The heterotrimeric G protein r Stgg1 is required for conidiation, secondary metabolite production and pathogenicity of Setosphaeria turcica

Pan Li, Xinjie Zhang, Yibin Lin, Shen Shen, Yulan Zhao, Jingao Dong and Zhimin Hao

ABSTRACT
Heterotrimeric G proteins are best known for their role in the transduction of extracellular signals to various downstream effectors. G proteins in higher eukaryotes are intensively studied; however, their roles in foliar pathogens are still elusive. In this study, we cloned the gene Stgg1 encoding G protein γ subunit in Setosphaeria turcica and investigated its function by RNA interference technology. Three independent Stgg1 targeted RNAi mutants R3, R5 and R6 with diverse silencing efficiency were generated. Knock-down of Stgg1 resulted in a significant reduction in mRNA levels of the genes encoding Gα (Stga1, Stga2, Stga3) but not for Gβ (Stgb1). Stgg1 RNAi mutants exhibited significantly elongated hyphal cells with blocked conidium production. In addition, Stgg1 RNAi mutants all appeared in lighter colony colour compatible with inhibited secondary metabolites. Further assays demonstrated that Stgg1 was required for biosynthesis of melanin and HT-toxin activity. Furthermore, down-regulation of Stgg1 largely inhibited the infection capacity. Thus, we proposed that Stgg1 played crucial roles in conidiation, secondary metabolite production and pathogenicity of S. turcica and is, therefore, an ideal target for drug design against foliar pathogens.

Introduction
Setosphaeria turcica is the causative pathogen of the northern corn leaf blight, a severe disease affecting maize growth worldwide [1]. This disease has caused devastating yield losses of maize during its periodic epidemics. Developing maize varieties of disease resistance is an efficient strategy for prevention of this disease, however, challenged by the frequent variation of S. turcica [2]. The signaling pathways essential for pathogenicity are therefore the focus of disease control.

The guanine nucleotide-binding protein (G protein) signaling pathway is evolutionarily conserved and composed of seven-transmembrane G protein-coupled receptors (GPCRs), heterotrimeric G proteins and diverse downstream effectors [3]. GPCRs represent the largest protein family of transmembrane receptors, which can transmit a variety of environmental signals, such as hormones, nutrients, light, odours, and chemoattractants, to the intracellular heterotrimeric G proteins [3–5]. Heterotrimeric G protein consists of α, β and γ subunit encoded by independent genes. It is widely reported in fungi such as Penicillium camemberti and Pestalotiopsis microspora that the Gα-cAMP signaling pathway controls growth, conidiation, development, secondary metabolism and stress response [6,7].

Most released filamentous fungal genomes possess five G protein-encoding genes, three for Gα, one for Gβ and one for Gγ [4,8]. Activated by ligands bound to GPCRs, Gr acts together with Gα as a dimer, while Gγ functions separately as the GTP hydrolase. The uniform characteristics of all Gr proteins is the CaaX box motif at the carboxy terminus as the regulatory domain [9].

Compared to G6 and Gβ subunits, the function of the Gr subunit has only been reported in fewer species of filamentous fungi. In N. crassa, a G protein γ subunit GNG-1 forming a Gαγ dimer with GNB-1 is required for normal female fertility, asexual development and mRNA expression of G6 proteins [10]. In A. nidulans, the sole G protein γ subunit gpgA is essential for normal vegetative growth and developmental progression [11]. Recently, a putative Gr subunit gene MGG1 in M. oryzae was reported to be involved in asexual/sexual sporulation, appressorium formation and pathogenicity [12].

In this study, we present the identification and characterization of a Gγ subunit named Stgg1 in S. turcica. To
explore the function of Stgg1, we took advantage of the RNA interference (RNAi) approach and generated stable Stgg1 silenced transformants. Our data proved that Stgg1 is involved in the regulation of hyphal morphology and conidiation, but is dispensable for colony growth. Moreover, Stgg1 is also required for the biogenesis of essential secondary metabolites related to the pathogenesis of S. turcica, such as melanin and HT-toxin. In addition, infection assay with stgg1 mutants further confirmed the involvement of Gr subunit in pathogenicity.

Materials and methods

Fungal strains and culture conditions

All strains used in this study were derived from wild type (WT) 01–23 of Setosphaeria turcica. Fungal strains were grown on potato dextrose agar (PDA, 2% glucose, 20% potato, 1.5% agar) at 25 °C as described previously.

Isolation and identification of Stgg1 gene

Degenerate primer pair GPz-L1 and GPz-R, GPz-L2 and GPz-R, designed from the G-protein sequence [16], were respectively used to amplify the homologous fragment of the target gene using WT genomic DNA as the template. Specific primers GPzW-3–1, GPzW-5–1 and GPzW-5–7 were employed to acquire the 5’ and 3’ flanking sequence according to the manufacturer’s instruction of GenomeWalker Universal Kit (Clontech Laboratories, Inc.). The primers used in this study are listed in Supplementary Table S1.

Generation of Stgg1 RNAi transformants

Fungal transformation vector pSilent-1 [17], which possessed a hygromycin B phosphotransferase gene as a selection marker, was used to construct the Stgg1 RNAi vector according to the DNA assembly methods previously described [18]. Specific primers Gprz-up1 and Gprz-up2, Gprz-down1 and Gprz-down2 (Supplementary Table S1) were employed to amplify two fragments of Stgg1, GrF1 (350 bp) and GrF2 (700 bp), from WT genomic DNA. Both fragments were respectively subcloned into SnaBl-HindIII and KpnI-BglII sites of pSilent-1. The construct was introduced into the protoplast of 01–23 strain with the polyethyl eneglycol (PEG)-mediated transformation strategy as described previously [15]. Transformants were preliminarily screened on PDA medium containing hygromycin B (50 μg/mL) for at least three generations and further confirmed by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) with tubulin and 18 s rRNA as the internal control as described previously [13,14].

Melanin analysis, pathogenicity and HT-toxin assays

Intracellular melanin from 0.1 g dried mycelia of 10-day-old materials was extracted and estimated according to the method modified in our lab [14]. Pathogenicity assay was performed using fungal discs (III = 5 mm) of the indicated strains following the method described previously [15]. Toxicity analysis of crude HT-toxin was conducted using the method established in our laboratory [15]. Seedlings of five-day-old corn cultivar OH43 were used for these assays.

Data analysis

Data are mean values with standard deviation (±SD) from three independent experiments with triple replications. Data analysis was performed using DPS V9.50 software.

Results and discussion

Isolation and characterization of the Stgg1 gene

A fragment of around 400 bp size was amplified with the indicated degenerate primers listed in Supplementary Table S1. The result of blastX showed that it shared a similarity of 82% and 80% with genes encoding the G protein γ subunit (Gγ) of Phaeosphaeria nodorum and Podospora anserine, respectively, indicating that it is a Gγ homologue in S. turcica, and was named as Stgg1. Then, the 398 bp downstream fragment and the 355 bp upstream fragment were obtained through genome walking. All these fragments were spliced into a 1129-bp sequence that included the 457 bp open reading frame (ORF) of Stgg1, which was divided into two introns and three exons. It showed an identity of 99.3% with the corresponding region in the genome of sequenced S. turcica strain Et28A and was deposited in GenBank with the accession number KT692660. The nucleotide sequence of the translation initiation site (TIS) was 5’-CACCATG-3’, which was coincident with the conserved sequence 5’-CA(C/A)(A/C)ATG-3’ of TIS in eukaryotes.

The predicted Stgg1 protein consists of 100 amino acid residues with a molecular mass of 11.32 kDa. Stgg1 showed relatively moderate identity with Gγ proteins in other filamentous fungi (Figure 1): 68.1% to Botrytis cinerea, 64.4% to Aspergillus nidulans, 65.6% to Neurospora crassa, 61.1% to Magnaporthe grisea, but only 30.8% identity with Saccharomyces cerevisiae and 13.9% with Schizosaccharomyces pombe. Identified by Conserved
Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), it possessed a GGL superfamily domain which was located between 29 and 100 aa (E-value = 1.64e-16) and is a typical characteristic of G protein.

BLAST searches revealed that only one G protein subunit exists in S. turcica, named as Stgg1. The predicted Stgg1 protein possesses the conserved CAAX box motif (CAAM in S. turcica) at the carboxy terminus, which is subject to posttranslational modification [8].

**Generation of Stgg1 knock-down mutants**

In this study, we adopted the RNA interference approach to further identify the role of Stgg1 in the development and pathogenicity control of S. turcica. We have previously reported that the cAMP pathway is essential for the pathogenicity of Setosphaeria turcica [14]. To gain more details of the G protein and cAMP signaling pathway mediated pathogenicity, we generated silenced clones of the G protein $g$ subunit (G $g$) gene Stgg1.

To select the Stgg1 knock-down mutants, we initially screened transformants that could grow on the hygromycin B selective media, among which 10 positive mutants were obtained. According to the semi-quantitative RT-PCR screen, we selected three independent silenced clones R3, R5 and R6 (Figure 2(A,B)). In these transformants, the silencing efficiency for Stgg1 increased in the order of R3 > R6 > R5 as seen from the semi-quantitative RT-PCR (Figure 2(A,B)). Intriguingly, we found that the mRNA levels of Stga1, Stga2 and Stga3 genes encoding G protein $\delta$ subunits were significantly decreased when Stgg1 expression was silent (Figure 2(C, D)). This phenomenon further confirmed the stable silencing of Stgg1 in the indicated transformants, which is also found in N. crassa and M. oryzae [10,12]. However, the expression of the Stgb1 gene encoding the G protein $\beta$ subunit was not affected (Figure 2(C,D)). Further studies must be carried out to understand the molecular mechanisms underlying the inter-regulation between G protein subunits.

**Stgg1 regulates hyphal morphology and is required for conidiation**

To determine whether Stgg1 regulates the growth and colony phenotype, the growth patterns of the indicated mutants were compared with the WT strain. As seen from Figure 3(A), no significant defect on the radial growth rates of the mutants was observed compared to the WT strain. However, the colony colour of the Stgg1 RNAi transformants was dramatically affected. As seen in Figure 3(A), WT strain colony exhibited greyish black colour, whereas the three independent transformants were pale. The aerial mycelia of the transformants were low, smooth-spread and flocculent, while that of the WT strain was compact and fascicular. In addition, the hyphal colour of the transformants was lighter than that of WT strains (Figure 3(B)). Furthermore, the silenced strains produced elongated hyphal cells (Figure 3(C)). Significantly, all the hyphal morphology defects in Stgg1 RNAi transformants mutants are highly compatible with the silencing efficiency. Moreover, 10-day-old transformants did not form any conidia, while the WT already produced a great deal of spores. We therefore inferred that Stgg1 played an important role in hyphal morphogenesis and was crucial for conidiation.

As demonstrated by some fungal pathogens, conidia germinate on the host plants, form germ tubes and...
differentiate into specialized infection structures called appressoria in response to physical and chemical signals [19]. Conidiogenesis and development of appressoria are key phases in host plant colonisation. Previous studies showed that G protein β subunit SfaD plays a crucial role in negative regulation of conidiation under submerged culture conditions [20], but α subunit GpgA seems not involved in this control process [11]. In this study, we showed that gene silencing of G protein γ subunit in S. turcica Stgg1 blocks conidia generation. As the silencing of Stgg1 completely abolished the ability of S. turcica to produce conidia, the same size of fungal disc instead was placed on the leaf to test the pathogenicity of Stgg1 mutants compared with the WT strain. The results showed that when the G γ subunit was silenced, the scab areas resulting from Stgg1 mutants were significantly smaller than that of the WT strain. These results provide evidence that S. turcica is able to cause foliar disease via hyphae-driven appressoria, once sensing environmental cues. Our studies will help to unveil the regulatory mechanisms involved in conidiation and virulence secondary metabolites that contribute to development of efficient strategies for Northern Corn Leaf Blight disease control.

Stgg1 is required for melanin biosynthesis and pathogenicity
Melanin was found to be an essential secondary metabolite related to the pathogenesis of S. turcica. The dramatic change in the colony colour of Stgg1 silenced transformants prompted us to check whether Stgg1 is essential for melanin biosynthesis. We extracted the intracellular melanin from the indicated strains. As shown in Figure 4(A,B), the melanin yields of R3, R5 and R6 were 0.064 g/L, 0.095 g/L and 0.116 g/L, respectively, which were significantly lower than 0.347 g/L in the WT strain. The defect of melanin biosynthesis was positively related with the silencing efficiency of Stgg1. This finding suggests that Stgg1 has a positive role in the regulation of melanin biosynthesis.

Another pathogenesis related secondary metabolite HT-toxin was also assayed. Crude HT-toxin extracted from the indicated strains was inoculated on the host leaves with wounded epidermis in vitro. After 24 h, the colour of tissues around the wound turned light brown. As shown in Figure 4(C), the affected areas on the leaves inoculated with the toxin from silenced transformants were all smaller compared to WT. The result above suggested that G protein γ subunit is required for HT-toxin generation.
We also performed a leaf infection assay to further check the dependency of \textit{stgg1} on the pathogenicity to the host. Since \textit{stgg1} is essential for conidiation, we then used fungal discs (\( \text{III} = 5 \text{ mm} \)) of the indicated strains to infect the corn leaves. After being inoculated on the susceptible hosts, both WT and \textit{Stgg1} silent mutants could cause elongated lesions. However, the lesions caused by the mutant strains were significantly smaller than that caused by WT. The higher \textit{Stgg1} silencing efficiency the mutants showed, the smaller lesions were caused (\textit{Figure 4}(D,E)). These results illustrated that the \textit{Stgg1} gene was essential for pathogenicity.

For some fungal pathogens in plants and animals, DHN melanin is considered to be an essential virulence factor. Melanin deficient mutants losing the ability to invade host cuticle eventually lose their pathogenicity [21,22]. In this study, the reduction of melanin content was positively related with the silencing efficiency of \textit{Stgg1}. During the infection process, the melanin deposit created the turgor pressure of appressorium [23]. Our results showed that the colony colour of the silent transformants was lighter and the amount of the melanin produced in the three silent transformants was decreased compared with the WT strain. In parallel, the activity of the other virulence factor HT-toxin was weaker in the silent \textit{Stgg1} transformants.

**Conclusions**

Our results provide evidence that G protein gamma subunit \textit{Stgg1} is crucial for the pathogenicity of \textit{S. turicica}. It is essential for the sporulation, which is a key step in disease epidemic of foliar pathogens, and it also positively regulates the biosynthesis of cellular melanin and HT-toxin, both of which are important pathogenic factors for \textit{S. turicica}. Although the downstream signal pathway and molecular mechanisms involved in these events are not well understood yet, this research still helps us illustrate the regulatory function of G-protein mediated signal pathways for the pathogenicity of pathogenic fungi.
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Disclosure statement

The authors declare no conflict of interests.

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ORCID

Yibin Lin http://orcid.org/0000-0003-0608-7875

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Figure 4. Intracellular melanin and pathogenicity analysis of Stgg1 knock-down mutants. (A) Melanin yield in Stgg1 knock-down mutants and WT. (B) Quantification of relative melanin yield in Stgg1 knock-down mutants compared to WT. (C) HT-toxin virulence analysis from Stgg1 knock-down mutants. (D) Pathogenicity test for Stgg1 knock-down mutants. (E) Quantification of the lesion area.
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