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Cochliobolus heterostrophus T-toxin Gene Expression Modulation via G Protein and MAPK Pathways

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Abstract

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The role of G-protein and mitogen-activated protein kinase (MAPK) in filamentous fungi has been studied for over a decade, but downstream elements are less known. Here, we used microarray and Northern blot analysis to examine the involvement of these signalling pathways in controlling the known *Cochliobolus heterostrophus* T-toxin biosynthetic gene, *DEC1*, whose control is important in epidemic prevention. Comparison of the expression profile in wild-type strains and in G-protein and MAPK signalling deficient mutants revealed a unique, environmental-dependent control mechanism for the *DEC1* gene. The results suggest, for the first time, a possible role in pathogenicity for the G-protein α2 subunit in this organism, and hint to a common role of G-protein α1 and β1 subunits and MAPK in maintaining accurate levels of this toxin during pathogenesis.

Keywords: *Cochliobolus heterostrophus; DEC1* gene; fungal RNA; maize; signal transduction; Southern corn leaf blight

In eukaryotic cells, the small guanosine triphosphate (GTP)-binding protein Ras and heterotrimeric G proteins are involved in the transmission of external signals. In response to receptor occupancy, the G-protein α subunit (*CGA1* gene product) replaces bound guanosine diphosphate (GDP) with GTP and dissociates from the G-protein complex. Both Gα-GTP and the free Gβγ subunits can now positively activate downstream effectors, which results in the induction of gene transcription and stimulation of morphological and virulence changes. Upon GTP hydrolysis, Gα blocks further signalling via its reassociation with Gβγ (for review, see Clapham Neer 1993; Bolker 1998). In *Cochliobolus heterostrophus*, the agent of Southern corn leaf blight, G-protein subunits, Gα (*CGA1*) and Gβ (*CGB1*), as well as a mitogen-activated protein kinase (MAPK, *CHK1*), participate in several developmental pathways. Disruption studies of these signalling components result in severe phenotypes, including loss of the normal meandering hyphal growth pattern on hard surfaces, lack of appressorium formation and defects in mating and virulence (Horwitz *et al.* 1999; Lev *et al.* 1999; Degani *et al.* 2004; Ganem *et al.* 2004; Degani 2013a,b). An epistatic relationship may exist between the two branches of the G-protein pathways (Degani 2013a). MAPK is a known target of Gβγ in budding yeast and mammalian cells (Crespo *et al.* 1994; Gudermann 2008), and new lines of evidence indicate that some crosstalk may exist between the MAPK and the G-protein signalling pathways in *C. heterostrophus* as well (Degani 2013a).

Data on G-protein subunits and MAPK involvement in the regulation of gene expression in fungal species are constantly growing. Nevertheless, few genes have been shown to be regulated by signal transduction in *C. heterostrophus* to date. Among these are two cellulase genes whose expression is modulated by the MAPK pathway (Degani 2014a; Lev & Horwitz 2003) and four hydrophobins (Degani *et al.* 2013a,b). The expression of key genes involved in melanin biosynthesis in two *C. heterostrophus* MAPKs, Chk1 and Mps1, has also been shown to be induced significantly under hyperosmotic conditions

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compared to invariably high expression in the wild type (WT) (Eliahu *et al.* 2007).

One potential gene that might be a target for the G-protein and/or MAPK signalling hierarchies is the T-toxin gene, *DEC1*, which is important in epidemic prevention**.** Interest in this gene was sparked when, in 1970, a previously unknown variant of *C. heterostrophus* caused a severe epidemic in all corn hybrids containing the Texas cytoplasmic male sterility gene (T-cms, used for efficient crossing and production of corn hybrids) and destroyed about 15% of all corn produced in the United States that year (monetary lost estimation of 1 billion dollars) (Agrios 2005). The variant strain, which is strongly virulent to this corn line (T-cms), is classified as race T. The other most common strain is moderately virulent to all corn lines and is classified as race O (Carson 1998). Biochemical and genetic analyses have shown that the high virulence of race T relative to race O is due to a single metabolic difference – race T's production of a family of linear long-chain (C_{35} to C_{41}) polyketides collectively is known as T-toxin (Yoder *et al.* 1997). This T-toxin has been shown to inhibit mitochondrial function specifically in T-cms maize (Rhoads *et al.* 1995) by binding to a protein (URF13) unique to the inner mitochondrial membrane (Rhoads *et al.* 1995). Binding of T-toxin causes the formation of pores in the inner membrane (Siedow *et al.* 1995) and leakage of NAD^+ along with other solutes necessary for normal mitochondrial function (Matthews *et al.* 1979). Thus, specific interaction between a unique protein in the plant and a unique polyketide from the fungus leads to susceptibility. Plant resistance is due to the absence of URF13 rather than to an active defense mechanism.

Genes at two unlinked loci (*Tox1A* and *Tox1B*) are required for production of the polyketide T-toxin by race T (Kodama *et al.* 1999). *Tox1A* encodes a polyketide synthase (PKS1) required for T-toxin biosynthesis and high virulence. *Tox1B* includes two genes, *DEC1* (similar to acetoacetate decarboxylase-encoding genes) and *RED1* (similar to genes encoding members of the medium-chain dehydrogenase/reductase superfamily) (Rose *et al.* 2002). *DEC1* and *RED1* map within 1.5 kb of each other on the *Tox1B* chromosome 6;12 (Figure 2B) and are unique to the genome of race T, an observation consistent with the hypothesis that these genes were acquired by *C. heterostrophus* via a horizontal transfer event (Yang *et al.* 1996). Targeted disruption of *DEC1* drastically reduced both T-toxin production and virulence of race T to T-cytoplasm maize, whereas specific inactivation of *RED1* had no apparent effect on T-toxin production or virulence (Rose *et al.* 2002).

Table 1. *Cochliobolus heterostrophus* strains used

Strain	Genotype	Reference or Source	Comments
WT C4 (Race T) WT C5 (Race O)	*MAT1-2*; *Tox1*$^+$ ATCC 48331 *MAT1-1*; *Tox1*$^-$ ATCC 48332	Leach *et al.* (1982)	wild-type strains obtained after six backcrosses and that are nearly isogenic
cga1	*MAT1-1*; *Tox1*$^-$ ATCC 48332	Horwitz *et al.* (1999)	G-protein α1 subunit disrupted mutant (in the WT C5 background), created by insertion of the hygromycin cassette into the coding region, combined with an 18-bp deletion
C4cga1	*MAT1-2*; *Tox1*$^+$ ATCC 48331	Horwitz et al. (1999)	G-protein α1 subunit disrupted mutant (in the WT C4 background), created by complete deletion of the coding region and insertion of the hygromycin cassette
Cga2	*MAT1-2*; *Tox1*$^+$ ATCC 48331	Giloh M. [MSc Thesis] (in Ganem *et al.* 2004)	G-protein α2 subunit disrupted mutant (in the WT C4 background), having no known phenotypes
cgb1	*MAT1-2*; *Tox1*$^+$ ATCC 48331	Ganem *et al.* (2004)	G-protein β subunit disrupted mutant (in the WT C4 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
chk1	*MAT1-2*; *Tox1*$^+$ ATCC 48331	Lev *et al.* (1999)	MAPK disrupted mutant (in the WT C4 background), created by replacement of the coding region with the hygromycin resistance cassette

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Here, we used microarray and Northern blot analysis to identify the transcriptional profile of the *Tox1B* (*DEC1* and *RED1*) genes in *C. heterostrophus* WT, G-protein α and β subunits and MAPK signal-deficient strains. The results may define the unique contribution of each signalling unit to the regulation of this biosynthesis pathway. Moreover, the transcriptional profile may point to distinct and shared roles of the signalling components.

MATERIAL AND METHODS

Strains. The *C. heterostrophus* strains used are listed in Table 1.

Sample preparation for microarray. WT and signalling mutants were grown on different liquid media: complete medium with xylose (CMX, 48 h) or maltose (CMM, 48 h), minimal medium with xylose (MMX, 72 h), and also inoculated on maize cv. Grand Jubilee (Pop Vriend Seeds B.V., Andijk, the Netherlands, supplied by Eden Seeds, Reut, Israel) plants (as described previously Lev & Horwitz (2003) and Degani (2014b)). The intact leaves of the plants were dipped into homogenised mycelial suspension in order to obtain widespread lesions. Infected plants were incubated in a growth chamber, in a moist atmosphere, at a temperature of 30°C, under continuous fluorescent white light, for different periods of time according to virulence of mutant strains in order to produce high and as similar as possible amounts of fungal RNA. Wild-type and *cga2*-infected plants were incubated for 24 h, and plants infected by *chk1*, *cgb1* and *cga1* were incubated for 48 hours. The portion of fungal RNA in samples isolated from infected tissue varies from about 23% (inoculation by *chk1*) to 50% (inoculation by wild type). Ethidium bromide staining of gel-electrophoresed RNA enables relative estimation of the plant RNA portion in each sample: the intensity of bands representing plant chloroplast rRNA varies significantly among samples. The very few samples that did not meet the strict criteria of the microarray preparation were eliminated from the final microarray chip.

Northern blot analysis. The *DEC1* probe was synthesised by PCR using the primers pair: DEC1-for 5' CTCCCTCAATCCTCAACATGACTATC 3'; DEC1-rev 5' GTTTCAGAAGCTATAGACGTTCAATAACCTC 3'. The resultant product (760 bp long) was purified and verified by sequencing (Expect = 5e-15, identity to *DEC1* in the gene bank) (Figure 2A). Total RNA was isolated using the phenol-SDS procedure (Ausubel *et al.* 1987), precipitated with lithium acetate, and denatured with formamide and formaldehyde. Separation was carried out on a formaldehyde-containing agarose gel and blotting onto Hybond N membrane (Amersham). Hybridisations were carried out at 65°C according to Church and Gilbert (1984) with ^{32}P-labelled DNA.

Normalisation of fungal RNA quantities. As a control for normalization of RNA expression levels, we hybridised blots with an oligonucleotide probe complementary to the A *GAPDH* housekeeping gene (encoding glyceraldehyde-3-phosphate dehydrogenase), according to Lev and Horwitz (2003) and Degani (2014b). A *GAPDH* fragment generated by PCR with primers 5' CCCTCGCCTGACGCCCCCAT 3' and 5' CGAGGACACGGCGGGAGTAA 3' was used to quantify fungal RNA.

Microarray search and results processing. The *C. heterostrophus Tox1B* genes (*DEC1* and *RED1*) were used to search an Affymetrix microarray chip containing *C. heterostrophus* WT strains and different signalling deficiency mutant strains that were grown on different media, including plant infection as described above (Syngenta Biotechnology Inc., North Carolina, USA; Affymetrix chip containing 25 215 sequences based on 13 309 801 bases). This Affymetrix microarray chip is currently the property of Syngenta Biotechnology Inc. and its database is not accessible to the public. However, we were allowed to search the array and publish several scoring results. This includes the *C. heterostrophus Tox1B* genes (*DEC1* and *RED1*) described here and two other genes (*BPH* and *CIPA)* that have already been published elsewhere (Degani 2014b). The search was done using the BLASTN 2.2.6 program (Altschul *et al.* 1997). The expression of oligonucleotides showing high homology to the predicted gene was normalised and analysed with GeneSpring® software (Silicon Genetics, Redwood City, USA) (Wildsmith & Elcock 2001). The selected gene was identified by BLASTX against the gene bank database using NCBI BLAST sequence analysis services at http://blast.ncbi.nlm.nih.gov/Blast.cgi.

RESULTS

We used the known T-toxin, *Tox1B* locus gene *DEC1*, to study the role of G-protein α and β subunits and of MAPK in regulating this biosynthetic pathway and in controlling its expression. The sample preparation for the microarray (dipping the intact

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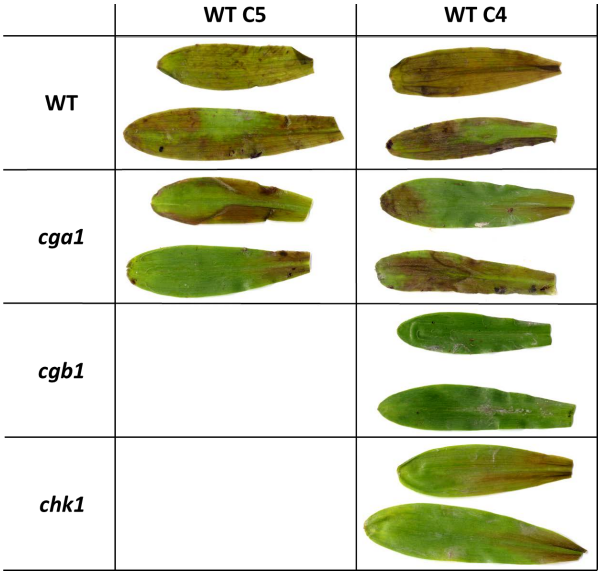

Figure 1. Disease symptoms on maize leaves. Intact leaves were inoculated with mycelial suspensions of the indicated *Cochliobolus heterostrophus* strains. Dark areas indicate necrotic regions (leaves were photographed 36 h post-infection). Left column, WT C5 (Race O, *MAT1-1; Tox1*$^{-}$) and mutants in the C5 background; right column, WT C4 (Race T, *MAT1-2; Tox1*$^{+}$) and mutants in the C4 background; mutant strains: *cga1* (Δ*Gα*1), *cgb1* (Δ*G*β1), and *chk1* (ΔMAPK1) (Table 1); for each fungal strain, two representative infected maize first leaves are shown; the *cga2* (Δ*Gα2*) strain was not included in this photo but its virulence is the same as for the WT strains

leaf in mycelia suspension of different fungal strains and incubating the infected plants for 24–48 h) can also be used as a virulence assay. Wild-type and *cga2*-infected plants were incubated for 24 h; during this time, the plant tissue was completely destroyed (Figure 1). The *cga1* and *cgb1* mutants were incubated for 48 h and caused a similar degree of infection: a large portion of maize leaf area became necrotic. The chk1 signalling deficiency mutant caused chlorosis in the leaves but with only few or no apparent necrotic patches, as described previously (Lev & Horwitz 2003) (Figure 1). This procedure enabled us to classify the mutants in a series of decreasing virulence beginning with the wild-type and *cga2* strains, followed by *cga1* and *cgb1* strains, and finally the *chk1* strain, in which the production of symptoms on the leaf is virtually abolished (Figure 1 illustrated this assay after 36 h).

The *Tox1B* sequence was taken from the gene bank and used to conduct an alignments search within the array database using the BLASTN 2.2.6 program (Altschul *et al.* 1997). Four oligonucleotides were found to be part of the *Tox1B* locus (designated as ctrl fungal 35 , Ch006021, Ch007342, and ctrl fungal 23). These four DNA segments overlap each other and all contain parts of the *Tox1B* locus (Figure 2B). Three of these oligonucleotides have both a *DEC1* and *RED1* encoding sequence while one (ctrl fungal 23) has only the *RED1* site.

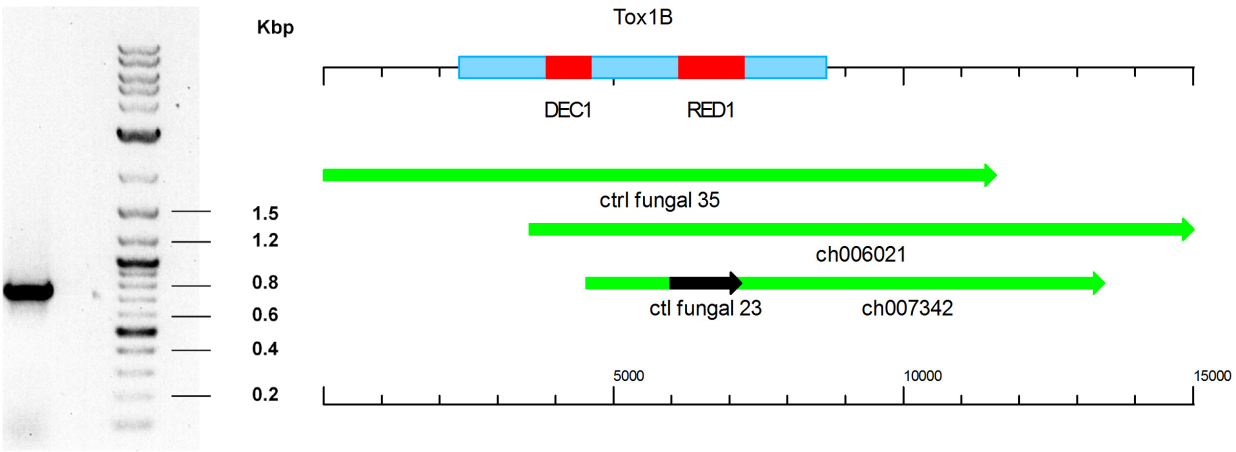

Figure 2. The *DEC1* probe synthesis and a schematic diagram of the four array oligonucleotides sets in the *Tox1B* locus: (A) The *DEC1* probe resultant product was 760 bp long; (B) Schematic diagram of the four array oligonucleotides sets in the *Tox1B* locus, required for the production of the polyketide T-toxin by *C. heterostrophus* race T. *DEC1* and *RED1* are two genes located on the *Tox1B* locus located on chromosome 6;12 (Rose *et al.* 2002). The scheme was created using Clone Manager Version 7.03 after alignment of the nucleotide against each other and against the *Tox1B* locus from the gene bank. Alignments were done using the NCBI-Align two sequences (BL2SEQ) service at http://blast.ncbi.nlm.nih.gov/Blast.cgi

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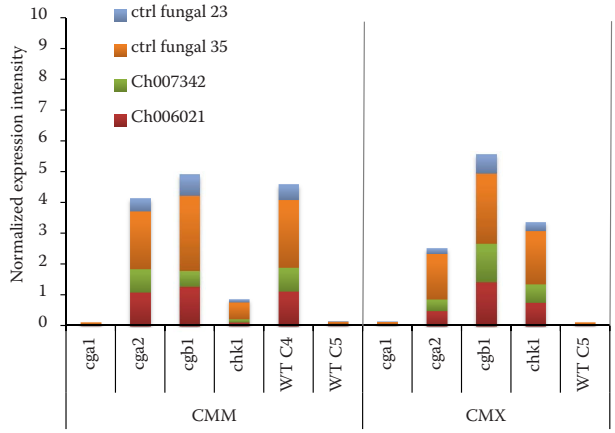

Figure 3. *C. heterostrophus Tox1B* expression profile in CMM and CMX media cultures. The WT and signalling mutants were grown on different liquid complete medium with xylose (CMX) or maltose (CMM) for 48 hours. Microarray (Affymetrix chip) normalised expression results indicate transcript levels of WT and the signal deficiency mutant strains: *cga1*, *cga2*, *cgb1,* and *chk1*. Curves indicate the expression of four array oligonucleotides that together comprised the *Tox1B* locus (as illustrated in Figure 2); strains abbreviations as in Figure 1

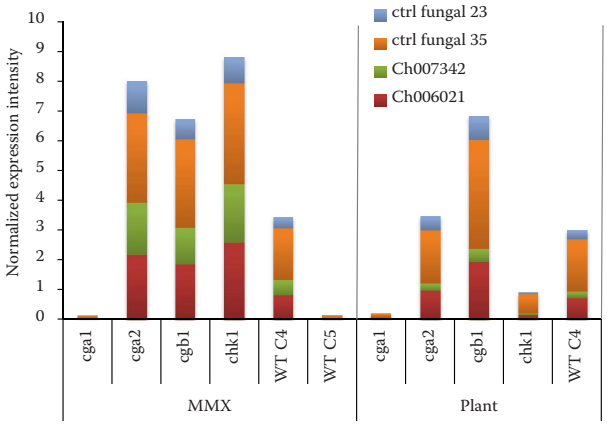

Figure 4. *C. heterostrophus Tox1B* expression profile in MMX medium cultures and in intact maize plants infected leaves. Cultures grew on minimal medium with xylose (MMX) for a period of 72 h. In the infected maize plants, the incubation periods varies from 24–48 h, according to the strains specific virulence (see Figure 1). Transcript levels (normalised expression results) were obrained using the same microarray (Affymetrix Chip) as in Figure 2. Each bar indicates the expression sum of four array oligonucleotides that together comprised the *Tox1B* locus (as illustrated in Figure 2); strain abbreviations as in Figure 1

The four array oligonucleotide sets showed similar expression profile in all media and strains, and differed only in overall expression intensity (Figures 3 and 4). A slight difference was observed in the ch007342 oligonucleotide, which showed a reduced expression level in *cgb1* on CMM medium in comparison to *cga2*. Two array oligonucleotide sets, ctrl fungal 35 and Ch006021 (which are also the longest of all four oligonucleotide tested), showed the highest expression level in all media and strains. The *C. heterostrophus* C4 (race T) differs from C5 (race O) because it produces the T-toxin (Yoder *et al.* 1997). Nevertheless, these two strains have similar virulence on maize (cultivar without T-cytoplasm) leaves (Figure 1) as stated earlier (Degani 2013a). WT C5 and *cga1* (which was created in the WT C5 background) strains showed no expression of the *Tox1B* genes under all experimental conditions, as expected (Figures 3 and 4). In contrast, WT C4, *cga2* and *cgb1* (which are in the C4 background) showed evident levels of expression. Interestingly, *chk1* (which is also in the C4 background) showed very low expression levels on CMM medium and on infected plant leaves, and reduced levels on CMX medium (Figures 3 and 4). The *chk1* expression pattern was inverted on MMX medium in which it was the highest of all strains (Figure 4). The *cga2* showed C4 WT levels of *Tox1B*, except for MMX medium in which an elevation in all mutant strains in the C4 background was observed (Figures 3 and 4). *cgb1 Tox1B* expression levels were higher than WT C4 levels in all media, but increased to more than two-fold in the poor MMX medium and on plant tissues (Figure 4).

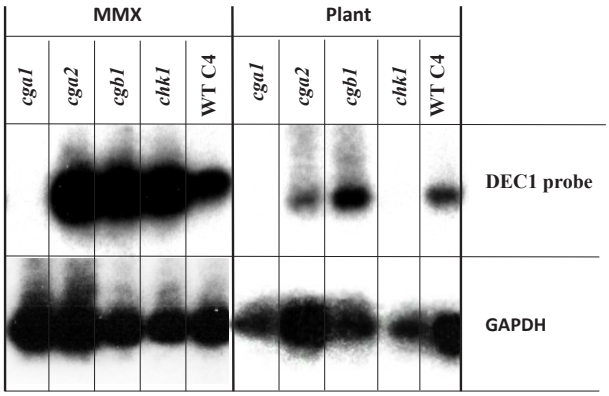

Figure 5. *C. heterostrophus Tox1B* expression profile in Northern blot analysis. Northern blot analysis indicates transcript levels of WT (C4), and signal deficiency mutant strains in MMX medium (minimal medium with xylose) cultures and in intact maize plants infected leaves (strains abbreviation as in Figure 1). GAPDH gene-encoding glyceraldehyde-3-phosphate dehydrogenase, used here as positive control

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The *Tox1B* sequence was taken from the gene bank and used as a template for the synthesis of *DEC1* probe (Figure 2A). The probe was labelled and used for Northern blot analysis of the same RNA samples used for the array (Figure 5). For the two selected growth conditions (MMX media and maize-infected leaves), the Northern blot analysis results were in complete agreement with the array analysis plots (Figures 3 and 4).

DISCUSSION

Deciphering the signalling mechanisms that lead to fungal development and pathogenicity has been studied intensively over the past decade. Although a considerable amount of data exist on the key player in these conserved pathways, the G-protein-encoding genes, and the mitogen-activated protein (MAP) kinase, less is known about downstream effectors, and the remaining components of these pathways still need to be revealed (Bolker 1998). At the output of these cascades there are sets of target genes, whose expression began to be studied in recent years. We previously identified roles for the individual G-protein Gα and Gβ subunits (Horwitz *et al.* 1999; Degani *et al.* 2004; Ganem *et al.* 2004; Degani 2013a,b) and for the MAPK (Lev *et al.* 1999) in *C. heterostrophus* development, reproduction, and pathogenicity (summarised in Degani 2013a). To date, little knowledge exists on downstream signalling elements of these cascades in this species. The influence on target genes expression was demonstrated in only a few instances (Lev & Horwitz 2003; Eliahu *et al.* 2007; Degani *et al.* 2013; Degani 2014a,b).

Here, we identified the gene encoding for the acetoacetate decarboxylase (*DEC1*) that is under the control of *C. heterostrophus* signal transduction pathway components. This gene is involved directly in the pathogenesis process and its controlling is important in epidemic prevention. Genes at two unlinked loci (*Tox1A* and *Tox1B*) are required for production of the polyketide T-toxin by *C. heterostrophus* race T, a pathogenic fungus that requires T-toxin for high virulence to maize with T-cytoplasm (Kodama *et al.* 1999). Previous studies indicated that *Tox1A* encodes a polyketide synthase (PKS1) and *Tox1B* locus containing the *DEC1* and *RED1* genes required for polyketide (T-toxin) biosynthesis and high virulence on T-cytoplasm maize (Kodama *et al.* 1999; Rose *et al.* 2002). To date, no role has been identified for G-protein and MAPK signalling components in controlling the *Tox1* associated genes.

Here, we reported that depending on growth conditions, these elements may be important for polyketide synthesis. Similar results in microarray analysis and Northern blot repetition indicate that the G-protein beta subunit and MAPK disruption altered the *Tox1B* locus expression profile. The *cgb1* showed a significant elevation of *Tox1B* transcript levels on MMX medium and in plant infection cultures. In contrast, *CHK1* mutation led to reduced *Tox1B* mRNA levels on CMM medium and plant infection cultures, and increased mRNA levels in cultures that grew on MMX medium. It appears from these results that during *C. heterostrophus* host infection, the MAPK mediation pathway upregulated the T-toxin synthesis while the Gβ associate cascade maintained normal synthesis levels by down-regulation. This may suggest that *Tox1B* expression, like other traits such as melanin production, sporulation, and appressoria formation, is a response to environmental sensing and is mediated by the signalling cascades. *CGA2* mutation caused a slight reduction in *Tox1B* expression on CMX medium and an elevation in expression on MMX medium. Interestingly, it is the first recognised role in pathogenicity of *CGA2* in this organism. It will be interesting to see if *CGA1* disruption (in the WT-C4 background) will lead to impaired T-toxin production during plant infection. An examination of the *Tox1A* locus expression profile in the various signalling mutant strains may provide us with another indication of the signalling role in Tox1 production.

CONCLUSIONS

The key players in signal transduction pathways, G-protein and MAPK, are mediators of environmental sensing responses that lead, through as yet unknown cascades, to an alteration in target gene expression. This study showed that these genes, however, share an environmental-dependent response, mediating the *Tox1B* expression profile. Furthermore, the signalling components act as inducers or repressors depending on the target gene and environmental conditions, indicating a complex role. It will be most interesting to understand how the same signal mechanism can fine-tune these distinct and sometimes opposite responses, and at the same time, maintain a fine balance with the other signal cascades.

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