Construction of a Constitutively Activated Ga Mutant in the Maize Pathogen Cochliobolus heterostrophus

Ofir Degani1,2

1Tel-Hai College, Upper Galilee, Israel; 2Migal—Galilee Research Institute, Kiryat Shmona, Israel.

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ABSTRACT

Conserved eukaryotic signaling proteins participate in development and disease in plant pathogenic fungi. Mutants in CGA1, a heterotrimeric G protein Ga subunit gene of the maize pathogen Cochliobolus heterostrophus, are defective in several developmental pathways. Conidia from CGA1 mutants germinate as abnormal, straight-growing germ tubes that form few appressoria, and the mutants are female-sterile. The CGA1, Ga subunit, is also thought to act as a down regulator of hydrophobin expression and secretion in this fungus and in related Ascomycete species. Although cga1 mutants can cause normal lesions on plants there are host physiology conditions under which full virulence requires signal transduction through CGA1-mediated pathways. A Ga activated mutant, cga1Q204L was created to help establish the role of CGA1 in growth and development, and in mediating hydrophobin secretion and expression. The activated Ga allele was transformed into a cga1 mutant strain. The transgenic lines showed phenotypes resembling the null mutant in development, sporulation and hydrophobicity, indicating a possible role for CGA1 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 Ga encoding genes, one Gβ and one Gγ gene. Deletion of the MAP kinase gene CHK1 [3] or the Gβ gene CGB1 [1] has a vast effect on growth and development and is drastically reducing virulence under all conditions tested. Mutants in CGA1, a heterotrimeric G protein Ga 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 CGA1 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, CGA1 disruption and deletion mutants are considerably less virulent [6]. In addition disruption of the CGA1 gene causes aerial growth formation and spores aggregation that indicates a possible role for CGA1 in regulation of hydrophobin secretion [5]. Determination of Cochliobolus heterostrophus hydrophobins expression in cga1 mutants provided the molecular evidence for the role of CGA1 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 Ga signaling was reported in C. parasitica [7], M. grisea...
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 Ga allele (cga1\(^{Q204L}\)) to investigate the role of CGA1 in developmental processes. In particular, we examined its influence on hydrophobin associated 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 \(\alpha\) subunit gene *CGA*1 was: C5\(\Delta\)cga1 (*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 ca\(g\)) to leucine (L, coded by ct\(g\)) 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. *Apa*1, *Bst*\(x\)1 and *Hind*III were used in order to confirm the construction of the plasmid and the presence of the CGA1 insert. First examination was done by addition of 1 \(\mu\)l *Apa*1 to 2 \(\mu\)l reaction buffer \#4 (New England Biolabs) 0.5 \(\mu\)l BSA, 2 \(\mu\)l DNA (pCGA1-Bar plasmid) and 14.5 \(\mu\)l DDW, and incubation at 25\(\deg\)C for 1 hour. Alternatively, 1 \(\mu\)l *Bst*\(x\)1, was added to 2 \(\mu\)l reaction buffer \#3 (NEB), 2 \(\mu\)l DNA (pCGA1-Bar plasmid) and 15 \(\mu\)l DDW, and the reaction was incubated at 55\(\deg\)C for 1 hour. Third examination was done by adding 1\(\mu\)l *Hind*III to 2 \(\mu\)l reaction buffer \#2 (NEB), 2 \(\mu\)l DNA (pCGA1-Bar plasmid) and 15 \(\mu\)l DDW, and incubation at 37\(\deg\)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 5’ GATGTCGGTGGTCTCGATCAGAGC 3’ and Q-204L Reverse 5’ GCTCTGATCGCAGACCAGCACGAGC 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 \(\mu\)L LB + Amp: 3 \(\mu\)L DNA, were added to 40 \(\mu\)L XL-1 suspended cells. Heat shock treatment was admitted by incubating cells on ice for 30 minutes, and then transferring them to 42\(\deg\)C heated bath for 45 seconds. Cells were transferred to regeneration broth containing LB and incubated at 37\(\deg\)C, for 45 min, then plated on color screening plates and incubated at 37\(\deg\)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 CGA1 primers: Cga1-f: 5’ GAGTCGCTCGAG-CTCCCGC 3’ and the Cga1-r: 5’ GCATAGTATCCGT-GGCCGAGG 3’ (Figure 1(a)). DNA fragments length was determined using gel electrophoresis. Size marker ladders were of two types. 5% Hyperladder/lane; HL was determined using gel electrophoresis. Size marker.

Fungal transformation. Transformation to the WT and the cga1 strains were 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-3’ (Figures 1(a), 2). The sequence of the PCR products was determined (Figure 3).

3. Results

cga1Q204L Mutation

cga1Q204L construction and verification. In order to construct a constitutively activated CGA1 mutant we used site-directed mutagenesis (Q204L mutation) to change glutamine to leucine at position 204. A plasmid carrying the CGA1 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 CGA1 mutated gene and by sequencing. Vectors containing the Q204L mutation were prepared from the plasmid using two unique restriction sites ApaI or BstXI (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 cga1. The mutated colonies were grown on selective media (CM-Bar) for several transfers. DNA extracted from both strains, cga1 and the WT, was obtained by PCR amplification. Mutant in the background of cga1 was verified by amplifying the trans-

Figure 2. PCR confirmation of a cga1Q204L integration event in the cga1 strain. Genomic DNA samples from one cga1Q204L mutant strain (QL18a), from the plasmid pCGA1-Bar and from cga1 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 cga1Q204L integration event in the cga1 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 does not expresses the insert gene. Bottom two rows: sequencing of strain QL18 (cga1 background) with the same primers. As mentioned in the text, cga1Q204L 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 CGA1 gene base pair is present yet the gene is inactive.
formed sequence using the forward Cga1 B4xho primer, and the Cga1-r reverse primer (Figure 1(a)). Since the cga1 mutation was created by deleting 18 bp upstream to the CGA1 promoter after Xho restriction site and insertion of a hygromycin cassette instead, an unsuccessful transformation will result in the presence of a sole copy of the disrupted CGA1 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 CGA1 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 cga1Q204L 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 cga1 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 CGA1Q204L 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).

cga1Q204L phenotype characterization. The cga1Q204L 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 cga1 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 cga1 and the cga1Q204L mutants and both strains showed hydrophobic colony surface.

Former examination showed that cga1 strains have a significant sensitivity to Sorbitol (1M) osmotic stress in comparison to the WT strains [4]. The cga1Q204L 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 cga1 in mediating osmotic stress response. Although this expectation, the constantly activated cga1 mutant showed the same sensitivity to
Sorbitol (1M) osmotic stress as CgA1 disruption strains (data not shown).

4. Discussion

In most published works employing activated Ga alleles, it has not been shown, biochemically, that the transgene encodes a protein lacking GTPase activity, or that it activates downstream effectors such as adenylyl cyclase (an example is [7]). Such work can support the resulting phenotype conclusion. Nevertheless, phenotypes characterized, based on site specific mutagenesis (such as Q204-L, G42-R and R178-C), designed to constitutively activate Ga signaling, has been widely used to investigate the role of genes encoding heterotrimeric G-protein a subunits (Ga) in filamentous fungi. Constitutively activated mutants in this gene were used to study the Ga’s role in Schizosaccharomyces pombe [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 Ga alleles has been used by a number of laboratories to identify putative signaling-related functions [7-10]. Free Gβγ may cause a signal in both the Ga null and activated mutant strains and the phenotypic traits of both strains are affected mainly by the manipulated Ga gene. Indeed in some instances phenotypes of the Ga null and activated mutant strains were different. In N. crassa [9] the Ga activated mutant (gna-1R178C and gna-1Q204L) 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 Δgna-1 presents the opposite phenotypes. Furthermore, the permanent activation of Δgna-1 abolished the osmotic sensitivity, the lower extension rate and the female sterility of the null Δgna-1 mutant. In A. nidulans [10], constitutive signaling of α-subunit of G protein (fadA4Δ28) resulted in proliferation and block of sporulation in contrast to the null mutant (fadA4) that was characterized by reduced growth with normal sporulation. Other studies demonstrated that some similar phenotypes result from constant activation or silencing of the Ga subunit. In M. grisea activated and null mutants of the Ga 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 Ga (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 cga1 allele, Q204L. Although the success of the mutagenesis was proved by PCR and sequencing, this mutant presents similar phenotypes to the null cga1 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 CgA1 as a stabilizer of these traits.

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REFERENCES


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