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Research Paper

Crosstalk between the cAMP-PKA pathway and the β-1,6-endoglucanase in *Verticillium dahliae*

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Summary. In plant pathogenic fungi, different signalling pathways operate to control responses to nutrient availability during plant infection. A candidate from the cAMP-PKA signalling pathway, the cAMP-dependent protein kinase A gene, *pka*C1, and the β-1,6-endoglucanase gene, *veg*B, involved in cell wall degradation, were studied in *V. dahliae*. Double mutants of the fungus were constructed, with insertional inactivation in the *pka*C1 and *veg*B genes. Different developmental traits and virulence towards eggplant were evaluated in single and double disruption mutants. In all media tested, double mutants showed better radial growth but less conidia and microsclerotia than the wild type. An interaction between *veg*B and *pka*C1 in controlling virulence on eggplants was recorded, as double mutants were slightly less virulent than the single mutant *veg*B$^-$, but more virulent than the single mutant *pka*C1$^-$. Concomitant or independent function of the two genes and the signaling pathways they operate in for the different growth parameters and virulence are discussed.

Keywords. Protein kinase A, cAMP-mediated PKA, cell wall degrading enzyme.

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INTRODUCTION

Fungi, and especially plant pathogens, can produce many plant cell wall degrading enzymes (CWDEs) for penetration and successful infection of their hosts (Kubicek *et al*., 2014). Involvement of these enzymes in penetration, plant defense induction, and symptom expression has been studied extensively (Cooper, 1987; Walton, 1994; Di Pietro *et al*., 2003). However, although found to contribute to pathogenesis, in most cases no specific roles have been directly attributed to specific genes coding for CWDEs, and since the activities of these enzymes from different fungi show preferences for different types of plant biomass and adaption to pathogen lifestyles, their roles in pathogenesis remains unclear (King *et al*., 2011; Couturier *et al*., 2012).

Probable explanations for this are: (a) that CWDEs are indirectly involved in pathogenesis, and (b) that due to the organization in large multigene families of CWDEs in fungal pathogens, and the functional specialization that each enzyme exhibits, the unequivocal identification of a particular gene involved in pathogenicity is prevented by the masking of its function by the corresponding function of other genes in the same family (Walton, 1994; Coutinho *et al*., 2003; Zhao *et al*., 2013; CAZy website (http://www.cazy.org/CAZY/)). There is sufficient evidence for masking from a number of studies with mutants of inactivated CWDE genes that always retained at least some residual enzyme activity. Examples are: the xylanase genes of *Cochliobolus carbonum* (Apel-Birkhold and Walton, 1996); the polygalacturonase genes of *C. carbonum* (Scott-Craig *et al*., 1990, 1998); the pectate lyase genes of *Nectria haematococca* (*Fusarium solani* f. sp. *pisi*), (Rogers *et al*., 2000); and the xylanase genes of the rice pathogen *Magnaporthe grisea* (Nguyen *et al*., 2011). In the case of *C. carbonum*, double or triple xylanase mutants were shown to retain full pathogenicity on maize (Apel-Birkhold and Walton, 1996). Similarly, in *V. dahliae*, disruption of a trypsin protease gene did not affect pathogenicity (Dobinson *et al*., 2004), while an insertional mutant with the single copy β-1,6-endoglucanase gene showed only a minor reduction in virulence (Eboigbe *et al*., 2014).

Beta-1,6-endoglucanase function has been little studied in plant pathogenic fungi. These enzymes are produced by several fungal species under conditions of carbon starvation, together with other hydrolytic enzymes to release carbon for survival by cell autolysis (Adams, 2004; Martin *et al*., 2007). In mycoparasitic fungi, the role of β-1,6-endoglucanases in degradation of chitin and cell wall β-glucan has been demonstrated using gene disruption studies (Amey *et al*., 2003). In mutualistic fungi (*Neotipodium* sp.) a β-1,6-endoglucanase protein was found to be secreted in the host plant apoplast, conferring a role in fungal nutrition. Based on the absence of β-1,6-glucosidic bonds (the main substrate of β-1,6-endoglucanases) in plants, Moy *et al*., (2002) hypothesized that β-1,6-endoglucanases may be involved in the degradation of cell walls of other fungi to prevent them from infecting host plants. However, in plant pathogenic fungi β-1,6-endoglucanases are considered to also break β-1,3-glucosidic bonds present in plant cell wall callose during parasitic attack (Martin *et al*., 2007). Furthermore, β-glucanases secreted by fungi or produced by hosts aid β-glucan degradation, releasing hydrolytic enzymes from fungal hyphae. In support of this hypothesis, growth of *Botrytis cinerea* on media containing β-glucanases (glucanex, β-1,3-glucanase) resulted in altered morphology of the mycelium and increased activity of hydrolytic enzymes, including peroxidases, laccases, and catalases (Gil-ad *et al*., 2001).

To avoid the problems caused by the masking effects of gene expression in multigene families, attention was focused on the transcriptional regulation of CWDE genes expression and the signals that control whole sets of pathogenicity genes (Roncero *et al*., 2008; Kubicek *et al*., 2014). Tonukari *et al*. (2000) earlier reported that one way to overcome the problem of functional redundancy was to abolish expression of an entire class of CWDE enzymes by knocking out components of signal transduction pathways or transcriptional activators. The sucrose non-fermenting gene *SNF1* that regulates catabolite repression in *C. carbonum* was found necessary for the expression of several CWDEs (Tonukari *et al*., 2000). Mutation of *SNF1* in this fungus led to varying levels of repression of CWDE genes, reduced growth on complex polymers such as xylan and pectin, and also reduced virulence on its maize host. Similarly, the disruption of *SNF1* in the vascular wilt fungus *F. oxysporum* resulted in strongly impaired pathogenicity that was attributed to the low induction of CWDE genes (Ospina-Giraldo *et al*., 2003). Thus, the *VdSNF1* gene was essential for the induction of CWDEs and virulence in *V. dahliae*, confirming the significant role of these genes in pathogenicity of the fungus (Tzima *et al*., 2011).

Although the implication of the *SNF1* pathway in regulation of CWDEs in plant pathogenic fungi is well documented, there is little information on whether CWDEs are also influenced by the cyclic AMP- Protein kinase A (cAMP-PKA) and/or mitogen-activated protein kinase (MAPK) signalling pathways. Both pathways are necessary for fungal pathogenesis and play important roles in the formation of appressoria (Tudzynski and Tudzynski, 2001; Lee *et al*., 2003). The implication of the MAP kinase pathway in modulation of CWDEs was clearly indicated by reduced production of polygalacturonases and pectate lyase enzymes in the *Fusarium oxysporum* f. sp. *lycopersici* MAP kinase disruption mutant *fmk*1 (di Pietro *et al*., 2003). In *Magnaporthe grisea,* activity of the cAMP-dependent protein kinase (PKA) increased during germination of conidia and appressorium formation on hydrophobic surfaces, whereas in mutants lacking the catalytic subunit of PKA (*cpk*a), appressorium formation was impaired (Kang *et al*., 1999). Similarly, disruption of the catalytic subunit protein kinase A gene *VdPKAC1* (hereafter, *pka*C1) of *V. dahliae* caused reduced virulence, although mutants caused typical disease symptoms (Tzima *et al*., 2010). Therefore, since *V. dahliae* does not possess specialized infection structures that in other fungi (including *M. grisea*) are essential for

host invasion (Madhani and Fink 1998), we have tested if cAMP-dependent genes including *pka*C1 influence host penetration by *V. dahliae*, in combination with the β-1,6-endoglucanase gene (*veg*B) that was recently shown to affect the virulence of the fungus (Eboigbe *et al*., 2014).

MATERIALS AND METHODS

Fungus isolates and culture conditions

A *V. dahliae* race 2 isolate from tomato, obtained in Greece, (isolate ID number 123wt-r2; hereafter referred to as wild type or wt), and the *veg*B$^-$ disruption mutant derived from the wild type (Eboigbe *et al*., 2014) were used in this study. For long term storage, the wild type and mutant strains were stored at -80°C, as conidial suspensions in 25% glycerol. Cultures were reactivated on freshly made potato dextrose agar (PDA).

Fungus growth experiments on different carbon sources were performed using a basal medium containing mineral salts and trace elements as in minimal medium (Puhalla and Mayfield, 1974). Carbon sources were added individually to the basal medium (at final concentrations of 2% w/v) and the amended media were adjusted to pH 6.5. Radial growth assays were performed on basal medium containing 2% agar, supplemented with 2% carboxycellulose, pectin, glucose and sucrose. Growth experiments were also performed on PDA (provided by two different suppliers; Difco and BP), that was prepared according to the manufacturers' instructions. Plates were each inoculated by placing 10 μL of conidial suspension (10^7 conidia mL^{-1}) of each strain at the centre of each inoculated plate. Fungal cultures were incubated at 23°C and the colony diameters were recorded at intervals of 2 to 3 d, while colony morphology was also observed and recorded.

For conidia production, 5 mm culture plugs from the edge of actively growing mycelia of each fungal strain were transferred to the centre of PDA plates. After 10 d of incubation, 3 mL of sterilized water was added to each plate to harvest conidia. Conidia were released by scraping off fungal cultures with a glass rod. The plates were then shaken gently and a small aliquot (10 μL) of the suspension from each plate was placed on a microscope slide haemocytometer to determine number of conidia.

Construction and verification of vegB/pkaC1 double disruptants

For the construction of *veg*B$^-$/*pka*C1$^-$ double disruptants, the catalytic subunit of the protein kinase A gene, *pka*C1, was disrupted in the *veg*B$^-$ mutant strain by *Agrobacterium tumefaciens* mediated transformation (ATMT), as described by Tzima *et al*. (2010). Mutant *veg*B$^-$, is a race 2 *V. dahliae* strain in which the β-1,6-endoglucanase gene has been disrupted by insertional inactivation, using hygromycin B as a selection marker gene (Eboigbe *et al*., 2014). For ATMT of mutant *veg*B$^-$, the *Agrobacterium* strain AGL1 was used, which carries the binary vector pGK-Gen, harbouring the geneticin resistance cassette. The binary vector pGPK-Gen was constructed in a previous study, and consists of a pGKO2 backbone (Khang *et al*., 2005), in which the *V. dahliae pka*C1 mutant allele was cloned. The *pka*C1$^-$ mutant allele was constructed from a 3 kb fragment including the *pka*C1 gene, in which the 0.73 kb *Bsr*GI/*Bbv*CI portion, located 274 nucleotides downstream from the start codon, was replaced with the 1,480 bp geneticin resistance gene cassette by blunt ending both vector and insert.

Double disruptants were constructed by ATMT of the *veg*B$^-$ strain as previously described (Mullins *et al*., 2001; Tzima *et al*., 2010). Colonies of double mutants appeared after 3-4 d incubation on PDA supplemented with 50 μg mL^{-1} geneticin, 50 μg mL^{-1} hygromycin B and 50 μM F2dU (5-Fluoro-2-deoxyuridine; Sigma), and were subjected to single conidium isolation. The binary vector pGPK-Gen harbours the HSV*tk* gene (Herpes Simplex Virus Thymidine Kinase). The nucleoside analog F2dU is converted by the HSV*tk* gene product to a toxic compound against *V. dahliae*, selecting against ectopic transformants, thus increasing transformation efficiency (Khang *et al*., 2005).

The confirmation of *pka*C1 disruption in the *veg*B$^-$ mutant, thus creating *pka*C1$^-$/*veg*B$^-$ double disruptants, was achieved by PCR using primers VdPK1400bp-up (5'-AGCCCAACAGCCCCATTACCC-3') and VdPK-1400bp-dn (5'-GCCCAGGCGCTTCGTCAGA-3').

Pathogenicity assays on eggplants

Virulence of the *V. dahliae* wt, *pka*C1- and *veg*B$^-$ single disruption mutants and double disruptants 123Δ*VP1*, 123Δ*VP2*, 123Δ*VP3*, 123Δ*VP4* was evaluated on eggplant ('Black Beauty'). Although the wt was a tomato strain, we carried out the pathogenicity assays on eggplant because this species is more susceptible than tomato to *V. dahliae* (e.g. more rapid disease development, and increased incidence and severity of disease), thus allowing precise evaluation of plant responses to the wt and mutant isolates.

For inocula preparation, fungal cultures grown for 5 to7 d in SSN medium (Sinha and Wood, 1968) were

passed through several layers of cheesecloth (to remove mycelia). The concentration of conidia in resulting suspensions was adjusted to approx. 1×10^7 mL^{-1}. Ten plants at the second true leaf stage were inoculated by drenching the roots with 10 mL conidialsuspensions of each isolate. Virulence assays were performed twice.

Disease severity at each observation was expressed by the percent of leaves that showed wilting symptoms and was recorded for 39 d post inoculation. Disease ratings were plotted over time to generate the disease progress curves, and the areas under the disease progress curve (AUDPC) were calculated using the trapezoidal integration method (Campbell and Madden, 1990). Disease severity was expressed as percentage of the maximum possible AUDPC for the whole period of the experiment, and is referred to as relative AUDPC (Korolev *et al*., 2001). Relative AUDPC values calculated for each treatment were subjected to ANOVA, and means were separated by Duncan's multiple range test.

RESULTS

*Construction of double mutants pka*C1$^-$/*veg*B$^-$

Gene *pka*C1 was previously cloned from a *V. dahliae* tomato race 1 strain and found to have a coding sequence of 1,787 bp (Tzima *et al*., 2010). Since the *veg*B$^-$ knockout mutant contained the hygromycin resistance gene, the binary vector pGPK was altered to carry the geneticin resistance cassette (pGPK-Gen), and was used for ATMT transformation of *veg*B$^-$ (Figure 1A; Tzima *et al*., 2010). Double resistant to hygromycin and geneticin transformants were isolated on appropriate selective media and were examined for stability of the genotypes for several generations. Single colony derived stable transformants were subsequently tested by PCR to examine the sizes of amplicons for the *pka*C1 gene. Representative putatively double-disruptants producing a larger amplicon (2,183 bp) in comparison with the wild

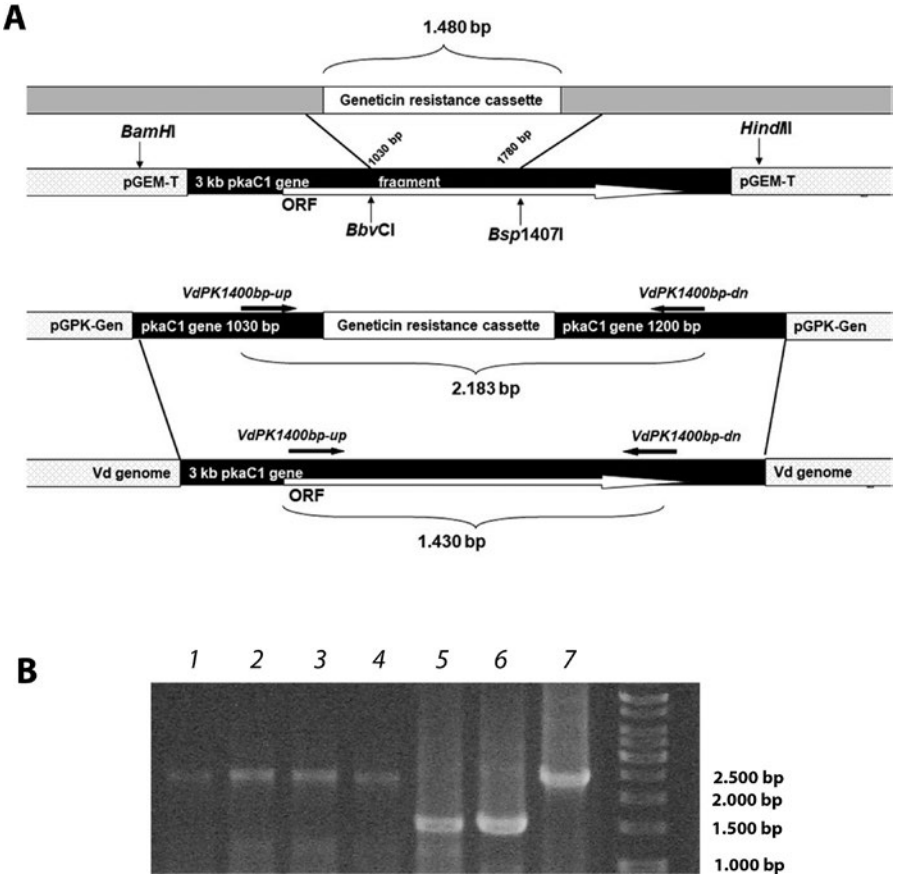

Figure 1. Generation of *Verticillium dahliae pka*C1/*veg*B double disruption mutants. A) Schematic presentation of *pka*C1 mutant allele construction and integration in the fungal genome by a double homologous recombination event. B) Disruption of *pka*C1 verified by amplification of the gene from four double disruptants 123Δ*VP1,* 123Δ*VP2,* 123Δ*VP3,* and 123Δ*VP4* (lanes 1-4; 2.183 bp), genomic DNA (lane 5) and plasmid DNA (lane 6) of clone pPKA3kb, with native fragment 1430 bp), and plasmid DNA of clone pGPK-Gen (lane 7).

type strain 1,430 bp amplicon are shown in Figure 1B. Transformants 123Δ*VP1*, 123Δ*VP2*, and 123Δ*VP4* were further examined for the possible genetic interaction between *veg*B with *pka*C1 in double knock-out *V. dahliae* mutants (*veg*B-/*pka*C1-), and for their pathogenicity in comparison with that of the single knock-out mutants and the wild type strain.

Production of conidia and utilization of carbon sources

The physiology and development of wild type *V. dahliae, veg*B$^-$ and double disruptants were examined by estimating conidium production, and their ability to degrade different carbon sources. Growth of mutant *pka*C1$^-$on glucose, sucrose and xylose was reduced compared to the wild type strain, while on pectin, carboxycellulose and Difco PDA this reduction was less apparent. Mutant *veg*B$^-$ and the double disruptants 123Δ*VP1*, 123Δ*VP2*, and 123Δ*VP4* showed slightly increased radial colony growth compared to the wild type strain and mutant *pka*C1$^-$, which was more pronounced on pectin, carboxycellulose and sucrose (Figure 2).

Colony morphology of all strains was similar in all carbon sources, except growth on Difco PDA. The *vegB*$^-$ mutant and the double disruptants formed abundant microsclerotia after 1 week of growth, while colonies of the wild type and *pka*C1$^-$ showed no microsclerotia at the same time and very few after 20 d. The use of PDA from another supplier (Scharlau) affected colony morphology of all strains with variable intensities of pigment formation. Mutant *veg*B$^-$ displayed increased and resuscitate growth compared to all other strains, while the double disruptants showed a fluffy growth phenotype with different patterns of microsclerotium production between the double disruptants. Colonies of 123Δ*VP1* were white, while 123Δ*VP2* produced microsclerotia at the colony centres and edges, and 123Δ*VP4* produced microsclerotia in internal rings within the colonies. This indicated the presence and absence of microsclerotium formation stimuli in the different PDA preparations.

In comparison with the wild type strain, production of conidia was reduced in all the other strains, at about 40-50%. The exception was *pka*C1,$^-$which showed an approx. 30% reduction.in production of conidia.

Pathogenicity assays on eggplant

Infection assays on eggplants were performed to determine the effects of single mutants *pka*C1$^-$, *veg*B$^-$, and double disruption mutants 123Δ*VP1*, 123Δ*VP2* and

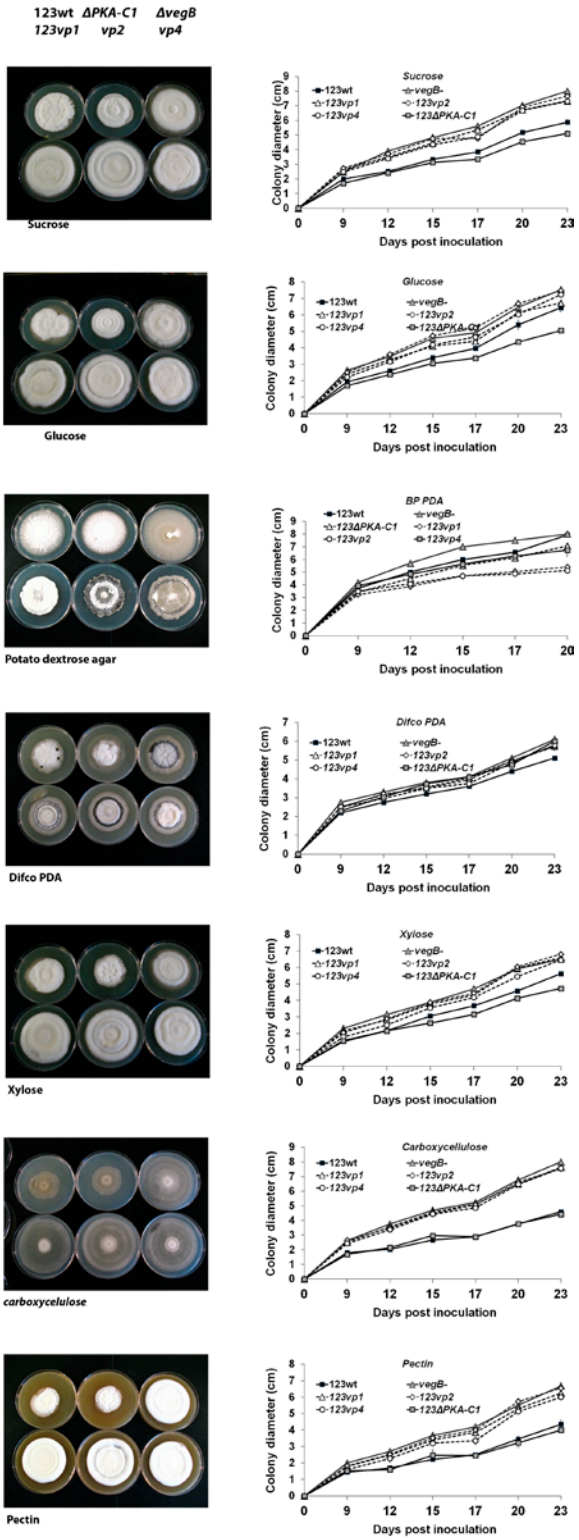

Figure 2. Radial growth of *Verticillium dahliae* wild type (123wt-r2), mutant *veg*B$^-$ and double disruptants 123Δ*VP1*, 123Δ*VP2* and 123Δ*VP4* on different carbon sources. Left: Images of fungal colonies on carbon sources after 23 d of growth. Right: Graphs presenting radial growth of the strains at specific time points.

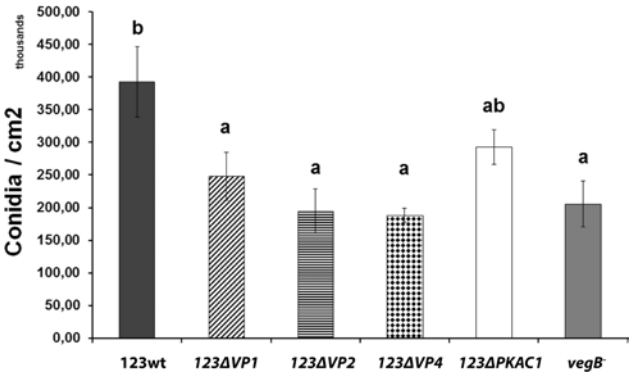

Figure 3. Production of conidia by *Verticillium dahliae* wild type (123wt-r2), mutant *veg*B- and double disruptants 123Δ*VP1*, 123Δ*VP2* and 123Δ*VP4*. Error bars indicate standard errors calculated for five replicates. Values calculated for each treatment were subjected to analysis of variance and means were separated by Fishers's least significant deifference (LSD) procedure. Columns with different letters are statistically different at $P \leq 0.05$.

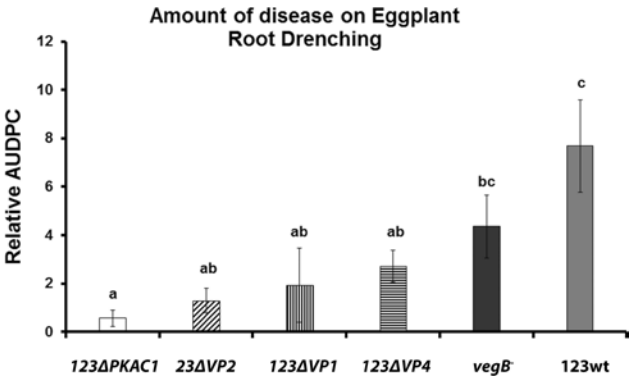

Figure 4. Disease severity caused by the *Verticillium dahliae* disruption mutants *veg*B-, *pka*C1-, double disruptants 123Δ*VP1*, 123Δ*VP2*, 123Δ*VP4* and the wild type 123wt-r2 on eggplants infected by root drenching. Disease severity was expressed as relative AUDPC. Bars indicate standard errors calculated for 20 replicates. Relative AUDPC values calculated for each treatment were subjected to analysis of variance and means were separated by Fishers's least significant deifference (LSD) procedure. Columns with different letters are statistically different at $P \leq 0.05$.

123Δ*VP4* on pathogenicity of *V. dahliae* in comparison with the wild type strain.

Mutant *pka*C1- caused significantly less disease (relative AUDPC of 0.55%) compared to the wild type strain (7.7% relative AUDPC). Disruption of *veg*B caused a smaller non-significant reduction in virulence of the *veg*B- mutant (4.4% relative AUDPC) compared to the wild type strain. Double disruptants 123Δ*VP1*, 123Δ*VP2* and 123Δ*VP4* caused intermediate amounts of disease compared to that caused by mutants *pka*C1- and *veg*B- (relative AUDPCs were 1.9% for 123Δ*VP1*, 1.3% for 123Δ*VP2* and 2.7% for 123Δ*VP4*; Figure 4).

DISCUSSION

The cAMP-PKA pathway has been extensively studied in yeast, but to a much lesser extent in plant pathogenic fungi. In plant pathogens, this signaling pathway has been implicated in morphological and physiological traits (conidiation, branching, growth), and in virulence (Yamauchi *et al*., 2004; Tzima *et al*., 2010; Kim *et al*., 2011). Disruption of the catalytic subunit protein kinase A gene, *pka*C1, in a *V. dahliae* tomato race 1 strain resulted in reduced conidiation and growth, increased branching and reduced invasive growth (Tzima *et al*., 2010).

Beta-1,6-endoglucanases, secreted by fungi, degrade mainly β-1,6-glucosidic bonds (present in fungus cell walls). However, there is accumulating evidence that these enzymes can also degrade β-1,3-glucosidic (laminarin and cell wall callose) and β-1,4-glucosidic bonds (chitin) in the environment, to achieve survival of the fungi under conditions of carbon starvation, by cell wall autolysis, as well as during plant- and myco- parasitism (Stahmann *et al*., 1992; Amey *et al*., 2003; Martin *et al*., 2007). In plant pathogenic fungi, β-1,6-endoglucanases probably aid in plasticity of hyphae during plant-microbe interactions, and release of hydrolytic enzymes entrapped in the cell walls of hyphae by the presence of β-glucan (Gil-ad *et al*., 2001). Disruption of the β-1,6-endoglucanase gene *veg*B in a *V. dahliae* race 2 isolate was reported by Eboigbe *et al*. (2014). Thus, to detect possible crosstalk between the β-1,6-endoglucanase gene and the PKA signalling pathway in *V. dahliae* race 2, double disruption mutants were created by a sequential approach.

In agreement with previous results (Tzima *et al*., 2010; Eboigbe *et al*., 2014), both single disruption mutants in *V. dahliae* race 2 that were examined here, *veg*B- and *pka*C1-, showed marked reduction in the production of conidia, which was more pronounced in *veg*B- than in *pka*C1-. However, no additional differences in the production of conidia were observed in the double mutants that were constructed. The *pka*C1- mutant had reduced growth on the different carbon sources tested, both in comparison with the wild type and also with the *vegB*- and the double disruption strains. V*eg*B- and the three double mutants showed similar growth on fermentable (sucrose, glucose) and alternative (xylose, pectin, carboxycellulose) carbon sources, which was increased in comparison with the wild type strain. This

indicates that the *veg*B$^-$ disruption phenotype was dominant over the *pka*C1$^-$ phenotype. A possible explanation for this reduced growth and conidiation phenotype of *pka*C1$^-$ is that the mutant fungal strain had impaired perception of appropriate signals from the environment, thus failing to initiate conidiation and growth processes. This has been demonstrated in several other similar cases (Kronstad *et al*., 1998; Casas-Flores *et al*., 2006; Doehlemann *et al*., 2006; Skamnioti and Gurr, 2007). In such mutants, the additional absence of β-1,6-endoglucanase may elevate the composition of fungal hyphae in its substrate, β-glucan (Martin *et al*., 2007). As endoglucanases have been shown to participate in hydrolysis and re-construction of the fungal cell walls during growth and morphogenesis (Adams *et al*., 2004), this may also further interfere with perception and response to environmental signals, thus explaining the dominant phenotype of *veg*B$^-$ over *pka*C1$^-$ disruption.

Glucose signalling in yeasts and filamentous fungi is controlled by different pathways including the cAMP-PKA pathway that regulates growth in response to glucose availability, whereas sucrose non-fermenting gene product (SNF1), induces transcriptional changes in the presence of low glucose concentrations or during growth on alternative carbon sources (Tonukari *et al*., 2000; Hong *et al*, 2003; Santangelo, 2006; Zaman *et al*., 2009). In yeasts, it has been shown that PKA activity controls the localization of the β subunit isoform (Sip1) of the Snf1 protein kinase and its complex with the Snf1 catalytic subunit in response to carbon source availability, indicating interference between the two pathways (Hedbacker *et al*., 2004). Furthermore, microarray analysis in yeast revealed that the PKA pathway centrally controls cell growth and transcriptional changes of the majority of genes involved in response to glucose availability. SNF1 modulates the expression of a small number of genes, a significant portion of which is independent of the PKA pathway, including the majority of glucose repressed genes (Zaman *et al*., 2009). The expression of *veg*B was reduced in *VdSNF1* mutants (Eboigbe *et al*., 2014), indicating that *veg*B is under catabolite repression controlled by SNF1 in *V. dahliae*. Therefore, similar to its role in yeast, the SNF1 ortholog gene may also operate in *V. dahliae* and *veg*B may belong to a group of genes that function independently from the PKA pathway.

Previous results for plant–*V. dahliae* interactions (Tzima *et al*., 2010; Eboigbe *et al*., 2014) have shown that *pka*C1 is implicated in invasive growth of *V. dahliae* race 1, whereas *veg*B has only a minor effect on virulence in *V. dahliae* race 2. It was therefore expected that the disruption of *pka*C1 in *V. dahliae* race 2 would cause reduction in virulence. Disruption of both genes in race 2 of *V. dahliae* resulted in double mutants causing less disease in the sensitive eggplant-*V. dahliae* pathosystem than the single mutant *veg*B$^-$, and more disease than the single mutant *pka*C1$^-$. These results, though not strongly supported statistically, indicate a possible interaction between *pka*C1 and *veg*B in controlling virulence. Pathogenicity is complex, which includes growth on alternative carbon sources (e.g. pectin induces the production of hydrolytic enzymes), ramification into plant tissues, and confrontation with host defense mechanisms (Agrios, 2005). It is therefore difficult to speculate in which of these pathogenicity factors the functions of *pka*C1 and *veg*B may overlap, to fully explain the phenotypes observed in the double disruptant mutants of *V. dahliae* race 2. However, as disruption of *veg*B is thought to affect the activity of other hydrolytic enzymes (Gil-ad, 2001), and disruption of *pka*C1 possibly causes deregulation of the expression profiles of genes involved in similar processes, the combined action of these genes may form the basis for an explanation of the phenotypes observed. In support of this hypothesis, as well as to add to the complexity of the mechanisms involved, the disruption of a Ga subunit gene in *Botrytis cinerea* caused reduced expression of endoxylanase and endoglucanase genes, and increased expression of a pectate lyase gene, all of which aided plant cell wall degradation (Gronover *et al*., 2001). Further investigation of the interaction of *pka*C1 and *veg*B in *V. dahliae* and the downstream targets affected is required to clarify pathogenicity mechanisms of this important plant pathogen.

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