Ras GTPases Modulate Morphogenesis, Sporulation and Cellulase Gene Expression in the Cellulolytic Fungus Trichoderma reesei

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Abstract

Background: The model cellulolytic fungus Trichoderma reesei (teleomorph Hypocrea jecorina) is capable of responding to environmental cues to compete for nutrients in its natural saprophytic habitat despite its genome encodes fewer degradative enzymes. Efficient signalling pathways in perception and interpretation of environmental signals are indispensable in this process. Ras GTPases represent a kind of critical signal proteins involved in signal transduction and regulation of gene expression. In T. reesei the genome contains two Ras subfamily small GTPases TrRas1 and TrRas2 homologous to Ras1 and Ras2 from S. cerevisiae, but their functions remain unknown.

Methodology/Principal Findings: Here, we have investigated the roles of GTPases TrRas1 and TrRas2 during fungal morphogenesis and cellulase gene expression. We show that both TrRas1 and TrRas2 play important roles in some cellular processes such as polarized apical growth, hyphal branch formation, sporulation and cAMP level adjustment, while TrRas1 is more dominant in these processes. Strikingly, we find that TrRas2 is involved in modulation of cellulase gene expression. Deletion of TrRas2 results in considerably decreased transcription of cellulolytic enzymes upon growth on cellulose. Although the strain carrying a constitutively activated *TrRas2<sup>G16V</sup>* allele exhibits increased cellulase gene transcription, the *cbh1* and *cbh2* expression in this mutant still strictly depends on cellulose, indicating TrRas2 does not directly mediate the transmission of the cellulose signal. In addition, our data suggest that the effect of TrRas2 on cellulose gene is exerted through regulation of transcript abundance of cellulase transcription factors such as Xyr1, but the influence is independent of cAMP signalling pathway.

Conclusions/Significance: Together, these findings elucidate the functions for Ras signalling of T. reesei in cellular morphogenesis, especially in cellulase gene expression, which contribute to deciphering the powerful competitive ability of plant cell wall degrading fungi in nature.

Introduction

Cellulose is one of the primary structural components in lignocellulosic materials that represent the major components of plant cell wall. Degradation of cellulose plays a key role in the global carbon cycle and this process mainly depends on the synergistic effects of many kinds of cellulolytic enzymes (e.g., cellobiohydrolases, endo-β-1, 4-glucanases and β-glucosidases) [1,2]. Many filamentous fungi, such as *Trichoderma reesei*, could secrete a broad range of cellulolytic enzymes which are needed in breakdown of cellulose to smaller, soluble sugars [3,4,5,6]. With respect to the energy crisis and the global warming problems, conversion of cellulose to biofuels as an alternative fuel source has become the focus of world attention. To obtain a highly efficient cellulase complex used for biofuels production, the expression and secretion mechanism of cellulolytic enzymes has been subjected to study for decades [7,8,9,10,11]. Cellulase gene expression strictly depends on the induction by cellulose or its derivatives [12]. It is recognized that several transcriptional factors, e.g., the transcriptional activators XhR/Xyr1 [13], Ace2 [14], and the HAP2/3/5 complex [15] as well as the repressors CreA/Cre1 [16], Ace1 [17], are involved in the transcriptional regulation of cellulase genes. However, little is known about the signal pathway that cells sense and transmit extracellular cellulose signal to stimulate the transcription of cellulolytic enzyme genes.

Wang and Nuss [18] discovered that the expression of cellulbiohydrolase I gene was regulated by a GTP-binding-protein-linked signalling pathway in the fungal pathogen *Cryptococcus parasitica*. In *T. reesei*, stimulation of cellulolytic enzyme gene expression by light involves the function and activation of the G-Alpha Proteins GNA1 and GNA3 [19]. Cellulase induction in *T. reesei* by sophorose can be enhanced by increasing intracellular cAMP levels, which implies that cAMP signalling pathway probably modulates the transcription of cellulase genes [20].
The lower number of cellulase and hemicellulase encoding genes in the genome of *T. reesei* than other sequenced biomass-degrading fungi also reveals that adequate signal transduction machineries are required during regulation of sensing cellulase [21].

Ras-like proteins are members of Ras subfamily of GTPase proteins that function as the molecular switch through cycling between inactive GDP-bound and active GTP-bound forms, which play important roles in various signal transduction pathways controlling cell proliferation, morphogenesis, oncogenic transformation, vesicular trafficking and gene expression [22,23,24]. In *Saccharomyces cerevisiae*, Ras1 and Ras2 sense extracellular glucose to regulate cell cycle progress through cAMP signalling pathway, and Ras2 also controls pseudohyphal differentiation via both MAPK and cAMP pathways [25,26]. Expression of the dominant activated Ras2 allele triggers filamentous growth in maize pathogen *Ustilago maydis*, while similar change in cell morphology was not found in the Ras1 dominant activated strain. Moreover, both Ras1 and Ras2 are involved in inducing pheromone gene expression in this fungus [27,28]. In human pathogens *Candida albicans* and *Cryptococcus neoformans*, Ras1 has been shown to control filamentation and virulence via MAP kinase and cAMP-PKA signalling pathway [29,30]. Through a TBLASTX search using nucleotide sequences of *S. cerevisiae* Ras1 and Ras2 as queries, we found two putative homologues of the Ras-subtype GTPase, TrRas1 and TrRas2, in the genome of the cellulolytic model fungus *T. reesei*. However, detailed functions of these two Ras signal proteins in this organism remain unknown.

It is also known that in many fungi, e.g., *S. cerevisiae*, *C. neoformans* and *C. albicans*, Ras plays an important role in activating the cAMP pathway to regulate cell morphology and cell cycle [32]. Meanwhile, Schuster et al. [31] discovered that two crucial components of cAMP pathway, adenylyl cyclase and protein kinase A, were involved in light modulated cellulase gene expression and regulation of vegetative growth in *T. reesei*. With respect to the knowledge mentioned above, it would be attractive to explore the role of Ras signalling in the regulation of morphological development and cellulase gene expression and to study the relationship between Ras and cAMP pathway in *T. reesei*. In this work, we have found that both TrRas1 and TrRas2 play similar roles in morphogenesis and adjusting cAMP level, while TrRas1 is more dominant than TrRas2. Moreover, we also provide the evidence that TrRas2 is involved in regulation of cellulase gene expression.

**Results**

**Characterization of TrRas1 and TrRas2**

Inspection of *T. reesei* genome sequences with TBLASTX revealed two putative Ras GTPases, named TrRas1 and TrRas2, with high homology to *S. cerevisiae* Ras1 and Ras2. The corresponding *TrRas1* gene (GenBank accession no. JX114947) consists of a predicted 967 bp open reading frame interrupted by three introns and encodes a protein of 213 amino acids, which shares 41.0% and 39.3% amino acid sequence identity to *S. cerevisiae* Ras1 and Ras2 respectively. While the putative 237-amino-acid TrRas2 protein (GenBank accession no. AFQ23948) is encoded by a 994 bp open reading frame interrupted by a 280 bp intron, which has the identity of 31.4% and 30.5% to *S. cerevisiae* Ras1 and Ras2 respectively. Sequencing of *TrRas1* and *TrRas2* cDNA from RT-PCR confirmed the model provided above. Transcripts of *TrRas1* and *TrRas2* could be detected in both conidiophores and hyphal cells (data not shown).

Alignment of the amino acid sequences of TrRas1 and TrRas2 along with those of their orthologues of other fungi revealed that all the conserved domains, GTP or GDP binding site, GAP effector binding site, the GTPase domain and the CAAX box for membrane association [22], are included in both TrRas1 and TrRas2. The amino acid sequence of TrRas1 has high identity to the Ras1/RasA proteins of *Penicillium marneffei* (81.9%), *A. fumigatus* (81.9%), *Schizosaccharomyces pombe* (73.0%), *U. maydis* (71.0%) and *N. crassa* (67.6%), while TrRas2 shares great identity to the Ras2/RasB proteins from *N. crassa* (72.3%), *P. marneffei* (67.1%), *A. fumigatus* (65%) and *U. maydis* (57.0%). As shown in Figure 1, phylogenetic analysis using Ras-related Rho orthologues as an outgroup demonstrated that TrRas1 and TrRas2 cluster well with the corresponding Ras proteins from other organisms. The designations of *TrRas1* and *TrRas2* were chosen based on their subclass loci in the phylogenetic tree.

**Transcription of TrRas1 and TrRas2 is not induced by specific carbon source**

In order to be able to relate the Ras signalling proteins to carbon source utilization, the transcription of *TrRas1* or *TrRas2* itself on various carbon sources was firstly investigated. To this end, the strain QM9414 was pre-grown with glycerol, and then the mycelia were transferred to liquid minimal medium with glycerol, glucose or cellulose as the sole carbon source and incubated for 6, 24 or 48 h. No significant differences in *TrRas1* transcript formation were observed for the cellulase-repressing substance glucose and for the cellulase-inducing carbon source cellulose. These transcript levels never exceed those observed on glycerol. Meanwhile, statistical analyses also revealed the similar expression levels of *TrRas2* for three different carbon sources (Figure 2A). These data strongly indicate that the transcriptions of *TrRas1* and *TrRas2* are neither significantly influenced by glucose repression nor by cellulose induction.

*TrRas1* has high amino acid identity (45.5%) to TrRas2, which indicates that they may possess overlapping functions in the cellular processes. To support this, we have detected the transcriptional abundance of *TrRas1* or *TrRas2* gene in Δ*TrRas2* or Δ*TrRas1* mutant strain respectively. From the data, we found that transcript of *TrRas1* in the Δ*TrRas2* mutant increased by 50%–80% compared to the wild-type strain, while *TrRas2* in the Δ*TrRas1* strain increased by 60%–90% (Figure 2B). In addition, *TrRas1* and *TrRas2* showed significantly differences in mRNA levels regardless of which carbon source was used in media, reaching more than 75-fold enhanced transcript abundance of *TrRas1* compared to *TrRas2* (Figure 2A). Similar phenomena was found in *Mucor ramannus*, in which the different transcript levels between Δ*MRas1* and Δ*MRas3* were observed, suggesting these two proteins play distinct roles during morphogenesis in this fungus [33]. These data suggest that *TrRas1* and *TrRas2* may play distinct roles in addition to overlapping functions in *T. reesei*.

As mentioned above, transcriptions of Ras GTTPases TrRas1 and TrRas2 themselves do not respond to certain carbon source signals. Nevertheless, it cannot be rejected that they are still involved in signalling pathways by which *T. reesei* cells regulate carbon source sensing. Consequently, their putative functions in carbon source utilization and morphogenesis would be investigated in the next studies through gene targeting technology.

**Growth of mutants, ΔTrRas1 and ΔTrRas2, on different carbon sources**

Null mutation at the *TrRas1* or *TrRas2* locus was introduced into the parental strain TU-6 by gene replacement using *petA* or *pyG* gene [34,35] as the selective marker respectively. No putative other genes were contained in the flanking regions of the deletion
cassette to ensure that only the relevant target genes were replaced. PCR and Southern blot analysis revealed that TrRas1 or TrRas2 was indeed deleted without ectopic integration of the deletion cassette (Figure S1B, C; Figure S2B, C). Detection of mRNA expression by RT-PCR further confirmed the absence of TrRas1 or TrRas2 transcript in relevant mutants (Figure S1D; Figure S2D).

To examine the influence of TrRas1 and TrRas2 on carbon source utilization and morphological development, a series of growth experiments were conducted in agar plates. In detail, the parental strain TU-6 and the deletion strains DTrRas1 and DTrRas2 were cultured on MM plates containing glucose, glycerol, lactose or cellulose for 4 days. The results were shown in Figure 3. Both mutants showed dramatically reduced colony size on all carbon sources investigated in this work while the parental strain was able to form the normal colonies, indicating that the growth of T. reesei on agar plate is directly influenced by deletion of TrRas1 or TrRas2 no matter what carbon source is used in the medium. Moreover, disruption of TrRas1 results in a more severe growth deficiency than that of TrRas2, suggesting that TrRas1 plays more dominant regulatory roles in fungal development. Interestingly, DTrRas2 could not produce clear zone around the colony on cellulose plate as compared with the parental strain and ΔTrRas1, which suggests that TrRas2 may be involved in regulation of cellulase production.

TrRas1 is essential for polarized apical growth, branching and sporulation

When cultured on complete medium PDA (supplemented with glucose as the carbon source), the ΔTrRas1 mutant formed dramatically decreased colonies with dense mycelia and no conidiospores (Figure 4A) which was similar to that observed on minimal medium agar plate in Figure 3. Retransformation experiment cannot be carried out for the DTrRas1 mutant due to the severe growth defects of disruption of TrRas1. Therefore, we constructed the cbh1-TrRas1 mutant, in which the native TrRas1 promoter was replaced with the cellulose-inducible T. reesei cbh1 promoter, through homologous recombinant experiments to further confirm the role of TrRas1 in fungal development. The cbh1-TrRas1 transformant was confirmed by PCR and Southern blot analysis (Figure S3B, C, D). The same as ΔTrRas1, cbh1-TrRas1 showed no sporulation and small and dense colony that could not expand under repressing conditions (glucose), whereas it returned to the wild-type colonial phenotypes when shifted to

![Image of phylogenetic analysis of Ras proteins](https://example.com/phylogenetic_analysis.png)

**Figure 1.** Phylogenetic analysis of Ras proteins. The Ras-related Rho orthologues were used as an outgroup. The analysis was performed using Neighbor-joining method in the MEGA4.0 software and 1000 Bootstrap replications as test of phylogeny. GenBank accession numbers for the proteins are as follows: S. cerevisiae-Ras1, AAA34958; S. cerevisiae-Ras2, AAA34959; C. albicans-Ras1, AAF03566; U. maydis-Ras1, AAO19640; U. maydis-Ras2, AAO19639; S. cerevisiae-Rho1, AAA34977; N. crassa-Rho1, ACD01425; C. albicans-Rho1, XP_715825; A. nidulans-RhoA, AAK08118; P. marneffei-RhoA, XP_002144340.

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inducing conditions (cellulose) (Figure 4A). Although null mutation of TrRas1 results in severe defects on morphogenesis and sporulation, the ∆TrRas1 strain still maintains the ability in degrading cellulose to form clear zone (Figure 4A).

The TrRas1 mutants were examined microscopically to assess whether there were defects in hyphal morphogenesis. In contrast to the parental strain TU-6, ∆TrRas1 exhibited highly branched, swollen and misshapen hyphal cells after 5 days growth on plates containing either glucose or cellulose (Figure 4B). As expected, when cultured on glucose medium, the cbl1-TrRas1 mutant displayed the same hyphal morphological defects as in ∆TrRas1, whereas it became to normal filamentous fungal cells under cellulose-inducing conditions (Figure 4B). Taken together, these results suggest that (a) TrRas1 is an important signal protein that controls hyphal cell formation and polarized apical growth in T. reesei; (b) deficiency of the hyphal growth subsequently results in a small and dense colony in the TrRas1 deletion mutant; (c) TrRas1 is involved in controlling asexual development in T. reesei. Although similar roles of Ras1 during filamentous growth have been reported in many ascomycetous fungi, e.g., U. maydis and P. marneffei [28,37], deletion of this GTPase could not be generated in these fungi because such a mutation might be either lethal or leads to severe defects. In this work, deletion of TrRas1 was successfully carried out and resulted in severe defects in either hyphal polarized apical growth or sporulation in T. reesei, which provide the new evidence that Ras1 is essential for hyphal growth in ascomycetous fungi.

TrRas2 also modulates polarized apical growth and hyphal branch formation

T. reesei strain with null mutation in the TrRas2 gene displays reduced growth rate in comparison to the parental strain TU-6 on minimal medium agar plates. Meanwhile, we have also found that the ∆TrRas2 strain showed decreased cellulase activity on cellulose plate (Figure 3). To genetically confirm the phenotypes in the ∆TrRas2 mutant, retransformation of TrRas2 was carried out as described in the methods. PCR and RT-PCR analysis revealed the regain of TrRas2 (Figure S2D, E). Phenotype detection revealed that ReTrRas2 could completely complement the defects of the ∆TrRas2 mutant and grow similarly to wild-type (Figure 5A, B, C). In addition, to study the role of Ras2 in regulation of morphogenesis and cellulose-cellulase signalling pathway in detail, we constructed a mutant strain PAnigpdA-TrRas2G16V which carried an active TrRas2 allele (TrRas2G16V) whose product was defective in GTPase activity, thus resulting in permanent signal transmission. In many fungi, such mutations have been considered dominant over the wild-type allele in vivo and applied to study the function of Ras GTPase [27,29,36,37]. PCR and Southern analysis revealed the insertion of gpdA::TrRas2G16V as single-copy integration in the genome of TU-6 (Figure S4B, C, D). Sequencing of the TrRas2 cDNA from the PAnigpdA-TrRas2G16V strain indicated the successful expression of the mutant allele (data not shown). Transcription of the TrRas2 gene in the PAnigpdA-TrRas2G16V mutant was analyzed under cellulose-inducing conditions. Statistical analysis by quantitative real-time PCR showed 7.7-, 33.8- and 13.8-fold up-regulation of the TrRas2 gene transcript in the PAnigpdA-TrRas2G16V mutant compared to the parental strain after inducing for 9 h, 20 h and 40 h respectively (Figure S4E). This increased transcript accumulation in the mutant may be because the expression of TrRas2G16V allele is under the control of strong AnigpdA promoter. Boyce et al. have found that overexpression of RasA did not affect the phenotypic effects of the mutant Ras2G16V allele in P. marneffei [37]. Indeed, the dominant activated TrRas2G16V mutant strain which expresses TrRas2G16V under control of its endogenous promoter displays similar morphological phenotypes as in PAnigpdA-TrRas2G16V (as shown in Text S1 and Figure S6). Therefore, it is believed that the phenotypic changes in the PAnigpdA-TrRas2G16V transformant were attributed to the activated form of TrRas2G16V but not the overexpression of this gene.

The effects of TrRas2 on morphological phenotypes were analyzed by culturing the parental strain TU-6-Z and relevant mutants on PDA plates or liquid minimal medium (LMM) with glucose as the carbon source. The parental strain TU-6-Z, which was constructed by transforming TU-6 with the ppgG cassette and could be cultured independent of uracil, was applied as a control in functional analysis of TrRas2. Neither the growth and morphological phenotype nor the cellulase activity is affected by the integration of the ppgG cassette in the TU-6-Z transformant (data not shown). After 5 days growth on PDA plates, colonies of...
the parental strain TU-6-Z comprising hyphae and conidia were visible. In contrast, the ΔTrRas2 strain exhibited reduced colonies with irregular boundaries and greatly decreased aerial hyphae (Figure 5A). Despite no spores were found in the ΔTrRas2 strain on PDA plate at early stages, conidiation was visible after 9–10 days growth (data not shown). Interestingly, the dominant activated PAnigpdA-TrRas2G16V strain also showed reduced colonies with no conidia and aerial hyphae, but their colonies exhibited regular borders (Figure 5A). In contrast to the wild-type, the ΔTrRas2 mutant formed aggregated hyphae with a hyper-branching phenotype while dominant activation of the TrRas2 resulted in more dispersive hyphae with fewer branches and enhanced polarized apical growth when cultured in LMM (Figure 5B, C). These findings are similar to those in N. crassa and A. fumigatus, in which disruption of Ras2/RasB results in a series of phenotypes, e.g., decreased conidiation, reduced hyphal growth rate on agar plate, irregular colonial boundaries and increased branching [46,47]. Obtained data on the phenotypes of the TrRas2 mutants suggest that correct cycling between GDP-bound and GTP-bound TrRas2 is required for growth. Although growth rates are both delayed for ΔTrRas2 and PAnigpdA-TrRas2G16V strains on either PDA plates or LMM, the biomass of ΔTrRas2 could reach the similar level as that of the parental strain after 60 h of growth in LMM while that is not for PAnigpdA-TrRas2G16V (Figure S5A, B). Similarly, deletion of RasB does not influence the total hyphal mass accumulation in liquid culture in A. fumigatus [46]. The fewer biomass accumulations in the PAnigpdA-TrRas2G16V mutant may be due to the decreased rate of branch formation. These results demonstrate that TrRas2 also acts to modulate polarized apical growth and branch formation in T. reesei, albeit the morphological defects are clearly less severe than that of ΔTrRas1 mutant.

Cellulase formation is influenced by TrRas2

The fact that cultivation of the ΔTrRas2 strain on cellulose plate leads to no clear cellulolytic zone formation prompted us to detect the cellulolytic enzyme activity in the relevant mutants. The parental strain TU-6-Z, the TrRas2 deletion strain and the dominant activated PAnigpdA-TrRas2G16V strain were grown to exponential phase, thus keeping the growth rate at the same level, and then equal amounts of mycelia were transferred to medium with cellulose as the sole carbon source and induced designated periods for cellulase production. Cellobiohydrolase activity (Figure 6A) and secreted protein concentration (Figure 6B) in supernatant samples of all cultivations were measured. As it can be
inferred from the results, both celllobiohydrolase activity and protein concentration were strikingly reduced in the TrRas2 deletion strain compared to the parental strain during the cultivation periods. In contrast, the PAnigpdA-TrRas2G16V strain secreted more extracellular protein and showed higher celllobiohydrolase activity than the parental strain (Figure 6A, B). SDS-PAGE analysis of the culture supernatants also confirmed the results mentioned above (Figure 6C). Obtained data reveal that the signal protein TrRas2 plays important roles in the cellulolytic enzyme formation in T. reesei.

TrRas2 modulates expression of major cellulase genes on cellulose

Having found that the cellulase activity is greatly decreased in the ΔTrRas2 strain, we wondered whether null mutation of TrRas2 leads to down-regulation of the cellulolytic enzyme gene expression. To address this question, the expression levels of two major cellulase genes (cbh1 and cbh2) were investigated by quantitative real-time PCR (Figure 6 D, E). Similar to the previous reports [38,39], we found that both cbh1 and cbh2 were greatly expressed in the parental strain upon induction by cellulose, but not by glucose. In the presence of cellulose, only marginal transcripts of both cbh1 and cbh2 were detectable in the TrRas2 deletion strain at early time of cultivation. Then the transcription levels of cbh1 and cbh2 in the ΔTrRas2 strain increased at the end of the fermentation (induced for 44 h) but only reached to 20% of that at 20 h in the parental strain (Figure 6D, E). These data allowed us to assume that TrRas2 may be involved in transmitting the signal from cellulose to cellulase gene expression, i.e. the mutant with the constitutively activated TrRas2 should form cellulase in the absence of cellulase inducer.

To test the hypothesis, the expression of cbh1 and cbh2 in the dominant activated PAnigpdA-TrRas2G16V strain on noninducing carbon source glucose was examined. However, no apparent cellulase genes expression (cbh1 and cbh2) was detected in the PAnigpdA-TrRas2G16V strain on glucose (Figure 6D, E), indicating that constitutive activation of TrRas2 does not result in the inducer-independent cellulase gene expression, i.e., TrRas2 does not directly transmit the extracellular cellulose signal to stimulate cellulase gene expression. Only cbh1 but not cbh2 is subjected to Cre1-dependent carbon catabolite repression [39], thus exclude the interference of glucose catabolite repression in this experiment.

Although the possibility of the direct cellulose signalling by TrRas2 was rejected, we found that cultivation of the PAnigpdA-TrRas2G16V strain on cellulose resulted in an early and increased (increase by 73% for cbh1 and 128% for cbh2 at 20 h) cellulase formation.
gene transcription compared to the parental strain (Figure 6D, E), thus indicating that this GTPase could modulate cellulase gene expression in the presence of cellulose. These data suggest that TrRas2 may sense the signals except cellulose and act upstream of the transcription regulators to modulate cellulase gene expression. This observation further confirms that TrRas2 is a cellulose sensor that also regulates the cellulase transcription factors.

**The expression of the cellulase gene transcription factors is influenced by TrRas2**

It is believed that the expression of cellulolytic gene is regulated by the cellulase transcription factors (e.g., Xyr1, Ace1, Ace2 and Cre1) in *T. reesei* [13,14,17,40]. To investigate whether TrRas2 influences cellulase gene transcription through modulating the expression of these regulators, we tested the transcript abundance of *xyr1*, *ace1*, *ace2* and *cre1* in the TrRas2 mutants. The results were shown in Figure 7. Similar to the reports of Portnoy et al. [40], we found that *xyr1* transcript accumulation in the parental strain on cellulose was higher (7.9-fold at 20 h) than that on glucose. In the presence of cellulose, *xyr1* transcript abundance in the ΔTrRas2 strain was strikingly lower than that of the parental strain, whereas the relative expression of this gene in the *PAnigpdA-ΔTrRas2* strain was apparently higher (2.6-fold at 20 h) compared to the parental strain TU-6-Z (Figure 7A). Similar results were also found when the mutants were cultured on glucose (Figure 7A), suggesting TrRas2 positively influences the transcript level of the major regulator *xyr1* independent of inducing carbon source. Although constitutive activation of TrRas2 leads to the increase of *xyr1* transcript abundance on glucose, cellulase gene expression in *PAnigpdA-ΔTrRas2* is still undetectable on this repressing carbon source. This might be due to the lower levels and the inactive status of Xyr1 relative to that on cellulose. Additionally, we also found TrRas2 has a negative effect on transcript level of *ace1* but a positive influence on *cre1* transcription only in the presence of cellulose, while only has a small effect on transcript abundance of *ace2* (Figure 7B, C, D).

It has been shown that Xyr1 acts as an activator while Ace1 as a repressor in regulation of cellulase gene expression [13,17]. The transcript patterns of *xyr1* and *ace1* in TrRas2 mutants are strictly in accordance with that of *zbb1/zbb2*, suggesting that TrRas2 modulates cellulase gene expression via regulation of the abundance of *xyr1* and *ace1* on cellulose. Since Ace1 possesses a negative [41] while Cre1 plays a positive role [40] in the transcription of *xyr1*, our data indicate that TrRas2 may regulate the abundance of the major transcriptional factor Xyr1 through regulating the expression level of *ace1* and *cre1*.

**Regulation of cellulase gene transcription by TrRas2 requires Xyr1**

The finding that TrRas2 positively regulates the expression of the major transcriptional regulator Xyr1 raised the question of whether Xyr1 is indeed the downstream target of TrRas2 required for regulation of cellulase gene expression. In order to address this question, we constructed a mutant strain which overexpresses the *xyr1* gene under control of the constitutive *AnigpdA* promoter in the ΔTrRas2 background (Figure S7A and B). Mutant *Oxyr1.11* was used for the following experiments. Real-time PCR analysis showed that the expression of *xyr1* in this mutant strain increased by 27% compared to TU-6-Z, while was 17.6-fold higher than that of ΔTrRas2 (Figure S7C). This mutant did not display altered
growth compared to the ΔTrRas2 strain (Figure 8A). Cellulase gene expression strikingly decreased in the ΔTrRas2 mutant at 10 h and 22 h, and then increased at 34 h. However, the transcription of cbhl could be detected at 10 h and reach to wild-type level at 22 h in the Oxyr1 strain (Figure 8B). These data therefore indicate that overexpression of xyr1 suppresses the cellulase gene expression defect of the ΔTrRas2 mutant, suggesting that Xyr1 acts as one of the major downstream targets of TrRas2 during regulation of cellulase gene transcription. Overexpression of xyr1 could not completely rescue the effect of TrRas2 deletion on cbhl expression, indicating that other targets may exist.
downstream of TrRas2 in the pathway regulating cellulase gene expression.

TrRas1 and TrRas2 play similar roles in increasing cAMP level

In many fungi, such as *S. cerevisiae*, *C. neoformans* and *C. albicans*, Ras1/2 GTPase plays a prominent role in morphogenesis and gene expression through regulating cAMP level [32]. It has been found that cAMP pathway is involved in regulation of vegetative growth and light modulated cellulase gene expression in *T. reesei* [31]. In order to study whether Ras proteins modulate morphogenesis and cellulase gene expression via cAMP pathway in *T. reesei*, the intracellular cAMP levels in the parental strain TU-6-Z, the ΔTrRas2 strain and the *PanigpdA-TrRas2G16V* strain were shown. Transcripts of the target genes were detected just before inducing (BF, 0 h) and 12 h, 20 h, 28 h, 36 h, 44 h after the beginning of the cultivation on cellulose and 10 h, 20 h on glucose. The ratio obtained from BF was set to 1.

![Figure 7. Analysis of the influence of TrRas2 on expression of the transcription factors encoding genes.](https://example.com/figure7.png)

Relative expression of *xyl1* (A), *ace1* (B), *cre1* (C) and *ace2* (D) from the parental strain TU-6-Z, the ΔTrRas2 strain and the *PanigpdA-TrRas2G16V* strain were shown. Transcripts of the target genes were detected just before inducing (BF, 0 h) and 12 h, 20 h, 28 h, 36 h, 44 h after the beginning of the cultivation on cellulose and 10 h, 20 h on glucose. The ratio obtained from BF was set to 1.

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(Figure 9B), thus leading us to reject the possibility that TrRas2 modulates cellulase gene expression through cAMP signalling. However, expression of the dominant activated *TrRas2G16V* allele resulted in 34% and 50% increase in cAMP levels on glucose and cellulose respectively (Figure 9B), which suggested that TrRas2 also plays a role in increasing cAMP concentration. An alternative explanation for no changes in cAMP level in the strain lacking TrRas2 could be that TrRas1 might be able to complement the role of TrRas2 in adjusting cAMP level. From these data, one could assume that filamentation and sporulation defects in the ΔTrRas1 mutant could be due to the decrease in cAMP levels. We consequently supplemented the PDA media with cAMP to investigate the growth of ΔTrRas1 mutant. As shown in Figure 9C, the results showed that the filamentous growth and the aerial hyphae growth of the ΔTrRas1 mutant were greatly enhanced by addition of exogenous 2 mM cAMP, although not to the wild-type level. It would be concluded that TrRas1 regulates filamentation program through cAMP signalling pathway. Simi-
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Figure 8. Influence of Xyr1 overexpression in the TrRas2 deletion strain on regulation of cellulase gene transcription. (A) Phenotypes of the TU-6-Z, ΔTrRas2 and oxy1 stains upon growth on PDA plates. Strains were grown for 5 days at 30 °C. Scale bar = 1 cm. (B) Northern blot analysis of cbh1 transcription in the TU-6-Z, ΔTrRas2 and oxy1 stains. Strains were induced with 1% Avicel cellulose. A total of 2 μg RNA was loaded per lane. 28S rRNA and 18S rRNA were used as the control.

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Discussion

Ras GTPases are critical binary switches in signalling processes that convey signals from extracellular environment to nucleus and regulate cell growth, proliferation and differentiation in eukaryotes from human to yeast [22,23]. Ras genes are firstly identified as oncogenes in human tumors and mutations of these genes exist in almost one third of all human cancers [44]. Recently, the functions of Ras involved in fungal development, pathogenesis and gene expression have been well documented [32]. In this study, our results clearly indicate that both TrRas1 and TrRas2 play similar and distinct roles during morphogenesis and cellulase gene expression in the model cellulolytic fungus T. reesei. Indeed, TrRas1 and TrRas2 are both involved in the regulation of polarized apical growth of hyphal cells, branch emergence and increasing cAMP level. However, TrRas1 is more dominant than TrRas2 during controlling these cellular processes. Specially, TrRas2 is also involved in the regulation of cellulase gene expression in the presence of cellulose.

Polarized apical growth is crucial for filamentation and asexual development in filamentous fungi, and Ras GTPases emerge as key regulators in this process [45,46]. In S. cerevisiae, Ras1 and Ras2 are correlated with cell elongation, cell adhesion, agar invasion and the pseudohyphal growth [25,47]. In the dimorphic pathogenic fungi, C. albican, CaRas1p is required for polarized growth and thereby contributes to transition from yeast-like mode of growth to filamentous growth [29]. As in C. albican, RasA regulates polarized growth of yeast cells and hyphae in P. marneffei and also regulates initiation of asexual development and branch emergence in this fungus [37]. Deletion of A. fumigatus RasB results in a lag in germination, a hyper-branching phenotype and a deficient colony with decreased peripheral growth and irregular borders on solid agar [49]. Kana-uchi et al. [49] discovered that Nc-ras2 regulates polarized growth, cell wall synthesis, aerial hyphae formation and conidiation in N. crassa. In U. maydis, signalling pathways mediated by Ras2 regulate filamentous growth while Ras1 does not influence cell morphology [27,28]. Similarly, both TrRas1 and TrRas2 play important roles in controlling polarized apical growth in T. reesei. The mutant with a deletion in TrRas1 fails to produce normal filamentous hyphae but produces a cluster of swollen and short hyphal cells, while expression of the dominant activated TrRas2G16V allele promotes polarized growth greatly. Although deletion of TrRas2 results in a reduced colony with decreased aerial hyphae and scalloped borders, the mutant still can maintain the filamentous growth, suggesting TrRas1 and TrRas2 may be functional redundant in controlling polarized apical growth and TrRas1 is more dominant. This is similar to S. cerevisiae and C. neoformans, where Ras1 and Ras2 possess redundant cellular functions [26,30,50]. In addition, we also found TrRas1 and TrRas2 may play overlapping roles in increasing cAMP concentration. Despite significant efforts, a T. reesei strain with double deletion of TrRas1 and TrRas2 could not be generated suggesting such mutation may be lethal like that of S. cerevisiae, in which lacking Ras1 and Ras2 simultaneously is inviable [51,52].

Having found the critical roles of TrRas1 and TrRas2 in polarized apical growth, we wonder what are the signalling mechanisms that are regulated by TrRas1 and TrRas2 during filamentous growth of T. reesei. Schuster et al. [31] have found that cAMP signalling is involved in vegetative growth in T. reesei. In this work, our results demonstrate that deletion of TrRas1 leads to a decrease in cAMP level and that addition of exogenous cAMP could partially rescue the filamentation defect of ΔTrRas1, suggesting TrRas1 may act upstream of adenyl cyclase to regulate morphogenesis in T. reesei. However, the morphological defects in the ΔTrRas1 strain are more severe than those in Δacy1 and Δpkac1 which display dramatically decreased growth rate on solid agar but normal hyphal cells and conidia formation [31]. Moreover, although deletion of TrRas2 leads to no change in cAMP level, defects of phenotype are found in the ΔTrRas2 mutant. These facts clearly indicate that other pathways besides cAMP signalling are regulated by TrRas1 and TrRas2 during morphogenesis. It has been shown that the maintenance of polarized growth is regulated by Cdc42 or its homologue CIB which co-localizes with actin at the hyphae apex to organize the actin cytoskeleton and that the Cdc42 or CflA activation is which lacking Ras1 and Ras2 simultaneously is inviable [51,52].

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pathways controlling filamentous growth exist in *T. reesei* and depend upon TrRas1 and TrRas2. By analogy with *S. cerevisiae*, we found homologues of the MAP kinase signalling components involved in filamentous growth, TrSte20 (tre104364), TrSte11 (tre4945), TrSte7 (tre75872) and TrSte12 (tre36543), are existed in *T. reesei* genome. Consequently, we proposed the model of TrRas1 and TrRas2 signalling (as illustrated in Figure 10A) that may be involved in filamentation, vegetative growth and asexual development in *T. reesei*. In fact, the relationships between TrRas1 and TrRas2 signalling and MAP kinase cascade in controlling filamentous growth are being studied in our lab.

The mutant ΔTrRas1 still can degrade cellulose to form a clear zone on cellulose plate despite it shows a severe growth defect, while deletion of ΔTrRas2 interrupts the cellulose degrading on the plate. Therefore, we focus our study on exploring the influence of TrRas2 on cellulase gene expression. Deletion of TrRas2 leads to a great decrease in the transcription of major cellulase genes, suggesting that TrRas2 is involved in cellulase gene expression. However, constitutive activation of TrRas2 does not lead to cellulase gene transcript independent of inducer, indicating that TrRas2 is not directly involved in transmitting the signal from cellulose to cellulase gene expression. It has been shown that light acts through signalling pathway involving heterotrimeric G-proteins/cAMP/PKC1 to modulate cellulase gene transcription in *T. reesei* [19,31,55]. In silico analysis of 2 kb upstream of the TrRas2 translational start codon reveals two single EUM1-binding motifs (EUM1, envoy upstream motif 1) [55] (positions −1228 to −1223 and positions −1748 and −1743) which have been described to occur in genes regulated by light. Thus, it seems that TrRas2 signalling may transmit the light or other signals to modulate cellulase gene expression.

It has been discovered that cAMP signalling is involved in regulation of cellulase gene expression [19,31]. Moreover, the adenylate cyclase, which produces the cAMP as a secondary...
Xyr1 has been demonstrated as a central transcriptional regulator that controls xylanolytic as well as cellulolytic enzyme genes expression in *T. reesei* [13]. The transcription of almost all cellulase genes is significantly impaired or reduced in the *xyr1*-konc stout strain under inducing condition [56]. In this work, *xyr1* transcription is greatly decreased in the Δ*TrRas2* mutant while increased in the *TrRas2*Δ*trras1* transformants, indicating that *TrRas2* may modulate cellulase gene expression by regulating the abundance of Xyr1. The fact that overexpression of *xyr1* in the Δ*TrRas2* background could rescue the defect of cellulase gene expression further confirmed that Xyr1 acts as one of the major downstream targets of *TrRas2* during regulation of cellulase gene transcription. In addition, we also find that *TrRas2* has a negative effect on *ace1* transcript while a positive effect on *cre1* expression in the presence of cellulose. Since *Ace1* is shown as a repressor in the expression of the *xyr1* [41] and *Cre1* is necessary for the full induction of *xyr1* transcript on cellulose [40], our data suggest the possibility that *TrRas2* also modulate the abundance of Xyr1 via modulating transcription of *ace1* and *cre1*. Taken together, it is convincible that *TrRas2* senses extracellular signals (e.g., light) and acts through an unidentified pathway to modulate the expression of transcriptional regulators which further regulate the cellulase gene transcription (Figure 10B).

In conclusion, our results show that *TrRas1* and *TrRas2* play similar and distinct roles in morphogenesis in the model cellulolytic fungus *T. reesei*. Moreover, signalling pathways, but not the cAMP signalling, mediated by *TrRas2* are involved in modulating cellulase gene expression through regulating the transcription of cellulase gene transcriptional regulators. Identification of the downstream components of *TrRas1* and *TrRas2* during filamentation and cellulase gene expression will be a major challenge for future studies. In addition, it will be fascinating to study the true extracellular signals transmitted by *TrRas2* during modulation of cellulase production.

**Materials and Methods**

**Strains, cultural conditions and microscopy**

* T. reesei strains QM9414 (ATCC 26291), TU-6 (ATCC MYA-256; uridine auxotroph [57], *pyrF*), TU-6-Z (the strain transformed with *pyrG* cassette from pAB1-35 [35], *pyrG*), Δ*TrRas1* (*ΔTrRas1::ptrA*), *cbh1-TrRas1* (*cbh1:: (ptrA)); Δ*TrRas1::ptrA*), Δ*TrRas2* (*ΔTrRas2::pyrG*), Re*TrRas2* (Re*TrRas2::ptrA*, Δ*TrRas2::pyrG*), P~Nit~*TrRas2ΔG16V* [gpdA(p)-Δ*TrRas2::ptrA*], and *Oxyr1* [gpdA(p)-*xyr1::ptrA*, Δ*TrRas2::pyrG*] were used throughout this study. All the strains were maintained on potato dextrose agar (PDA) plates, or supplemented with 10 μM mL−1 uridine when necessary (for strains TU-6, Δ*TrRas1* and *cbh1-TrRas1*). *Escherichia coli* DH5α was used for cloning of constructs and cultured in LB broth supplemented with appropriate antibiotics at 37 °C.

To analyze the transcript levels of *TrRas1* and *TrRas2* on different carbon sources, replacement experiments were designed. Pregrown mycelia of 0.6 g were transferred to 150 ml minimal medium containing different carbon sources (e.g. 2% glucose, 2% glycerol and 1% Avicel cellulose) and grown for additional 6 h, 24 h and 48 h. To detect the expression level of *TrRas2* in Δ*TrRas1* (and vice versa), equivalent squares of agar with growing strains, TU-6-Z, Δ*TrRas1* and Δ*TrRas2*, were grown for 12 h, 24 h and 48 h at 30°C and 200 rpm in 150 ml liquid glucose minimal medium. Then mycelia were harvested and used for RNA extraction.

For assays of growth on different carbon sources, equivalent squares of agar with growing strains were inoculated on plates with
minimal medium supplemented with 1% (w/v) of the corresponding carbon source and 2% agar for 4 days at 30°C.

Strains TU-6-Z, ΔTrRas2 and PAnigpdA-ΔTrRas2G16V were cultured in liquid glucose minimal medium or on PDA plates to determine growth rate according to the method of Aro et al. [14].

For induction experiments [56], equivalent squares of agar with growing strains, TU-6-Z, PAnigpdA-ΔTrRas2G16V and ΔTrRas2, were grown for 30 h, 36 h and 48 h at 30°C and 200 rpm in 200 ml liquid glucose minimal medium (LMM). Mycelia were collected by filtration, washed twice with sterilized water, and equal amounts of mycelia (1 g) were transferred to 200 ml minimal medium with 1% Avicel cellulose (w/v) as the inducer or 1% glucose (w/v) as the control. For RNA extraction, induction was performed for 12 h, 20 h, 28 h, 36 h and 44 h on Avicel cellulose or 10 h and 20 h on glucose at 30°C and 200 rpm. For cellulase activity assay, culture medium samples were collected at 24 h, 36 h, 48 h, 60 h, 72 h and 84 h after induction. For detection of cbh1 expression in the Oxyr1 strain, RNA was extracted after induced for 10 h, 22 h, and 34 h.

Strains TU-6, ΔTrRas1 and cbh1-TirRas1 were inoculated on plates with PDA or minimal medium containing 1% (w/v) Avicel cellulose as the sole carbon source for 5 days at 30°C to determine the morphological phenotypes. Strains TU-6-Z, ΔTrRas2, PA-nigpdA-ΔTrRas2G16V and Oxyr1 were grown on PDA plates or liquid minimal medium with 2% glucose (w/v) as the carbon source for morphological phenotype assays. Photographs of colonies were taken with a Samsung Digimax S500 camera. Microscopic images were captured on Nikon eclipse 80i light microscope (Nikon, Japan).

Molecular techniques

T. reesei genomic DNA isolation was carried out as described previously [58]. PCR experiments were performed with standard protocols using a T1 Thermocycler (Biometa, Gottingen, Germany) unless otherwise indicated. Prime design was carried out using the primer premier 5.00 software (PREMIER Biosoft). DNA fragments were purified using Gel Extraction Kit (Omega, USA). Oligonucleotides synthesis and DNA sequencing were performed at Sangon Inc. (Shanghai, China). Oligonucleotides used in this study are listed in Table S1 in supplementary materials. Using chromosomal DNA and cDNA of T. reesei as the templates, TirRas1 or TrRas2 genes were amplified with primer pairs Ras1-RT-S/Ras1-RT-A or Ras2-RT-S/Ras2-RT-A respectively. Then the fragments were cloned into pMD18-T (Takara, Japan) to sequence the wild-type TirRas1 and TrRas2 genes. The related DNA sequence data have been deposited in GenBank. Standard procedures were applied for other molecular manipulations [59]. Multiple alignments of protein sequences were performed with ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Phylogenetic analysis was inferred using the Neighbour-Joining method and the software MEGA4.0. The T. reesei QM6a DNA sequence and protein predictions of the T. reesei genome database ver2.0 were used in this study (http://genome.jgi-psf.org/Trire2/Trire2.home.html).

Construction of T. reesei mutants

Fusion PCR and gene targeting were used for producing the mutants of this study as described previously [58,60]. To construct TrRas1 deletion strain (ΔTrRas1), a deletion construct ΔTrRas1::ptrA yielded a 1.5 kb fragment in the wild-type strain. Then two fragments were fused with chloral2W (http://www.eki.ac.uk/Tools/msa/chvalor2/). Phylogenetic analysis was inferred using the Neighbour-joining method and the software MEGA4.0. The T. reesei QM6a DNA sequence and protein predictions of the T. reesei genome database ver2.0 were used in this study (http://genome.jgi-psf.org/Trire2/Trire2.home.html).

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For expression of a constitutively activated version of TrRas1G16V, which had a single amino acid mutation at codon 16 (glycine to valine), PA-nigpdA-ΔTrRas2G16V (gpdA(p)-TrRas2G16V::pyrG) strain was constructed. Primers pairs Ras2-nest-S/O3Ras2-A and O3Ras2-S/Ras2-nest-A containing the mutation to be introduced into the wild-type template DNA were used to amplify the 5’ portion and 3’ portion of TrRas2 respectively. Then these two fragments were fused with primer pair Ras2-RT-S/Ras2-RT-A and cloned into pMD18-T to make pTrRas2G16V. Coding region of TrRas2G16V was completely sequenced to ensure that only the desired mutations had been introduced. The AnigpdA promoter generated from pAn2-S1 using primer pair Pgpds-A+Sgpds-A was digested with EcoRI and ligated to the EcoRI-Smal sites of pTrRas2G16V to make pTrRas2G16V. A 2.7 kb pyrG fragment from pBAD-1 using primer pair pyrG- EcoR1-S/pyrG- EcoR1 was inserted into pTrRas2G16V, digested with EcoRI to generate pO8ras2. The pO8ras2 was used for transformation of T. reesei TU-6. Transformant with an integration of pO8ras2 was firstly tested with primer pair YORas2-S/YORas2-A (YORas2-S binds within the AnigpdA promoter region and YORas2-A binds...
within the TrRas2G16V codon region), which resulted in a 2.0 kb product only in the transformant.

For overexpression of syr1 in the ΔTrRas2 mutant, the Oxyr1:: ptrA cassette was constructed as following. Firstly, a 2.1 kb pyrithiamine resistance cassette ptrA, a 1.2 kb AnigpdA promoter from pAN7-1 and a 3.6 kb wild-type syr1 gene were amplified using primer pairs ptrA-S/ptrA-A, Oxyr1-001/FpgdA-A and Oxyr1-002/Oxyr1-003 respectively. Then the Oxyr1:: ptrA cassette was obtained through fusion of these three fragments by using primer pair ptrA-S/Oxyr1-004. The Oxyr1:: ptrA cassette was applied to transform the ΔTrRas2 strain and pyrithiamine resistant Oxyr1 transformants were chosen for the Southern analysis.

RNA extraction and quantitative real-time reverse transcription PCR

For RNA extraction, mycelia were harvested by filtration, homogenized in Mini-BeadBeater (Biospec, USA) with 0.5 mm Zirconium/Silica beads at 4°C, and then total RNA were isolated with Trizol reagent kit (Life Technologies, USA).

Synthesis of cDNA from total RNA was performed using PrimeScript RT reagent Kit (Takara, Japan) as the manufacturer’s instructions. Real-time PCRs were carried out in a LightCycler 480 System (Roche Diagnostics, Germany). All PCRs were performed in triplicate in 20 μl reaction mixtures containing 1x SYBR Premix Ex Taq TM, 0.2 μmol L−1 forward primer, 0.2 μmol L−1 reverse primer, and 2 μl cDNA template (100-fold diluted) using the SYBR Premix Ex Taq TM (Tli RNaseH Plus) kit (Takara, Japan). Real-time PCR protocols were as following: 1 min initial denaturation at 95°C, followed by 40 cycles of 5 s at 95°C, 20 s at 60°C. Melting curve analysis with a temperature gradient of 0.1°C s−1 from 65°C to 95°C was performed. LightCycler480 software 1.5.0 was used to calculate Ct value. Transcript levels of target genes were normalized against the level of actin gene with ddCt method [61].

Northern hybridization analysis

Northern blotting analysis was performed using DIG Northern Starter Kit (Roche Diagnostics, Germany) according to the manufacturer’s instruction. The chb1 fragment obtained from PCR using primer pair NT7-CBH1-S/NT7-CBH1-A was used as the template for labeling RNA probe with digoxigenin.

Southern hybridization analysis

Transformants constructed in this study were confirmed by Southern blotting analysis using DIG Easy Hyb kit (Roche Diagnostics, Germany) according to the manufacturer’s instruction. Probe A, B and C generated from PCR using primer pairs Ras1-probe-s/Ras1-5-A, Ras1-3-S/Ras1-5-A and Ras2-Nest-S/Ras2-5-A were used for hybridization for ΔTrRas1, chbl1-TrRas1 and ΔTrRas2 respectively. Hybridization of BamHI-digested genomic DNA using probe A yielded a 0.9 kb fragment in a strain with the ΔTrRas1 allele, whereas a 10 kb fragment was observed in the wild-type strain. Hybridization of BalI or Apal-digested genomic DNA with probe B resulted in a 3.5 kb or 2.0 kb fragment in the wild-type strain while a 3.7 kb or 2.4 kb fragment in the chbl1-TrRas1 mutant respectively. Similarly, Southern hybridization of BamHI-digested genomic DNA with probe C yielded a 2.2 kb fragment in the wild-type strain and a 3.3 kb fragment in the ΔTrRas2 strain. In addition, the integration of the PlagigdaA-TrRas2G16V allele into the T. reesei genome was analyzed by Southern hybridization using XhoI-digested genomic DNA with PCR probe D obtained using primer pair Ras2G16V-probe-S/
in the relevant mutants. (E) PCR analysis indicated the regain of *TrRas2* expression cassette in the *RcTrRas2* transormants.

(TIF)

**Figure S3** Replacement of the native *TrRas1* promoter with the regulatable *cbh1* promoter. (A) Schematic representation of the genomic organization of the *TrRas1* promoter locus in *TU-6* and *cbh1-TrRas1* mutant. Primer pairs and relative positions of the *Apal* and *BalII* restriction sites are given. Probe C used for Southern analysis is shown as black box. (B)/C) PCR analysis showed that the native *TrRas1* promoter had been replaced by *cbh1* promoter successfully and that there was no ectopic integration of the replacement cassette. (D) Southern blot of the chromosome digested with *Apal* or *BalII* confirmed the replacement of the native *TrRas1* promoter in the mutant.

(TIF)

**Figure S4** Construction of *PAnigpdA-TrRas2G16V* strains expressing the dominant activated *TrRas2* allele. (A) Graphical representation of the *TrRas2* genomic locus from the wild-type strain *TU-6* and *PAnigpdA-TrRas2G16V* strains. Primer pairs and relative positions of the *SacI* and *XhoI* restriction sites are given. Probes D and E used for Southern analysis are shown as red boxes. (B) PCR analysis showed the successful integration of the pORas2 plasmid into the genome of *TU-6*. (C) Southern blot of the chromosome digested with *XhoI* confirmed the integration of the *gdA(p)-p-TrRas2G16V* cassette into the genome of *TU-6*. (D) Southern blot of the chromosome digested with *SacI* confirmed that the *gdA(p)-p-TrRas2G16V* cassette integrated ectopically. (E) Quantitative real-time PCR analysis showed that the *TrRas2* mRNA levels in the *PAnigpdA-TrRas2G16V* strain was significantly increased when compared to that of the wild-type strain. The relative mRNA levels were presented by setting the amount of *TrRas2* mRNA at 9 h in wild-type strain as 1. Actin gene was used as the reference. (TIF)

**Figure S5** Radial growth and biomass of the *TU-6-Z*, *ΔTrRas2* and *PAnigpdA-TrRas2G16V* strains. (A) Radial growth rates of the *TrRas2* mutants. Equivalent squares of agar with growing strains were cultured on PDA plates at 30°C. (B) Biomass accumulation in LMM at 200 rpm and 30°C with equivalent squares of agar with growing relevant strains as the inoculation.

(TIF)

**Figure S6** Construction of the dominant active *TrRas2G16V* mutant under the control of its own promoter. (A) Schematic representation of the genomic organization of the *TrRas2* locus in the *TU-6* and *TrRas2G16V* strains. Relative positions of the *BamHI* restriction sites are given. Probe G used for Southern analysis is shown as red box. (B) Southern blot of the chromosome digested with *BamHI* confirmed that the *TrRas2G16V* cassette integrated ectopically. (C) Growth phenotypes of strains *TU-6-Z* and *TrRas2G16V* on PDA plates. One *TrRas2G16V* mutant was chosen as the representative. Scale bar = 1 cm.

(TIF)

**Figure S7** Overexpression of Xyr1 in the *ΔTrRas2* mutant. (A) Schematic representation of the genomic organization of the *xyl1* locus in the *ΔTrRas2* and *Oxyr1* strains. Relative positions of the *HindIII* restriction sites are given. Probe F used for Southern analysis is shown as red box. (B) Southern blot of the chromosome digested with *HindIII*. The 1.68 kb wild-type band is present in all the strains. An additional band longer than 0.87 kb indicates the presence of *Oxyr1*-tag cassette. (C) Quantitative real-time PCR analysis of *xyl1* in the *TU-6-Z*, *ΔTrRas2* and *Oxyr1* strains. Strains were induced on 1% Avicel cellulose for 22 h. The relative mRNA levels were presented by setting the amount of *xyl1* mRNA in *ΔTrRas2* as 1. Actin gene was used as the reference. (TIF)

**Text S1** Expression of the dominant active *TrRas2G16V* allele under control of its own promoter.

(DOCX)

**Table S1** Primers used in this study.

(DOCX)

**Author Contributions**

Conceived and designed the experiments: JZ. TW. Performed the experiments: JZ. Analyzed the data: JZ Y. Zhang. Contributed reagents/materials/analysis tools: TW YQ JZ. Wrote the paper: JZ. Advise on the writing of the paper: Y. Zhang Y. Zhong.
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