Pectate lyase affects pathogenicity in natural isolates of *Colletotrichum coccodes* and in *pelA* gene-disrupted and gene-overexpressing mutant lines

BAT-HEN BEN-DANIEL^{1,2}, DUDY BAR-ZVI^{2,*} AND LEAH TSROR (LAHKIM)¹

¹Department of Plant Pathology, Agricultural Research Organization, Gilat Research Center, M. P. Negev 85280, Israel

²Department of Life Sciences, Ben-Gurion University, Beer-Sheva 84105, Israel

SUMMARY

Colletotrichum coccodes (Wallr.) S. Hughes, the causal agent of black dot on potato and anthracnose on tomato, reduces yield and crop quality. We explored the role of secreted pectate lyase (PL), a cell wall-degrading enzyme, in the aggressiveness of *C. coccodes*. *In vitro*-cultivated highly aggressive isolates secreted immunologically detectable PL levels 6 h after transfer to secondary medium versus 12 h for mildly aggressive isolates, suggesting that secreted PL is a virulence factor. The gene encoding PL, *CcpelA*, was cloned and used for the genetic manipulation of highly (US-41 and Si-72) and mildly (Si-60) aggressive isolates. *CcpelA* gene-disrupted mutants showed reduced aggressiveness towards tomato fruits and impaired PL secretion and extracellular activity. Conversely, overexpression of *CcpelA* in the Si-60 isolate increased its aggressiveness and PL secretion. Comparison of *CcpelA* cloned from isolates US-41 and Si-60 revealed that both encode identical proteins, but differ in their promoters. Bioinformatics analysis for *cis*-acting elements suggested that the promoters of the US-41 and Si-60 isolates contain one and no AreA-binding site (GATA box), respectively. AreA has been suggested to be involved in fungal aggressiveness; therefore, *CcpelA* may be a key virulence factor in *C. coccodes* pathogenicity, and the differences in isolate aggressiveness might result from promoter activity. Quantitative reverse transcriptase-polymerase chain reaction analyses confirmed the higher level of *CcpelA* transcript in isolate US-41 versus Si-60.

INTRODUCTION

Colletotrichum coccodes (Wallr.) S. Hughes has been isolated from a large number of host plants worldwide. Its major hosts are potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*), in which it causes black dot and anthracnose, respectively. Potato black dot is characterized by small black sclerotia developing on senescing or dead plant tissue, roots, stems, stolons and daughter tubers, thereby reducing tuber quality and yield. Initial tomato

anthracnose symptoms appear on ripe fruit as light-brown flecks, which develop into small, slightly sunken, water-soaked circular lesions. The lesions increase in size, become more depressed and the central portion darkens. As the fungus spreads within the fruit, semi-soft decay occurs (Dillard, 1992). The maceration of host tissue indicates the activation of fungal cell wall-degrading enzymes (CWDEs) (Wei *et al.*, 1997). Among the CWDEs of plant pathogens, the pectinases polygalacturonase, pectin lyase and pectate lyase (PL) have been widely studied. Pectinases, which degrade the pectic component found in the middle lamella and primary cell wall of most plants, have been postulated to be involved in plant penetration and colonization by phytopathogens (Shih *et al.*, 2000): the disruption of *Botrytis cinerea* endopolygalacturonase reduces its colonization on tomato (ten Have *et al.*, 1998), disruption of *Aspergillus flavus* polygalacturonase decreases its ability to invade and colonize cotton balls (Shieh *et al.*, 1997) and disruption of *pglA*, which encodes a polygalacturonase in *Xylella fastidiosa*, compromises this pathogen's ability to systemically colonize grapevine (*Vitis vinifera*) (Roper *et al.*, 2007). In addition, *Colletotrichum gloeosporioides* has been found to produce PL (*pelB*) during colonization of infected avocado tissue (Wattad *et al.*, 1997), and its disruption via homologous recombination reduces fungal virulence by approximately 40% (Yakoby *et al.*, 2001). In *C. coccodes*, a direct relationship has been observed between extracellular serine protease activity and the induction of disease symptoms in tomato (Redman and Rodriguez, 2002).

In this study, we explored the relationship between *C. coccodes* pathogenicity and PL secretion. Highly aggressive isolates were found to secrete PL earlier than mildly aggressive isolates. *Colletotrichum coccodes pelA* (*CcpelA*) was cloned and used for the genetic manipulation of *C. coccodes*: overexpression of *CcpelA* in a mildly aggressive isolate increased its aggressiveness, and disruption of *CcpelA* by homologous recombination in highly aggressive isolates decreased the aggressiveness of the transgenic fungi. In addition, sequence analysis of *CcpelA* genes from highly and mildly aggressive isolates showed that both express identical proteins, but differ in their promoter sequences, in agreement with the differences observed in the expression rates of these isolates.

*Correspondence: Email: barzvi@bgu.ac.il

RESULTS

PL secretion from wild-type (WT) *C. coccodes* isolates

We have a collection of *C. coccodes* isolates from diverse geographical origins and vegetative compatibility groups, and from a number of hosts (Ben-Daniel *et al.*, 2009, 2010). The aggressiveness of these isolates was determined using a recently developed bioassay, which is more sensitive and less laborious than other available bioassays (Ben-Daniel *et al.*, 2009). For this study, we selected eight *C. coccodes* isolates, four from tomato and four from potato. Each subgroup contained two highly aggressive and two mildly aggressive isolates (Fig. 1A). These eight isolates were assayed for PL secretion by *in vitro*-cultured mycelia. PL protein could be immunologically detected using antibody raised against PL of *C. gloeosporioides* in cultures of the highly aggressive isolates US-41, US-39, Si-120 and Si-72 as little as 6 h after transfer to secondary medium (Fig. 1B). In contrast, PL was only detected in media of the mildly aggressive isolates Bsor, Eshel, Si-60 and US-11 after 12 h (Fig. 1B). The correlation between aggressiveness (Fig. 1A) and early PL secretion (Fig. 1B) suggested that PL might be a key virulence factor in *C. coccodes*.

Cloning of *CcpelA* from a highly aggressive isolate

We chose to clone the PL gene *CcpelA* from the highly aggressive *C. coccodes* isolate US-41, as detailed in Experimental procedures. The full-length, 2398-bp DNA sequence was sequenced (GenBank accession number GU206360) and analysed using Eukaryotic GeneMark.hmm software (Lomsadze *et al.*, 2005). The coding sequence contained three exons and two introns for a deduced

325-amino-acid protein (Fig. S1, see Supporting Information). The deduced protein sequence showed high homology to PL sequences from other fungi and lower homology to bacterial PL proteins. Highest homology (89% identity and 95% similarity) was found to PL from *Glomerella graminicola* M1.001 (accession number EFQ25829). This high amino acid sequence homology with the deduced amino acid sequence of CcPelA supports the exon–intron structure suggested in Fig. S1. However, the 824-bp and 465-bp sequences upstream and downstream of the coding sequence, respectively, did not show significant homology to any sequences deposited in GenBank.

CcpelA gene disruption

To examine the role of *CcpelA* in *C. coccodes* aggressiveness, we disrupted this gene in the highly aggressive isolate US-41. We applied the targeted gene disruption method using a linear minimal element (LME) construct developed for *Alternaria brassicicola* (Cho *et al.*, 2006). This method makes use of a linear construct of the target gene and an antibiotic resistance selectable marker. The construct ends are most probably digested in the host fungus by exonuclease, followed by circularization and incorporation into the genome by a single homologous recombination event, leading to gene disruption. Plasmid pGH1-1240PL was constructed as described in Experimental procedures. In this construct, the CcPelA-encoding sequence is not fused to the promoter sequence, and thus is not expected to be expressed in the target genome.

Germinating conidia of isolates US-41 and Si-72, originating from tomato and potato, respectively, were transformed with *Hind*III-linearized pGH1-1240PL. Twenty-four hygromycin-resistant

Fig. 1 Aggressiveness and pectate lyase secretion of wild-type *Colletotrichum coccodes*. (A) Wild-type *C. coccodes* isolates originating from tomato (US-41, US-39, Eshel and Bsor) and potato (Si-120, Si-60, Si-72 and US-11) were tested for aggressiveness using stem scar inoculation of mature green tomato fruits, as described in Ben-Daniel *et al.* (2009). (B) Western blot analysis of pectate lyase secreted by *C. coccodes* isolates following transfer to secondary medium. Culture media were collected at the indicated time after hyphal transfer to secondary medium. Samples were processed and analysed as described in Experimental procedures.

transformant colonies were obtained from each isolate. Genomic DNA was prepared from conidia of each of the transformants, as well as from the nontransformed parental isolates. The integration site of the introduced DNA was analysed by polymerase chain reaction (PCR), using primers derived from linker DNA-pGEM sequences within the transformed DNA (construct-specific primers) and from flanking sequences of *CcpelA* (gene-specific primers) which were not included in the transformation construct (Fig. 2B). Two sets of primers were used: the first pair consisted of For-new-PL4 and Rev-new-PL4, whose template sequences reside in sequences 5' and 3' to the *CcpelA* sequence used for the transformation, and thus are not present in the transformation construct.

Fig. 2 Targeted gene disruption of *CcpelA*. (A–C) Schematic diagram of the strategy used for targeted gene disruption of *CcpelA* encoding pectate lyase (PL). (A) *CcpelA* in wild-type (WT) genome. (B) Linearized pGH1-1240PL plasmid containing *CcpelA* sequence. (C) Genome organization of *CcpelA* disrupted via homologous recombination. Box colours: dark and light grey, *CcpelA* gene sequence (sequence marked in dark grey was included in the disruption construct); black, sequences derived from pGEM vector; white shading, hygromycin resistance gene; white, other vector sequences. Arrows indicate the positions and orientations of the polymerase chain reaction (PCR) primers used for verification of the transformants: 1, For-new-PL4; 2, Rev-new-PL4; 3, For-pGEM; 4, Rev-pGEM. (D–F) PCR analysis of *CcpelA* gene-disrupted transformants. Genomic DNA isolated from wild-type (WT) isolates US-41 and Si-72, *CcpelA* gene-disrupted transformants US-41/7.1 and Si-72/2.1, and representatives of ectopic transformants US-41/5.1 and Si-72/5.1 were analysed using the following primer sets: (D) For-new-PL4 and Rev-new-PL4 (1 and 2 in A and C); (E) For-new-PL4 and For-pGEM (1 and 3 in C); (F) Rev-new-PL4 and Rev-pGEM (2 and 4 in C). All PCR products were run on a 1% agarose gel and stained with ethidium bromide. Mutants were numbered according to electroporation number/colony number.

PCR using these primers is expected to amplify 1.8- and 5-kb DNA fragments from the nondisrupted and disrupted *CcpelA* gene, respectively (Fig. 2A,C). As the reaction conditions employed in this PCR analysis do not permit the amplification of long templates, PCR products were only expected for the nondisrupted (WT) gene (Fig. 2D). In most transgenic mutant lines obtained from either isolate, the DNA construct used for transformation was integrated into the fungal genome by nonhomologous recombination, thus leaving the endogenous *CcpelA* gene intact (not shown). We isolated two independent transformants (US-41/7.1 and Si-72/2.1) resulting from homologous recombination, one in each of the isolates.

The introduced DNA was incorporated into the host genome by homologous recombination in one transformant for each isolate (c. 4%), resulting in the expected gene disruption (Fig. 2D, lanes 2 and 5). To verify the homologous recombination events, genomic DNA of the putative PL-disrupted transformants was further analysed using the secondary primer sets For-pGEM + For-new-PL4 and the complementary Rev-pGEM + Rev-new-PL4. The sequences of the pGEM linkers are unique to DNA introduced by the transformation procedure, whereas the sequences corresponding to the For-new-PL4 and Rev-new-PL4 primers are not included in the transformed DNA sequences, and thus originate from fungal DNA. Thus, only homologous recombination can produce DNA sequences in the genome of the transgenic fungi that can be amplified using both primer sets (Fig. 2C). As expected, 1.4- and 1.5-kbp DNA fragments could be identified by the For-pGEM + For-new-PL4 and Rev-pGEM + Rev-new-PL4 primer pairs, respectively, using DNA isolated from gene-disrupted transformants US-41/7.1 and Si-72/2.1 (Fig. 2E,F, lanes 2 and 5, respectively). No products were obtained when these primer sets were used in the presence of template DNA prepared from either WT parental isolates (Fig. 2E,F, lanes 1 and 4) or ectopic transformants (Fig. 2E,F, lanes 3 and 6). DNA sequencing of these amplified DNA fragments confirmed that the linker pGEM sequences that are unique to the pGH1-1240PL construct were integrated into the fungal genome adjacent to the *CcpelA* gene (data not shown).

Aggressiveness and PL secretion of *CcpelA*-disrupted transformants

Transgenic mutant lines of *C. coccodes* were stable, with no change in morphology or growth rate on potato dextrose agar (PDA) medium (data not shown). The aggressiveness of the *CcpelA*-disrupted transformants was evaluated by mature green tomato stem scar injection bioassay. *CcpelA*-disrupted mutant lines of both isolates showed reductions in all aggressiveness parameters (Fig. 3A): lesions caused by the *CcpelA*-disrupted transformants were reduced by 20%–25% in size relative to those obtained following injection of a conidial suspension of the respective parental strains. Furthermore, fungal colonization at the

Fig. 3 Analyses of *CcpelA* gene-disrupted mutants. (A) Aggressiveness of *Colletotrichum coccodes* wild-type (WT) isolates and *CcpelA* gene-disrupted mutants. (B, C) Pectate lyase (PL) secreted from WT gene-disrupted transformants. Hyphal mats from the indicated lines were transferred into the secondary media and shaken at 150 rpm, 25 °C. Culture medium was harvested 24 h later, concentrated and analysed for the presence of PL by Western blotting (B) (16 µg protein loaded per lane) or PL enzymatic activity (C). *Mean is significantly different ($P < 0.05$) from WT in a paired *t*-test.

blossom end of the fruit was reduced approximately 10-fold in the *CcpelA*-disrupted mutant lines (not shown).

Western blot analysis of secreted proteins confirmed that the reduced aggressiveness in the *CcpelA*-disrupted transformants resulted from a reduction in CcPelA secretion and not from a secondary effect. Secreted PL could not be detected by Western blot, even 24 h after transfer of *CcpelA*-disrupted mutant lines to the secondary medium (Fig. 3B). PL enzymatic activity assayed in the secondary medium (Fig. 3C) was correlated with the reduction in aggressiveness (Fig. 3A).

Complementation of *CcpelA*-disrupted transformants

To verify that the phenotype of the mutants obtained by homologous recombination resulted from knocking out of the *CpelA* gene, we complemented these mutants by transforming the two *CcpelA*-disrupted mutant lines with the complementation construct. The construction of plasmid pOE-NET is described in Experimental procedures. This plasmid, containing the CcPelA-encoding sequence driven by the strong fungal promoter PgpD and the nourseothricin (NET) resistance gene, was linearized using *HindIII* (Fig. 4A), and employed to transform germinating conidia of the *CcpelA*-disrupted US-41/7.1 and Si-72/2.1 mutants. Transformants were selected on 100 µg/mL NET, and assayed for aggressiveness

Fig. 4 Complementation of *CcpelA*-disrupted mutants. (A) Schematic diagram of the construct used for *CcpelA* complementation and overexpression. Box colours: grey, PgpD promoter; dotted black and white, *CcpelA* genomic sequence including protein-encoding exons and intron; black, ampicillin resistance gene for selection in *Escherichia coli*; white, NET (nourseothricin) resistance gene; striped, GACAC microsatellite DNA. (B) Aggressiveness assay. WT, wild-type isolates; KO, *CcpelA* gene-disrupted mutants (US-41/7.1 and Si-72/2.1); CO1–3, independent transformants of gene-disrupted mutants complemented with *CcpelA*. *Mean is significantly different ($P < 0.05$) from WT in a paired *t*-test.

using the mature green tomato fruit injection bioassay. The aggressiveness of the *CcpelA*-disrupted mutants was complemented by ectopic overexpression of *CcpelA* (Fig. 4B), confirming that the decreased aggressiveness of both US-41/7.1 and Si-72/2.1 *CcpelA*-disrupted mutants is caused by disruption of the *CcpelA* gene.

Overexpression of *CcpelA* in a mildly aggressive isolate

To determine whether *CcpelA* expression is a limiting factor in mildly aggressive isolates of *C. coccodes*, we examined whether the aggressiveness of a mild *C. coccodes* isolate could be enhanced by the overexpression of *CcpelA*. Plasmid pOE, described in Experimental procedures, containing the CcPelA-encoding sequence driven by the strong fungal promoter PgpD and a hygromycin-selection marker, was linearized by *HindIII* digestion (Fig. 5A) and electroporated into germinating conidia of the mildly aggressive Si-60 isolate. Twelve hygromycin-resistant transformant colonies were analysed using For-gpd2 and Rev-old-PL2 primers, designed for the PgpD promoter and *CcpelA* coding sequence, respectively (Table 1), expecting to amplify a 1.4-kb fragment from transgenic fungi but not from the WT parental isolate, independent of the DNA integration site. Analysis of all transformants resulted in the expected amplicon (not shown).

Fig. 5 Overexpression of *CcpelA* in the moderately aggressive Si-60 isolate. (A) Schematic diagram of the construct used for the overexpression of *CcpelA*. Box colours: grey, *Pgpd* promoter; dotted black and white, *CcpelA* genomic sequence including protein-encoding exons and intron; black, ampicillin resistance gene for selection in *Escherichia coli*; white, *hph* TrpC (hygromycin B resistance gene); striped, GACAC microsatellite DNA. (B) Aggressiveness of wild-type (WT) *Colletotrichum coccodes* isolate Si-60 and five independent transgenic *CcpelA*-overexpressing Si-60 mutants (OE1–5). (C, D) Pectate lyase (PL) secreted from *C. coccodes* isolate Si-60 WT and *CcpelA*-overexpressing mutants. WT Si-60 and three transgenic *CcpelA*-overexpressing Si-60 mutants were cultured in secondary medium. Culture medium was collected 24 h later, concentrated and analysed for the presence of PL by Western blotting (20 µg protein loaded per lane) (C), or for PL enzymatic activity (D). *Mean is significantly different ($P < 0.05$) from WT in a paired *t*-test.

Aggressiveness and PL secretion of overexpressing mutants

As the introduced DNA is expected to largely integrate randomly within the genomic DNA (see above), the activity of the inserted gene is expected to be affected by neighbouring genes of the host organism, also known as the positional effect. We thus evaluated the aggressiveness of the confirmed transformants using the lesion size parameter of the mature green tomato bioassay (Ben-Daniel *et al.*, 2009). The lesions obtained with the different transformants were larger than those obtained with the parental isolate (Fig. 5B). This variability most probably resulted from a positional effect of the introduced DNA within the fungal genome.

Table 1 Primers employed in this study, their sequences and annealing temperatures used.

Primer	Sequence	Annealing temperature (°C)
For-1	GTCTGGGTCGACCACT	50
For-1240-PL	TCCCATTCCTCAGAACTTCG	58
For-2350-PL	TCACAGAAGTATGCCAAGCG	58
For-AreA1	CGTGCACTCCGAGTC	60
For-new-PL4	GGTGATGATACCCTAGTTGTC	58
For-pGEM	TGTTCTAGAGCGGCCGCGGAATTC	67
For-gpd2	CCAATTGCTTCGATCGGTA	58
For-OE	AATCCATGGAAGACGTTGCCG	60
Rev-1240-PL	CGGTGTGACTGTACTAAGTG	58
Rev-2	TGTTTTCGCTAGGTGACG	50
Rev-AreA1	ACAGCACCACGACAACAACG	60
Rev-new-PL4	CTGTGCCACAACAGAGATCT	58
Rev-OE2	AACATCGATTCCAGCTCTGGC	60
Rev-old-PL2	CAGAGATCCCTTCCACTGAA	60
Rev-pGEM	AATGCTAGCAGGCGGCCGGAATTC	67
RT For-qPL2	GAGAAGGGGAGC TCCCTGAC	60
RT Rev-qPL2	CCTTGACACCGCCGATCTT	60
RT For-18S	ACGGTTGACGTAGGCCCTTA	60
RT Rev-18S	TCCAGTGCGAGACGTTAGTT	60
TSP 1.1A	GAGATCCCTTCCACTGAATG	55
TSP 1.2A	CACGTAAGTTTGTCTACGA	55
TSP 1.3A	ACTCGTCAATGCCGATGG	55
TSP 1.4A	GGCTTCTGAGTTAGAGTAGA	55
TSP 2.1A	GTTAGTTTCCGACCGAGGATA	60
TSP 2.2A	CTATCTCGGTCGGAACATAA	60
TSP 2.3A	CCGCCGATCTTCAAGTTGCG	60
TSP 2.4A	GTCACCTGTGATGCTCGAG	60
TSP 3.1A	TCGTGAGCAAACTACGTG	60
TSP 3.2A	GCATTCAGTGAAGGGATCTC	60
TSP 3.3A	TGGCGGACGTAGAGACCGAT	60
TSP 3.4A	CCACGCCGACAACACTAGGGTA	60

Nevertheless, lesion size values obtained from five transformants were significantly larger (27%–44%) than those from the parental WT accession. In accordance with the increased aggressiveness of the transgenic mutants, *in vitro*-grown cultures of three of the mutant lines with highly increased aggressiveness secreted higher levels of CcpelA (Fig. 5C) and exhibited higher levels of PL activity (Fig. 5D).

Cloning of *CcpelA* from a mildly aggressive *C. coccodes* isolate

To determine whether the level of aggressiveness in *C. coccodes* WT isolates might result from differences in *CcpelA*, this gene was cloned from the mildly aggressive isolate Si-60 by PCR, using primers For-2350-PL and Rev-new-PL4 (Table 1). The resulting DNA was sequenced (GenBank accession number GU206361) and its nucleotide sequence was compared with that of the *CcpelA* gene cloned from the highly aggressive isolate US-41 (GenBank accession number GU206360). The genes cloned from both isolates encoded identical 325-amino-acid-long putative proteins (Lomsadze *et al.*, 2005). Moreover, both 1109-bp coding regions

were interrupted by two introns of 70 and 61 bp in length. All introns included GTRNGY and YAG consensus 5' and 3' splice sites, respectively, and the internal CTRAY consensus sequence (Unkles, 1992). In contrast, DNA sequence comparison of the promoter sequences of both isolates showed 19 base switches and three base gaps. Both promoters contained two potential TATA boxes and potential binding motifs for the transcription factors TEA/ATTS, CreA, PacC, MPG1, XlnR, ACEI and ACEII (Fig. 6). However, two major differences were found between the promoters: (i) the *CcpelA* promoter from the highly aggressive isolate US-41 contained an AreA-binding site (GATA box), which was mutated in the mildly aggressive isolate Si-60 (Fig. S2, see Supporting Information); (ii) the studied promoters differed in the number of tentative Hap-binding sites (CAAT)—five in the highly aggressive isolate and only four in the mildly aggressive isolate (Fig. 6).

Neither isolate's 3' noncoding region contained the consensus AATAAA polyadenylation sequence; however, ATAA, which functions as a polyadenylation signal in a number of fungal genes (Unkles, 1992), was present in both isolates.

The difference in gene sequence between the highly aggressive US-41 and mildly aggressive Si-60 suggested that the expression level of the *CcpelA* gene should be higher in isolate US-41. To test this, we determined the relative levels of *CcpelA* transcript in these isolates after transfer to secondary medium. As predicted, the *CcpelA* transcript level in the highly aggressive isolate was almost twice that observed in the mildly aggressive strain 5 h after transfer to secondary medium (Fig. 7A). Interestingly, transcript levels of the *areA* gene encoding the GATA-box binding transcription factor discussed above (Fig. 6) were also twice as high in US-41 than in Si-60 (Fig. 7B).

DISCUSSION

PL secretion from WT *C. coccodes* isolates

Both saprophytic and plant-parasitic fungi produce extracellular enzymes which can degrade plant cell wall components. These fungi not only digest plant cell wall polymers to obtain an important nutrient source, they also degrade the cell wall to aid in cell penetration and spread throughout the plant tissue. Of the numerous CWDEs produced by plant-pathogenic fungi, research has concentrated on the pectin-degrading enzymes, including polygalacturonase, PL and pectin methylesterase. This is because the pectinases are typically produced first, in the largest amounts, and are the only CWDEs capable of macerating plant tissue and killing plant cells on their own. Fungi produce different types of pectinases that are classified by their substrates, type of lysis and mode of action on the pectin polymer (Annis and Goodwin, 1997).

PL secretion was assayed following transfer to a secondary medium described for the secretion of PL from *C. gloeosporioides* (Drori *et al.*, 2003; Kramer-Haimovich *et al.*, 2006) using anti-

C. gloeosporioides PL antibody (Wattad *et al.*, 1994). We found 58% identity in amino acid sequence between CcPelA and PL from the avocado pathogen *C. gloeosporioides*, which is assumed to be sufficient for antibody recognition. This antibody also cross-reacts with PL secreted from *C. gloeosporioides* attacking mango and papaya, and with PL secreted from *Colletotrichum musae* attacking banana (Wattad *et al.*, 1997). We found that, although all examined *C. coccodes* isolates secreted PL into the medium, the rates of PL accumulation correlated with the level of aggressiveness of the isolates (Fig. 1). Secreted PL could be detected in the secondary medium as early as after 6 h in highly aggressive isolates, but only after 12 h in cultures of mildly aggressive fungi. These differences in secretion rate postulated PL as a virulence factor. Differences in the secretion kinetics of cellulase, another CWDE, have been described for *Verticillium dahliae*, in which a highly aggressive isolate secreted cellulase earlier than a mildly aggressive one (Novo *et al.*, 2006), suggesting that the earlier secretion contributes to the isolate's aggressiveness via earlier penetration.

CcpelA gene disruption and overexpression

The aggressiveness analyses of the WT isolates (Fig. 1) suggested that early secretion of CcPelA is essential for full virulence. This was further demonstrated by manipulating *CcpelA* expression in transgenic *C. coccodes*. Gene-specific disruption was achieved by homologous recombination events in a construct containing a partial target gene and a selection marker gene (Fig. 2B). Previous studies have demonstrated that linear DNA is more effective than circular DNA in genetic transformation of phytopathogenic fungi (Cho *et al.*, 2006; Horowitz *et al.*, 2002). We therefore used a linearized DNA construct in our transformation protocols. In *C. coccodes*, most transformation events result in nonhomologous recombination of the foreign DNA. Nevertheless, homologous recombination events were detected in about 4% of the transformation events. The virulence of the highly aggressive accessions was reduced to levels comparable with that of the mildly aggressive WT accessions on disruption of *CcpelA* (Fig. 3A). The reduced aggressiveness of the knockout mutant could be complemented by transformation with *CcpelA* (Fig. 4B), confirming that the reduced aggressiveness in the knockout mutants is a result of interference in *CcpelA*. Accordingly, CcPelA did not accumulate in the medium of gene-disrupted transformants following transfer to the secondary medium (Fig. 3B). Plant-pathogenic fungi are known to encode multiple PL genes (reviewed by Reignault *et al.*, 2008). Our data suggest that *C. coccodes* encodes more than one *pel* gene, and that CcPelA is the major PL secreted in *C. coccodes* or, alternatively, that CcPelA is the primary PL orthologue of *C. coccodes* that is recognized by the anti-*C. gloeosporioides* PL antibody. The latter explanation is less likely because there is only 58% amino acid sequence identity between CcPelA and the *C. gloeosporioides* orthologue used for antibody production (Wattad *et al.*, 1994),

US-41	1	TCGGACCTTCAACGTTGCTCGCATGCTGCCAGATGGGGT -CGGTTGGCTCGTCTCTTCT	59
Si-60	1	TCGGACCTTCAACGTTGCTTGCCTGCTGCCAGAT -GGGGTCCGGCTGGCTCGTCTCTTCT	59
US-41	60	<div>CreA</div> <div>CTGGGG</div> <div>XlnR</div> <div>CTAGCC</div> <div>CTAGCAACGACAGCCGAATGAGCCTATTCCCAACC</div>	119
Si-60	60	<div>CTGGGG</div> <div>CTAGCC</div> <div>CTAGCAACGACAGCCGAATGAGCCTATTCCCAACC</div>	119
US-41	120	<div>CAAT</div> <div>CCTGCTACCCCCATCCCGAGTCC</div> <div>CAAAT</div> <div>ACCCCGTACCGTGAACGGCTTGTTTA</div> <div>CreA</div> <div>CTCCG</div>	179
Si-60	120	<div>C-AAT</div> <div>CCTGCTACCCCCATCCCGAGTCC</div> <div>C-AAT</div> <div>ACCCCGTACCGTGAACGGCTTGTTTA</div> <div>CTCCG</div>	178
US-41	180	<div>CATAACAAGTCGCTATTCAAGAACTCCCTCACTTCTTGACGCCATTTTGGGAG</div> <div>CreA</div> <div>GTGGA</div>	239
Si-60	179	<div>CATAGCAAGTTGCTATTCAAGAACTCCCTCACTTCTTGACGCCATTTTGGGAG</div> <div>GTGGA</div>	238
US-41	240	<div>GTGCGCCAGTCACCTGGTTCGGGCAACCTAGTCGACTTGGGATGGATCAAGACGAGATTT</div>	299
Si-60	239	<div>GTCGCGCCAGTCACCTGGTTCGGGCAACCTGGTCGACGTGGGATGGATCAAGACGAGATTT</div>	298
US-41	300	<div>CAAT</div> <div>TTCCTTAGTCGCGCCGGTGATTTCGATGTGACGGTCTCGTTCGAGGC</div> <div>CAAT</div> <div>CACCTGTGCG</div>	359
Si-60	299	<div>CAAT</div> <div>TTCCTTAGTCGCGCCGGTGATTTCGATGTGACGGTCTCGTTCGAGGC</div> <div>CAAT</div> <div>CACCTGTGCG</div>	358
US-41	360	TCACACGCGTCTGTGGCAAGTGCTTCGCCCATGGGATTTTCGTCTGAGCGTCTACACC	419
Si-60	359	TCGCACGCGTCTGTGGCAAGTGCTTCGCCACGGGATTTTCGTCTGAGTGTCTACACC	418
US-41	420	<div>TEA/ATTS</div> <div>CTGGAATG</div> <div>TTTTGGAGGCTATCCCGGCTCCCGCAGATGGCGACA</div> <div>CAAT</div> <div>CAAGGGCTTCAA</div>	479
Si-60	419	<div>CTGGAATG</div> <div>TTTTGGAGGCTATCCCGGCTCCCGCAGATGGCGACA</div> <div>CAAT</div> <div>CAAGGGCTTCGA</div>	478
US-41	480	<div>CAAT</div> <div>TGGGGACCGGGACCATATGCG</div> <div>ATTG</div> <div>GAAACGCTCGGTGATGTACCCTAGTTGTGCGG</div> <div>CreA</div> <div>GTG</div>	539
Si-60	479	<div>ATTG</div> <div>TGGGGACCGGGACCATATGCG</div> <div>ATTG</div> <div>GAAAGGCTCGGTAATGTACCCTAGTTGTGCGG</div> <div>GTG</div>	538
US-41	540	<div>ACEI</div> <div>GGGTAGCCTGCGCTCTCAAGCATCACGAGTGACACACCATCTACTCTAACTCAGA</div>	599
Si-60	539	<div>GGGTAGCCTGCGCTCTCAAGCATCACGAGTGACGACCATCTACTCTAACTCAGA</div>	598
US-41	600	<div>PacC</div> <div>AGCCAAG</div> <div>GCTCAAAGCTTTACCC</div> <div>TTC</div> <div>TATATAA</div> <div>GACGTTGCCGTGGAACATCAGACCAAC</div>	659
Si-60	599	<div>AGCCAAG</div> <div>GCTCAAAGCTTTACCC</div> <div>TTC</div> <div>TATATAA</div> <div>GACGTTGCCGTGGAACATCAGACCAAC</div>	658
US-41	660	<div>MPG1</div> <div>AGTTTCT</div> <div>CCCCTCT</div> <div>CCTCATCAGCCTCATCAGCCTTT</div> <div>TEA/ATTS</div> <div>CATTCT</div> <div>TTCTTCTGTTTCATCCCA</div>	719
Si-60	659	<div>AGTTTCT</div> <div>CCCCTCT</div> <div>CCTCATCAGCCTCATCAGCCTTT</div> <div>CATTCT</div> <div>TTCTTCTGTTTCATCCCA</div>	718
US-41	720	<div>TEA/ATTS</div> <div>TTCC</div> <div>TCAGAACTTCGGTAGTGCTAGTCACCCGCCTTTCCAGCTTTTGCATCGTATTCCGT</div>	779
Si-60	719	<div>TTCC</div> <div>TCAGAACTTCGGTAGTGCTAGTCACCCGCCTTTCCAGCTTTTGCATCGTATTCCGT</div>	778
US-41	780	<div>TATA box</div> <div>CCGAAC</div> <div>TATATATT</div> <div>CAACAAAC</div> <div>AGATA</div> <div>ACCTCGCACACCCTCAAG</div>	824
Si-60	779	<div>TATATATT</div> <div>CCGAAC</div> <div>TATATATT</div> <div>CAACAAACAAATAACCTCGCACACCTTCAAG</div>	823

Fig. 6 Promoter analysis of *CcpelA* gene cloned from wild-type isolates US-41 and Si-60. Potential binding sites for known transcription factors are marked.

Fig. 7 *CcpelA* (A) and *CcareA* (B) mRNA levels in isolates US-41 and Si-60. Total RNA was isolated from the highly aggressive US-41 and mildly aggressive Si-60, 5 h after transfer to secondary medium. cDNA was prepared and relative transcript levels of *CcpelA* and *CcareA* were analysed by real-time polymerase chain reaction (PCR) as described in Experimental procedures, using 18S rRNA as an internal standard. Expression levels of isolate US-41 were given the value of unity.

suggesting that the antibody will cross-react with other PL orthologues. Similarly, the virulence of the mildly aggressive isolate could be enhanced to the level of the highly aggressive isolates by the overexpression of PL (Fig. 5). The decrease in aggressiveness as a result of gene disruption and the increase in virulence caused by overexpression led us to conclude that PL is a virulence factor in *C. coccodes*, and that it is a limiting factor in mildly aggressive *C. coccodes* isolates.

Differential aggressiveness of *C. coccodes* results from differences in *CcpelA* promoter structure and activity

Highly aggressive *C. coccodes* isolates secreted PL earlier than mildly aggressive ones (Fig. 1). In addition, *CcpelA* transcript levels were approximately twice as high in highly aggressive versus mildly aggressive isolates (Fig. 7A), suggesting that the transcription rates of this gene are higher in the highly aggressive accession. As expected, promoters of the *CcpelA* genes isolated from US-41 and Si-60 were highly homologous. At the nucleotide level, the respective promoter sequences were 824- and 823-bp long, and identical in more than 97% of their nucleotide sequences. Both promoters contained two potential TATA boxes at nucleotides 625 and 785 (Fig. 6). Analysis of the promoter sequences for binding motifs of transcription factors suggested that both promoters share the potential for a large number of transcription factors. Some of these suggested sites, e.g. binding motifs for ACEI, ACEII, CreA, MPG1, PacC, TEA/ATTS and XlnR (Fig. 6), have been shown to regulate fungal CWDE gene expression (Aro *et al.*, 2005). These numerous binding motifs suggest a multifactorial regulation of *CcpelA* that might allow the gene to be expressed under different environmental conditions.

Two of these transcription factors, PacC and CreA, are likely candidates for the virulence-related modulation of gene expression: PacC has been reported as a transcription activator regulating gene expression in a pH-dependent manner. In *C. gloeosporioides*, *pac*-deleted mutants are reduced in their *pelB* expression, PL secretion and virulence towards avocado fruits (Miyara *et al.*, 2008).

Accordingly, promoter analysis of *pelB* revealed five potential PacC-binding motifs. For *C. coccodes*, ammonia and pH have been shown to facilitate fungal virulence (Alkan *et al.*, 2008).

CreA has been shown to be involved in glucose-dependent down-regulation of numerous pectinases (Aro *et al.*, 2005). Nevertheless, not all pectinases are subjected to glucose repression, e.g. P2c of *A. flavus*, Bcpg1 of *B. cinerea* and *pelB* of *Fusarium solani* f.sp. *pisi* (Guo *et al.*, 1995; Shieh *et al.*, 1997; ten Have *et al.*, 1998). Recently, Miyara *et al.* (2008) reported that *pelB* expression and PL secretion in *C. gloeosporioides* are up-regulated in the presence of glucose. Accordingly, CreA-binding motifs were not found in the *pelB* promoter. Although four CreA-binding motifs were found in the *CcpelA* promoter of both isolates (Fig. 6), we suggest that these sequences are not involved in glucose repression, as PL was secreted by *C. coccodes* in the presence of glucose (Fig. 1). In agreement with this, Orejas *et al.* (2001) reported that the CreA-binding sites found in the promoter *xlnB* are nonfunctional. However, CreA might act as a down-regulator of *CcpelA* under certain environmental conditions that have yet to be studied.

The two promoters showed minor sequence differences: 19 base differences and three gaps. These differences appeared to affect two potential *cis*-acting elements: although the US-41 promoter contained five CAAT (CCAAT) boxes, the Si-60 promoter contained only four intact CAAT motifs, whereas one binding site was removed as a result of the deletion of adenine corresponding to location 478 on the corresponding aggressive US-41 isolate (Fig. 6). CCAAT sequences have been found in the 5' region of approximately 30% of eukaryotic genes. A conserved multimeric protein complex (Hap) recognizes and binds to this sequence (Aro *et al.*, 2005). CCAAT sequences have been found in the promoters of many cellulase and pectinase genes (Unkles, 1992), and Hap has been shown to play a role in the formation of an open chromatin structure on several of these, indicating that CCAAT-binding complexes are necessary for full transcriptional activation of certain promoters (Narendja *et al.*, 1999).

The second difference between promoters was the G-to-A mutation in the *CcpelA* proximal promoter in a sequence corresponding to position 803 of US-41 (Figs 6 and S2), leading to removal of a potential AreA-binding site (GATA box) in the mildly aggressive Si-60 isolate. Many phytopathogenic fungal genes are up-regulated under conditions of nitrogen starvation. Regulation of nitrogen metabolism in *Aspergillus nidulans* and *Neurospora crassa* has been described to involve the GATA family transcription factors AreA and Nit2, respectively (Basse and Farsing, 2006). In *C. gloeosporioides*, nitrogen starvation causes transcriptional activation of *areA*, and *nit* mutants (which cannot utilize nitrate) do not secrete PL (Kramer-Haimovich *et al.*, 2006). In the bean pathogen *Colletotrichum lindemuthianum*, *cln1* mutants (AreA homologue) are nonpathogenic and are unable to switch to the necrotrophic stage of colonization (Pellier *et al.*, 2003). In *C. coccodes*, *areA* and *nit* mutants are impaired in ammonia secretion

and show reduced virulence towards tomato (Alkan *et al.*, 2008). This suggests that nitrate present in the secondary medium might play a role in the induction of *CcpelA* via AreA binding to the GATA sequence on the promoter of the gene of the highly aggressive US-41. In the present study, PL secretion occurred in the presence of KNO_3 (Fig. 1), a secondary nitrogen source that might facilitate AreA expression. The *CcpelA* promoter of US-41, but not Si-60, contains a putative AreA-binding motif (GATA box). Point mutation in the Si-60 GATA box (Fig. S2) might prevent positive regulation of *CcpelA* by the AreA transcription factor, leading to slower gene expression and delayed CcpelA secretion. Interestingly, *areA* transcript levels were also higher in the highly aggressive US-41 than in the moderately aggressive Si-60 (Fig. 7B). Our data thus suggest that the less aggressive isolate has both reduced *areA* levels and a concomitant loss of the AreA-binding site in the promoter of the *CcpelA* gene, resulting in a reduction and/or delay in the expression levels of *CcpelA* (Fig. 7A), and thus in reduced aggressiveness.

Overexpression of *CcpelA* in Si-60 under the regulation of the promoter Pgp_d thus enhanced PL secretion and aggressiveness towards tomato fruits (Fig. 5), supporting our assumption that differences in CcpelA secretion rates and/or dynamics between highly and mildly aggressive *C. coccodes* isolates are caused by differential gene regulation. The role of individual transcription factors in the modulation of *CcpelA* expression remains to be demonstrated.

EXPERIMENTAL PROCEDURES

C. coccodes isolates

Natural *C. coccodes* isolates were isolated from infected tomato (US-41, US-39, Eshel, Bsor) or potato (Si-120, Si-60, Si-72 and US-11). North American *C. coccodes* isolates (US-##) were obtained from D. Johnson, Washington State University, Pullman, WA, USA.

Aggressiveness assay

The bioassay of fungal aggressiveness was conducted as described previously (Ben-Daniel *et al.*, 2009). Briefly, 10- μL suspensions containing 1×10^5 conidia/mL were injected into the scar tissue of mature green tomato fruits, weighing c. 150–200 g, of the *C. coccodes*-susceptible variety cv. 1402 (Zeraim Gadera, Gadera, Israel). Fruits were sliced open vertically in a laminar hood at 7 days post-inoculation (dpi), and the necrotic tissue was measured. The extent of fungal colonization was determined in non-necrotic tissues from the blossom end of the fruit, opposite the inoculation site. Two biological replicates were performed.

In vitro PL secretion

The detection of secreted PL protein was performed essentially as described previously (Kramer-Haimovich *et al.*, 2006). Liquid M₃S medium

(40 mL) [2.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.7 g/L KH_2PO_4 , 1 g/L Bacto peptone, 1 g/L Bacto yeast extract (Difco, Lawrence, KS, USA) and 10 g/L sucrose] was inoculated with 1 mL containing 1×10^6 conidia. The cultures were incubated at 25 °C in a shaking incubator at 150 rpm for 4 days, and harvested by filtration through a sterile Büchner funnel fitted with filter paper. Collected hyphae were washed with sterile distilled water and transferred to 45 mL of secondary medium (4 g/L K_2HPO_4 , 2 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5 g/L KNO_3 , 0.3 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 10 mg/L FeCl_3 and 10 g/L glucose, adjusted to pH 6.0 with NaOH). Cultures were incubated under the same conditions, and samples were collected at the indicated times (6–24 h), and filtered through a sterile Büchner funnel fitted with filter paper to remove hyphae. The filtrate was further centrifuged for 10 min at 12 000 g to remove conidia. The resulting supernatant was dialysed in a SnakeSkin (Pierce, Rockford, IL, USA) pleated dialysis tube (10 000 molecular weight cut-off) for 48 h against 50 mM Tris-HCl, pH 8.5. The filtrate was lyophilized, resuspended in 1 mL sterile double-distilled water and stored at –20 °C. Protein samples were quantified by Bradford reagent protein assay (Bio-Rad, Hercules, CA, USA) using bovine serum albumin (Sigma, St. Louis, MO, USA) as the standard. For immunological determination of PL secretion, proteins were precipitated from 100- μL samples with 900 μL acetone at –20 °C for 12 h. Pellets were resuspended in sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (Laemmli, 1970) and resolved by electrophoresis on a 10% SDS-polyacrylamide gel (Mini Protean II, Bio-Rad). Proteins were electroblotted onto nitrocellulose membranes, and PL was immunologically detected using anti-*C. gloeosporioides* PL antibody (kindly provided by D. Prusky, Agricultural Research Organization, Bet-Dagan, Israel), as described previously (Wattad *et al.*, 1994). Experiments were repeated three times and blots from one representative experiment are presented.

PL activity assay

Fungi grown in liquid culture were transferred to secondary medium as described above. Growth medium was collected 6 h later, lyophilized and resuspended in 1:50 of the initial volume. Samples of 0.1 mL were analysed for PL activity in a total volume of 0.4 mL, as described previously (Collmer *et al.*, 1988). The increase in absorbance at 232 nm was measured for 2 min. One activity unit was defined as an increase of 0.01 optical density (OD)/min. Experiments were repeated two or three times.

DNA manipulation

Fungal genomic DNA was isolated from water-washed conidia collected from *C. coccodes* cultured for 3 days on PDA medium at 25 °C, following the instructions for the MasterPure Yeast DNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA). DNA was cloned using *Escherichia coli* strain DH5 α . DNA walking was performed using a SpeedUp Premix Kit (Seegene, Seoul, South Korea). PCRs were performed with ReddyMix PCR MasterMix (Thermo Scientific, Waltham, MA, USA) unless otherwise specified. PCR primer sequences and annealing temperatures are given in Table 1. Reactions were repeated twice and a photograph from one representative reaction is shown.

Cloning of *CcpelA* from isolate US-41

Degenerate primers (For-1 and Rev-2, see Table 1) were designed to a highly conserved sequence in three PL genes from other *Colletotrichum*

species (accession numbers AF156983, AF156985, AF052632). A 224-bp fragment was obtained by PCR using the For-1 and Rev-2 primers and US-41 genomic DNA template. The amplified fragment was verified by DNA sequencing, and target-specific primers were designed for DNA walking. A 341-bp upstream sequence that extended the primarily cloned sequence by 210 bp was cloned using a DNA walking SpeedUp Premix Kit (Seegene) with the target-specific primers TSP1.1A, TSP2.1A and TSP3.1A (Table 1) and general primers supplied by the manufacturer. Primers TSP1.3A, TSP2.3A and TSP3.3A were used for a 772-bp extension, and TSP1.4A, TSP2.4A and TSP3.4A for another 601-bp extension. The same kit was used to clone the 3' sequence of the gene using target-specific primers TSP1.2A, TSP2.2A and TSP3.2A and general primers supplied by the manufacturer, yielding a 788-bp fragment. The full-length assembled 2398-bp DNA sequence was cloned by PCR using *C. coccodes* (US-41) genomic DNA and primers For-2350-PL and Rev-new-PL4 (Table 1).

Construction of gene disruption plasmid pGH1-1240PL

A linear construct containing a partial target gene and an antibiotic resistance selectable marker gene was designed by amplifying the genomic DNA sequence encoding *CcpelA* (positions 715–1954, Fig. S1) by PCR using primers For-1240-PL and Rev-1240-PL (Table 1). The amplified product was subcloned into pGEM-T-Easy vector (Promega, Madison, WI, USA), and the resulting plasmid was used as a template for a secondary PCR using primers For-pGEM and Rev-pGEM (Table 1). The resulting PCR product, including a core of partial *CcpelA* DNA sequence flanked by short pGEM sequences, was digested with *NheI* and *XbaI*, and ligated into the compatible *XbaI* site of the pGH1 vector, which contains *hphB* as a selectable marker (Horowitz *et al.*, 2004; kindly provided by S. Freeman, Agricultural Research Organization, Bet-Dagan, Israel), yielding the pGH1-1240PL gene disruption construct.

Construction of plasmids pOE and pOE-NET

A 1460-bp DNA fragment, composed of the *CcpelA* coding sequence, including introns (positions 630–2089, Fig. S1), was amplified using genomic DNA template prepared from the highly aggressive *C. coccodes* isolate US-41, using primers For-OE and Rev-OE2 (Table 1) and the high-fidelity Ex-taq polymerase (Takara Bio Inc., Shiga, Japan). The enhanced product was cloned into pGEM-T-Easy vector, digested with *Clal* and *NcoI*, and ligated into plasmid gGFP-CA (Horowitz *et al.*, 2002; kindly provided by S. Freeman), in which the green fluorescent protein (GFP) gene was removed by digestion with the same restriction enzymes. The resulting plasmid pOE contained the *CcpelA* coding sequence driven by the strong fungal promoter *Pgpd* and a hygromycin selection marker (Fig. 5). An *EcoRI* fragment harbouring the NET resistance gene from the pNV15 plasmid (Levy *et al.*, 2008) was subcloned into the *EcoRI* restriction site of pOE, yielding the pOE-NET plasmid.

Fungal transformation

Germinating conidia were transformed by electroporation essentially as described previously (Horowitz *et al.*, 2002). The respective isolates were cultivated on solid PDA medium for 4 days at 25 °C, and conidia were

collected in water, washed twice by centrifugation and adjusted to 1×10^7 conidia/mL. Pea juice (25 mL) in a 500-mL flask was inoculated with 1 mL washed conidial suspension, and conidia were germinated at 25 °C for 5 h (at 50 rpm). The germinating conidia were collected by centrifugation, washed with cold sterile electroporation buffer [1 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES)-NaOH pH 7.5, 50 mM mannitol] and suspended in the same buffer at 1×10^8 conidia/mL. Aliquots (0.1 mL) were placed into cold 0.2-cm electroporation cuvettes (Bio-Rad) and linearized DNA plasmid (5–10 µg) was added. Electroporation was performed with a gene pulser (Bio-Rad) set at 1.4 kV, 800 Ω and 25 µF. Following the electrical pulse, the conidia were plated on Petri dishes containing regeneration medium (per litre: 145.7 g mannitol, 4 g yeast extract, 1 g soluble starch, 50 mL pea juice and 15 g agar) and incubated at 25 °C. An overlay of 1% (w/v) water agar with 80–100 µg/mL hygromycin B (Sigma) or NET (Jena Bioscience GmbH, Jena, Germany) was added 10 h later. Colonies of transgenic fungi appearing 7 days later were transferred to 5-cm PDA plates containing 100 µg/mL hygromycin B or NET.

RNA extraction and analysis of gene expression

Fifty conidia were grown in liquid M₃S medium for 2 days; 200 mg mycelium was transferred to the secondary medium, harvested 5 h later and RNA was extracted using the EZ-RNA kit (Biological Industries, Kibbutz Beit Haemek, Israel) according to the manufacturer's instructions. cDNA was synthesized using a ProtoScrip M-MuLV First Strand cDNA Synthesis Kit (New England Biolabs, Beverly, MA, USA) and random primer mix, according to the manufacturer's directions. Relative transcript levels were assayed by real-time PCR analysis using a RotorGene 3000 system (Corbett Research, Sydney, Australia). Reaction mix contained (20 µL total volume) Absolute Blue SYBR Green ROX mix (Thermo Scientific), 200 nm of each of the forward and reverse primers, and 2–20 ng cDNA. *CcpelA* transcript levels were determined using RT-For-qPL2 and RT-Rev-qPL2 primers (Table 1). Primers For-AreA1 and Rev-AreA1 (Alkan *et al.*, 2008; Table 1) were used for the quantification of *areA* transcripts. 18S rRNA (Table 1) was used as the internal standard. The following programme was used: 15 min at 95 °C, followed by 40 cycles of 94 °C for 10 s, 60 °C for 15 s and 72 °C for 20 s. The samples were subjected to melting curve and RNA relative quantification analyses using Rotor-Gene 6 software (Corbett Research).

Statistical analysis

Data were analysed statistically with JMP Software (SAS Institute Inc., Cary, NC, USA). Data were subjected to one-way analysis of variance (ANOVA), and means were separated by Student's multiple-range test ($P = 0.05$).

ACKNOWLEDGEMENTS

We thank S. Freeman (Agricultural Research Organization, Bet-Dagan, Israel) for providing pGH1 and gGFP-CA plasmids, D. Prusky (Agricultural Research Organization, Bet-Dagan, Israel) for the anti-*C. gloeosporioides* PL antibody, D. Johnson (Washington State University, Pullman, WA, USA) for the North American isolates and C. Vainstein for editing the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Nucleotide sequence of the *CcpelA* gene from the highly aggressive *Colletotrichum coccodes* isolate US-41 (GenBank accession number GU206360). Coding sequence is in capital letters; the deduced amino acid sequence is indicated underneath. The putative signal peptide is indicated in bold letters.

Fig. S2 DNA sequencing chromatogram from *CcpelA* promoter of US-41 (A) and Si-60 (B) wild-type isolates. GATA box marked in US-41 promoter and altered base is marked by an asterisk.

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