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Biomolecular Engineering xxx (2007) xxx–xxx
Biomolecular Engineering
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Expression of the yeast *cpd1* gene in tobacco confers resistance to the fungal toxin cercosporin

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Received 12 October 2006; received in revised form 13 December 2006; accepted 18 December 2006

Abstract

Many phytopathogenic species of the fungus *Cercospora* produce cercosporin, a photoactivated perylenequinone toxin that belongs to a family of photosensitizers, which absorb light energy and produce extremely cytotoxic, reactive oxygen species. The *cpd1* (cercosporin photosensitizer detoxification) gene of yeast (*Saccharomyces cerevisiae*), which encodes for a novel protein with significant similarity to the FAD-dependent pyridine nucleotide reductases, confers resistance to cercosporin when over-expressed in yeast. The aim of this work was to investigate the potential ability of *cpd1* gene to confer resistance to cercosporin when expressed in tobacco plants (*Nicotiana tabacum*). Transgenic tobacco plants were produced using *Agrobacterium tumefaciens*, with *cpd1* integrated as the gene of interest. We report here that expression of *cpd1* gene in tobacco can mediate resistance to cercosporin. The involvement of *cpd1* gene in the detoxification of the cercosporin reinforces previous observations, which suggested that resistance to cercosporin is mediated by a mechanism involving toxin reduction.

Keywords: Cercosporin detoxification; *Cpd1* gene; Transgenic tobacco

1. Introduction

Fungi of the genus *Cercospora* cause leaf spot and blight diseases in a wide range of economically important crops, such as sugar beet, tobacco, banana, coffee, corn, peanut and soybean (Daub, 1982; Fajola, 1978; Lynch and Geoghegan, 1977; Venkataramani, 1967). The pathogenicity factor is believed to be a red polyketide toxin known as cercosporin (Daub, 1982; Daub and Ehrenshaft, 2000). When cercosporin absorbs light energy it is converted to an electronically excited triplet state. In this state it can react with molecular oxygen, producing the activated oxygen species, radical-like superoxide, hydrogen peroxide and hydroxyl radical and non radical-like singlet oxygen (Spikes, 1989). Reactive oxygen species (ROS) production induced by cercosporin in the infected plants results in lipid peroxidation and membrane rupture (Daub, 1982), leading to leakage of nutrients from cells and thus allowing fungal growth (Daub and Ehrenshaft, 2000).

Cercosporin has a principal role in host plant infection and virulence (Daub and Ehrenshaft, 1993). Although it is most toxic to bacteria, many fungi, plants and animals (Daub, 1987), fungi species of the genus *Cercospora* are resistant to cercosporin, tolerating concentrations 1000-fold higher than concentrations considered lethal for other organisms. Although the mechanisms providing cercosporin resistance to *Cercospora* species or other microbes are not fully understood, it has been shown (Sollod et al., 1992; Daub et al., 1992; Leisman and Daub, 1992), that living hyphae of cercosporin-resistant fungi are capable of reducing cercosporin. Furthermore, Daub and her colleagues have proposed a model for cercosporin self-resistance in which the toxin is transiently and reversibly reduced at *Cercospora* hyphae (Daub et al., 1992). In addition, Jenns et al. (1995) have shown that cercosporin-sensitive mutants of *Cercospora nicotianae* are unable to reduce cercosporin. *Cercospora* species are a very successful group of pathogens, since there is only one report for resistance in

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1389-0344/$ – see front matter © 2007 Published by Elsevier B.V.
doi:10.1016/j.bioeng.2006.12.003

rice. In this case, the resistance is due to the presence of carotenoids and lack of cercosporin uptake (Batchvarova et al., 1992).

A cercosporin photosensitizer detoxification gene *cpd1* was isolated for the first time from *Saccharomyces cerevisiae* (Ververidis et al., 2001). *Cpd1* encodes a novel protein of 378 amino acids. It shows significant similarities to the FAD-dependent pyridine nucleotide reductases, especially in the conserved motifs such as the FAD and NAD(P)H binding domains. It is likely to be plasma membrane-associated, as it is predicted to have one to three hydrophobic segments. Although *cpd1* is not essential for yeast growth, it confers resistance to cercosporin when over-expressed in yeast.

The Cpd1 protein is similar to both extended sequence and short sequence motifs with FAD pyridine nucleotide reductases like glutathione reductase (GR), trypanothione reductase (TR) and thioredoxin reductase (THR) (Ververidis et al., 2001). These reductases catalyze the reduction of oxidized glutathione, which plays a role in the defence of the cell against oxidative stress and xenobiotic toxicity (Izawa et al., 1998). THR reduces thioredoxins, which have been shown to have diverse functions in bacteria (Holmgren, 1985). GR, TR, and THR reductases are important in cellular defence against oxidative stress (Arrick et al., 1981; Muller, 1996). The *cpd1* gene has been shown to confer resistance to cercosporin and other ROS-generating photosensitizers when over-expressed in *S. cerevisiae* (Ververidis et al., 2001).

We describe here the development and testing of transgenic tobacco transformed with the *cpd1* gene from yeast. The results indicate that the expression of yeast *cpd1* confers resistance to pure cercosporin in transgenic tobacco plants.

2. Materials and methods

2.1. Plant material and culture

Tobacco seeds of the commercial aromatic cultivar "Basmas" were surface sterilized for 2 min in 70% ethanol and 15 min in 1.3% sodium hypochlorite solution with 0.025% Tween 20 added, then washed three times with sterile distilled water, dried on sterile filter papers and placed under aseptic conditions on Petri dishes containing MS medium (Murashige and Skoog, 1962), solidified with 0.8% agar–agar (Sigma, USA). The pH was adjusted to 5.8 with KOH or HCl before autoclaving (121 °C for 20 min). Petri dishes were placed in a SANYO MLR-350H growth chamber (USA) at 25 °C in the dark. After the seeds had germinated, they were transferred to Magenta® vessels GA-7 (Sigma, USA) containing the same medium and were incubated in a growth chamber at 25 °C with a 16 h light (100 μE m^{-2} s^{-1})/8 h dark period.

3. Methods

3.1. Agrobacterium transformation

The plasmid pBin/Hyg/Cpd1, which harbors the *cpd1* gene driven by the CaMV 35S promoter and the selectable gene *hph*, which confers resistance to hygromycin, was isolated from bacteria by alkali lysis (Sambrook et al., 1989). Transformation of *Agrobacterium tumefaciens* strain GV2260 was achieved by electroporation at 3.0 kV at 50 μF. Resistant *Agrobacterium* cells were then grown in 900 μL sterile YEB medium (5 g beef extract, 1 g yeast extract, 5 g peptone, 5 g sucrose, 500 mg $MgSO_4$, made up to 1 L) at 28 °C for 3 h, and subsequently bacteria were spread on Luria Broth plates containing kanamycin, carbenicillin and rifampicin 100 mg/L each, finally they were incubated at 28 °C. Transformed *Agrobacterium* colonies appeared on the selection plates after 2–3 days.

3.2. Generation and verification of transgenic plants

Agrobacterium-mediated plant transformation was used to produce transgenic tobacco plants (Horsch et al., 1985). Leaf discs were placed in Petri plates containing 5 mL of MS regeneration medium (MS_R—MS supplemented with 1 mg/L BA and 0.1 mg/L NAA). The leaf discs were co-cultivated with 1 mL of an overnight grown liquid culture of *A. tumefaciens* (carrying the pBin/Hyg/Cpd1 plasmid), and kept in the dark for 16 h at 25 °C. They were then blotted dry and placed abaxial side up in Petri dishes containing MS_R medium solidified with 0.8% agar and supplemented with 3% sucrose, without selection agents (hygromycin). The leaf discs were incubated for 24 h in a growth chamber at 25 °C with a 16 h light (100 μE m^{-2} s^{-1})/8 h dark period and then they were transferred to selection medium (MS_R containing 15 mg/L hygromycin, 500 mg/L cefotaxime and 250 mg/L carbenicillin) and placed back in the same growth chamber until they have regenerated. In 3–4 weeks, resistant shoots appeared and were transferred to MS medium without growth regulators, but containing 15 mg/L hygromycin, 500 mg/L cefotaxime and 250 mg/L carbenicillin. After 2 weeks, growing plants were transferred to rooting medium (MS supplemented with 1 mg/L NAA, 15 mg/L hygromycin, 500 mg/L cefotaxime and 250 mg/L carbenicillin), prior to their transfer to soil.

Genomic DNA was isolated from leaves using the DNeasy kit (Qiagen, USA). The following primer pair was used for PCR detection of the specific *cpd1* gene: "cpd1L" 5′-CGCGGATCCCAGGAAAGAGCAGAGAAAGG-3′ (29 mer) and "cpd1R" 5′-CGCGGATCCCGCTGCAGTTCATATTTTAGTC-3′ (31 mer). Amplification was performed for 32 cycles of 1 min at 94 °C, 1 min at 58 °C and 1 min at 72 °C. PCR products were separated by gel electrophoresis on 1% agarose. For Southern blot hybridizations, genomic DNA samples (15 μg) were digested with *Hin*d III (Roche, England) restriction enzyme and the resulting fragments were resolved on a 0.8% agarose gel at 20 V for 16 h. The DNA was transferred to a GeneScreen nylon membrane (Perkin-Elmer, USA). *Cpd1* inserts amplified via PCR were labeled with α^{32}P-dCTP using the High Prime labelling Kit (Roche, UK) and were used as hybridization probes. Hybridization was performed at 65 °C for 12 h. The membrane was washed with 0.1% SSC and 0.1% SDS in sterile distilled H_2O at 60 °C, then dried on sterile filter paper. Membrane blots were exposed with Kodak BioMax medical X-ray Film (Kodak, USA) with intensifying screens at −80 °C (Sambrook et al., 1989).

3.3. Transgene expression and inheritance

Total RNA was isolated from leaves using the RNeasy Kit (Qiagen, USA). Transcripts were detected by RT-PCR. For reverse transcription the "cpd1R" (31 mer) primer was used. The 15 μL amplification mixture contained 75 units of reverse transcriptase M-MLV (GIBCO, BRL®), 0.5 mM of each dNTP, 1× buffer, 0.5 μM downstream primer, 150 ng/μL DTT, 150 ng/μL Rnase inhibitor (GIBCO, BRL®) and 0.2 μg total RNA. Reverse transcription was performed for 1 h at 43 °C followed by 5 min at 94 °C for enzyme inactivation. One microliter of each RT reaction was used for PCR amplification as described above.

T_1 seeds, produced after selfing T_0 plants, were surface sterilized in ethanol for 30 s followed by 10 min in NaOCl. Seeds were then rinsed five times with sterile distilled water. Sterilized seeds were germinated on MS medium supplemented with 15 mg/L hygromycin. Three replications with approximately 100 seeds of each transformed line were used for statistical analysis. The data were analyzed by the X^2-test at $p < 0.05$ or $p < 0.01$.

3.4. Determination of tolerance of transgenic plants to cercosporin

Ion leakage, an indicator of tissue damage in response to the presence of irradiated cercosporin, was measured according to Gwinn et al. (1987), with a Crison Model Micro CM 2201 conductivity meter. Specifically, following this method, round leaf discs (6 mm in diameter) were taken from 1-month-old soil

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grown plants. We used five plants per each transgenic line and for each control, taking the 3rd and 4th leaf from each plant. Prior to the experiment, we tested tobacco leaf discs of "Basmas" cultivar with a range of cercosporin solutions (0.8, 1.0, 1.2, 1.4 μM) and we found that 1.2 μM to be the concentration of choice for the electrolyte leakage experiment (data not shown). Fifteen leaf disks from each one of the transgenic lines and each one of the controls were cut with a leaf borer and were suspended in 25 mL test tubes containing 10 mL of 1.2 μM cercosporin (Sigma, USA) in 2% aqueous methanol kept in the dark for 1 h at 25 °C for the cercosporin to be absorbed by the leaf discs. After 1 h the leaf discs were exposed to light (fluorescent light intensity 115 μE m^{-2} s^{-1}) at 25 °C. Measurements were taken at 0, 1, and 6 h time points (time 0 represents the time point when the leaf discs were placed in the cercosporin medium and time 1 when they were first exposed to light).

The differences in conductivity of the solutions between 1 and 6 h were determined for each transgenic line and the controls. For statistical analysis (ANOVA) of the conductivity results, three sets of 15 leaf discs from each independent T_0 and T_1 line and from each control were used. The experiment for T_0 and T_1 generations was performed separately.

In addition to the electrolyte leakage experiment with leaf discs, cercosporin tolerance of *cpd1* transgenic plants was measured in whole tobacco plants *in vivo*. In order to further investigate the resistance of the transgenic lines carrying *cpd1* to cercosporin, we used 1-month-old tobacco plants grown in soil. The controls and transgenic plants were grown in a SANYO growth chamber under 16/8 h light/dark, respectively, at 25 °C, light (fluorescent light intensity 115 μE m^{-2} s^{-1}). The soil was covered with plastic wrap and the potted plants were dipped and fully immersed into a beaker (covered with aluminium foil) containing a 1.2 μM cercosporin solution (cercosporin was diluted in a 2% aqueous methanol solution in dark). The beaker was placed in a vacuum chamber (Nalgene, Rochester, USA, General Europe Vacuum, Milano, Italy) and subjected to −100 kPa, for 3 min to facilitate cercosporin penetration into the plant tissues. The pressure was released slowly over a 5 min period and no detectable mechanical damage to tissue was observed. Initially relatively high humidity was maintained around the plants by bagging pots and plants in large black plastic bags. The bagged pots were placed in the growth chamber for 24 h in the dark and then the bags were removed and the plants exposed to light again. Leaves were observed for 10 days with the first symptoms being visible 48 h after the bags were removed and on day 10 post-application, lesions were counted and the leaves were photographed (Upchurch et al., 2005). For both experiments we included three controls: (a) wild type (wt) treated with cercosporin and subjected to the same treatment as the transgenic plants (PC), (b) wt treated with methanol and treated as the transgenic plants (NC^1), and (c) wt treated with cercosporin but kept in the dark (NC^2).

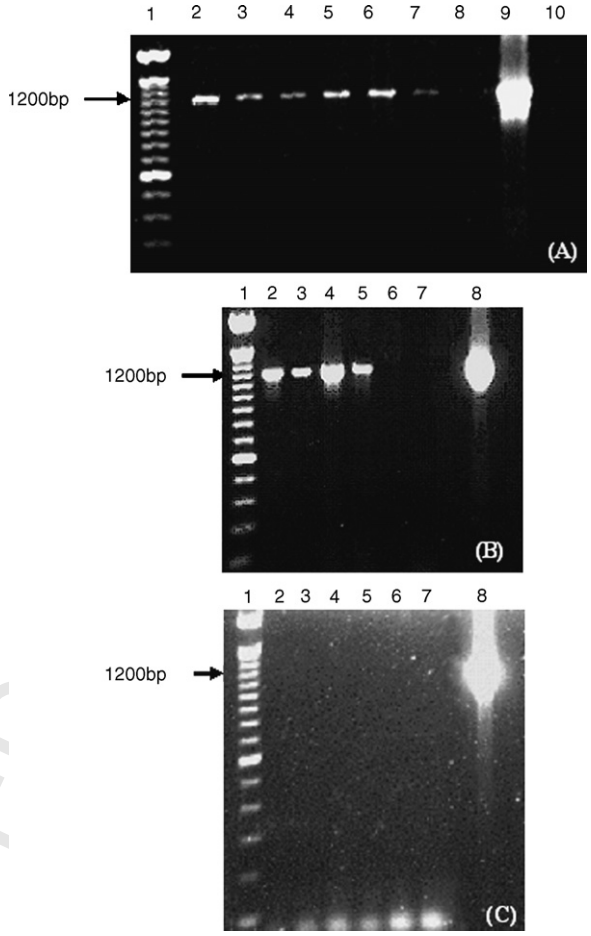

Fig. 1. Verification of T_0 transgenic plants by PCR. (A) Lane 1: 100 bp DNA ladder; lanes 2–7: transgenic lines (A, B, H, J, I and Z) with *cpd1* gene; lane 8: negative control (DNA from non-transformed plant); lane 9: positive control (pBin/hyg/Cpd1 DNA); lane 10: negative control (no DNA); (B) expression analysis of *cpd1* gene by RT-PCR and (C) PCR analysis of isolated RNA (except for positive control, no other amplification was observed, verifying the positive bands in 1-B). Lane 1: 100 bp DNA ladder; lanes 2–5: transgenic lines (A, B, H, Z) with *cpd1* gene; lane 6: negative control (DNA from wild type non-transformed tobacco plant); lane 7: negative control (no DNA); lane 8: positive control (pBin/hyg/Cpd1 DNA).

4. Results

4.1. Verification of transgenic plants, transgene expression and inheritance

Putative transgenic T_0 plants that had been selected for rooting on hygromycin were initially screened by PCR. As shown in Fig. 1(A), the *cpd1* gene can be detected with specific primers that amplify a 1200 bp band. Six plants (A, B, H, J, I and Z) produced a 1200 bp PCR product identical to the one produced by pBin/Hyg/Cpd1 binary vector used for the transformation experiments.

Active transcription of the *cpd1* gene in T_0 transgenic plants was confirmed by RT-PCR, as shown in Fig. 1(B). PCR amplification of positive control DNA (pBin/Hyg/Cpd1 binary vector) produced a fragment of 1200 bp, as did the first strand cDNA of four T_0 plants (plants A, B, H and Z). Plants I and J were also tested for *cpd1* transcription but did not produce the 1200 bp fragment (data not shown). Untransformed plants and RNA free control samples did not have any products (Fig. 1(B)). Positive RNA samples were subjected to PCR without reverse transcription and no signal was detected, confirming that the samples were not contaminated with DNA (Fig. 1(C)).

Seeds from self-pollinated T_0 transgenic plants were collected and germinated on selection medium (MS + 15 mg/L hygromycin). We observed the typical 3:1 segregation ratio in T_1 progenies from two of the T_0 plants line progenies, B and Z, (Table 1). PCR analysis in T_1 plants confirmed the stable inheritance of the transgene. As shown in Fig. 2(A), the T_1 plants (lines A, B, H, and Z) produced a 1200 bp PCR product representative of the transgene *cpd1* insert. The observed segregations were checked with X^2-criterion and lines B274 ($X^2 = 0.64$) and Z274 ($X^2 = 6.06$) followed the expected 3:1 ratio for a single dominant gene. Progeny lines from A and H also showed a Mendelian 1:1 segregation ratio.

Southern blot analysis of T_1 plants confirmed the transformation events. A *cpd1* gene specific probe was used

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Table 1
Segregation of hygromycin resistance T_1 seeds produced after self-pollination of transgenic tobacco plants

Transgenic lines	Seeds germinated in hygromycin	Seeds not germinated in hygromycin	Total number of seeds	Germination in hygromycin (%)	Segregation ratio
A	153	157	310	51.16 d[a]	1:1
B	219	81	300	71.43 b	3:1[a]
H	161	136	297	54.33 d[a]	1:1
Z	213	96	309	68.93 c	3:1[b]
PC[c]	300	0	300	100 a	0
NC[d]	0	300	300	0	0

Seeds were germinated on MS medium supplemented with 15 mg/L hygromycin. Positive control (PC) stands for seeds from non-transgenic tobacco plants germinated on MS medium without antibiotic selection and negative control (NC) stands for seeds from non-transgenic tobacco plants germinated on MS medium with antibiotic selection (15 mg/L hygromycin).

[a] Means within a column followed by the same letter are not significantly different at $p = 0.05$ and observed segregation rates are not significantly different from the expected rates at $p = 0.05$.

[b] Observed segregation rates are not significantly different from the expected rates at $p = 0.01$.

[c] Seeds from non-transgenic tobacco plants germinated on MS medium without antibiotic selection.

[d] Seeds from non-transgenic tobacco plants germinated on MS medium with antibiotic selection.

for Southern hybridization. *Hin*d III-digested genomic DNA from PCR-positive plants was subjected to hybridization; the different pattern of the observed bands confirms the independent nature of the transformation events. *Hin*d III linearizes the pBin/Hyg/Cpd1 plasmid (~14 kb). The blot on the *cpd1* transgenic plants is presented in Fig. 2(B). Four lines were identified that contain the *cpd1* gene and were used for further experiments. Southern blot analysis revealed multiple insertions for each of the four transgenic lines, which were all phenotypically normal and set seeds. Southern analysis of the four PCR-positive plants proved the independency of four transgenic lines but also suggested that two of the PCR-positive lines failed to produce a positive RT-PCR fragment and a positive Southern. That was probably due to the presence of the *Agrobacterium* or the plasmid used for the transformation, which had not been integrated into the plant genome in the original putatively transformed T_0 plants tested. Molecular data from PCR and Southern analysis confirmed that the hygromycin-resistant lines A, B, H and Z contained the *cpd1* gene (Figs. 1(A), and 2(A) and 2(B)).

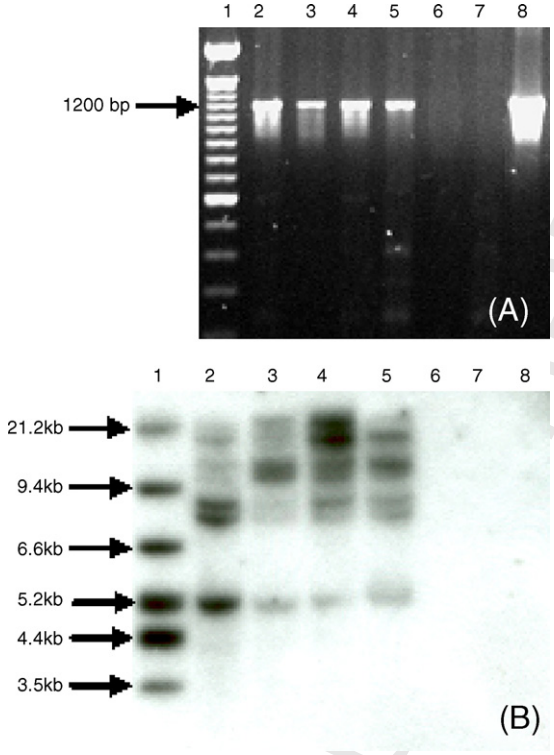

Fig. 2. PCR amplification of *cpd1* gene in T_1 progeny and verification by Southern analysis. (A) PCR amplification. Lane 1: 100 bp DNA ladder; lanes 2–5: transgenic lines (A, B, H, Z) with *cpd1* gene; lane 6: negative control (DNA from non-transformed plant); lane 7: negative control (no DNA); lane 8: positive control (pBin/hyg/Cpd1 DNA). (B) Southern DNA blot analysis. Lane 1: λ *Hin*dIII plus λ *Hin*dIII–*Eco*RI DNA ladder; lanes 2–5: transgenic lines (A, B, H, Z) with *cpd1* gene; lanes 6 and 7: PCR-positive lines I and J failed to produce a positive Southern band (escapes); lane 8: negative control (DNA from wt non-transformed tobacco plant).

4.2. Determination of tolerance of transgenic plants to cercosporin

Cercosporin-induced cell damage can be qualitatively and statistically assessed by conductivity measurements indicative of electrolyte leakage from leaf tissue (Gwinn et al., 1987). The differences in electrolyte leakage between 1 and 6 h measurements as conductivities of three different controls and the four transgenic lines described above treated with 1.2 μM cercosporin are summarized in Fig. 3(A) and (B) for T_0 and T_1 generations, respectively. We performed statistical analysis of the electrolyte leakage at 0 and 1 h time points of the transformed lines and the controls which showed that there were no statistical significant differences. It is known that light plays an important role in the induction of the cercosporin toxicity (Fajola, 1978). Statistical analysis showed a highly significant difference between the transgenic lines and the controls in cercosporin in the light (T_0, $F = 11.4$, $p = 0.0003$ and T_1, $F = 3.1$, $p = 0.02$). Thus expression of *cpd1* leads to elevated resistance against cercosporin. All four lines were statistically different from the PC control and all revealed higher resistance to cercosporin, both in T_0 and T_1 generations compared to the PC control. Also A, H and Z lines were not statistically different amongst them and from NC[1], showing the

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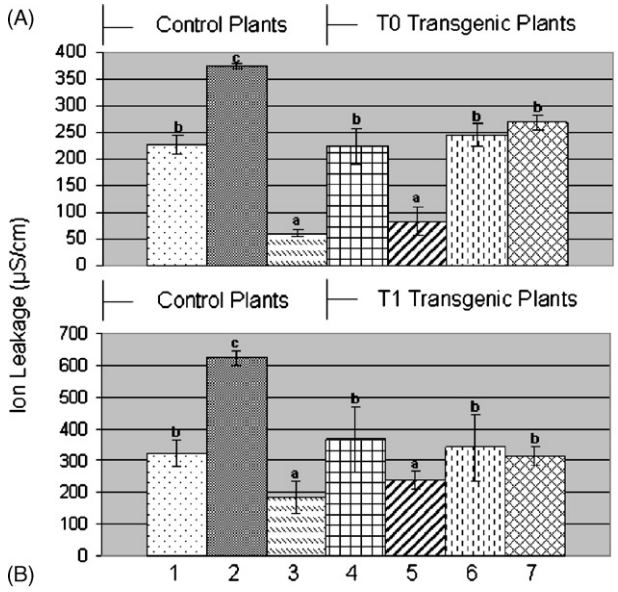

Fig. 3. Phenotypic expression of T_0 and T_1 transgenic tobacco plants harboring *cpd1* gene. (A) Conductivity of the 1.2 μM cercosporin solution bathing leaf discs from T_0 transgenic lines. Column 1: wt tobacco plants in methanol (NC^1); column 2: wt tobacco plants in cercosporin (PC); column 3: wt tobacco plants in cercosporin kept in the dark (NC^2). All the controls have been regenerated along with the transgenic plants. Columns 4–7: transgenic lines (A, B, H and Z, respectively); LSD = 62, $p \leq 0.0003$. (B) Conductivity of the 1.2 μM cercosporin solution bathing leaf discs from T_1 transgenic lines. Column 1: wt tobacco plants in methanol (NC^1); column 2: wt tobacco plants in cercosporin (PC); column 3: wt tobacco plants in cercosporin kept in the dark (NC^2). All the controls have been regenerated along with the transgenic plants. Columns 4–7: transgenic lines (A, B, H and Z, respectively); LSD = 16.6, $p \leq 0.02$.

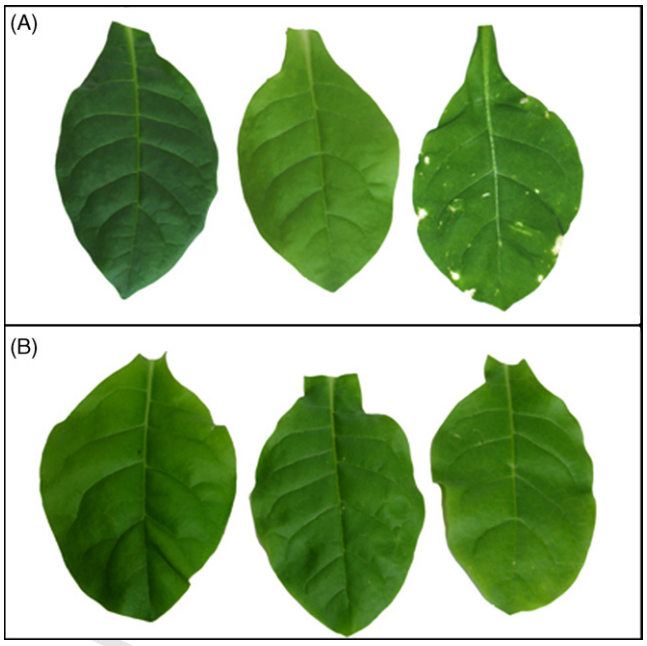

Fig. 4. Phytotoxicity of cercosporin (1.2 μM solution) on "Basmas" leaves under vacuum infiltration. Pictures were taken 10 days post-application. (A) Non-transformed control leaves from wild type tobacco plants, NC^1 (treated with 2% aqueous methanol solution), NC^2 (treated with cercosporin and kept in the dark), and PC (treated with cercosporin); (B) transgenic lines A, B and H (treated with cercosporin).

same electrolyte leakage as measured by the conductivity measurements. One line in particular (B) was the most resistant to cercosporin between the four transgenic lines, as it had the same conductivity measurements as the NC^2 both in T_0 and in T_1 generations (Fig. 3(A) and (B)).

Three plants from each of the *cpd1* transgenic lines A, B, H and Z and three non-transgenic "Basmas" control plants were tested for sensitivity to a 1.2 μM of cercosporin in 2% aqueous methanol solution by vacuum infiltration. Table 2 shows that on day 10 post-application, the mean number of lesions was significantly increased in PC compared to the transgenic lines. The controls NC^1 and NC^2 were free of lesions. Transgenic lines showed reduced cercosporin induced lesions, having from 2.3 to 4.7 mean number of lesions per plant, which was significantly lower than PC (33.7 mean number of lesions per plant). These results demonstrate that in the light, the *cpd1* expressing transgenic lines show significantly reduced necrotic lesions compared to the non-transgenic (Fig. 4(A) and (B)).

Table 2
Leaf lesion count for wt and T_1 *cpd1* transgenic tobacco plants ("Basmas") treated with a 1.2 μM cercosporin solution under vacuum infiltration

	Mean number of leaves per plant	Mean number of leaves with lesions per plant	Mean number of lesions per plant
A	13.3	1.7	2.3
B	13.3	1.7	2.7
H	13.3	2.0	3.0
Z	11	2.3	4.7
PC	11	5.0	33.7
NC^1	13	0	0
NC^2	10	0	0

We used three plants of each of the above *cpd1* transgenic lines (A), (B), (H) and (Z) treated with cercosporin, and the controls; PC, a non-transgenic wt treated with cercosporin; NC^1, a non-transgenic wt treated with 2% aqueous methanol solution; NC^2, a non-transgenic wt treated with cercosporin and kept in the dark during the experiment.

5. Discussion

Many *Cercospora* species produce a unique photoactivated and photo-induced perylenequinone toxin (cercosporin) which is toxic via the generation of ROS (Knox and Dodge, 1985). ROS destroy the membranes of host plants, providing nutrients to support the growth of these intercellular pathogens (Daub and Ehrenshaft, 2000). Like many other organisms, plants have evolved a wide range of enzymatic mechanisms to detoxify oxygen species. One such enzymatic mechanism of great importance is the superoxide dismutase (SOD) enzyme that converts superoxide to hydrogen peroxide. In many cases it appears that SOD is a key enzyme for providing protection against oxidative stress (Bannister et al., 1987). As has been shown in various laboratories, including ours, expression of superoxide dismutase (SOD) transgenes has led to resistance to oxidative stress in tobacco (Bowler et al., 1991), pepper (Zambounis et al., 2002), and sugarbeets, which is also resistant to the fungus *Cercospora beticola* (Tertivanidis et al., 2004).

Daub and Ehrenshaft (2000) have shown that *CRG1* and *SOR1* genes, which were isolated from cercosporin sensitive mutants of *Cercospora*, could restore the cercosporin resistance in the fungus. Pyridoxine (vitamin B6) has been recently (Bilski et al., 2000) linked with cercosporin resistance through chemically quenching singlet oxygen. *PDX1* encodes for a protein that functions in a novel pathway for pyridoxine biosynthesis and thus confers resistance to cercosporin (Ehrenshaft et al., 1999).

Carotenoids have also been suggested as another mechanism leading to *Cercospora* resistance (Batchvarova et al., 1992), but Ehrenshaft et al. (1995) found that carotenoids do not mediate cercosporin and $^1O_2$ resistance in the fungus.

Moreover, other genes have been shown to confer resistance to cercosporin. The *snq2*, an ABC-type efflux transporter mediated resistance to *S. cerevisiae* when over-expressed on high-copy plasmids (Ververidis et al., 2001). An MFS-like protein CFP, which is believed to export the toxin, has also been successfully used to develop cercosporin resistance tobacco plants (Upchurch et al., 2005). The *cpd1* gene has been shown to confer resistance to cercosporin and other oxygen-generating photosensitizers when over-expressed in *S. cerevisiae* (Ververidis et al., 2001). In this paper we have demonstrated that constitutive expression of *cpd1* gene mediates cercosporin resistance in tobacco.

We generated four independent transgenic tobacco lines (A, B, H and Z) expressing the *cpd1* gene, coding for a putative reductase protein. Molecular data from PCR and Southern analysis confirmed that the hygromycin-resistant plants A, B, H and Z contained the *cpd1* gene. RT-PCR analyses with specific primers yielded product of the predicted size, showing the expression of the gene in the transgenic plants.

Segregation analysis of T_1 progeny from the four independent transgenic plants (Table 1) demonstrated that the *hph* gene was stably inherited by T_1 progeny as a single Mendelian trait (3:1) for lines B and Z, which has been commonly observed in other segregation studies of transgenic plants (Bano-Maqbool and Christou, 1999). We also observed a Mendelian 1:1 segregation ratio for A and H plants. A 1:1 segregation ratio is an indication of instability through either gene silencing or rearrangement and loss of the transgene. Although the Southern blot suggests that there might be more than one copies inserted for each transgenic line, the segregation analysis shows that they are inherited as a single copy, which suggests that they might be linked or there are position effects. Chromosomal position effects, caused by the random integration of the transgenic DNA in the plant genome, also contribute to the variability of transgene expression.

Electrolyte leakage from leaf tissue has been used before to measure the cell damage caused by cercosporin (Gwinn et al., 1987). All four transgenic lines showed higher levels of cercosporin resistance due to the expression of *cpd1* gene. The statistical analyses of T_0 and T_1 generations revealed that three transgenic lines were as resistant to cercosporin as NC^1 and that; one was as resistant to cercosporin as NC^2.

Furthermore cercosporin induced lesions are reduced in leaves of *cpd1* transgenic lines (A, B, H and Z) compared to PC. Lesions only form in the presence of light. NC^1 and NC^2 controls were free of lesions. Since cercosporin is a major toxin (Daub et al., 1992) produced by the fungal pathogen *C. nicotianae*, expression of *cpd1* in transgenic plants might reduce the severity of necrotic lesions.

According to Daub et al. (1992) cercosporin self-resistance of *Cercospora* species includes, amongst other mechanisms, the reversible reduction of cercosporin. The results presented here, of transgenic tobacco plants expressing *cpd1* gene being resistant to cercosporin, reinforce the above hypothesis. Since *cpd1* gene is a putative reductase, reduction of cercosporin could be a way to confer resistance to the toxin. Moreover, disruption of CTB1 and CZK3, which play key roles in cercosporin biosynthesis in *C. nicotianae* (Choquer et al., 2005), results in the loss of cercosporin synthesis and decrease in virulence of the pathogens. The *ctb1* and *czk3* null mutants incited fewer and smaller lesions on host leaves, suggesting that strategies that avoid the toxicity of cercosporin could reduce the disease incidence caused by *Cercospora* spp. (Choquer et al., 2005). The observed high levels of resistance to cercosporin suggest that *cpd1* may confer resistance to *Cercospora* species too, as the cercosporin molecule plays an important role in plant infection and virulence (Upchurch et al., 1991; Daub et al., 1992; Daub and Ehrenshaft, 1993).

Thus the *cpd1* gene represents a promising candidate to be used, through genetic engineering, to enhance cercosporin resistance and may make a valuable contribution to crop protection.

Acknowledgments

The authors are grateful to Dr. Anil Day (University of Manchester) and the two anonymous reviewers for their invaluable suggestions towards the improvement of this manuscript, Dr. Paschalis Akritidis for technical assistance to the experiment of the vacuum infiltration. This work was supported by a grant from the General Secretariat for the Research and Technology (Greek Ministry of Development) to A.T. P.M. is the recipient of a National Scholarship Foundation (IKY), PhD Scholarship.

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Please cite this article in press as: Madesis, P. et al., Expression of the yeast *cpd1* gene in tobacco confers resistance to the fungal toxin cercosporin, Biomol Eng (2007), doi:10.1016/j.bioeng.2006.12.003