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Scientific Research

Construction of a Constitutively Activated G*α* Mutant in the Maize Pathogen *Cochliobolus heterostrophus*

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ABSTRACT

Conserved eukaryotic signaling proteins participate in development and disease in plant pathogenic fungi. Mutants in *CGA*1, a heterotrimeric G protein Gα subunit gene of the maize pathogen *Cochliobolus heterostrophus*, are defective in several developmental pathways. Conidia from *CGA*1 mutants germinate as abnormal, straight-growing germ tubes that form few appressoria, and the mutants are female-sterile. The *CGA*1, G*α* subunit, is also thought to act as a down regulator of hydrophobin expression and secretion in this fungus and in related Ascomycete species. Although *cga*1 mutants can cause normal lesions on plants there are host physiology conditions under which full virulence requires signal transduction through *CGA*1-mediated pathways. A G*α* activated mutant, *cga*1^{Q204L} was created to help establish the role of *CGA*1 in growth and development, and in mediating hydrophobin secretion and expression. The activated G*α* allele was transformed into a *cga*1 mutant strain. The transgenic lines showed phenotypes resembling the null mutant in development, sporulation and hydrophobicity, indicating a possible role for *CGA*1 as a stabilizer of these traits.

Keywords: *Cochliobolus*; Constitutively Activated; G-Alpha Subunit; G-Protein; Fungal; Signal Transduction

1. Introduction

Mutants in genes encoding conserved eukaryotic signal transducing proteins have been very helpful in efforts to understand the environmental mediated control of development and the sensory pathways needed to detect the host and establish invasive growth. Several such mutants have been constructed for the maize pathogen *Cochliobolus heterostrophus*, agent of Southern corn leaf blight [1-4]. In Ascomycetes for which sufficient sequence information is available, there are three G*α* encoding genes, one G*β* and one Gγ gene. Deletion of the MAP kinase gene *CHK*1 [3] or the G*β* gene *CGB*1 [1] has a vast effect on growth and development and is drastically reducing virulence under all conditions tested. Mutants in *CGA*1, a heterotrimeric G protein Gα subunit, produce conidia that germinate as abnormal, straight-growing germ tubes forming few appressoria [5]. Nevertheless, these mutants can cause normal lesions on plants, unlike other filamentous fungal plant pathogens in which functional homologues of *CGA*1 are required for full virulence [2]. This demonstrates that appressorium formation is not essential for virulence. Indeed, inoculation with mycelium results in growth on the leaf surface followed by penetration into the leaf without noticeable appressorium formation: sometimes aggregates of mycelia localize to stomatal apertures, but it seems that direct penetration of the epidermis is also possible. Detailed examination indicated that under some host physiology conditions, *CGA*1 disruption and deletion mutants are considerably less virulent [6]. In addition disruption of the *CGA*1 gene causes aerial growth formation and spores aggregation that indicates a possible role for *CGA*1 in regulation of hydrophobin secretion [5]. Determination of *Cochliobolus heterostrophus* hydrophobins expression in *cga*1 mutants provided the molecular evidence for the role of *CGA*1 in suppression of hydrophobins expression [5].

Although, Gene disruption studies are an efficient way to identify the role of signaling components such as the G-protein subunits and the MAPK cascade, a constant activation of desired genes became, in recent years, a powerful genetic tool to accomplish the information resulting from the disruption experiment and to identify new functions. Site specific mutagenesis (such as Q204-L, G42-R and R178-C) designed to constitutively activate G*α* signaling was reported in *C. parasitica* [7], *M. grisea*

[8], *N. crassa* [9] and in *A. nidulans* [10]. Conversion of one of these amino acids abolishes GTPase activity, which in turn would constitutively activate G protein signaling. Here we constructed a constitutively activated Gα allele (*cga*1^{Q204L}) to investigate the role of *CGA*1 in developmental processes. In particular, we examined its influence on hydrophobin assosiated traits.

2. Materials and Methods

2.1. Strains

Wild type *C. heterostrophus* strain was C4 (*MAT*1-2; *Tox*1 + ATCC 48331; abbreviated in figures as WT C4). Mutant, previously developed [2], in the G protein *α* subunit gene *CGA*1 was: C5Δ*cga*1 (*MAT*1-1 *tox*-cga1, created by insertion of the hygromycin cassette into the coding region, combined with an 18 bp deletion).

2.2. Construction of the Q204L Mutation

A specific sequence change in the pCGA1-Bar plasmid (**Figure 1(a)**) was done using primed amplification by the polymerase chain reaction (PCR). The method is based on the amplification of the entire plasmid using primers that include the desired changes. The site directed mutagenesis (Q204L mutation) was done here to change glutamine (Q, coded by c**a**g) to leucine (L, coded by c**t**g) by replacing the nucleic acid adenine (a) with thymine (t). As result of this point mutation the GTPase activity should be abolished and the CGA1 gene is constantly activated.

Vector preparation. A plasmid (pCGA1-Bar, 6338 bp, **Figure 1(a)**) containing pBluescript (2918 bp), *CGA*1 gene (2387 bp) and bar expression cassette (1015 bp) for Bialaphos antibiotic resistance, was used as a template for the synthesis of the desirable vector.

Enzyme restriction reaction. ApaI, BstxI and HindIII were used in order to confirm the construction of the plasmid and the presence of the *CGA*1 insert. First examination was done by addition of 1 μl ApaI to 2 μl reaction buffer #4 (New England Biolabs) 0.5 μl BSA, 2 μl DNA (pCGA1-Bar plasmid) and 14.5 μl DDW, and incubation at 25˚C for 1 hour. Alternatively, 1 μl BstxI, was added to 2 μl reaction buffer #3 (NEB), 2 μl DNA (pCGA1-Bar plasmid) and 15 μl DDW, and the reaction was incubated at 55˚C for 1 hour. Third examination was done by adding 1μl HindIII to 2 μl reaction buffer #2 (NEB), 2 μl DNA (pCGA1-Bar plasmid) and 15 μl DDW, and incubation at 37˚C for 1 hour (**Figure 1(b)**).

Site directed mutagenesis. Site mutagenesis was conducted with "QuikChange® site-directed Mutagenesis Kit" (Qiagen) according to the manufacturers protocol. Two complimentary oligonucleotides were synthesized to contain the Q204L mutation. Primers: Q204L forward

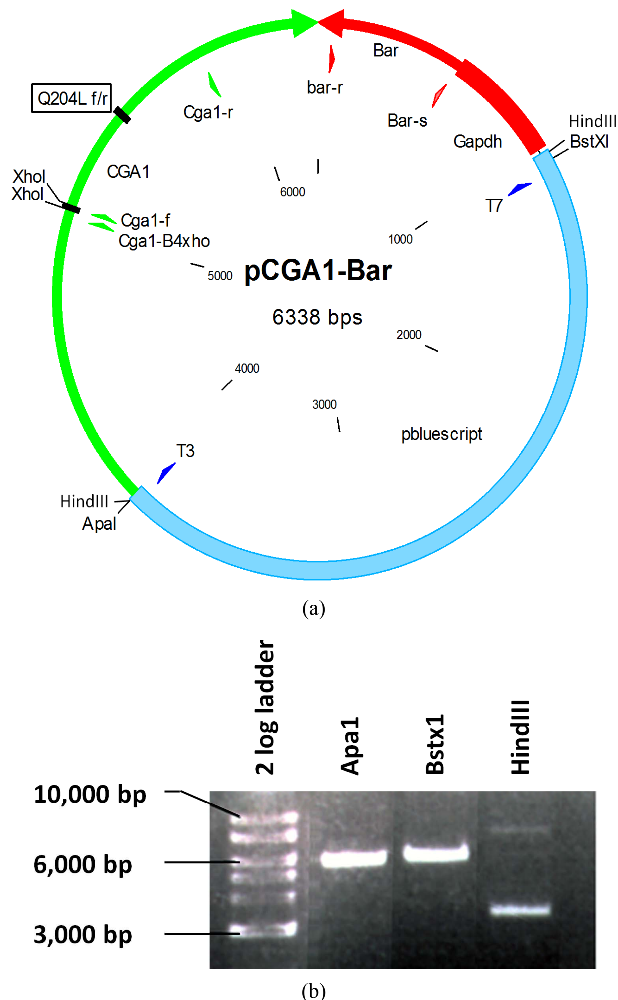

Figure 1. Q204L gene replacement constructs. (a) Restriction map of the construct pCGA1-Bar vector. The specific sequence change in the plasmid is marked by black frame (Q204L f/r). (b) Digestion of the plasmid with Apa I, Bstx I or Hind III was done before it was used for fungal transformation. Restriction reactions conditions are described in materials and methods. 2 Log DNA ladder (New England Bioloabs).

5' GATGTCGGTGGTC**T**GCGATCAGAGC 3' and Q-204L Reverse 5' GCTCTGATCGC**A**GACCACCGACA-TC 3' (**Figure 1(a)**, black frame). The pCGA1-Bar plasmid was used as a template for the reaction.

Cloning and amplification. The transformation was conducted using XL-1 Blue MRF' (Stratagene) super competent *E. coli* cells, suspended in 5 μl LB + Amp: 3 μl DNA, were added to 40 μl XL-1 suspended cells. Heat shock treatment was admitted by incubating cells on ice for 30 minutes, and then transferring them to 42˚C heated bath for 45 seconds. Cells were transferred to regeneration broth containing LB and incubated at 37˚C, for 45 min, then plated on color screening plates and incubated at 37˚C overnight.

Transformed E. coli colonies selection and verification. Since transformed colonies have selection markers for pBluescript, blue colonies were used as a template for PCR reactions using Bio-X-Act (stratagene) polymerase, with the *CGA*1 primers: Cga1-f: 5' GAGTCGCTCGAG-CTCCCGC 3' and the Cga1-r: 5' GCATAGTATCCGT-GGCGCAGG 3' (**Figure 1(a)**). DNA fragments length was determined using gel electrophoresis. Size marker ladders were of two types. 5% Hyperladder/lane; HL (Bioline), 2 Log DNA ladder (New England Bioloabs).

Fungal transformation. Transformation to the WT and the *cga*1 strains was performed as described previously (Turgeon *et al.*, *Molecular and Cellular Biology*, 7(9), 1987). Each strain was transformed with linear DNA excised from the plasmid using different restriction enzymes: ApaI, BstxI, or HindIII (DNA kept in 50 µl STC buffer). After first overnight incubation period, an agar over layer, containing bialaphos antibiotics to a final concentration of 100 µg/ml, was added. Plates were incubated for an additional 3 - 8 days at 30˚C in the light, until colonies appeared. Bialaphos resistant colonies were isolated and their DNA was extracted using a "Miniprep" Kit (Qiagen) according to the manufacturers' protocol. The purified DNA was used for PCR with the primers Cga1-f and Cga1-r or with the primers pairs: Cga1-f/Cga1 B4xho (5' CCGTTGTTGCCTCCATTAGC 3') and CGA1-r or Bar-r 5' GGTACCGGCAGGCTG-AAGTC 3' and Bar-s 5' CGGCCGTCTGCACCATCG 3' (**Figures 1(a)**, **2**). The sequence of the PCR products was determined (**Figure 3**).

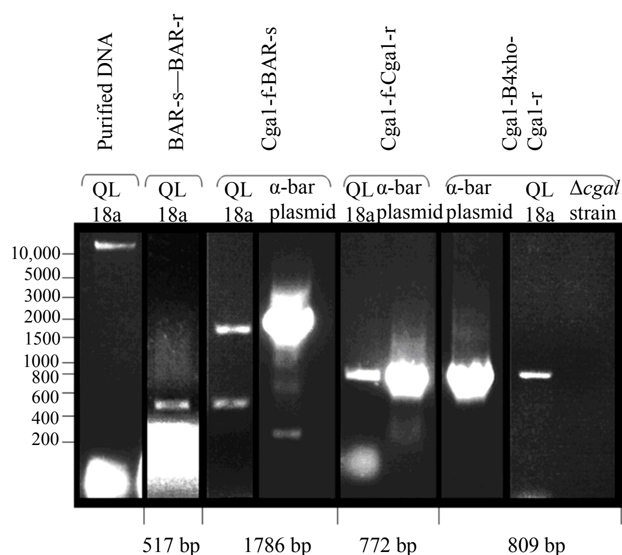

Figure 2. PCR confirmation of a *cga*1^{Q204L} integration event in the *cga*1 strain. Genomic DNA samples from one *cga*1^{Q204} mutant strain (QL18a), from the plasmid pCGA1-Bar and from *cga*1 strain were used as templates for amplification with the indicated primer pairs. Numbers at the bottom indicates the expected band size.

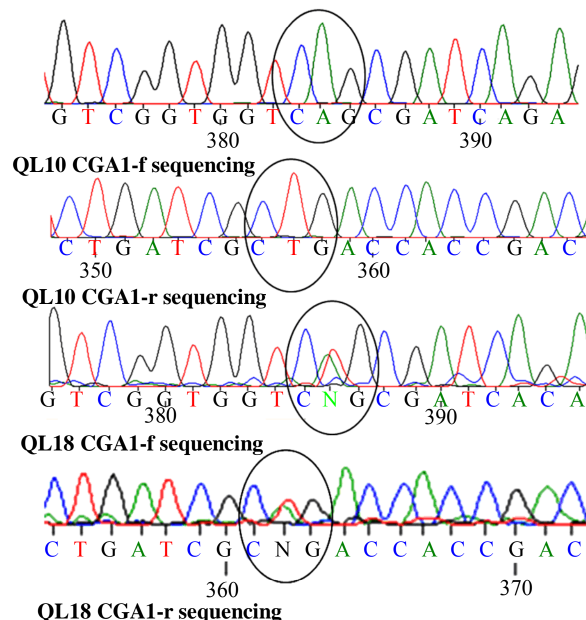

Figure 3. Sequencing confirmation of a *cga*1^{Q204L} integration event in the *cga*1 strain. Encircled bases are targeted for site mutation resulting in the Q204L mutation. Top Two rows: sequencing of strain QL10 with Cga1-f (top row) and Cga1-r (second row). The results show that this mutant dose not expresses the insert gene. Bottom two rows: sequencing of strain QL18 (*cga*1 background) with the same primers. As mentioned in the text, *cga*1^{Q204L} carrying two copies of the CGA1 gene (the activated one and the original disrupted one). This explains the finding of two different bases on the allocated Q204L site. The original *CGA*1 gene base pair is present yet the gene is inactive.

3. Results

*cga*1^{Q204L} Mutation

*cga*1^{Q204L} *construction and verification.* In order to construct a constitutively activated *CGA*1 mutant we used site-directed mutagenesis (Q204L mutation) to change glutamine to leucine at position 204. A plasmid carrying the *CGA*1 gene followed by the Bar resistance cassette (pCGA1-Bar, **Figure 1(a)**) was used as a template for Site-directed mutagenesis (**Figure 1(a)**, black frame) and afterward for cloning in *E. coli* cells. The mutagenesis and the cloning success were verified by PCR amplification of the *CGA*1 mutated gene and by sequencing. Vectors containing the Q204L mutation were prepared from the plasmid using two unique restriction sites Apa1 or Bstx1 (6 kb linear fragment) or HindIII that excluded the pBluescript vector (3 kb linear fragment) (**Figure 1(b)**).

The plasmid was then transformed into two strains of *C. heterostrophus*, WT and *cga*1. The mutated colonies were grown on selective media (CM-Bar) for several transfers. DNA extracted from both strains, *cga*1 and the WT, was obtained by PCR amplification. Mutant in the background of *cga*1 was verified by amplifying the trans-

formed sequence using the forward Cga1 B4xho primer, and the Cga1-r reverse primer (**Figure 1(a)**). Since the *cga*1 mutation was created by deleting 18 bp upstream to the CGA1 promoter after Xho restriction site and insertion of a hygromicin cassette instead, an unsuccessful transformation will result in the presence of a sole copy of the disrupted *CGA*1 gene. So a PCR reaction with these primers will result in a 2854 bp product (772 bp CGA1 minus 18 bp of the coding region plus 2100 bp Hyg resisting cassette). Successful transformants are expected to be carrying an additional copy of the complete *CGA*1 gene, with the point mutation. So the same PCR will produce additional band, 809 bp long, which will allow us to distinguish between successful and unsuccessful transformants (**Figure 2**).

Since the new *cga*1^{Q204L} mutant has two copies of the cga1 gene (the activated one and the original disrupted one) a PCR with the primers Cga1-f and Cga1-r will result in a mixture product of both genes. In other words a mixture of adenine (A) and thymine (T) is expected. Several isolated mutants (WT and *cga*1 in the background) showed resistance to bialaphos antibiotics, and proved by PCR to have the bar expression cassette together with the CGA1 gene (as shown for the QL18a mutant strain, **Figure 2**). Nevertheless, only one mutation, QL18a (CGA1 in the background, created by plasmid digested with Hind III) showed mixture of adenine (A) and thymine (T) in a Cga1-f—Cga1-r PCR reaction product (**Figure 3**).

The WT strain transformation with the same linear plasmid led to at least one bar resistance mutant named QL10. This mutant proved by PCR to carry the bar expression cassette and the additional *CGA*1^{Q204L} gene but the resultant products that were sent for sequencing didn't contain the mixture of adenine (a) and thymine (t) as expected (**Figure 3**).

*cga*1^{Q204L} *phenotype characterization*. The *cga*1^{Q204L} mutation strain (QL18a) was characterized by appearance of white aerial hyphae (**Figure 4**), a WT proximately sporulation (**Figure 5**) but hyphal straight growth, with no apparent appressorium formation (**Figure 5**). Interestingly these phenotypes resemble the *cga*1 phenotypes. A "water drop assay" conducted to test the colony hyphae absorbency phenotype that may indicate hydrophobins secretion pattern (**Figure 4**, lower panel). Here also no obvious difference was found between *cga*1 and the *cga*1^{Q204L} mutants and both strains showed hydrophobic colony surface.

Former examination showed that *cga*1 strains have a significant sensitivity to Sorbitol (1M) osmotic stress in comparison to the WT strains [4]. The *cga*1^{Q204L} may show resistance similar to the WT or even more as described in *N. crassa* [9]. So this trait may provide us with a better insight of the role of *cga*1 in mediating osmotic stress response. Although this expectation, the constantly activated *cga*1 mutant showed the same sensitivity to

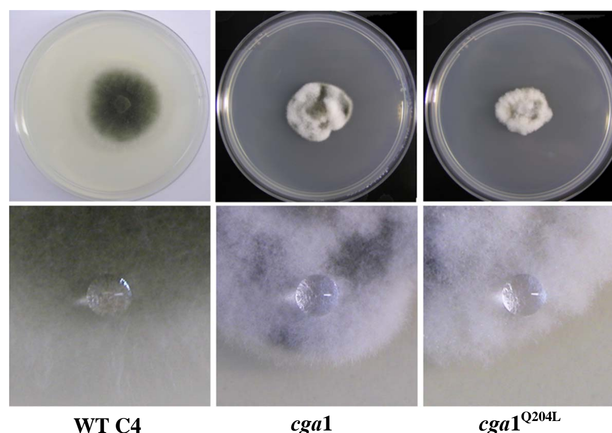

Figure 4. Colony phenotype and surface hydrophobicity drop assay for the WT, *cga*1 and *cga*1^{Q204L} strains. Cultures were grown for 3 days on CM in 37˚C at continuous whit fluorescent light. The lower panel is a magnification of the same cultures one hour after deposition of 10 μl drop of sterile DDW near the margins of the colony.

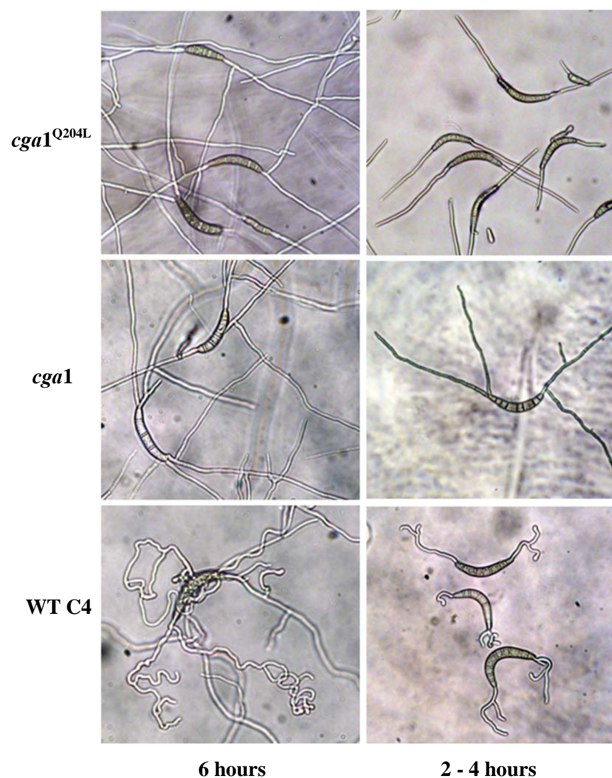

Figure 5. Conidiation and hyphal phenotypes of WT, *cga*1 and *cga*1^{Q204L} strains. While the WT hyphae grow in "wandering" way with appressoria formed at their tips, the *cga*1 has a straight growth without (or few) appressoria. The *cga*1^{Q204L} phenotype is similar to that of the *cga*1. It is growing in relatively straight patterns and has inability to produce appressoria.

Sorbitol (1M) osmotic stress as *CGA*1 disruption strains (data not shown).

4. Discussion

In most published works employing activated Gα alleles, it has not been shown, biochemically, that the transgene encodes a protein lacking GTPase activity, or that it activates downstream effectors such as adenylate cyclase (an example is [7]). Such work can support the resulting phenotype conclusion. Nevertheless, phenotypes characterization, based on site specific mutagenesis (such as Q204-L, G42-R and R178-C), designed to constitutively activate Gα signaling, has been widely used to investigate the role of genes encoding heterotrimeric G-protein α subunits (Gα) in filamentous fungi. Constitutively activated mutants in this gene were used to study the Gα's role in *Schizophyllum commune* [11], *Hypocrea jecorina* [12], *Ustilago maydis* [13], *Penicillium chrysogenum* [14] and *Cryphonectria parasitica* [7].

Comparative analysis of the phenotypic traits exhibited by fungal strains containing null or activated Gα alleles has been used by a number of laboratories to identify putative signaling-related functions [7-10]. Free G$\beta\gamma$ may cause a signal in both the Gα null and activated mutant strains and the phenotypic traits of both strains are affected mainly by the manipulated Gα gene. Indeed in some instances phenotypes of the Gα null and activated mutant strains were different. In *N. crassa* [9] the Gα activated mutant (*gna*-1^{R178C} and *gna*-1^{Q204L}) has longer, abundant aerial hyphae, less conidia per aerial hyphae, greater colony dry weight mass, lower carotenoid secretion, higher cAMP levels and increased sensitivity to heat and hydrogen peroxide-induced oxidative stress than wild-type strains while the null mutant Δ*gna*-1 presents the opposite phenotypes. Furthermore, the permanent activation of Δ*gna*-1 abolished the osmotic sensitivity, the lower extension rate and the female sterility of the null Δ*gna*-1 mutant. In *A. nidulans* [10], constitutive signaling of α-subunit of G protein (*fadA*^{G42R}) resulted in proliferation and block of sporulation in contrast to the null mutant (*fadA*) that was characterized by reduced growth with normal sporulation. Other studies demonstrated that some similar phenotypes result from constant activation or silencing of the Gα subunit. In *M. grisea* activated and null mutants of the Gα subunit *magB* exhibited similar phenotypes in terms of reduced conidiation, sexual reproduction and virulence [8]. These findings were supported by a recent work in *C. parasitica* [7]. Pigmentation, conidiation, and virulence negative regulation were completely compromised in both Gα (*cpg*-1) null mutant and activated *CPG*-1 strains (QL and RC). Nevertheless dissimilar responses were identified in the two *C. parasitica* mutant strains when subjected to a variety of stresses.

In this work we constructed an activated *cga*1 allele, Q204L. Although the success of the mutagenesis was proved by PCR and sequencing, this mutant presents similar phenotypes to the null *cga*1 mutant. These phenotypes include colony growth rate on CM or on CM containing 1.5 M sorbitol (hyper osmotic stress), sporulation, hyphae straight growth, aerial hyphae growth and hydrophobicity. The results were presented here indicating a possible role for *CGA*1 as a stabilizer of these traits.

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