African Journal of Microbiology Research Vol. 6(3), pp. 680-689, 23 January, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR11.1389
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Involvement of the putative G-protein α subunit gene *pngpa1* in the regulation of growth, sensitivity to fungicides, and osmotic stress in *Phytophthora nicotianae*

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Accepted 19 December, 2011

A G-protein α (Gα) subunit encoding gene (*pngpa1*) was isolated from an oomycete plant pathogen *Phytophthora nicotianae,* the causal agent of severe diseases in a wide variety of crops. Comparison on the genomic DNA and cDNA sequence of *pngpa1* reveals that it contains an intron and encodes a polypeptide of 355 amino acid residues. The deduced amino acid sequence of PnGPA1 shares a high degree of identity with Gα subunits from oomycetes of the genus *Phytophthora*. Reverse transcription PCR analysis of *pngpa1* revealed that, unlike *psgpa1* and *pigpa1* which were expressed in only sporangia and zoospores but not in mycelia, *pngpa1* was expressed not only in sporangia and zoospores but also in mycelia. A modified *Agrobacterium*-mediated transformation method was used to silence *pngpa1* gene expression in *P. nicotianae* and phenotypic effects were analyzed. Our results suggested that *pngpa1* influenced multiple physiological processes in *P. nicotianae* including hyphal growth, zoospore release, osmo-sensitivity and tolerance to the fungicide metalaxyl.

Key words: *Phytophthora nicotianae*, G-protein α subunit, gene disruption, osmotic stress, tolerance to metalaxyl.

INTRODUCTION

Black shank, caused by *Phytophthora parasitica* var. *nicotianae* (syn. *Phytophthora nicotianae*), is one of the most important diseases of tobacco (*Nicotiana tabacum*) in the world (Melton et al., 2005). The pathogen can infect roots, stems, and leaves at any stage of plant growth, resulting in root necrosis, wilting, chlorosis, stem lesions, stunting, and plant death. Disease develops rapidly under conditions of high temperature and high humidity (Lucas, 1975). The annual losses of tobacco from black shank vary from 1 to 3%, which results in millions of dollars in lost revenue (Melton et al., 2005). At present, black shank is managed through transplantation, breeding resistant cultivars, crop rotation, and the application of the fungicide metalaxyl (Lucas, 1975). Since the first introduction of metalaxyl in 1977 in Western Europe, this fungicide has been used extensively to control diseases caused by oomycetes (Schwinn and Urech, 1986; Shaw and Shattock, 1991). Metalaxyl belongs to the phenylamide fungicide group and specifically inhibits rRNA biosynthesis, targeting RNA polymerase complex I of the pathogens (Gisi and Sierotzki, 2008). When first

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introduced against many important plant pathogenic fungi, it displayed excellent performance as systemic fungicide with protective, curative, and long-lasting activity (Davidse et al., 1991). However, tolerance to metalaxyl rapidly developed in *Phytophthora infestans* as well as other oomycetes soon after its application (Davidse et al., 1981; Schwinn and Urech, 1986; Erwin and Ribeiro, 1996). The origin of such tolerance is largely unknown, but has been speculated to be due to simple mutation. Since metalaxyl acts at a single target site, a mutation might easily lead to tolerance by changing the target site affinity (Schwinn and Margot, 1991; Goodwin et al., 1992). However, genes responsible for tolerance have been shown to have different chromosomal locations in different isolates (Fabritius et al., 1997). The occurrence of metalaxyl tolerance in aggressive populations of *P. infestans* raised the possibility that metalaxyl tolerance might be associated with pathogen fitness (Cohen et al., 1983; Day and Shattock, 1997; Goodwin et al., 1996). However, subsequent analysis of oospore progeny indicated that metalaxyl tolerance and pathogen fitness were not readily linked (Gisi and Cohen, 1996). Although there have been many investigations on the genetic basis of phenylamide tolerance, the responsible genes and the nature of the mutations still remain unknown (Gisi et al., 2000; Shattock, 2002).

Heterotrimeric G proteins (G proteins) are known to play a vital role in the signal transduction cascades responding to a variety of environmental stimuli such as drugs, light, odor, neural transmitters and hormone perception in mammals (Li et al., 2007). Recently, the biological functions of the G-protein signaling pathways were partly revealed in the genus *Phytophthora* (Laxalt et al., 2002; Latijnhouwers et al., 2004; Hua et al., 2008). A Gα subunit encoding gene *pigpa1* and its Gβ analog *pigpb1* were isolated from the oomycete *P. infestans* both genes were found differentially expressed in the different developmental stages of *P. infestans*, with the highest expression in sporangia (Laxalt et al., 2002). Mutants lacking the Gβ subunit demonstrated defects in vegetative growth and sporulation (Latijnhouwers and Govers, 2003). Likewise, *Pigpa1*-silenced mutants suggested that PiGPA1 is crucial for zoospore motility and pathogenicity (Latijnhouwers et al., 2004). Recently, a Gα subunit gene *psgpa1* in *Phytophthora sojae* was found to negatively regulate calcium signaling pathway, thereby impacting zoospore chemotaxis (Hua et al., 2009). Further work suggested that Gα subunit of *P. sojae* not only participated in soybean isoflavone-mediated chemotaxis, but also contributed to locate penetration site in the host plant and influence the expression of regulators of G protein-controlled signaling pathways (Hua et al., 2008). Moreover, the *Ustilago maydis* cAMP-dependent protein kinase (PKA) was implicated in tolerance to the dicarboximide and aromatic hydrocarbon fungicides (Ramesh et al., 2001). Therefore, the G-protein signaling pathway may also be involved in the regulation of sensitivity to fungicide in *P. nicotianae*.

In this study, a Gα subunit gene *pngpa1* from *P. nicotianae* was cloned and characterized, and the gene *pngpa1* was disrupted by the *Agrobacterium*-mediated transformation. The phenotypic features of the mutant and the wild-type strain were compared, and the potential functions of the gene *pngpa1* in *P. nicotianae* were discussed.

MATERIALS AND METHODS

Strains and culture conditions

P. nicotianae isolates (62804, 2407) used in this study were provided by the Yunnan Tobacco Research Institute, and maintained in sterile distilled water as described by Liou et al. (2002). For preparation of DNA and RNA, isolates were grown on 5% V-8 juice medium (V8M) (Liou et al., 2002) at 25°C in the dark for 5 to 7 days. The mycelia were harvested by filtration according to the method of Mitchell et al. (2002).

Agrobacterium tumefaciens EHA105 was obtained from Dr. Zhang LM in our lab. Plasmid pPK2 was obtained from the Fungal Genetics Stock Center (Kansas, USA) as a gift.

Amplification of gene *pngpa1* and the corresponding cDNA

Genomic DNA was extracted from approximately 40 mg of fresh mycelia using the CTAB method (Zhang et al., 1996). The degenerate primers GpaS and GpaA were designed according to conserved regions of Gα subunit genes from other fungi (Table 1). PCR was carried out according to our previous report (Luo et al., 2008). A 570-bp conserved sequence was amplified and subsequently cloned into a PMD18-T vector for sequencing. Two sets of primers (TSP5-1/5-2/5-3 and TSP3-1/3-2/3-3) (Table 1) were designed according to the conserved sequence, and the 3' and 5' flanking sequences of the *pngpa1* gene were amplified using DNA Walking SpeedUp Premix Kit (Seegene, Korea). The complete *pngpa1* gene was obtained by assembling the sequences from the above PCR fragments using the DNAman software package (Version 5.2.2, Canada).

Total RNA was isolated from *P. nicotianae* according to the manual of TRIzol Reagent (Invitrogen, America). Total RNA was checked for DNA contamination and residual DNA was removed by DNase treatment. The 1st strand cDNA of the gene *pngpa1* was synthesized from sporangia using PrimerScript 1st strand cDNA synthesis kit (Takara, Japan) according to the manufacturer's instructions, and the cDNA was amplified using primers Gpa1 and Gpa2 (Table 1). The PCR product was cloned into a PMD18-T vector and sequenced. The intron was identified by aligning the DNA and cDNA sequences using the DNAman software package.

Expression analysis of the *pngpa1* gene

The expression patterns of the *pngpa1* gene during three developmental stages of *P. nicotinae* were analyzed using reverse transcription PCR (RT-PCR). Specifically, the total RNAs were isolated from mycelia, sporangia and zoospores, and the 1st strand cDNA was synthesized respectively. RT-PCR was then performed using primers Gpa1 and Gpa3 with the 1st strand cDNA obtained from mycelia, sporangia and zoospores as template, respectively.

Disruption of the gene *pngpa1*

To construct the plasmid for disrupting *pngpa1*, a 1132-bp fragment

Table 1. Primers used in this work.

Primers	Oligo sequence (5'-3')	Description
GpaS	AC(C/G)(A/G)T(C/T)(C/T/G)T(A/C/G/T)AAGCA(G/T)ATGA	Amplify the conserved sequence of *pngpa1*
GpaA	CAGTG(A/G)ATCCACTTCTT(A/T/C/G)CG	
TSP5-1	CCTTCAGTTTCATTTCCGTACACT	Amplify the 5' flanking sequence of *pngpa1*
TSP5-2	GTCACCACGTTGTTATAGATAATGG	
TSP5-3	CTCTTCGGTTAGAGCTGCACCATAC	
TSP3-1	GAGTTCCAGATCATCGAGTCAGTC	Amplify the 3' flanking sequence of *pngpa1*
TSP3-2	CAGGATATGTTGTACGCACGTGTG	
TSP3-3	ACCAGATCGATGGCGCTACCTTC	
Gpa1	ATGGGGCTCTGTGCTTCC	Amplify the cDNA of *pngpa1*
Gpa2	CTACATGAAGCCGGAGCCCTTG	
Gpa1	ATGGGGCTCTGTGCTTCC	Primers for RT-PCR
Gpa3	CCTTCAGTTTCATTTCCGTACACT	
5f	ACAGATATCTCATGGACATGCCACGTAGT*	Amplify the 5' fragment for constructing pPK2-1
5r	AACGATATCACGTCCTCGAAGCAGTGAAT	
3f	ACATCTAGAACGAGCTGGGAGTGCAGTAT	Amplify the 3' fragment for constructing pPK2-1
3r	AACAAGCTTCTGACGCAGCACCTTGTAGA	
5r	AACGATATCACGTCCTCGAAGCAGTGAAT	Confirmation of the insert direction of 5' fragment
Pgpd	ACCACCCCTCCAGTTCTTCT	
5S	GACCCATTCGGTTGTTTG	Confirmation of the transformants
5A	TCAGCCTGGAAAGCAAAT	
3S	GGTCGCCAACATCTTCTTC	
3A	CGACATTCCACGCTTGCT	

*The restricted sites are underlined.

containing the 5' end of the *pngpa1* gene was first amplified using primers 5f and 5r (Table 1). Upon restriction digest with *Eco*RV enzyme, the fragment was inserted into *Eco*RV-cut pPK2 plasmid harboring the hygromycin-resistant gene *hph* (Covert et al., 2001). Subsequently, the resulting plasmid was digested with *Xba*I/*Hin*dIII and a 940-bp fragment containing the 3' end of the *pngpa1* gene was amplified using primers 3f and 3r (Table 1) and inserted into the plasmid. The recombinant plasmid pPK2-1 was confirmed by PCR and restriction digestion. Finally, the disruption vector pPK2-1 was transformed into *A. tumefaciens* EHA105 and transformants were selected on Luria-Bertani agar (LB) supplemented with rifampicin (25 µg/ml) and kanamycin (50 µg/ml).

A fresh colony of *A. tumefaciens* EHA105 containing the disruption plasmid was cultured overnight at 28°C in LB broth supplemented with 25 µg/ml rifampicin and 50 µg/ml kanamycin. The *A. tumefaciens* overnight culture was diluted (1:100) into minimal medium (10 mM K_2HPO_4, 10 mM KH_2PO_4, 2.5 mM NaCl, 2 mM $MgSO_4$, 0.7 mM $CaCl_2$, 9 µM $FeSO_4$, 4 mM $(NH_4)_2SO_4$, 10 mM glucose) supplemented with 25 µg/ml rifampicin and 50 µg/ml kanamycin and incubated overnight at 28°C. The *A. tumefaciens* cells were diluted to an optical density of 0.15 (measured at 660 nm) in the induction medium (IM) [50 ml minimal medium, 40 mM 2-(N-morpholino) ethanesulfonic acid, 0.5% glycerol (w/v), 200 µM acetosyringone, pH 5.3] and cultured at 28°C for 4 to 6 h with gentle shaking until an OD_{660} of 0.6 to 0.8 was reached. Before co-cultivation, the cells were washed twice with an equal volume of sterile water.

Zoospores and mycelia were co-cultured with *A. tumefaciens*. 1 ml of *A. tumefaciens* containing the vector pPK2-1 was added to 50 ml of sterile water containing zoospores (approximately 3×10^7 zoospores). The mixture was incubated for 2 h at room temperature and harvested by centrifugation at 260 g for 5 min. The harvested mixture was plated on either IM agar plates covered with filters or 10% V8M plates and 400 µg/ml cefotaxim was added to kill the *A. tumefaciens* and 200 µg/ml hygromycin B to select the *P. nicotiane* transformants. After co-cultivation for 7 days in the dark the filters containing both zoospores and *A. tumefaciens* were transferred to 10% V8M plates. Meanwhile, mycelia were cultured in flasks containing 100 ml of 10% V8M at 25°C for 3 to 5 days with gentle shaking, and mycelia were harvested by filtration through sterile miracloth. Mycelia were inoculated with bacteria for 30 min and then transferred onto IM agar plates covered with cellophane for 7 days

Figure 1. Partial nucleotide and deduced amino acid sequences of the *Phytophthora nicotianan* Gα subunit gene *pngpa1*. Deduced amino acid sequence is shown below nucleotide sequence and numbered at left. The initiation and stop codons are underlined. Intron is displayed with a gray background. A putative promoter is displayed with a pane. The deduced locations of the G1-G5 regions of the GTPase domain (Gilman, 1987) are indicated by single lines below the alignment.

at 25°C, and then transferred to 10% V8M plates.

Selection and characterization of transformants

Transformants were further confirmed by PCR amplification using two pairs of primers (Table 1). The stability of transformants was tested according to our previously reported method (Zhang et al., 2008). The wild type strain and transformants were respectively transferred to oat meal agar (OMA) plates supplied with 200 µg/ml hygromycin B, and incubated for 7 days at 25°C. The growth rate and colony morphology of mutants were compared with the wild type strain on OMA plates. The tolerance to metalaxyl was determined by transferring mycelial plugs onto OMA plate containing metalaxyl at 0, 0.01, 0.05, 0.2 µg ml^{-1} concentrations. The sensitivity to osmotic stress was determined on OMA plate supplemented with NaCl at concentrations of 0, 2 and 4%. Each experiment was performed three times. Culturing and production of sporangia and zoospores from *P. nicotianae* were done according to previously reported methods (Mitchell and Hardham, 1999; Ambikapathy et al., 2002).

RESULTS

Cloning and analysis of the *pngpa1* gene

A 570 bp fragment corresponding to the conserved sequence of gene *pngpa1* was amplified using degenerate primers GpaS and GpaA (Table 1), and two fragments of 660-bp and 1258-bp were amplified from the 5' and 3' flanking regions of the conserved sequence by DNA walking technique. The full-length *pngpa1* gene was obtained by assembling the sequences from above fragments, yielding a 2728-bp fragment, which contains a 1150 bp open reading frame (ORF), and a 82 bp intron was identified by aligning the genomic DNA and cDNA sequences (Figure 1). A putative promoter from position at -256 to -305 bp upstream of the start codon ATG was identified using the BDGP Neural network promoter prediction interface (http://www.fruitfly.org/seq_tools/promoter.html

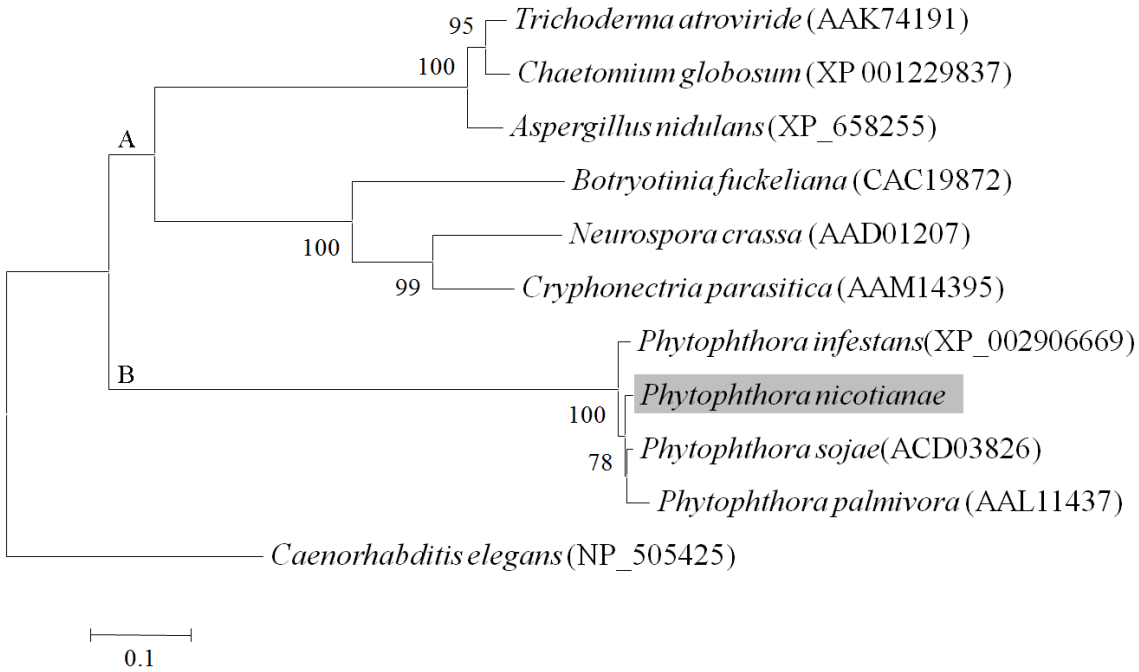

Figure 2. Phylogenetic tree of Gα subunit from different fungi and oomycetes. The tree was constructed with the MEGA version 4.0 program packages. UPGMA method was used with Poisson correction for multiple amino acid substitution and with 1000 random bootstrap replicates. The Gα subunit of *Caenorhabditis elegans* (NP_505425) was used as an outgroup in order to root the tree. The oomycete *P. nicotiane* was showed in gray.

(Reese, 2001). The gene *pngpa1* encoded a polypeptide of 355 amino acid residues. The calculated molecular mass and the p*I* of the putative protein were 40.8 and 5.24 kDa, respectively. The gene *pngpa1* and its corresponding amino acid residues are shown in Figure 1. The PnGPA1 has no signal peptide as predicted using the SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/). The nucleotide sequence of gene *pngpa1* had been deposited in GenBank, under the accession number HQ603677.

The deduced protein PnGPA1 of *P. nicotianae* shared a high degree of sequence identity (95-98%) to the Gα subunits from other oomycetes *P. infestans*, *P. sojae* and *P. palmivora*. The GTPase domain was predicted in PnGPA1 (Gilman, 1987). A phylogenetic tree (Figure 2) was constructed based on the deduced protein sequences of Gα subunits from oomycetes and fungi by the MEGA 4.0 program package. These Gα subunits were clustered into two clades, and the Gα subunits from fungi *Trichoderma atroviride*, *Chaetomium globosum*, *Aspergillus nidulans*, *Botryotinia fuckeliana*, *Neurospora crassa*, and *Cryphonectria parasitica* were clustered into clade A, while *P. nicotianae* and other three oomycetes were clustered into clade B.

RT-PCR analysis of gene *pngpa1*

According to the DNA and cDNA sequences of *pngpa1* (Figure 1), the specific primers for Gpa1 were designed (Table 1). RT-PCR was performed using the 1st strand cDNA as template, and a 250-bp PCR product was amplified from mycelia, sporangia and zoospores (Figure 3A), respectively. The results showed that the gene *pngpa1* was expressed not only in sporangia and zoospores but also in mycelia of *P. nicotianae*.

Disruption of the *pngpa1* gene

The disruption vector pPK2-1 (Figure 4A) was constructed, plasmid pPK2-1 consists of the pgpdA:hph:trpC cassette flanked by the 5' and 3' regions of the *pngpa1* gene. The pgpdA:hph:trpC marker cassette contains the promoter region of the gpdA gene encoding glyceraldehyde-3-phosphate dehydrogenase and the terminator region of the *Aspergillus nidulans* trpC gene, as well as antibiotic resistance selectable markers for both hygromycin B and kanamycin. The plasmid pPK2-1 was transformed into the competent cells of *A. tumefaciens* strain EHA105 and positive transformants were isolated on LB supplemented with rifampicin (25 µg/ml) and kanamycin (50 µg/ml).

Zoospores and mycelia were co-cultivated with *A. tumefaciens* containing the plasmid pPK2-1, respectively, and transformants (hygromycin-resistance isolates) were obtained under both conditions. The putative transformants were transferred to selective 10% V8M plates,

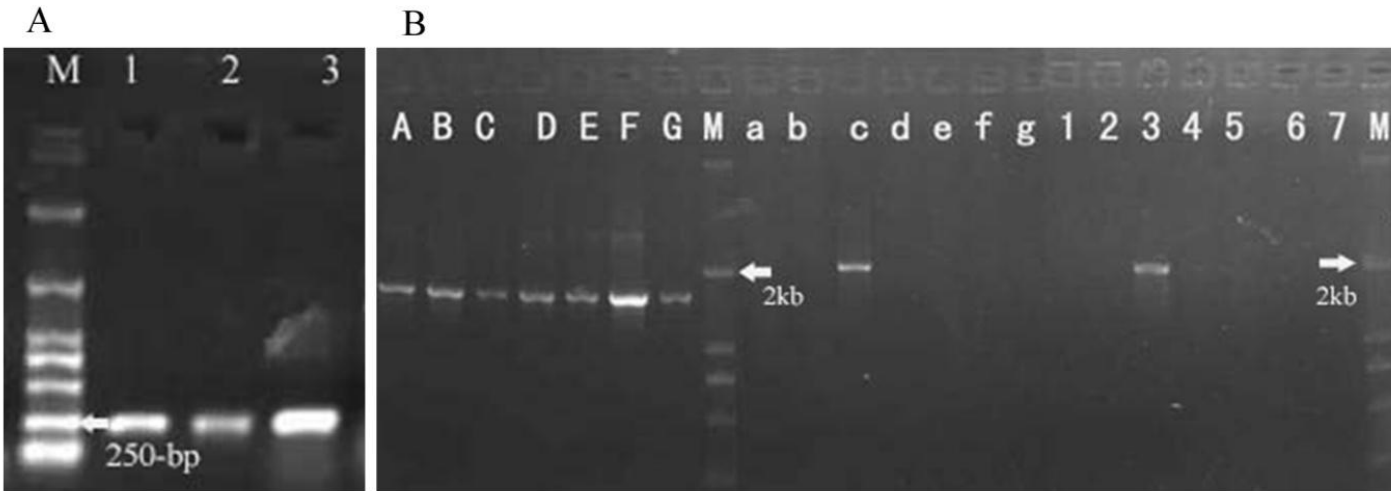

Figure 3. Agarose gel electrophoresis of PCR amplification products. A. RT-PCR analysis of gene *pngpa1* from *P. nicotiane*. Lanes 1, 2 and 3 were the RT-PCR results using total RNA from the mycelia, sporangia and zoospores as template, respectively. Lane M: DNA Marker. B. Screening and verification of positive transformants by PCR amplification. Genomic DNAs from seven transformants were used as template respectively, lane A-G with primers 5f+Pgpd, lane a-g with primers 5S+5A, Lane 1-7 with primers 3S+3A.

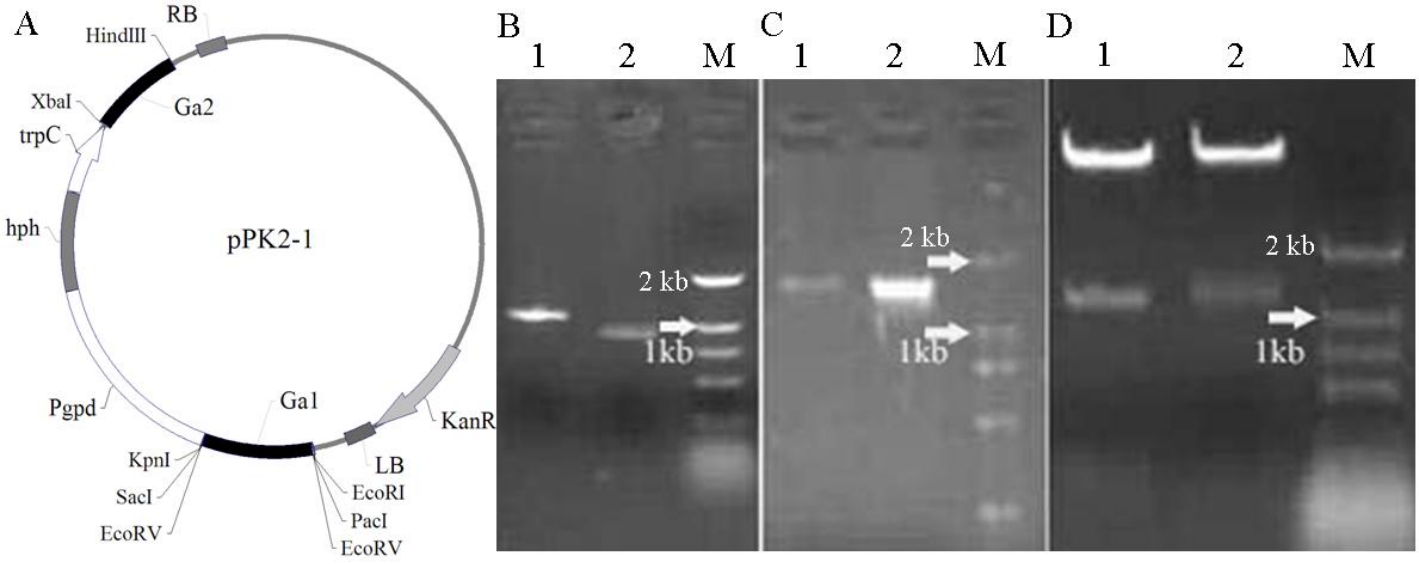

Figure 4. Construction of plasmid pPK2-1. A. The map of plasmid pPK2-1 generated by the program Redasoft visual cloning (version 3.2). The T-DNA is approximately 5 kb running clockwise in this drawing from the left border (LB) to the right border (RB). KanR, kanamycin resistance gene for selection in bacteria; Pgpd, *Aspergillus nidulans gpd* promoter; hph, hygromycin B resistance gene for selection in fungi; trpC, *A. nidulans trpC* terminator; Ga1, 5' terminal sequence of gene *pngpa1* (1132 bp); Ga2, 3' terminal sequence of gene *pngpa1* (940 bp). B. PCR amplification of Ga1 (Line 1) and Ga2 (Line 2). C. PCR amplification for verifying the inserted direction of Ga1 (Line 1 and Line 2). D. Plasmid pPK2-1 was digested using restriction enzyme. Line 1, Plasmid was digested by *Xba*I and *Hind*III. Line 2, was digested by *Eco*RV. M: DNA marker (DL2000).

and confirmed by PCR amplification using three pairs of primers. A 1.6-kb (1132+503) fragment (Figure 3B) was amplified from all the seven transformants using primers 5f and Pgpd (Table 1), while a 1.88-kb (1240+641) and a 2.1-kb (979+354+768) fragment was amplified from the positive transformants using primers 5S/5A and 3S/3A, respectively. One positive transformant (*Δpngpa1*) was chosen for subsequent phenotype analyses.

Phenotypic properties of the *Δpngpa1* mutant

The *Δpngpa1* deletion mutant showed a high degree of mitotic stability. Upon continuous incubation on OMA

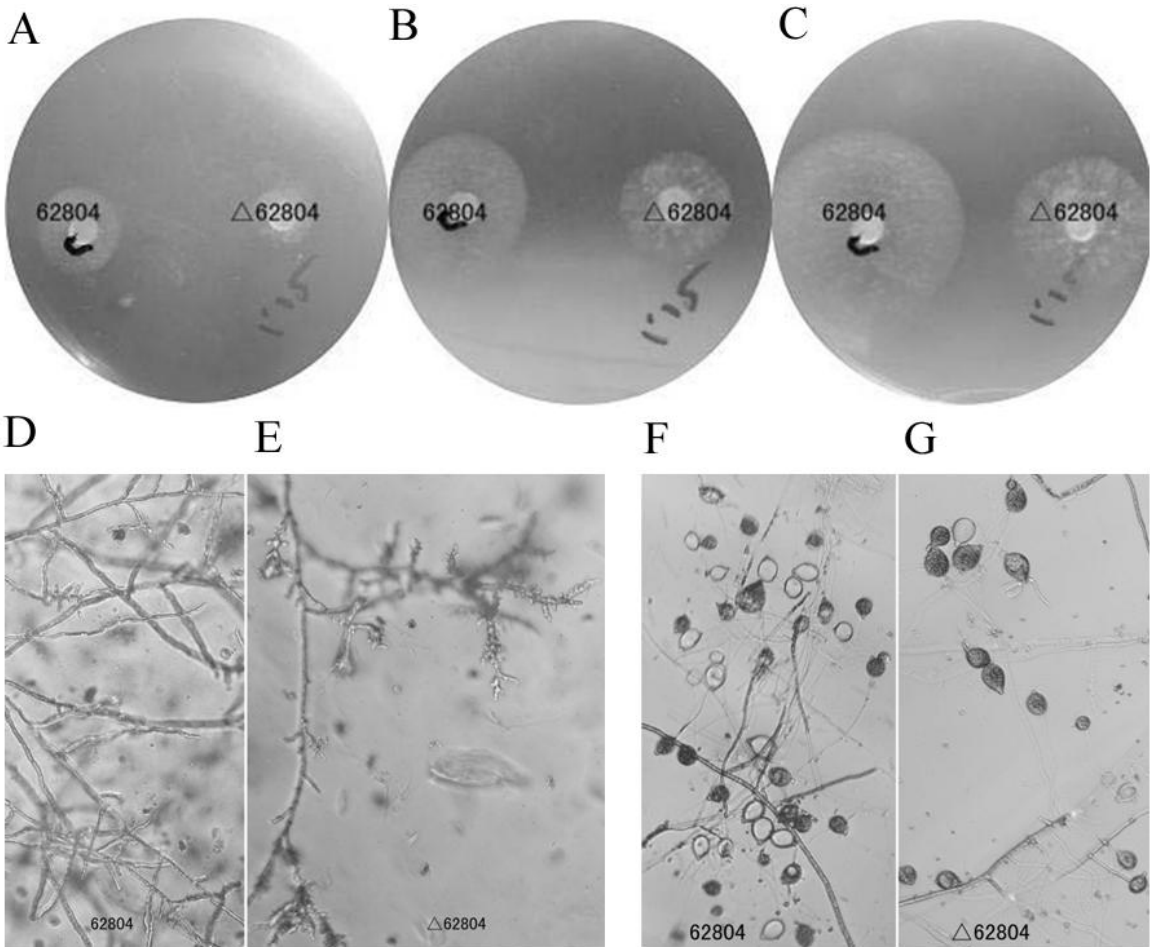

Figure 5. Comparison of growth, mycelia morphology and zoospores between the wild type strain and mutant (*Δpngpa1*). A, B and C. Mycelia grew on oat media for 2, 3 and 4 days, respectively. D and E. Mycelia morphology observed under a light microscope. F and G. Zoospores released from sporangia.

plates for five transfers, no change in genotype and drug resistance was found. Compared to the wild-type parental strain, the mutant showed similar morphological properties on the non-selective medium. However, its growth rate was much lower than that of the wild type strain (Figure 5A, B and C). After incubation for 7 days at 25°C, the colony diameter of the *Δpngpa1* deletion mutant was 5.0 cm, while that of the wild type was 9.0 cm. Under microscope, hyphae of the mutant showed less branching (Figure 5D, E), and both the sporangia producing ability and zoospore releasing ability decreased (Figure 5F, G). For example, the number of average sporangia under two visual fields (10 × 16) under the light microscope was 22.7 for the wild strain and only 9.3 in the *Δpngpa1* deletion mutant.

Growth of both the wild type strain and the *Δpngpa1* deletion mutant was inhibited on OMA plate containing NaCl (Figure 6A). However, the growth of the mutant decreased more than the wild type with almost complete inhibition on the OMA plate containing 4% NaCl (Figure 6B, C). A noticeable effect was also observed for metalaxyl tolerance, albeit in a reverse pattern. The growth of the wild strain was progressively inhibited with increasing metalaxyl concentration (Figure 6D, E, F and G), while the growth of the deletion mutant and metalaxyl-resistance strain (isolate 2407) showed no obvious difference among the tested metalaxyl concentrations. This result suggests that the gene *pngpa1* is involved in metalaxyl tolerance.

DISCUSSION

Since the first report of G proteins in filamentous fungi in 1993, it has been demonstrated that G proteins are essential for growth, asexual and sexual development, and virulence in both animal and plant pathogenic filamentous species (Li et al., 2007). G proteins are evolutionary conserved GTP-binding proteins that are composed of α, β, and γ subunits and participate in

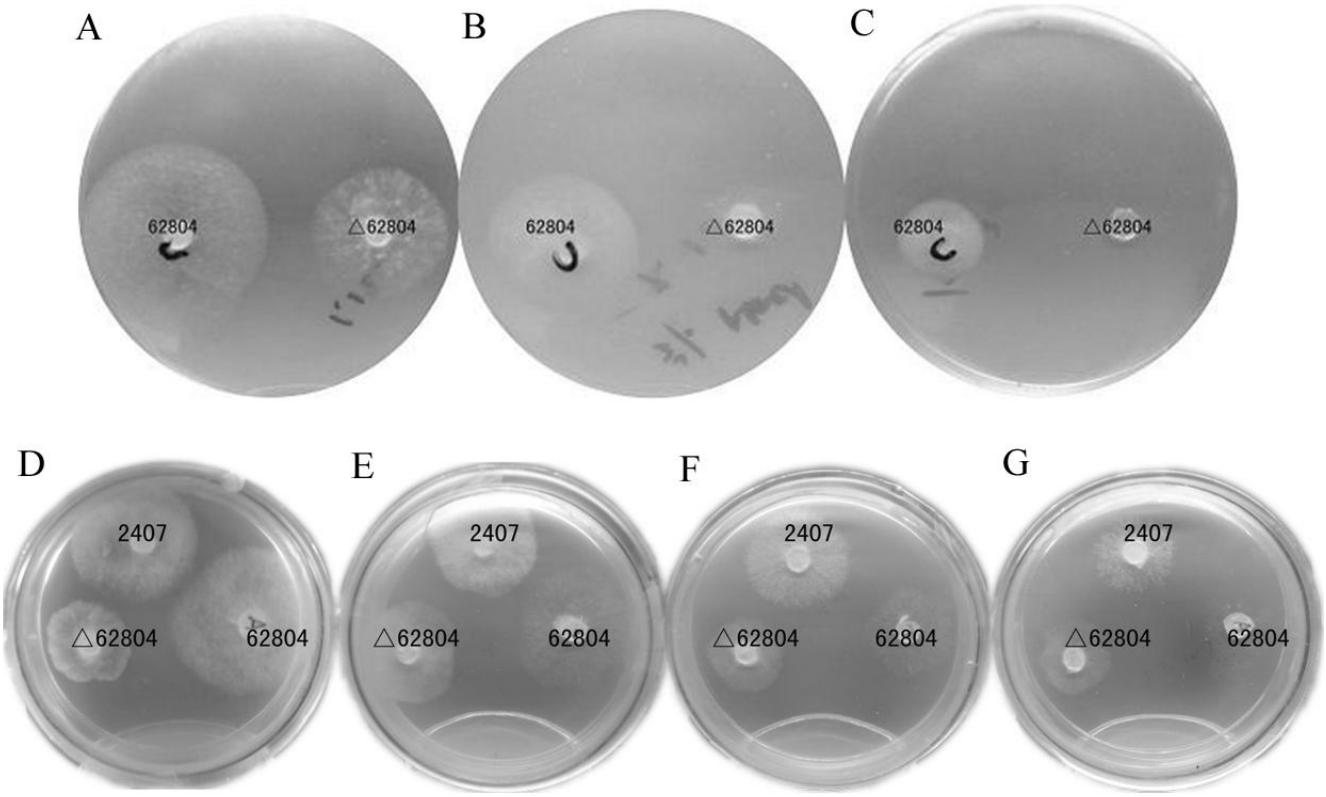

Figure 6. Mycelia grew on different concentrations of NaCl and metalaxyl. A, B and C. Sensitivity of mutant (*Δ*) and wild-type (62804) strains to osmotic stress. A, without NaCl; B, 2% NaCl; C, 4% NaCl. D, E, F and G. Resistance of mutant (*Δ*62804) and wild-type (62804, 2407) strains to metalaxyl. D, without metalaxyl; E, 0.01 μg/ml metalaxyl; F, 0.05 μg/ml metalaxyl; G, 0.2 μg/ml metalaxyl.

diverse signal transduction pathways (Neves et al., 2002; Li et al., 2007). Recently, several Gα and Gβ subunits were cloned from oomycete *Phytophthora*, and they were found involved in vegetative growth, sporulation, zoospore motility and pathogenicity (Laxalt et al., 2002; Latijnhouwers and Govers, 2003; Latijnhouwers et al., 2004; Hua et al., 2008). In this study, a putative Gα subunit gene *pngpa1* was isolated from *P. nicotianae*. The conserved sequence of *pngpa1* was amplified using a pair of degenerate primers designed according to the Gα subunit genes from *Phytophthora* and other fungi, and the 5' and 3' flanking sequences were amplified using DNA walking technique. The predicted amino acid sequence of PnGPA1 showed a high degree of similarity to reported Gα subunits from *Phytophthora*, and all conserved characteristic key structural motifs necessary for GTPase activity were present (Figure 1) (Gilman, 1987). The high sequence conservation of Gα subunits from *Phytophthora* suggested this gene is very important for growth and may play similar roles among these oomycetes.

Previous studies showed *Phytophthora* species possess only one Gα subunit gene and one Gβ subunit gene (Laxalt et al., 2002), and the Gα subunit gene *pigpa1* was differentially expressed among the different developmental stages of *P. infestans*, with the highest expression in sporangia (Laxalt et al., 2002). The *P. sojae psgpa1* gene was specifically expressed in sporangia and zoospores but not in mycelia. Whereas *psgpb1* and *pigpb1* show the opposite pattern, being expressed in mycelia but not in zoospores (Laxalt et al., 2002; Hua et al., 2008). Unlike these reports, the gene *pngpa1* was expressed not only in sporangia and zoospores but also in mycelia of *P. nicotianae* (Figure 3A), which suggested that it might play a role in the mycelia development. However, the potential reasons for the differential expression of Gα subunit gene in different *Phytophthora* species still remain to be elucidated.

The *Δpngpa1* mutant showed obvious physiological difference from the wild type strain: the hyphal growth was inhibited and the releasing of zoospores was reduced, a result similar to the functions of Gβ in *P. infestans* (Latijnhouwers and Govers, 2003). Interestingly, the *Δpngpa1* mutant was more sensitive to osmotic stress, while the tolerance to metalaxyl was increased, indicating that the *pngpa1* gene was involved in the regulation of osmotic stress response and tolerance to metalaxyl. Previous studies showed that the

adr1 gene, which encodes the catalytic subunit of the *U. maydis* cAMP-dependent PKA, is implicated in fungicide tolerance (Ramesh et al., 2001). The ***Δadr1*** mutant also showed hypersensitivty to osmotic pressure and tolerance to dicarboximide (Ramesh et al., 2001). The cAMP-dependent PKA is a downstream member of G protein signaling pathway (Li et al., 2007). In these fungi, defects in components in G protein signaling pathway may influence both resistance and osmotic sensitivity, these fungi may share similar mechanisms to respond to fungicides. Moreover, these results suggest that a general connection may exist between fungicide tolerance, osmotic stress, and signaling pathway. However, the modes of regulation of *pngpa1* in *Phytophthora* are largely unknown. Our results revealed the negative role of the gene *pngpa1* to metalaxyl tolerance in *P. nicotianae*. We thus speculate that the Gα subunit gene *pngpa1* may cause fungicide insensitivity by directly altering a target or by indirectly influencing the expression of the target factor so that elevated levels overcome inhibition.

In summary, our results provide novel insights into the role of Gα subunit gene *pngpa1* in *P. nicotianae*, providing a basis for further studies for identifying specific target(s) of fungicide and deciphering connections between these targets and G protein-mediated tolerance.

ACKNOWLEDGEMENTS

We are grateful to Prof. Jianping Xu (McMaster University, Canada) for his critical reading of this manuscript. This work was supported by projects from the National Natural Science Foundation of China (31060012), the West Light Foundation of the Chinese Academy of Sciences (to Jinkui Yang), and by projects from the Department of Science and Technology of Yunnan Province (approved no. 2009CI052).

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