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Accurate virulence test method for *Cochliobolus heterostrophus* wild-type and mutant strains in the post-genomic era

Ofir Degani [1,2]

[1] Tel-Hai College, Upper Galilee, 12210, Israel

[2] Molecular Phytopathology Laboratory, Migal – Galilee Research Institute, Southern Industrial Zone, P.O. Box 831, Kiryat Shmona 11016, Israel

Tel.: 972-54-6780114, Fax: 972-4-6817410, Email: d-ofir@bezeqint.net; ofird@telhai.ac.il

Abstract

Cochliobolus heterostrophus is an agriculturally important and emerging model pathogen for studying the signalling hierarchy's role during maize colonization. In particular, G-protein and MAPK-linked pathways play a major role during pathogenesis. Although gene disruption studies are an efficient method for identifying the role of these cascades, differentiating between the mutant strains' virulence ability may become an intricate task. For example, in *C. heterostrophus*, mutants in a G-protein α subunit gene, *cga1*, are defective in mating and appressorium formation, but unlike mutants in homologous genes in other fungal pathogens, *cga1* mutants remained highly virulent to corn under some host physiological conditions. Here, we used the *cga1* strain as a model for developing an *in vivo* sensitive and accurate pathogenicity assay. A detailed and well-controlled analysis of wild-type (WT) and *cga1* pathogenic behavior revealed that detached leaves are significantly more vulnerable to the disease than intact ones. In intact leaves, *cga1* mutants were less infective of maize under most conditions. This difference was maximized when the first seedling leaf was chosen for inoculation and when the infected leaves, with spores or mycelia fragments droplets, were incubated for a period of four days. This optimal condition set enabled us to classify the *C.*

heterostrophus G-protein signalling mutants (deficient in α, β or both subunits) in order of decreasing virulence: WT > *cga1* > *cgb1* > *cga1 cgb1*. The method presented here proves to be accurate and sensitive enough to identify even slight variations in virulence. Moreover, it could be modified for use in the studies of other foliar phytoparasitic fungi.

Keywords: *Cochliobolus heterostrophus*, G-protein, maize, MAPK, signal transduction, virulence

Introduction

Southern corn leaf blight (SCLB) disease caused by the fungus *Cochliobolus heterostrophus* (anamorphs *Bipolaris maydis, Helminthosporium maydis*) constitutes a considerable threat to corn production worldwide (Kump et al., 2011). No known genes confer complete immunity to this disease; instead, maize breeders rely on polygenic, quantitative resistance to SCLB (Holley and Goodman, 1989). Three races of *C. heterostrophus* known as O, T and C have been identified to date (Smith et al., 1970). Race O is considered to be the most common race in most areas and is controlled by nuclear genes. Race T, the cause of the 1970s epidemic in North America, is specific to maize containing Texas male-sterile cytoplasm (cms-T) and is controlled mainly by cytoplasmic factors. The most prominent difference between races O and T is that race O only infects leaves while race T infects leaves, stalks, leaf sheaths, ear husks, ears and cobs. Race C is a cms-C cytoplasm-specific race reported only in China (Wei et al., 1988).

Virulence test methods are an important means for investigating phytopathogenic fungi, therefore the development of these methods is a continuous effort, for example, in Lee et al. (1992) studying *Cryphonectria parasitica*. To study and evaluate *C. heterostrophus* strains virulence, an accurate, sensitive, rapid, convenient and reproducible pathogenicity test in the

post-genomic era is critical. The traditional test involves inoculation of living maize with fungal spores (Horwitz et al., 1999) or mycelia fragments (Ganem et al., 2004). Alternatively, the leaves of the plants were dipped into homogenized mycelial suspension in order to obtain widespread lesions (Lev and Horwitz, 2003). Other studies introduced a more precise method based on depositing mycelia fragments (Lev et al., 1999) or spore (Degani et al., 2004) drops on the leaves and qualitatively evaluating the symptom severity. However, the preliminary droplets technique, especially leaf spraying or dipping approaches, are inaccurate and incapable of differentiating between slight differences that are sometimes invisible to the naked eye.

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 that are needed to detect the host and establish invasive growth. Several such mutants have been constructed for the maize pathogen *C. heterostrophus* (Horwitz et al., 1999; Ganem et al., 2004; Lev et al., 1999; Degani, 2013a). In Ascomycetes, for which sufficient sequence information is available, there are three Gα-encoding genes, one Gβ and one Gγ gene (Hoffman, 2007). Deletion of the MAP kinase gene *chk1* (Lev et al., 1999) or the *G*β gene *cgb1* (Ganem et al., 2004) has a huge effect on growth and development, and drastically reduces virulence under all conditions tested. Comparison of the expression profile in wild-type strains and in *chk1* and *cgb1* signalling mutants revealed a unique, environmentally-dependent control mechanism for the known *C. heterostrophus* T-toxin biosynthetic gene, *DEC1*, whose restriction is important for epidemic prevention (Degani, 2015).

Cga1, a heterotrimeric G protein Gα subunit, is the *C. heterostrophus* orthologue of *Magnaporthe grisea MAGB* belonging to the fungal Gi class. Members of this class are often

required for full virulence, while disruption of other *Ga* genes confers no major phenotype as earlier observed (Gronover et al., 2001; Liu and Dean, 1997). Mutants in *C. heterostrophus* *cga1* produce conidia that germinate as abnormal, straight-growing germ tubes forming few appressoria (Horwitz et al., 1999). A Gα activated mutant, *cga1*^{Q204L}, showed phenotypes resembling the null mutant in development, sporulation and hydrophobicity, indicating a possible role for *cga1* as a stabilizer of these traits (Degani, 2013b). Nevertheless, *cga1* mutants can cause normal lesions on plants, unlike other filamentous fungal plant pathogens in which functional homologues of *cga1* are required for full virulence (Horwitz et al., 1999; Degani, et al., 2004). For *cga1* strains, it was demonstrated that appressorium formation is not essential for virulence (Horwitz et al., 1999). Indeed, inoculation with mycelium results in growth on the leaf surface followed by penetration into the leaf without noticeable appressorium formation. Interestingly, aggregates of mycelia sometimes localize at stomatal apertures, but apparent direct penetration of the epidermis is also possible (Horwitz et al., 1999). Detailed examination indicated that under some host physiological conditions, *cga1* disruption and deletion mutants are considerably less virulent (Degani et al., 2004). In addition, disruption of the *cga1* gene causes aerial growth formation and spores aggregation, indicating a possible role for *cga1* in the regulation of hydrophobin secretion (Degani, 2013a). Hydrophobins are small proteins that confer water-repellent properties to conidia, hyphae and fruiting structures, and are involved in the development and virulence of mycelial fungi, where they occur exclusively (Whiteford et al., 2002; Wosten, 2001). Determination of *C. heterostrophus* hydrophobins expression in *cga1* mutants provided the molecular evidence for the role of *cga1* in the suppression of hydrophobins expression (Degani et al., 2013).

A large number of fungal genes encoding α subunits of heterotrimeric GTP-binding proteins have been cloned in other fungi as well (for example Gronover et al., 2001; Liu and Dean, 1997; Turner and Borkovich, 1993). There is growing genetic evidence of the importance of

these genes in pathogenicity (Liu and Dean, 1997; Gao et al., 1996; Gronover et al., 2001). Signalling through the *cga1* G-protein pathway may be required for full fitness as a pathogen under some conditions but not others. For example, Liu and Dean (1997) showed that *MAGB* mutants have reduced virulence on young rice leaves but are fully pathogenic on older plants.

To test the conditions under which *cga1* mutants differ the most from the wild-type strain, we conducted a detailed, well-controlled study of the virulence of *C. heterostrophus cga1* mutants. The objective was to establish an assessing method that would enable us to define the virulence role of fungal signal transduction pathways. The developmental and physiological stages of the host leaf, the differences in susceptibility to the disease between the 1st, 2nd and 3rd seedling leaves, the infection method and incubation time could all be important factors in determining virulence differences.

Materials and Methods

Strains

The strains used in this study are listed in Table 1.

Culture conditions

Fungal strains were grown on complete medium (CM; Turgeon et al., 1985) with or without 50 µg/ml hygromycin B for about 10 days at room temperature (24-26°C) in continuous light from cool white and UVA-enriched fluorescent tubes (Philips, Eindhoven, the Netherlands). Conidia were collected by scraping the colony surface with a scalpel into sterile water containing 0.05% Tween 80. Spores were quantified microscopically by haemocytometer count. For inoculation with mycelia fragments, six culture agar disks, 6 mm in diameter, were cut from the margins of the colony and transferred to a 50 ml polycarbonate screw-capped test tube containing 20 ml liquid CM. The cultures were incubated diagonally in a rotary

shaker at 230 rpm and at a temperature of 30°C for 3-4 days. Mycelia were collected by centrifugation (10 min, 600 × *g*) and homogenized briefly (20 s, Polytron, Brinkmann Instruments, Westbury, NY). Fifty mg wet weight mycelia or $2x10^5$ spores (unless otherwise indicated) per ml of 0.05% Tween 80 was used to infect the leaves.

Table 1. Strains used in this study

Strain	Genotype	Reference	Comments
WT C4 (Race T)	MAT1-2; $Tox1^+$ ATCC 48331	Leach et al. (1982)	These strains were obtained after six backcrosses and are nearly isogenic.
WT C5 (Race O)	*MAT1-1*; $Tox1^-$ ATCC 42332	Leach et al. (1982)	
cga1	*MAT1-1*; $Tox1^-$ ATCC 42332	Horwitz et al. (1999)	G-protein α subunit disrupted mutant (WT C5 in background), created by insertion of the hygromycin cassette into the coding region, combined with an 18-bp deletion.
C4cga1 TSC17	*MAT1-2; Tox1*$^+$ ATCC 48331	Horwitz et al. (1999)	G-protein α subunit disrupted mutant (WT C4 in background), created by complete deletion of the coding region and insertion of hygromycin cassette.
cgb1	*MAT1-1*; $Tox1^-$ ATCC 42332	Ganem et al. (2004)	G-protein β subunit disrupted mutant (WT C5 in background), created by insertion of the hygromycin cassette into the coding region, combined with a 473 bp deletion. This strain contains an additional mutation but its phenotypic traits are similar in almost every aspect to the *cgb1* that contains only one mutation.
cga1 cgb1	*MAT1-1*; $Tox1^-$ ATCC 42332	Degani (2013a)	G-protein α and β subunits double mutant (WT C5 in background), created by insertion of the bar cassette into the *CGB1* coding region of *cga1* mutant strain, combined with a 473 bp deletion.
chk1	*MAT1-2*; $Tox1^+$ ATCC 48331	Lev et al. (1999)	MAPK disrupted mutant (WT C4 in background), created by replacement of the coding region with the hygromycin resistance cassette.

Maize plant growth conditions

Sweet maize cultivar (Grand Jubilee), available at the Institute of Cereal Crops Improvement, Tel Aviv University, was used throughout. Plants were grown in a greenhouse with a 14 h photoperiod at 22–25^{o}C. Maize seedlings were 9–12 days old, unless otherwise indicated; within this day range, plants were used when the 3rd leaf had emerged, remained partly rolled,

and started to expand. Each repetition included leaves from one individual plant, that is, the number of leaves is also the number of plants used for each experiment.

Virulence assays

Seedlings were inoculated by depositing drops (5 µl 0.05% Tween 80) of the above mycelia fragments (250 µg wet weight/drop) or conidia (1000 conidia/drop, unless otherwise indicated) suspensions on the leaves. Usually, in young seedlings (9–12 days old), one drop was deposited on the upper third part of the first three detached or intact leaves. Alternatively, detached leaves from the older plants were long enough for a series of 2-4 drops to be placed along the upper third part of the leaf. The plants, kept in a closed plastic bag with 100% humidity, or detached leaves in a closed Petri dish (with a wet Whatman no. 1 filter paper underneath), were then incubated in a growth chamber for 2-6 days at 30°C (unless otherwise indicated) under continuous white light.

Analysis of the virulence assay results

The pathogenicity of the fungal strains was determined as percentages of the infected plants (that is, plants that show any visible sign of disease) and by measuring the area of the necrotic patch (lesion area) caused by the pathogen. For quantitative analysis of the lesion area, leaves were scanned (300 dpi) and the lesion areas (calculated using Adobe Photoshop 6.0 and Tina 2.10g software, Raytest, Straubenhardt, Germany) were used to evaluate the severity of the infection. Statistical analysis, conducted using Student's *t* test, verified whether differences were statistically significant ($P \leq 0.05$).

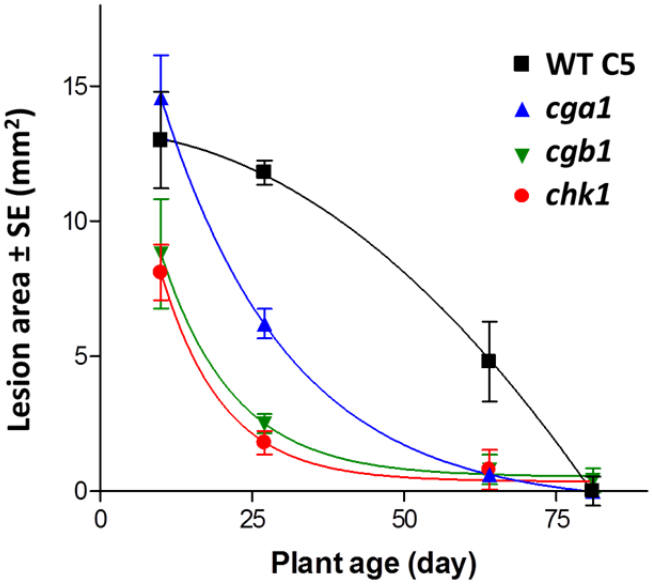

Figure 1. Infection of detached leaves as a function of plant age. Most developed leaves from maize plants of different ages (10, 27, 64 and 81 days old) were inoculated by depositing 2-4 drops (5 µl of 0.05% Tween 80) of spore suspension (10^2 spores/drop) of *Cochliobolus heterostrophus* wild type (WT C5, ■) or signal deficiency strain, *cga1* (G-protein Gα subunit, ▲) along the upper third part of the leaf. For the non-sporulating signal deficiency strains, *cgb1* (G-protein Gβ subunit, ▼) and *chk1* (Mitogen-activated protein kinase**,** MAPK, ●), 5 µl drops of 250 µg wet weight mycelia fragments per drop was used instead. Infected leaves were incubated in Petri dishes with wet filter paper for three days. Values represent an average lesion area (mm^2) of 5-7 leaves of the same age ± standard error.

Results

G-protein and MAPK mutants' ability to infect maize plants' leaves

We evaluated the ability of *C. heterostrophus* WT (C5), *cga1* (G-protein Gα subunit deficiency strain), *cgb1* (G-protein Gβ subunit deficiency strain) and *chk1* (Mitogen-activated protein kinase, MAPK, deficiency strain) (Table 1) to infect mostly developed, detached leaves from maize plants of different ages, up to 81 days (time course in Figure 1). Infection was done on the detached leaves, in Petri dishes with wet filter paper, by depositing 2-4 drops

(5 µl of 0.05% Tween 80, the number of drops was according to leaf size) of spore suspension (WT and *cga1* strains, 10^2 spores/drop) or mycelia fragments (*cgb1* and *chk1* strains, 250 µg wet weight per drop) on the upper third part of the leaf surface. Mycelial fragments, instead of spores, were used for the inoculation of *cgb1* and *chk1* mutants since they cannot produce spores efficiently (Ganem et al., 2004; Lev et al., 1999).

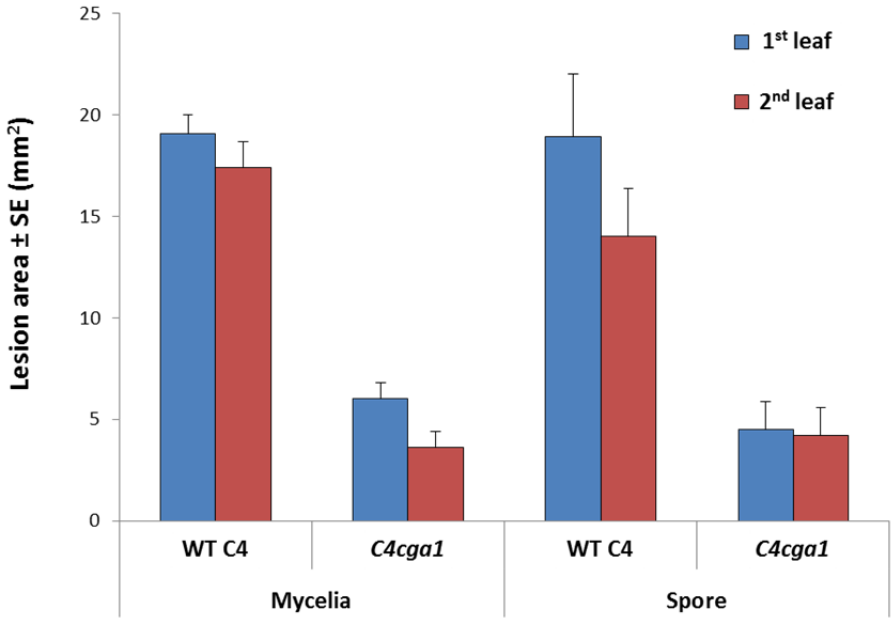

Figure 2. Mycelial inocula or conidial suspensions virulence assay for *C4cga1* mutant in comparison to the wild-type line (C4). Mycelial inocula or conidial suspensions of both strains were prepared as described in Figure 1. One drop (5 µl 0.05% Tween 80) was used to infect the upper third part of the 1st and 2nd intact leaves obtained from 9-12-day-old maize seedlings. Incubated time was three days. Values represent an average lesion area (mm^2) of at least seven leaves ± standard error.

The WT and *cga1* strains are capable of producing normal conidia, so a spore suspension was used instead of mycelia fragment suspension. This could be done since the two suspensions had similar capability to infect maize intact leaves in both strains (Figure 2, $P > 0.2$). Infected leaves were incubated for three days. Wild-type and the three signalling mutants were less

virulent on leaves from mature plants than they were on younger plants (Figure 1). This reduction was more pronounced for *cga1* and even more so for *cgb1* and *chk1*. Neither of the strains could infect leaves from 80-day-old plants. Interestingly, on leaves from 10-day-old seedlings, *cga1* had an infection ability similar to that of wild type ($P > 0.1$). Wild type and *cga1* caused significantly larger lesions (in diameter) on the upper part of the detached leaf than on the lower part ($p < 0.05$, data not shown).

A subsequent experiment was conducted in order to define the optimal measuring method to evaluate the vulnerable ability of the fungal strains. We estimated the percentages of the infected plants and measured the area of the necrotic patch caused by the pathogen. In both measuring methods, the wild-type and *cga1* mutants could infect detached leaves more effectively than the intact plant leaves (Figure 3; significant at $p < 0.01$). Nevertheless, the lesion area measuring method gave a better and more precise estimation of pathogen virulence ability. Therefore, this method was chosen for the subsequent experiments.

Interestingly, when we used 10-day-old detached leaves in Figure 1, the lesion area on detached 1st and 2nd caused by *cga1* mutant was comparable to WT, while in the subsequent experiments (Figures 2, 3) the lesion area caused by *cga1* was significantly ($p < 0.02$) smaller than WT. This difference may be explained by the use of one drop of inoculum (in Figures 2, 3) instead of two (Figure 1) to infect those relatively small leaves. It was previously shown that when 11-day-old seedlings were inoculated by a relatively massive amount of fungal spores, the symptoms caused by C4, C4*cga1* and C5*cga1* were nearly identical (Horwitz et al., 1999; Degani et al., 2004). Another interesting result was a noticeable difference between the susceptibility of the 1st and 2nd leaves (Figures 2, 3, significant at $p < 0.003$, found only for the WT strain). We examined this difference further in the subsequent experiments with intact leaves.

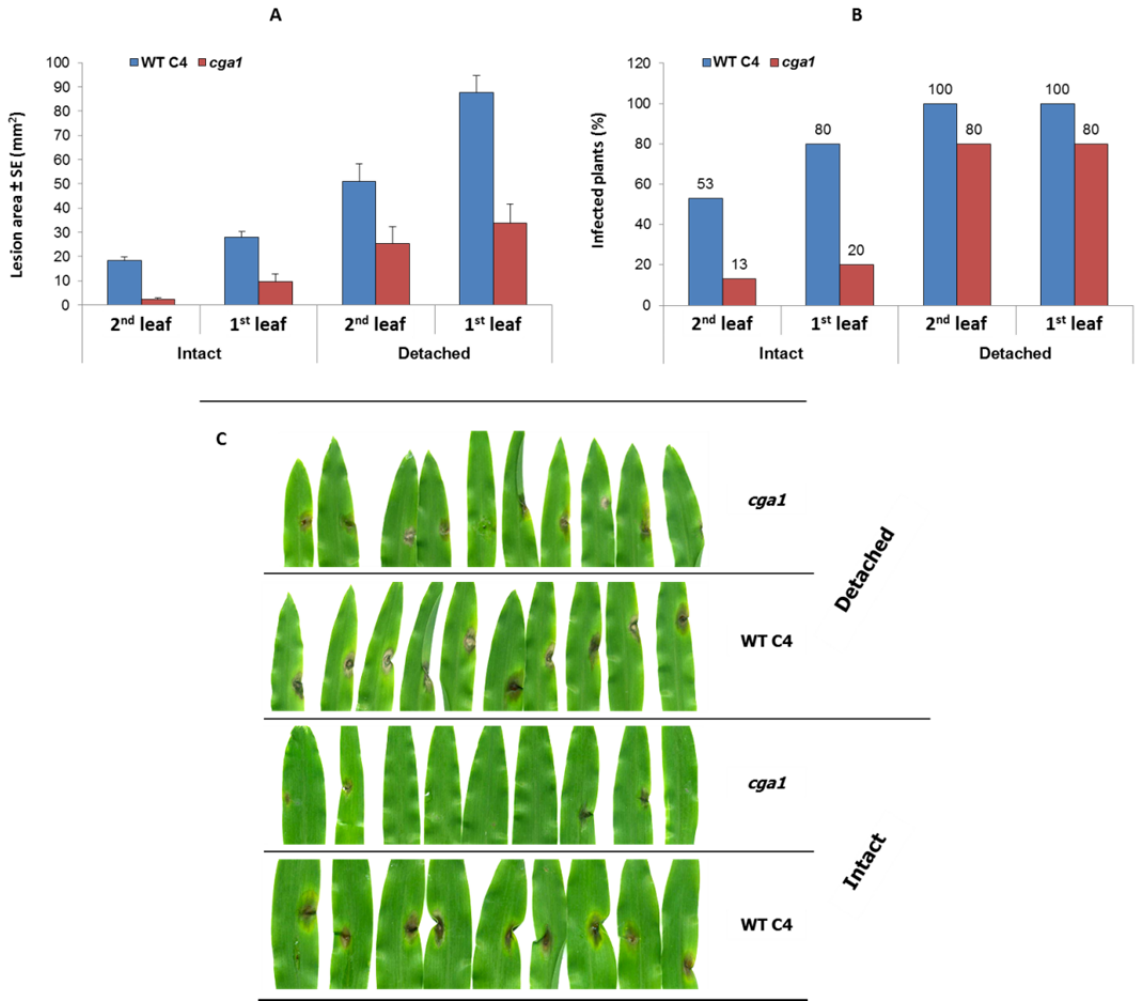

Figure 3. The infection pattern of *cga1* and WT in detached and intact leaves. Maize seedlings, 9-12 days old, were inoculated by depositing one drop (5 µl 0.05% Tween 80 solution of 10^2 spores/drop) on the upper third part of the first two detached or intact leaves. The infected plants in a closed plastic bag with 100% humidity or detached leaves in a closed Petri dish (with a wet filter paper underneath) were incubated for four days. **A**. Values represent average lesion areas (mm^2) of at least seven leaves ± standard error. **B**. Values represent infection (any visible injury) percentage calculated based on 10-14 leaves from the same age. **C**. The 2nd leaves were photographed four days post-infection. Dark areas indicate necrotic regions.

Adjustment of the intact leaves virulence assay conditions

Intact leaves are better for modelling host-pathogen interactions in the field. Furthermore, in the intact leaves pathogenicity assay, the fungus has to deal with the host's defense mechanisms, and develops in tissues that alter their structure and properties during plant growth and maturation. In addition, the balance of hormones in plant tissues were subjected to changes during growth, and at least two of them, cytokinin and ethylene, were found previously (Degani et al., 2004) to influence the development of the pathogen.

Here, we used the intact leaves pathogenicity assay to determine the conditions under which the difference between *cga1* and wild-type strains is greatest. As previously mentioned, these two strains produce conidia, so a spore suspension was used. The amount of spores (10^2 or 10^3 spores per 5 µl drop) used to infect the seedling's first three leaves was also a crucial parameter (Figure 4). A high concentration of spores may blur the differences between the leaves since, when using 1,000 spores/droplet, the pathogen causes a similar infection on the WT 2nd and 3rd leaf, with slightly more severe symptoms on the 3rd leaves than on the 2nd leaves (Figure 4). When using 100 spores/droplet, the disease symptoms on the WT 3rd leaves were less than half as severe in comparison to those on the WT 2nd leaves (Figure 4). A 10-fold reduced inoculum load reduced the extent of the lesions ($p < 0.05$), but the differences between wild-type and *cga1* mutants remained in both spore concentrations (Figure 4; $p < 0.05$).

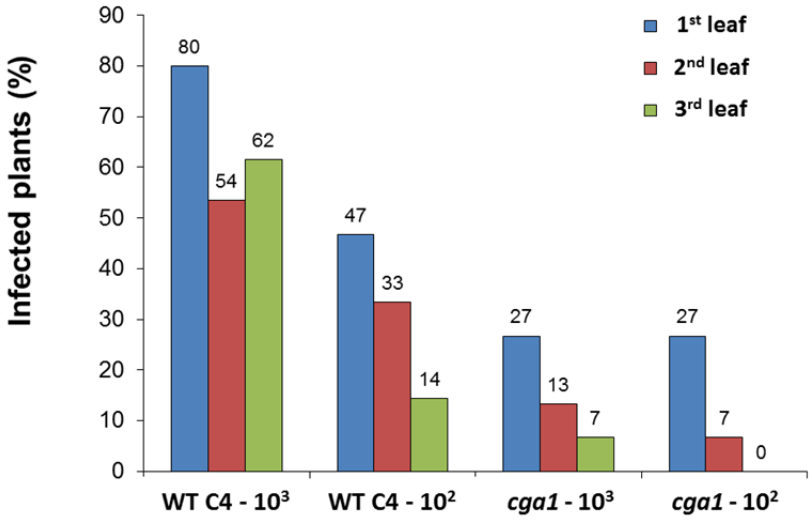

Figure 4. Influence of spore concentration on WT and *cga1* infection of different leaves. One drop of 5 µl containing 10^2 or 10^3 spores was deposited on the upper third part of the first three intact leaves (obtained from 9-12-day-old maize seedlings). The plants were then incubated in a moist chamber for three days at 30°C in continuous white light. Values represent infection (any visible injury) percentage calculated based on 15 leaves from the same age.

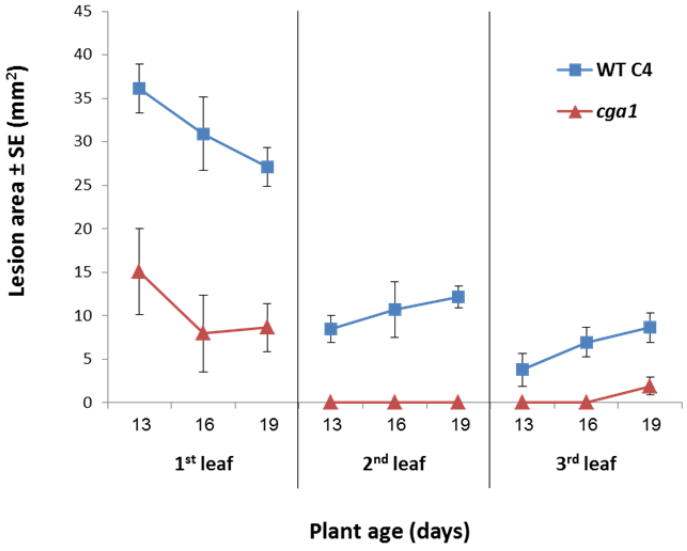

Figure 5. The effect of seedling age on infection severity. Wild-type and *cga1* spore suspensions (10^3 spores/drop) were used to infect intact leaves of 13-, 16- and 19-day-old

maize seedlings. (■), wild type; (▲), *cga1*. Incubated time was four days. Values represent average lesion areas (mm^2) of at least nine leaves of the same age ± standard error.

A clear difference in virulence between the first three intact leaves was observed when young seedlings (13-, 16- and 19-day-old maize seedlings) were inoculated (Figure 5). Both wild type and *cga1* were more easily able to infect the 1st leaf than the 2nd and 3rd leaves ($p < 0.05$). Symptoms decreased with seedling age on the 1st leaf but increased slightly with seedling age on the 2nd and 3rd leaves (Figure 5). This apparently reflects some unique physiological property of the 1st leaf. With increased incubation time, the lesions caused by the mutant increased in size faster than those caused by wild type, resulting in almost no difference when assayed on the first seedling leaf six days after inoculation (Figure 6; significant between the two strains existing at the first two days, $p < 0.0004$, and on the 2nd leaf at day 6; $p < 0.02$).

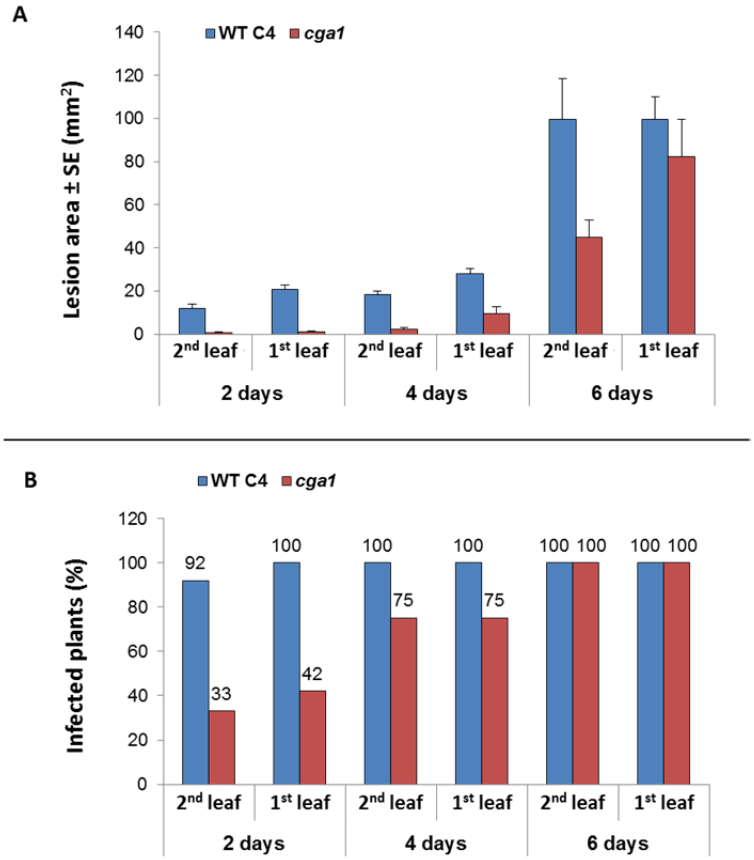

Figure 6. Infection time course for inoculation of intact leaves: effect of infection period on severity of lesions caused by wild-type C4 and *cga1*. A. Conidial suspensions (10^3 spores/drop) were used to infect intact maize first seedling leaves at the age of seven days; the seedlings were incubated for the indicated times at 25ºC. Values represent an average lesion size (mm^2) of at least 10 leaves for each time point ± standard error. **B**. Values represent infection (any visible injury) percentage of the same plants.

Virulence assays for *C. heterostrophus* G-protein signalling disrupted strains

Until now, we have primarily used spore suspension to inoculate the plant leaves. At this point, we intended to apply the conditions set determined in the above experiment and define the relative pathogenicity level of two additional signalling mutant strains, *cgb1* and the double mutant *cga1 cgb1* (G-protein Gα and Gβ subunit deficiency strain, (Degani, 2013a)). These strains are incapable of producing conidia, so the mycelia fragment infection method was used instead for all the strains. As described in Figure 2, the WT and *cga1* strains spore suspension or mycelia fragments suspension could infect intact leaves equally well. Finally, we used the well-defined method developed here to evaluate all of our G-protein signalling deficiency mutant strains in order to evaluate their role in determining the pathogenic behavior of *C. heterostrophus* (Figure 7).

The virulence assays condition set involved placing 5 µl drops of 50 mg wet weight mycelia per ml of 0.05% Tween 80 suspension on the upper third part of each of the first three intact leaves, incubation for four days in a wet chamber and measuring the lesion area. This experiment enabled us to classify the mutants in order of decreasing virulence: WT > cga1 > cgb1 > cga1 cgb1 (Figure 7, significant difference from the WT, $p < 0.05$ for the 1st leaf of *cga1*, the two first leaves of *C4cga1*, and for all three leaves of *cgb1* and the double mutant, *cga1 cgb1*). As can be clearly seen, symptoms production is virtually abolished on the

double, *cga1 cgb1* mutant leaves. A deeper examination of the results revealed an interesting difference in the pathogenic behaviour of the mutant strains (Figure 7). When infected with single mutant *cgb1*, the 1st and 3rd leaves displayed comparable resistance, which is greater than the 2nd leaf. This observation was similar to the scenario of infection with *cga1 cgb1* double mutant. In contrast, the two *cga1* mutant strains generated different results: the 1st and 2nd leaves became equally resistant to *cga1* mutant infection while the 3rd leaf was the most susceptible one.

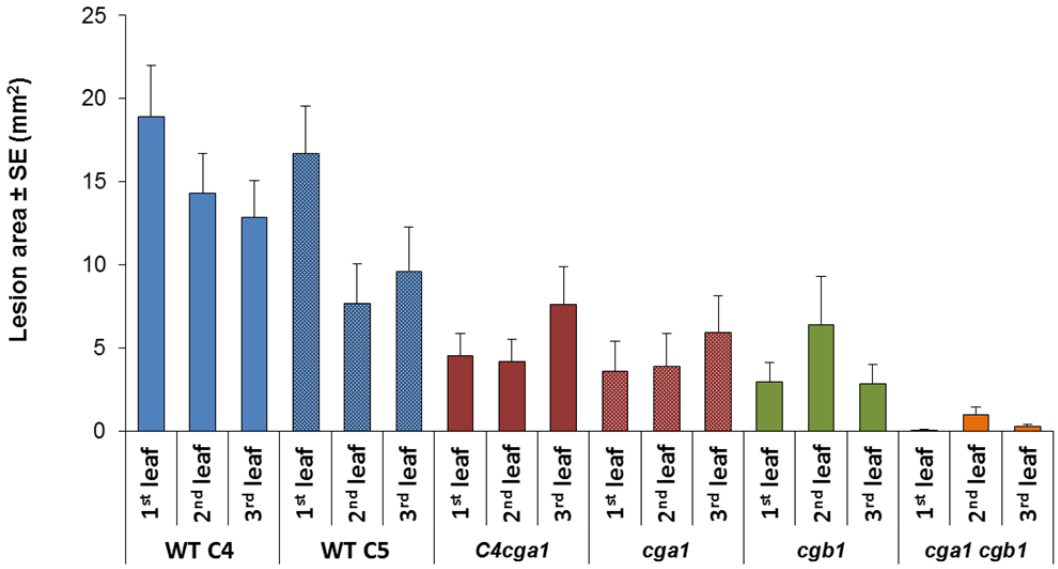

Figure 7. Virulence assays for the WT and G-protein signalling disrupted mutant strains. The first three intact leaves were inoculated by depositing drops (5 µl 0.05% Tween 80) of mycelial suspension (250 µg wet weight/drop) on the upper third part of each leaf. Incubated time was four days. Values represent an average lesion size (mm^2) of 18 leaves ± standard error.

Discussion

Mutants in the Gα subunit gene *cga1* cause lesions on the host leaf despite the abnormal development of germ tubes and decreased ability to form the small appressoria characteristic

of this species (Horwitz et al., 1999; Degani et al., 2004). Following spray inoculation and incubation for 6-7 days, symptoms caused by wild type and mutant were very similar. Here, we develop an *in vitro* pathogenicity assay that is based on conditions under which the wild type has a clear advantage over the mutant. The largest difference is especially noticeable on intact, young (9–12 days) leaves. On detached leaves (Figure 3) or on the first seedling leaf scored six days after inoculation (Figure 6), the *cga1* mutants cause symptoms that are almost as severe as those caused by the wild-type isolate. The simplest explanation for these differences is that the advantage conferred by an active *cga1* pathway is most evident under the conditions that are the most difficult for the pathogen.

One aspect might be the availability of nutrients. This hypothesis was also suggested as an explanation for the influence of culture conditions on saprophytic growth of G-protein mutants of *Cryphonectria parasitica* (Segers and Nuss, 2003). Detaching the leaf accelerates senescence and increases degradation of macromolecules (Degani et al., 2004). If the mutant is deficient in secreted enzymes, as reported for *Botrytis* (Gronover et al., 2001), a detached leaf is likely to be a more accessible nutrient source (Sinha, 1971). Consistent with this explanation, in saprophytic cultures, wild type and *cga1* show no major difference in growth rate (same culture diameter on complete medium plates). Indeed, in saprophytic culture, the G-protein α subunit mutant strains had WT levels of cellulase, pectinase and protease degradation activities, but grew significantly slower on minimal medium containing maltose (Degani, 2014). This weakened ability implies an essential role of the G-α subunit signalling in some poor nutritional environments. The 1st leaf may be acting as a source of carbon for the 2nd and 3rd leaves, which would act as a sink as they develop. This interpretation is supported by the observation of decreased infection of the 1st leaf and increased infection of the 2nd and 3rd leaves during early development of the seedlings (Figure 5). The 1st leaf may therefore be a favored nutritional surface for the growing fungus. Similarly, very young

seedling leaves may be a less accessible source of nutrients, e.g., sugars might not be as available since the photosynthetic rate has not yet peaked. Thus, easily available nutrients may minimize the difference between wild type and mutant.

Nutrient availability is probably not the only factor responsible for the difference in virulence between wild type and mutant. The deficiency in appressorium formation may also slow penetration of the mutant into the leaf. For example, it has recently been shown that *Curvularia lunata* deletion mutant of the *Clg2p* gene (a Ras protein homologous) had altered appressorium formation and conidial morphology. This mutant produced fewer, smaller lesions compared to the wild-type strain (Liu et al., 2016). The loss of difference between wild type and mutant with increased incubation time (Figure 6) is consistent with this second hypothesis. Indeed, the delay in infection by the MAPK mutant, *chk1* (Figure 1), and the double *cga1 cgb1* mutant (Figure 7), which completely lack appressoria, is much more extreme, and full wild-type virulence has never been observed (Lev and Horwitz, 2003). Nevertheless, when *chk1* penetrates the leaf, some necrosis does occur (Figure 1). The expression of two cellulase genes reaches wild type levels after a further delay that apparently results from both the slowed penetration and the direct modulation of cellulase gene expression by *chk1* (Lev and Horwitz, 2003; Degani 2014).

Senescence may enhance fungal infection, providing a third factor relevant to the difference between wild type and mutant. In *Arabidopsis* plants, detachment-associated physiological and molecular state changes of the leaf, such as initiation of salicylic acid- and ethylene-dependent senescence and inactivation of defense pathways, seems to contribute to the colonization of the plant by *Colletotrichum* spp. (Liu et al., 2016). In the work presented here, the *cga1* strain was less effective than the wild-type strain in infecting leaves detached from maize plants 27 days old or older. On leaves detached from seedlings younger than 10 days, *cga1* had a virulence similar to wild type. On intact leaves, however, *cga1* was always less

virulent than wild type. Stresses, e.g., nitrogen limitation and dryness, promote leaf senescence (Gan and Amasino, 1997). The *cga1* mutant was less virulent towards benzyladenine- (BA, a commercial cytokinin) treated detached leaves, which senesce more slowly, supporting the hypothesis that senescing leaves are more susceptible to attack by *cga1* (Degani et al., 2004).

A fourth explanation for the difference between wild-type and mutant strains might be the ability of the mutant to respond to plant surface signals, which play a major role in fungal infection (Kolattukudy et al., 1995). Topographical features of the plant surface and chemicals on the surface can trigger germination of fungal conidia and the differentiation of germ tubes into appressoria (Gilbert et al., 1996; Staples and Hoch, 1995). Despite their importance, the nature of the plant signals triggering this programmed differentiation is poorly understood. Thus, nutrient accessibility, the developmental and senescence stage of the host leaf, appressorium formation and surface sensing may all be important for determining the best method to study pathogenic variations in the fungal strains. The phenotypic variation that we have described here implies that the roles of fungal signal transduction genes must be defined in the context of the physiological state of the host. It is well known that host plants are more susceptible to fungal infection at some time in their growth cycle (Agrios, 2005). Fungal signal transduction pathways are therefore likely to be important in the timing of plant susceptibility to fungal disease.

The intact leaves droplets assay offers many advantages over other *C. heterostrophus* virulence tests. Within only four days of inoculation with the fungi, an accurate estimation of virulence can be made. The results are clear and consistent, and correlate well with results from other virulence test methods using the same fungal mutant strains published previously (Horwitz et al., 1999; Ganem et al., 2004; Lev et al., 1999; Degani, 2013). Additionally, it is economical, saves time and can be used to identify any possible genetic variability among *C.*

heterostrophus strains, even slight changes that are barely observed by the naked eye. The same approach, with some adjustments, could be used to evaluate pathogenic variations in other foliar fungal diseases.

Conclusions

Virulence test methods are an essential means for investigating phytopathogenic fungi, therefore the development of these methods is a continuous effort. In the post-genomic era, fungal mutant strains serve as a powerful means for identifying and investigating the role of specific genes and their involvement in pathogenesis. The present work is a detailed and well-controlled analysis of *Cochliobolus heterostrophus* pathogenic behavior and the involvement of conserved eukaryotic signal transducing proteins in the host inoculation outcome. The data presented revealed that detached leaves are significantly more vulnerable to the disease than intact ones. In intact leaves, *cga1* were less infective of maize under most conditions. This difference was maximized when the first seedling leaf was chosen for the inoculation and when the infected leaves were incubated for a period of four days. This optimal conditions set proved to be accurate and sensitive enough to identify even slight variations in virulence, and enabled us to classify the *C. heterostrophus* G-protein signalling mutants in order of decreasing virulence. This method could be modified for use in studies of other foliar phytoparasitic fungi.

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