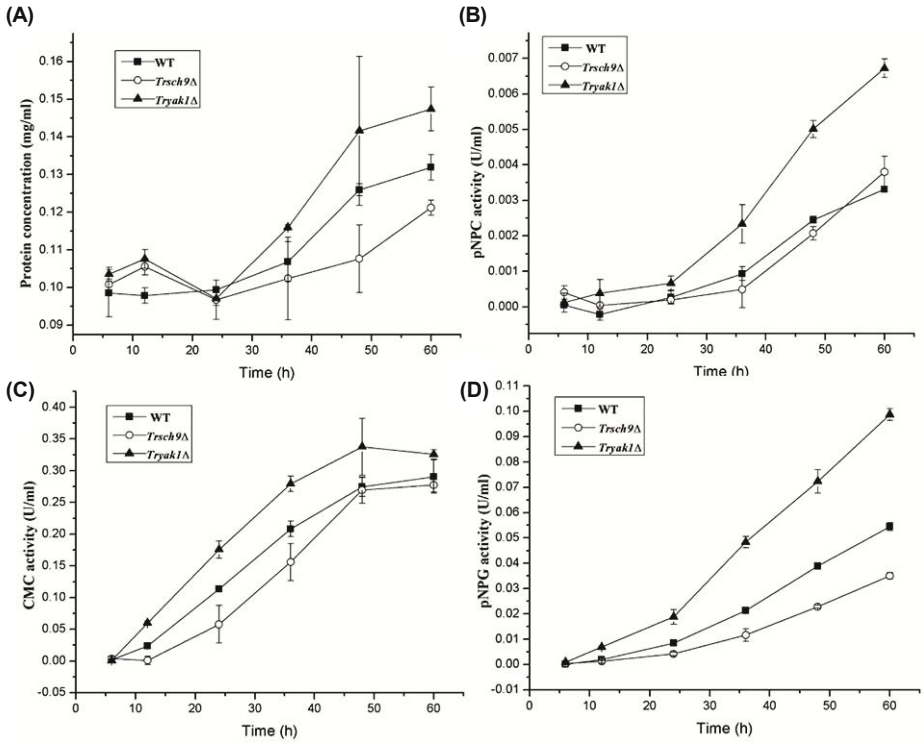

Fig. 6. The effect of the individual absence of TrSch9 and TrYak1 on cellulase production. Extracellular total protein (A), pNPC hydrolytic activity (B), CMC hydrolytic activity (C), and pNPG hydrolytic activity (D) were determined with the culture supernatant from WT, *Trsch9*Δ, and *Tryak1*Δ strains on Avicel. Equal amount of culture supernatant relative to biomass was measured for all the assays.

decrease in the production of extracellular cellulases was observed after incubation with Avicel (Fig. 6). By contrast, the extracellular cellulase activities were significantly higher in *Tryak1*Δ mutant compared with the WT strain. These results indicate that Sch9 and Yak1 not only have opposing effects in the regulation of the induced production of cellulases in *T. reesei* but they also have different functions in evolutionarily related filamentous fungi.

Acknowledgements

This work is supported by grants from the National Basic Research Program of China (2011CB707402), the National Natural Science Foundation (31270116 and 31470162), New Century Excellent Talents in University (NCET-10-0546), and Shandong Provincial Funds for Distinguished Young Scientists (JQ201108).

References

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.

Brown, N.A., de Gouvea, P.F., Krohn, N.G., Savoldi, M., and Goldman, G.H. 2013. Functional characterisation of the non-essential protein kinases and phosphatases regulating *Aspergillus nidulans* hydrolytic enzyme production. *Biotechnol. Biofuels* **6**, 91.

Budovskaya, Y.V., Stephan, J.S., Deminoff, S.J., and Herman, P.K. 2005. An evolutionary proteomics approach identifies substrates of the cAMP-dependent protein kinase. *Proc. Natl. Acad. Sci. USA* **102**, 13933–13938.

Fabrizio, P., Pozza, F., Pletcher, S.D., Gendron, C.M., and Longo, V.D. 2001. Regulation of longevity and stress resistance by Sch9 in yeast. *Science* **292**, 288–290.

Garrett, S., Menold, M.M., and Broach, J.R. 1991. The *Saccharomyces cerevisiae* YAK1 gene encodes a protein kinase that is induced by arrest early in the cell cycle. *Mol. Cell Biol.* **11**, 4045–4052.

Goyard, S., Knechtle, P., Chauvel, M., Mallet, A., Prevost, M.C., Proux, C., Coppee, J.Y., Schwartz, P., Dromer, F., Park, H., and *et al.* 2008. The Yak1 kinase is involved in the initiation and maintenance of hyphal growth in *Candida albicans*. *Mol. Biol. Cell* **19**, 2251–2266.

Gruber, F., Visser, J., Kubicek, C., and de Graaff, L. 1990a. Cloning of the *Trichoderma reesei pyrG* gene and its use as a homologous marker for a high-frequency transformation system. *Curr. Gen.* **18**, 447–451.

Gruber, F., Visser, J., Kubicek, C.P., and de Graaff, L.H. 1990b. The development of a heterologous transformation system for the cellulolytic fungus *Trichoderma reesei* based on a pyrG-negative mutant strain. *Curr. Gen.* **18**, 71–76.

Hartley, A.D., Ward, M.P., and Garrett, S. 1994. The Yak1 protein kinase of *Saccharomyces cerevisiae* moderates thermotolerance and inhibits growth by an Sch9 protein kinase-independent mechanism. *Genetics* **136**, 465–474.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., *et al.* 2007. Clustal W and clustal X version 2.0. *Bioinformatics* **23**, 2947–2948.

Lee, P., Cho, B.R., Joo, H.S., and Hahn, J.S. 2008. Yeast Yak1 kinase, a bridge between PKA and stress-responsive transcription factors, Hsf1 and Msn2/Msn4. *Mol. Microbiol.* **70**, 882–895.

Lee, P., Paik, S.M., Shin, C.S., Huh, W.K., and Hahn, J.S. 2011. Regulation of yeast Yak1 kinase by PKA and autophosphorylation-dependent 14-3-3 binding. *Mol. Microbiol.* **79**, 633–646.

Lynd, L.R., van Zyl, W.H., McBride, J.E., and Laser, M. 2005. Consolidated bioprocessing of cellulosic biomass: an update. *Curr. Opin. Biotechnol.* **16**, 577–583.

Lynd, L.R., Weimer, P.J., van Zyl, W.H., and Pretorius, I.S. 2002. Microbial cellulose utilization: Fundamentals and biotechnology. *Microbiol. Mol. Biol. Rev.* **66**, 506–577.

Mandels, M. and Andreotti, R. 1978. Problems and challenges in the cellulose to cellulase fermentation. *Proc. Biochem.* **13**, 6–13.

Morano, K.A. and Thiele, D.J. 1999. The Sch9 protein kinase regulates Hsp90 chaperone complex signal transduction activity *in vivo*. *EMBO J.* **18**, 5953–5962.

Parija, S.C., Shivaprakash, M.R., and Jayakeerthi, S.R. 2003. Evaluation of lacto-phenol cotton blue (LPCB) for detection of *Cryptosporidium*, *Cyclospora* and *Isospora* in the wet mount preparation of stool. *Acta Tropica* **85**, 349–354.

Pascual-Ahuir, A. and Proft, M. 2007. The Sch9 kinase is a chromatin-associated transcriptional activator of osmostress-responsive genes. *EMBO J.* **26**, 3098–3108.

Penttilä, M., Nevalainen, H., Rättö, M., Salminen, E., and Knowles, J. 1987. A versatile transformation system for the cellulolytic filamentous fungus *Trichoderma reesei*. *Gene* **61**, 155–164.

Ptacek, J., Devgan, G., Michaud, G., Zhu, H., Zhu, X., Fasolo, J., Guo, H., Jona, G., Breitkreutz, A., Sopko, R., *et al.* 2005. Global analysis of protein phosphorylation in yeast. *Nature* **438**, 679–684.

Ram, A.F.J. and Klis, F.M. 2006. Identification of fungal cell wall mutants using susceptibility assays based on Calcofluor white and Congo red. *Nat. Protocols* **1**, 2253–2256.

Roosen, J., Engelen, K., Marchal, K., Mathys, J., Griffioen, G., Cameroni, E., Thevelein, J.M., De Virgilio, C., De Moor, B., and Winderickx, J. 2005. PKA and Sch9 control a molecular switch important for the proper adaptation to nutrient availability. *Mol. Microbiol.* **55**, 862–880.

Santangelo, G.M. 2006. Glucose signaling in *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* **70**, 253–282.

Schuster, A., Tisch, D., Seidl-Seiboth, V., Kubicek, C.P., and Schmoll, M. 2012. Roles of protein kinase a and adenylate cyclase in light-modulated cellulase regulation in *Trichoderma reesei*. *Appl. Environ. Microbiol.* **78**, 2168–2178.

Suwunnakorn, S., Cooper, C.R. Jr., Kummasook, A., and Vanittanakom, N. 2014. Role of the *yakA* gene in morphogenesis and stress response in *Penicillium marneffei*. *Microbiology* **160**, 1929–1939.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28**, 2731–2739.

Thevelein, J.M. and de Winde, J.H. 1999. Novel sensing mechanisms and targets for the cAMP-protein kinase A pathway in the yeast *Saccharomyces cerevisiae*. *Mol. Microbiol.* **33**, 904–918.

Toda, T., Cameron, S., Sass, P., and Wigler, M. 1988. SCH9, a gene of *Saccharomyces cerevisiae* that encodes a protein distinct from, but functionally and structurally related to, cAMP-dependent protein kinase catalytic subunits. *Genes Development* **2**, 517–527.

Urban, J., Soulard, A., Huber, A., Lippman, S., Mukhopadhyay, D., Deloche, O., Wanke, V., Anrather, D., Ammerer, G., Riezman, H., *et al.* 2007. Sch9 is a major target of TORC1 in *Saccharomyces cerevisiae*. *Mol. Cell* **26**, 663–674.