

# Host Physiology and Pathogenic Variation of *Cochliobolus heterostrophus* Strains with Mutations in the G Protein Alpha Subunit, CGA1

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**Conserved eukaryotic signaling proteins participate in development and disease in plant-pathogenic fungi. Strains with mutations in *CGA1*, a heterotrimeric G protein G alpha 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. 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.  $\Delta$ *cga1* mutants of *C. heterostrophus* were less infective of several maize varieties under most conditions, but not all, as virulence was nearly normal on detached leaves. This difference could be related to the rapid senescence of detached leaves, since delaying senescence with cytokinin also had differential effects on the virulence of the wild type and the  $\Delta$ *cga1* mutant. In particular, detached leaves may provide a more readily available nutrient source than attached leaves. Decreased fitness of  $\Delta$ *cga1* as a pathogen may reflect conditions under which full virulence requires signal transduction through *CGA1*-mediated pathways. The virulence of these signal transduction mutants is thus affected differentially by the physiological state of the host.**

Eukaryotic organisms perceive and respond to the external environment through a variety of transmembrane signaling mechanisms. A well-conserved response pathway involves heterotrimeric ( $\alpha\beta\gamma$ ) GTP-binding proteins (6). A large number of fungal genes encoding alpha subunits of heterotrimeric GTP-binding proteins have been cloned (for examples, see references 7, 8, 14, and 21). There is genetic evidence for the importance of these genes in developmental processes, including mating (9, 17), sexual and asexual sporulation (1, 9), hyphal growth (1), and pathogenicity (4, 7, 14). In the maize pathogen *Cochliobolus heterostrophus*, strains with mutations in the G protein alpha-subunit gene *CGA1* are defective in mating and appressorium formation, but unlike strains with mutations in homologous genes in other fungal pathogens, the  $\Delta$ *cga1* mutants remained highly virulent to maize (8). *CGA1* is the *C. heterostrophus* ortholog of *MAGB*, and the *CGA1* product belongs to the fungal Gi class of heterotrimeric G proteins (8). Members of this genetic class often are required for full virulence, while disruption of other G $\alpha$  genes confers no major phenotype (for examples, see references 7 and 14).

Signaling through the Cga1 G protein pathway may be needed for full fitness as a pathogen under some conditions but not others. For example, Liu and Dean (14) showed that *Magnaporthe grisea* *MAGB* mutants have reduced virulence in young rice leaves but are fully pathogenic in older plants. When young rice seedlings (2 weeks old) were spray inoculated, germinated conidia of *magB*<sup>-</sup> transformants formed very few appressoria on leaves of young (2-week-old) rice seedlings, but in older rice plants (4 to 5 weeks old) appressorium formation increased with the age of the leaves.

Our objective was to determine if the virulence of the  $\Delta$ *cga1* mutants depended on host and environmental conditions. Our working hypothesis was that the status of the host plant is sensed by a fungal signal transduction pathway in which *CGA1* participates. This hypothesis was supported by our results and suggests that information on factors such as nutrient accessibility and the developmental and senescence status of the host might be important host characters for which data are transmitted by the fungal signaling pathway that contains *CGA1*.

## MATERIALS AND METHODS

**Strains.** Wild-type *C. heterostrophus* strains were C4 (*MAT1-2 Tox1*<sup>+</sup> [ATCC 48331]) and C5 (*MAT1-1 Tox1*<sup>-</sup> [ATCC 48332]). These strains were obtained after six backcrosses (11) and are nearly isogenic. Strains, previously developed (8), with mutations in the G protein alpha-subunit gene *CGA1* were C5 $\Delta$ *cga1* (*MAT1-1 tox cga1*; created by insertion of the hygromycin cassette into the coding region, combined with an 18-bp deletion) and C4 $\Delta$ *cga1* *TSC17* (*MAT1-2 Tox1*<sup>+</sup> GFP *cga1*; created by complete deletion of the coding region). C4 $\Delta$ *cga1* *TSC17* is abbreviated C4 $\Delta$ *cga1*.

**Culture conditions and virulence assays.** Fungal strains were grown on complete medium (CM) (20) with or without 50  $\mu$ g of hygromycin B/ml for about 10 days at room temperature in continuous light from cool white and UV A-enriched fluorescent tubes (Philips, Eindhoven, The Netherlands). Conidia were collected by scraping them off the colony surface with a scalpel into sterile water containing 0.05% Tween 80. Maize cultivars available at the Institute of Cereal Crops Improvement, Tel Aviv University, were tested (Table 1). Unless otherwise noted, cultivar Grand Jubilee was used throughout. Maize seedlings were 9 to 12 days old unless otherwise indicated; within the 9- to 12-day range, plants were used when the third leaf had emerged, remained partly rolled, and was beginning to expand.

Seedlings were inoculated by depositing drops (5  $\mu$ l each) of the suspension described above at a concentration of 1,000 conidia/drop on the leaves unless indicated otherwise. One drop was deposited on the upper third of the first three detached or undetached leaves. The plants in a moist chamber, or detached leaves in a closed petri dish (with a wet Whatman no. 1 filter paper underneath), were then incubated for 2 to 3 days at 30°C (unless otherwise indicated) under continuous white light in a growth chamber. For spray inoculation, plants were

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TABLE 1. Virulence assay comparing the C5 $\Delta$ *cga1* mutant to the C4 wild type on seedlings of different 12-day-old maize cultivars

Cultivar	Avg lesion			Area $\pm$ SE (mm <sup>2</sup> ) <sup>a</sup>		
	Wild-type C4			C5 $\Delta$ <i>cga1</i> mutant		
	1	2	3	1	2	3
Dwarfish	32 $\pm$ 8.3	14 $\pm$ 4.3		1.5 $\pm$ 1.3	1.1	
Bonus	22 $\pm$ 5.8	8.5 $\pm$ 1.8	2.9 $\pm$ 1.8	1.3 $\pm$ 0.0	1.3 $\pm$ 0.2	1.4 $\pm$ 0.2
Extra	19 $\pm$ 1.6	11 $\pm$ 3.1	4.3 $\pm$ 1.7	1.5 $\pm$ 0.3	1.6 $\pm$ 0.3	1.3 $\pm$ 0.4
Grand Jubilee	17 $\pm$ 2.8	6.8 $\pm$ 1.4	1.8 $\pm$ 1.1	1.7 $\pm$ 0.2	1.6 $\pm$ 0.3	1.3 $\pm$ 0.2
Royalty	13 $\pm$ 1.7	8.1 $\pm$ 2.2	2.1 $\pm$ 1.2	2.0 $\pm$ 0.3	1.6 $\pm$ 0.3	1.5 $\pm$ 0.3
Fodder	7.5 $\pm$ 5.6	6.2 $\pm$ 1.5	4.0 $\pm$ 2.1	1.5 $\pm$ 0.2	1.6 $\pm$ 0.4	1.5 $\pm$ 0.4

<sup>a</sup> Values were determined for at least seven leaves, except for the Fodder (four leaves) and Dwarfish (1 to 3 leaves) cultivars. The differences are significant ( $P < 0.05$ ;  $t$  test) between C4 and C5 $\Delta$ *cga1* in the first two leaves of the Bonus, Extra, Grand Jubilee, and Royalty cultivars. No third-leaf data were obtained for the Dwarfish cultivar because of its low rate of growth. 1, first leaf; 2, second leaf; 3, third leaf.

sprayed with a spore suspension containing  $10^5$  conidia/ml until runoff occurred, as described previously (8).

After inoculation, the plants were kept for 16 h in closed plastic cylinders with 100% humidity in a greenhouse with a 14-h photoperiod at 25°C. Photographs were taken 3 and 6 days after inoculation. For inoculation with mycelia, cultures were grown on CM for 14 days at room temperature (24 to 26°C) in continuous light from cool white and UV A-enriched fluorescent tubes. Six culture agar disks, each 1 cm in diameter, were cut from the margins of the colony of each plate and transferred to a 50-ml polycarbonate screw-cap test tube containing 20 ml of CM. The cultures were incubated diagonally in a rotary shaker at 230 rpm and at a temperature of 30°C. Mycelia were collected by centrifugation (10 min, 600  $\times$  g) and briefly homogenized (20 s; Polytron, Brinkmann Instruments, Westbury, N.Y.). Fifty milligrams (wet weight) of mycelia per ml of 0.05% Tween 80 was used to infect leaves. For quantitative analysis, leaves were scanned (300-dpi resolution), and the percentage of infected leaves, lesion diameter, or lesion area (calculated using Adobe Photoshop 6.0 and Tina 2.10 g software; Raytest, Straubenhardt, Germany) was used to evaluate the severity of the infection.

**Senescence effect.** Detached leaves were used to determine the influence of senescence on the infection ability of the  $\Delta$ *cga1* and wild-type strains, using senescence-associated hormones. The ethylene precursor amino-cyclopropane-1-carboxylic acid (ACC; 1 mM) in pure deionized water; 0.1 mM benzyladenine (BA), a commercial cytokinin (Duchefa); or pure deionized water alone was used for wetting the filter paper beneath infected detached leaves in a petri dish. All other conditions were as described for the virulence tests.

## RESULTS

**Virulence of  $\Delta$ *cga1* mutants.** Intact 12-day-old seedlings of six different cultivars were inoculated with conidia of the

TABLE 2. Virulence assay comparing C4 $\Delta$ *cga1* mutant to the wild-type line C4 from which it was derived<sup>a</sup>

Strain and leaf	Avg lesion area $\pm$ SE (mm <sup>2</sup> ) after infection with:	
	Mycelia	Spores
WT C4		
1	19 $\pm$ 3.1	19 $\pm$ 0.9
2	14 $\pm$ 2.4	17 $\pm$ 1.3
C4 $\Delta$ <i>cga1</i> mutant		
1	4.5 $\pm$ 1.3	6 $\pm$ 0.8
2	4.2 $\pm$ 1.4	3.6 $\pm$ 0.8

<sup>a</sup> Mycelial inocula or conidial suspensions of the mutant or the wild type (WT) were used to infect the upper third of the first (1) and second (2) leaves. Values represent an average for at least seven leaves.

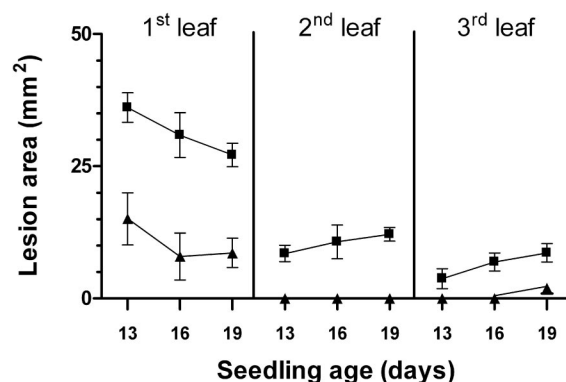


FIG. 1. Effect of seedling age on infection severity. C4 wild-type and C5 $\Delta$ *cga1* spore suspensions (1,000 conidia/drop) were used to infect leaves of 13-, 16-, and 19-day-old maize seedlings. ■, C4; ▲, C5 $\Delta$ *cga1*. Values represent averages of results for at least nine leaves of the same age. Bars indicate standard errors.

C4 and C5 $\Delta$ *cga1* strains of *C. heterostrophus*. On the first two leaves of all cultivars, virulence of C5 $\Delta$ *cga1* was strongly reduced (Table 1). There were variations in the average lesion size caused by C4 on different cultivars, and the first seedling leaf was more sensitive to infection than were the second and third leaves. The difference in results between C5 $\Delta$ *cga1* and C4 was significant ( $P < 0.05$ ) for four of the cultivars. The decreased virulence occurred in two independently constructed mutants (Table 2; see Fig. 2 and Table 4). The genetic backgrounds of these strains, like the nearly isogenic wild-type strains from which they were derived, differ in mating type and at the *Tox1* locus (see Materials and Methods).

We tested various conditions to determine when the difference between G protein alpha-subunit disruption mutants and wild-type strains is the greatest. Clear differences in virulence were observed when young seedlings (two- to three-leaf stage) were inoculated, with both C4 and C5 $\Delta$ *cga1* more able to infect the first leaf than the second and third leaves (Fig. 1). Symptoms decreased on the first leaf with seedling age but increased slightly with seedling age on the second and third leaves (Fig. 1). With time, the lesions caused by C5 $\Delta$ *cga1* increased in size faster than those caused by C4, resulting in almost no difference when they were assayed on the first seedling leaf 6 days after inoculation (Table 3). Reducing the inoculum load 10-fold also reduced the extent of the lesions, but the relationship between C5 $\Delta$ *cga1* and C4 was similar (data not shown).

Conidial suspensions of C5 $\Delta$ *cga1* and wild-type C5 were inoculated either as droplets or as a spray on young seedlings (two- to three-leaf stage; 8 days), and symptoms were recorded at 3 days after inoculation. The mutant was much less virulent than the wild type, and the difference was observed with either drop or spray inoculation with conidia (data not shown). Loss of virulence also occurred when mycelial fragments of C5 $\Delta$ *cga1* or wild-type C5 were used as the inoculum (Table 4). When 11-day-old seedlings were spray inoculated and incubated at 25°C (Fig. 2), the symptoms caused by C4, C4 $\Delta$ *cga1*, and C5 $\Delta$ *cga1* were nearly identical, as expected from earlier work (8), and independent of the maize variety which was different from that used in the previous study (8). C5 $\Delta$ *cga1* and C4 $\Delta$ *cga1* had a slightly reduced tendency to infect the upper part of the

TABLE 3. Infection time course for inoculation of intact leaves: effect of infection period on severity of lesions caused by wild-type C4 and the C5Δ*cga1* mutant<sup>a</sup>

Time after infection and leaf	Avg lesion area ± SE (mm <sup>2</sup> )	
	C4	C5Δ <i>cga1</i>
2 days		
1	21 ± 2.0	0.9 ± 0.4
2	12 ± 2.1	0.6 ± 0.3
4 days		
1	28 ± 2.3	9.5 ± 3.4
2	18 ± 1.6	2.4 ± 0.6
6 days		
1	99 ± 11	82 ± 17
2	99 ± 19	45 ± 8.3

<sup>a</sup> Conidial suspensions of C4 or C5Δ*cga1* were used to infect maize seedlings at age 7 days; the seedlings were incubated for the indicated times at 25°C. Values represent average results for at least 10 leaves for each time point. 1, first leaf; 2, 2nd leaf.

leaf, but this difference was much less noticeable at 6 days after inoculation (Fig. 2).

**Senescence and infection of detached leaves by the G alpha-subunit mutant C5Δ*cga1*.** Wild-type C4 and the C5Δ*cga1* mutant were less virulent on leaves from mature plants than they were on younger plants (Fig. 3). This reduction was more pronounced for C5Δ*cga1*. Neither C4 nor C5Δ*cga1* could infect leaves from 80-day-old plants. Leaves from 10-day-old seedlings were similar in susceptibility to both C4 and C5Δ*cga1*. Detached leaves from the older plants were long enough for a series of spore suspension drops to be placed along the leaf. Wild-type C4 and C5Δ*cga1* caused significantly larger lesions (in diameter) on the upper part of the detached leaf than at the lower part ( $P < 0.05$ ; data not shown). In addition, the first leaf, detached from 8-day-old seedlings, was more easily infected by C4 and C5Δ*cga1* than were the second and third leaves, as observed for the leaves of intact plants (e.g., with water inoculation) (Fig. 4). The C4 wild type could infect detached and intact leaves equally well, while the C5Δ*cga1* mutant could more effectively infect detached leaves than it could the leaves of intact plants (significant at a  $P$  value of  $<0.01$ ; calculation from additional data not shown).

Detaching leaves accelerates their senescence (15). We treated detached leaves with the synthetic cytokinin BA, which inhibits senescence, and the ethylene precursor ACC, which accelerates senescence, and evaluated C5Δ*cga1* infectivity. The hormone effects depend on leaf age. For the C4 wild type, BA had no significant effect on the first leaves, which are the most susceptible to infection, or the third leaves (Fig. 4; Table 5). On the second leaves, BA decreased the severity of infection (Fig. 4). ACC increased the severity of infection on the third leaves, but it had no effect on the second and first leaves. For C5Δ*cga1*, BA decreased infection of the first and second leaves but not the third leaves, while ACC had no significant effects. Note, however, that ACC promoted the development of aerial hyphae (Fig. 4), following infection with the mutant. BA had a more marked effect on C5Δ*cga1* ( $P$  value of  $<0.01$  for results for the first and second leaves) than on the wild type. The effect of BA was even more pronounced after longer incubation, as leaves that were not treated with BA had widespread chlorosis

and wilting, and the lesion size could no longer be determined accurately. Taken together, these results suggest that once leaves begin to senesce, the Cga1 pathway is of less importance in the pathogenicity process.

## DISCUSSION

Strains with mutations in the G alpha-subunit gene *CGAI* 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. Following spray inoculation and incubation for 6 days, symptoms caused by the wild type and mutant were very similar (Fig. 2) (8). We also identified other conditions under which the wild type has a clear advantage over the mutant. The largest difference is observed on intact, young leaves (9- to 12-day-old seedlings). On detached leaves (Fig. 3) or on the first seedling leaf when scored 6 days after inoculation (Table 3) the Δ*cga1* mutants cause symptoms that are 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.

Differences in nutrient availability could be responsible for observed differences in virulence, a hypothesis also suggested as an explanation for the influence of culture conditions on saprophytic growth of G protein mutant strains of *Cryphonectria parasitica* (18). Detaching the leaf accelerates senescence, increasing the degradation of macromolecules (15). The mutant may be deficient in secreted enzymes, as reported for G alpha-subunit mutants of *Botrytis cinerea*, which were deficient in proteases (7). *C. parasitica* lines where G protein signaling was suppressed by hypovirus infection were deficient in induction of cellobiohydrolase I (22). A detached leaf is likely to be a more accessible nutrient source than an intact leaf (19). Consistent with this explanation, in saprophytic cultures such as CM, the C4 wild type and C5Δ*cga1* mutant do not differ significantly in linear growth rate. Infection of the first leaf decreased with time, while infection of the second and third leaves increased with time during early seedling development (Fig. 2). The first leaf could serve as a carbon source for the second and third leaves, which would act as carbon sinks as they develop. The first leaf would initially be a favored nutritional source but with time would provide fewer nutrients as these compounds were translocated to the developing leaves.

TABLE 4. Comparison of C5Δ*cga1* mutant and wild-type C5 after inoculation with mycelia<sup>a</sup>

Strain and leaf	Avg lesion size ± SE (mm <sup>2</sup> )
WT C5	
1 .....	17 ± 2.8
2 .....	7.7 ± 2.4
C5Δ <i>cga1</i>	
1 .....	3.6 ± 1.8
2 .....	3.9 ± 2.0

<sup>a</sup> A suspension obtained by brief sonication of mycelia was used for inoculation. Values indicate mean lesion sizes for at least 7 leaves. WT, wild type; 1, first leaf; 2, second leaf.

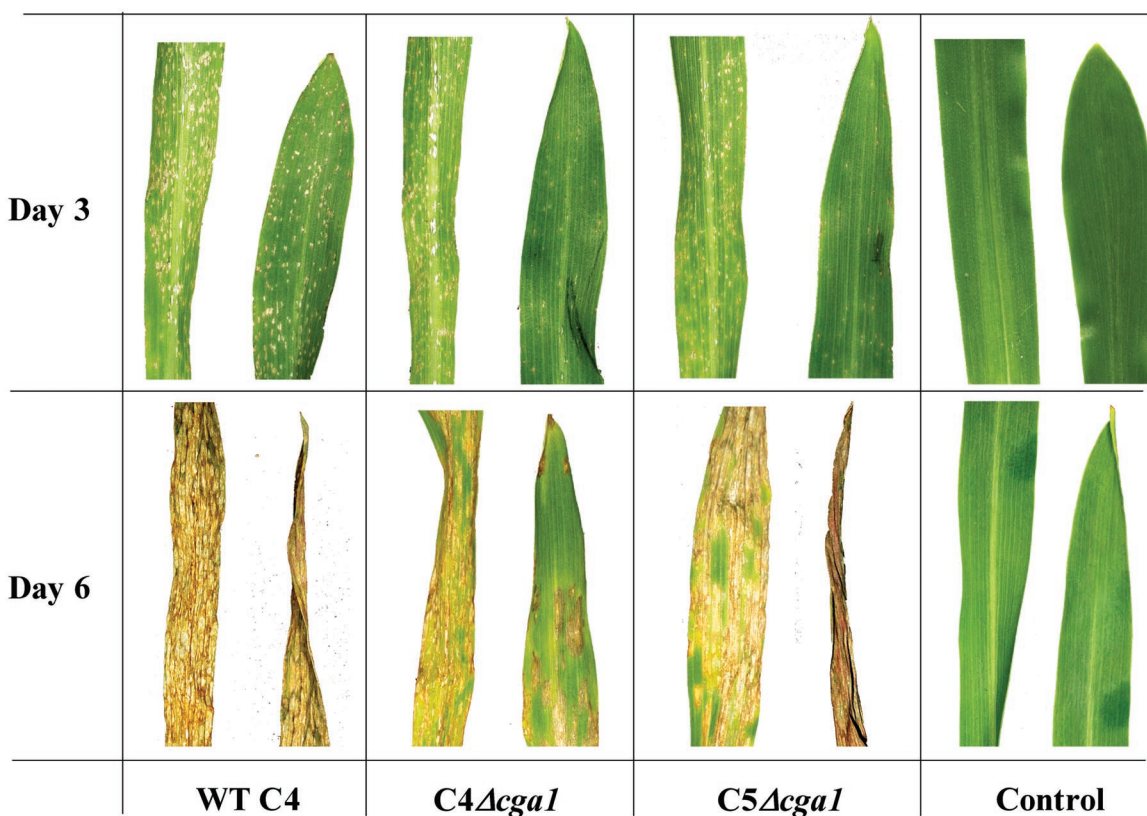


FIG. 2. Inoculation of intact leaves by spraying of conidial suspensions. Maize seedlings were inoculated at age 11 days by spray. Photographs were taken after 3 and 6 days. WT C4, wild-type *C. heterostrophus* strain C4.

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, the ready availability of nutrients may explain the decreased difference in

virulence observed between the C4 wild type and the *C5Δcga1* mutant.

Nutrient availability is probably not the only factor responsible for the difference in virulence between the wild type and the mutant. The deficiency in appressorium formation may slow penetration of the leaf by the mutant. The loss of the

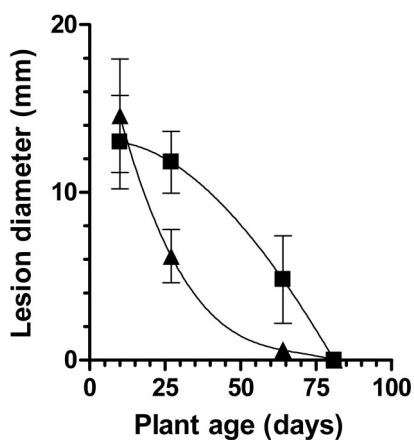


FIG. 3. Infection of detached leaves as a function of plant age. Leaves from maize plants of different ages were inoculated with *C. heterostrophus* wild-type C4 (■) and the G protein alpha-subunit mutant *C5Δcga1* (▲). Infection was done on the most-developed detached leaves in petri dishes with wet filter paper, by deposition of drops (5 μl) of spore suspension (100 conidia/drop). Values represent averages of results for five to seven leaves of the same age; each leaf had 2 to 4 drops placed on it. Bars indicate standard errors.

	<i>C5Δcga1</i>	WT C4
BA		
DDW		
ACC		

FIG. 4. Effect of leaf senescence on the virulence of the pathogen. Second leaves were detached and infected with drops of conidial suspension (1,000 conidia/drop). The filter paper in the plates was wetted with 0.1 mM BA, 1 mM ACC, or pure deionized water (DDW). White masses indicate aerial mycelia. Photographs were taken after 3 days of incubation.

TABLE 5. Effect of hormone treatments influencing leaf senescence on pathogen virulence<sup>a</sup>

Strain and leaf	Avg lesion area $\pm$ SE (mm <sup>2</sup> ) with:		
	BA	DDW	ACC
C5 $\Delta$ <i>cga1</i> mutant			
3	12 $\pm$ 1.7	11 $\pm$ 2.9	11 $\pm$ 4.5
2	9.1 $\pm$ 3.6	21 $\pm$ 2.3	19 $\pm$ 3.4
1	14 $\pm$ 2.4	24 $\pm$ 4.2	28 $\pm$ 6.4
WT C4			
3	10 $\pm$ 3.3	14 $\pm$ 3.9	25 $\pm$ 4.3
2	27 $\pm$ 4.4	39 $\pm$ 4.4	36 $\pm$ 3.1
1	45 $\pm$ 6.8	47 $\pm$ 3.2	51 $\pm$ 2.5

<sup>a</sup> The first three leaves (1, 2, 3) were detached and infected with drops of conidial suspension. The filter paper in the plates was wetted with 0.1 mM BA, 1 mM ACC, or pure deionized water. Representative second leaves are shown in Fig. 4. Values represent the average lesion areas for at least seven leaves.

difference between the wild type and the mutant with increasing incubation time (Table 3) is consistent with this second hypothesis. The mitogen-activated protein kinase mutant, the  $\Delta$ *chk1* strain, completely lacks appressoria but can still cause some necrosis when it penetrates a leaf, even though full wild-type virulence has never been observed (12, 13).

Senescence, which is enhanced by stresses such as nitrogen limitation and dryness (3), may also enhance fungal infection. C5 $\Delta$ *cga1* was less effective than the C4 wild type in infecting leaves detached from maize plants 27 days old or older. On leaves detached from seedlings <10 days old, C5 $\Delta$ *cga1* was always as virulent as the wild type. On undetached leaves, however, C5 $\Delta$ *cga1* was always less virulent than C4. The C5 $\Delta$ *cga1* mutant was less virulent towards BA-treated detached leaves, which senesce more slowly, supporting the hypothesis that senescing leaves are more susceptible to attack by  $\Delta$ *cga1*. As mentioned above, senescing leaves may provide a more readily accessible nutrient source but could also be the source of other senescence-specific signals, which the fungus could sense and respond to.

Finally, the wild-type and mutant strains might respond differentially to plant surface signals (10). For example, 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 (5, 20). Despite their importance, the nature of the plant signals that trigger such programmed differentiation is poorly understood.

Thus, the status of nutrient accessibility, developmental and senescence stages of the host leaf, appressorium formation, and surface sensing could be transmitted by the CGA protein in *C. heterostrophus*. In general, mutant genotypes are expressed as phenotypes in a way that depends on developmental stage, physiology, or environmental conditions. This dependency is apparent, for example, in the definition of the best conditions for scoring auxotrophic and other genetic markers in *Neurospora crassa* (16). The phenotypic variation described here implies that the roles of fungal signal transduction genes need to be defined in the context of the physiological state of the host. Host plants are more susceptible to fungal infection at particular times in their life cycle (2), and the signal transduction pathways of the pathogen might play a role in determining the timing of plant susceptibility to fungal disease.

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