Eur J Plant Pathol (2009) 125:355–365
DOI 10.1007/s10658-009-9485-4

Cisgenic melons over expressing glyoxylate-aminotransferase are resistant to downy mildew

Ido Benjamin · David Kenigsbuch · Mariana Galperin · Javier A. Abrameto · Yigal Cohen

Received: 26 November 2008 / Accepted: 23 April 2009 / Published online: 8 May 2009

Abstract Downy mildew caused by the oomycete *Pseudoperonospora cubensis*, is a devastating foliar disease of cucurbits. The wild melon PI 124111F (PI) is highly resistant to this disease while BU21/3 and Hemed are susceptible. In a previous study we showed that resistance in PI is metabolic, resulting from enhanced activity of glyoxylate aminotransferase encoded by the genes *At1* and *At2*. When either gene from PI was transformed into a susceptible plant it became resistant. Here we show that the nearly silent *At1* and *At2* in the susceptible Hemed confer resistance to downy mildew when overexpressed (by CaMV S35 promoter) in the susceptible BU21/3. The cisgenic plants, overexpressing either *At1* or *At2*, exhibited enhanced activity of glyoxylate aminotransferase and resistance against *P. cubensis*. Northern and western gel blot analyses suggested that the low expression of *At1* and *At2* in the susceptible melons is modulated by transcriptional inhibition.

I. Benjamin · M. Galperin · J. A. Abrameto · Y. Cohen (✉)
The Mina & Everard Goodman Faculty of Life Sciences,
Bar-Ilan University,
Ramat-Gan 52900, Israel
e-mail: ycohen@mail.biu.ac.il

D. Kenigsbuch
Department of Postharvest Science, Volcani Centre,
Beit-Dagan 50250, Israel

Keywords Aminotransferase · Cucurbits · Downy mildew · Enzymatic resistance (*eR*) · Peroxisome · Photorespiration

Introduction

Downy mildew caused by the oomycete *Pseudoperonospora cubensis*, is devastating to cucurbits in temperate regions of the world. Infected leaves exhibit pale green-yellow lesions with abundant sporulation, which eventually turn necrotic (Cohen 1981; Cohen and Eyal 1987; Cohen et al. 2003; Thomas et al. 1987).

A previously identified genotype of melon, Plant Introduction 124111 (=PI) from India exhibits resistance against all six pathotypes of downy mildew by producing small, water-soaked lesions, rich in callose and lignin, with almost no sporulation (Cohen et al. 1989). Genetic studies revealed that resistance of PI is controlled by two partially-dominant complementary loci, Pc_1 and Pc_2 (Kenigsbuch and Cohen 1992). Resistance in PI was associated with the presence of a tissue-specific cytoplasmic protein P45. This protein was not detected in the susceptible melons Hemed or Ananas-Yokneam (Balass et al. 1992, 1993). Two genes, designated *At1* and *At2* (aminotransferase) were deduced from the P45 protein, which has 80% homology with glyoxylate aminotransferase (GAT) from *Arabidopsis thaliana*, a photorespiration peroxisomal enzyme (Liepman and Olsen 1998; Somerville

and Ogren 1980). Over expression of *At1* or *At2* from the resistant PI (accession number AY066012 and AF461048 , respectively) in the susceptible melon BU21/3 resulted in resistance to *P. cubensis* (Taler et al. 2004). Genetic analysis showed that bacteria expressing *At1* and *At2* exhibit high GAT activity regardless of whether the genes were taken from the resistant PI or the susceptible Hemed (Taler et al. 2004).

In the present study, we examined the hypothesis that Hemed is susceptible to downy mildew because *At1* and *At2* are down-regulated. To confirm this, we cloned *At1* and *At2* from Hemed and incorporated them under the strong CaMV 35S promoter, into anther (regeneration competent) susceptible melon line, BU21/3. The produced cisgenic plants which over expressed *At1* and *At2* genes were indeed resistant to downy mildew.

Materials and methods

Plant material and transformation

The susceptible melon (Cucumis melo) cv. Hemed (Hazera Genetics, Mivhor, Israel), our susceptible inbred melon line BU21/3 highly competent for regeneration in culture (Galperin et al. 2003), and our resistant inbred melon line PI124111F (= PI), were used. Plants were grown in the greenhouse (19–34°C) from seeds in 0.25 l pots, and fertilised weekly with N: P: K (20:20:20) 0.5% (w/v).

Total RNA was extracted from leaves of 3 week-old susceptible Hemed plants using Tri-Reagent (Sigma). cDNAs of *At1* and *At2* were obtained by reverse transcription of the RNAs using the Reverse Transcription System (Promega, Madison, WI) and amplifying the specific genes by PCR. PCR for *At1* was performed with the forward primer 5′-AGATC TATGGATTACGTTTATGCA-3′ and the reverse primer 5′-CCCGGATCCTTAAATCCTTGAAGG-3′. PCR for *At2* was performed with the forward primer 5′-AGATCTATGGACTATGTTTATGGA-3′ and the reverse primer 5′-GGATCCTCACGGTTTCGAACC GAT-3′. The PCR annealing conditions were 10 cycles from 67–58°C (reducing by 1°C per cycle) followed by 25 cycles at 58°C. The two genes were subcloned into pGEM-T Easy Vector System (Promega), cut with BglII and EcoR1, and cloned into the binary vector pMON530.

Plant transformation was performed as described by Galperin et al. (2003). Briefly, BU21/3 seeds were peeled and germinated for 48 h in the dark on No. 1 Murashige & Skoog medium (4.4 g l^{-1} M&S, 3% sucrose [w/v], 0.8% agar [w/v] and vitamins). The cotyledons from each seed were cut in four, dipped for 15 min in a mixture of No. 2 Murashige & Skoog (4.4 g l^{-1} M&S, 3% sucrose [w/v]) and incubated in a dark chamber for 48 h. Co-cultivation was performed with *A. tumefaciens* EAH 105 bacteria, transformed with the gene *At1* / *At2* from the sensitive Hemed line, under the CaMV 35S promoter. The transformed cotyledons were placed in a dark chamber for 48 h on No. 3 Murashige & Skoog differentiation medium (4.3 g l^{-1} M&S, 3% sucrose [w/v], 1% agar [w/v], 1.6 mg l^{-1} 6-benzylaminopurine and vitamins) and subsequently transferred twice to No. 3 Murashige & Skoog selection medium (4.3 g l^{-1} M&S, 3% sucrose[w/v] , 1% agar [w/v], 1.6 mg l^{-1} 6-benzylaminopurine, cefotaxim 250 μg ml^{-1} + kanamicin 150 μg ml^{-1} and vitamins) for 5 days under light conditions. Cotyledons that developed calli were transferred to a No. 4 Murashige & Skoog elongation medium (4.3 g l^{-1} M&S, 3% sucrose [w/v] , 1% agar [w/v] , 0.05 mg l^{-1} 6-benzylaminopurine, cefotaxim 250 μg ml^{-1} + kanamicin 150 μg ml^{-1} and vitamins) for 2 weeks. Seedlings 5–10 mm long were planted in No. 5 Murashige & Skoog medium (4.3 g l^{-1} M&S, 3% sucrose [w/v] , 1% agar [w/v], 10.1 mg l^{-1} naphthalene acetic acid, and vitamins). T_0 cisgenic melon plants were planted in Jiffy-7 and subsequently planted in pots.

Pathogen, inoculation and disease assessment

A local isolate of the downy mildew causal agent *Pseudoperonospora cubensis* was used. The pathogen was propagated on intact Hemed plants or detached leaves in Petri dishes at 20°C (12 h light per day). Freshly-produced sporangia (1×10^3 sporangia ml^{-1}) were collected into ice-cooled distilled water and spray-inoculated onto the lower leaf surfaces on detached leaves or onto the upper leaf surfaces of intact potted plants. Inoculated plants or detached inoculated leaves (placed on moist filter paper in plastic trays (20×20×3 cm)) were kept in the dark at 17°C for 20 h and then transferred to growth chambers (12 h photoperiod, 100 μE m^{-2} s^{-1}) at 20± 1°C for two weeks for symptom production and sporulation of the pathogen.

To estimate plant resistance under natural conditions, plants were raised in a shade house and inoculated at the 2-leaf stage by spraying sporangial suspension (1×10^3 sporangia ml^{-1}) of the pathogen on their upper leaf surfaces. Resistance of T_0, and T_1 plants against *P. cubensis* was estimated at 7 days post-inoculation (dpi) using the method described before (Kenigsbuch and Cohen 1992; Thomas et al. 1987).

At1 and *At2* gene analysis

To obtain genomic DNA, 0.5 g leaf tissue was homogenised in liquid nitrogen, and heated with 800 µl CTAB buffer (2% CTAB [w/v], 1.4 M NaCl, 20 mM EDTA, 0.1 M Tris, and 1% 2-β mercaptoethanol [v/v]) at 60°C. Phenol:chloroform:isoamylalcohol (25:24:1) was added to the mixture, vortexed for 30 s and centrifuged for 5 min at 12,000 *g*. Supernatant was mixed with 800 µl chloroform: isoamylalcohol (24:1), vortexed for 30 s and centrifuged for 5 min at 12,000 *g*. Supernatant was mixed with an equal volume of 100% ethanol and incubated at room temperature for 5 min followed by overnight incubation at -20°C. The mixture was centrifuged for 10 min at 12,000 *g* and the supernatant discarded. The pellet was washed with 70% ethanol and centrifuged for 2 min at 12,000 *g*. The supernatant was discarded and the pellet dried at room temperature for 1 h and washed with 100–150 µl water. RNA was removed by incubation with 10U RNase in a 37°C water bath for 1 h. DNA concentration was measured with the aid of a spectrophotometer GENESYS 5 spectronic and by visual assessment on 1% agarose gel. Gel images were captured using the Video Documentation System for Gel Electrophoresis (Image Master VDS). cDNA was obtained using the Amersham Pharmacia Biotech First — Strand cDNA Synthesis Kit. The reaction was performed on 5 µg RNA with 0.5 µg ml^{-1} $Oligo(dT)_{15}$ primer. Identification of *At1* and *At2* in cisgenic T_0 and T_1 plants was carried out in a PCR reaction (2 µl DNA/cDNA, 2.5 µl 10×PCR buffer, 2.5 µl 2.5 mM dNTP mix, 1 µl 10 µM primer F, 1 µl 10 µM primer R, 2.5 µl Taq DNA polymerase and double distilled water (DDW) in 25 µl volume per reaction. PCR was performed using the Biometra T-Gradient with the following programme: 4 min 94°C, (45 s 94°C, 45 s 65°C, 1 min 72°C, <10°C touchdown gradient) ×9, (45 s 94°C, 45 s 55°C, 1 min 72°C) ×30, 7 min 72°C, 8°C. Bands were stained with 1 µg ml^{-1} ethidium bromide in 1% agarose gel. To identify *At1* the primer pairs F:5′- TGCCGAGCAATCACATTGT CCAAACCTCC-3′, R:5′- GCGACTGGGGTCA GGGTGCCAATCTTG-3′ were used. For *At2* identification the primer pairs F:5′- CCGCGGATGGACTA TGTTTATGGA-3′ and R:5′- GGATCCTCACGGT TTCGAACCGAT -3′ were used.

Northern gel blot analysis

Total RNA was extracted from plants using TRI reagent (Sigma, St Louis, MI). Final concentration of mRNA extracted was calculated as for DNA. Ten µg of total RNA was size-fractionated on formaldehyde agarose gel and transferred to Zeta probe membrane (Bio-Rad, Hercules, CA) overnight. Prehybridisation and hybridisation were performed with EZ-Hybridisation Solution (Biological Industries, Ltd, Israel). The blot was hybridised with three-random primed ^{32}P labelled fragments synthesised with the aid of Promega Prime-A Gene Random priming kit: 490 bp probe of *At1* (777-1267 in the coding region), 380 bp probe of *At2* (1027-1407) and 466 bp probe of 18S RNA (981-1447) synthesised on RT-PCR product template with downstream primer 5`-GTGTTG GCTTCGGGATCGG-3` and upstream primer 5`-CGCTCCACCAACTAAGAACGG-3`, for 2.5 h. Membranes were subsequently washed with 2×SSC (saline sodium citrate) and 0.1% SDS for 40 min followed by 0.1 SSC and 0.1% SDS for a further 40 min. Membranes were exposed to Agfa X-ray Corix film. Band intensity (arbitrary units) was calculated using Scion image (Scion Corporation, Frederick, Maryland).

Western blot analysis

For total protein extraction 100 mg leaf tissue was homogenised in liquid nitrogen and mixed with 200 µl extraction buffer (50 mM Tris-HCl, 0.3 M sucrose, 15 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT pH=7.5, and 2% PVP [w/v]). The mixture was centrifuged twice at 11,000*g* for 5 min. Protein concentration was determined by using a Bradford solution (Bio-Rad, Hercules, CA). Samples were fragmented on SDS-polyacrylamide gels and transferred to nitrocellulose membranes following staining with Pierce Gelcode Blue stain reagent (Pierce Biotechnology, Rockford, IL). Subsequently, membranes

were stained with Ponceau-S solution (Ponceau-S ×10: 2 g Ponceau-S, 30 g trichloroacetic acid, 30 g sulfosalicylic acid, and DDW to 100 ml) and washed with DDW. A polyclonal AT1 antigen, produced in a rabbit system was mixed with a blocking solution [5% (w/v) non-fat dried milk and autoclaved PBS×1 pH= 7.4 (8 g Nacl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, DDW to 100 ml)] at a ratio of 1:3700 in an 8 ml volume. Membranes were washed for 1 h at 37°C in this solution and then overnight in 4°C in 8 ml volume. After 10 min repeated washing with a solution of PBS ×1 and 0.05% Tween 20 at room temperature, membranes were washed a third time with PBS ×1. Membranes were incubated and washed similarly with Goat anti-Rabbit IgG HRP except diluted at a ratio of 1:3000. Membranes were washed in 5 ml PIERCE Super signal West Pico Chemiluminescent Substrate solution (Pierce Biotechnology, Rockford, IL) and exposed to Agfa X-ray Corix film for 30 s. Band intensity (arbitrary units) was calculated using Scion image (Scion Corporation, Frederick, Maryland).

GAT Enzyme assays

Homogenates from 0.3 g leaf tissue were mixed in 0.6 ml 50 mM HEPES pH 7, 0.2 mM EDTA, 2.5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 5 mM DTT, and 5 µl protease inhibitor, centrifuged for 10 min at 12,000 *g* at 4°C. For GAT activity assay 10 µl total protein (200 µg) were mixed with 890 µl AGT reaction solution (70 mM HEPES pH 7.0, 0.17 mM NADH, 0.1 mM pyridoxal-5-phosphate, lactate dehydrogenase 0.03 µ ml^{-1}, 1 mM glyoxylate, 20 mM L-alanine), and 100 µl 200 mM L-alanine in a final volume of 1 ml. AGT activity was measured at 340 nm by coupling the reduction of glyoxylate to the oxidation of NADH, in the presence of excessive lactate dehydrogenase. Enzymatic activity was calculated using NADH extinction coefficient of 6.2 cm^{-1} mM^{-1} and expressed in µmole $min^{-1}mg^{-1}$

Results

Cisgenic plants over expressing *At1* and *At2* from the susceptible Hemed are resistant to downy mildew

The genes *At1* and *At2* were separately cloned (see methods) from the susceptible Hemed and incorporated into the susceptible BU21/3 (Galperin et al. 2003) under CaMV promoter using the methods described before (Taler et al. 2004). When inoculated with sporangia of *P. cubensis*, 5 out of 27 and 6 out of 53 cisgenic T_0 plants carrying *At1* and *At2*, respectively were highly resistant to the disease. Resistant plants produced minute, water-soaked lesions with no sporulation, as did the resistant PI 124111F plants. Control, non-transformed plants produced large, chlorotic lesions with abundant sporulation, as did the susceptible control BU21/3 plants that were transformed with an empty binary vector. These highly resistant T_0 plants were self- pollinated to obtain T_1 progeny seeds, from which T1 plants were grown.

Disease resistance in T_1 cisgenic plants

The segregation for resistance to downy mildew in the cisgenic T1 plants is shown in Table 1. A ratio of 3:1 was observed between resistant and susceptible plants, indicating a single copy transformation by *A. tumefaciens*. Backcross progeny plants of T_1 X BU21/3 were all resistant (Table 1), further supporting the hypothesis of a single gene insertion. Resistant plants developed minute, water-soaked lesions with no sporulation upon inoculation with *P. cubensis* (Figs. 1 and 2). These lesions were embedded with callose and lignin depositions (not shown) as were the lesions produced by PI (Cohen et al. 1989).

PCR analysis of *At1* and *At2*

At1 and *At2* occur endogenously in melons (Taler et al. 2004). Therefore, the PCR assays, aimed to test the successful insertion of *At1* and *At2*, showed (Figs. 3a and 4a) two bands for each gene: a band corresponding to the cDNA (with no introns) transformed gene (505 bp for *At1*, 415 bp for *At2*) and a heavier band corresponding to the endogenous gene (DNA with introns, ~1500 bp for *At1*, ~800 bp for *At2*). Figures 3a and 4a show that T_1 plants resistant to downy mildew (lines No. 51 – 88, 103 – 137) carried the transformed genes in their genome.

Transcription of *At1* and *At2*

At1 and *At2* were each incorporated under the strong constitutive CaMV 35S promoter. Figures 3b and 4b

Table 1 Segregation of T_1 progeny plants for resistance to downy mildew

Generation	Line no.	Observed (R:S)	Expected ratio (R:S)	Chi-square	df	*P*
T_1	1	87:40	3:1	3.1803	1	0.07
T_1	6	47:22	3:1	2.7128	1	0.19
T_1	7	66:28	3:1	1.1489	1	0.28
T_1	13	54:24	3:1	1.3846	1	0.24
T_1	22	75:30	3:1	0.7142	1	0.40
T_1	30	51:24	3:1	1.9599	1	0.16
T_1	33	63:26	3:1	0.8426	1	0.36
T_1	38	96:38	3:1	0.8059	1	0.37
T_1	41	82:35	3:1	1.5071	1	0.22
BCs	T_1 (line 7) X BU21/3	64:0	All resistant	–	–	–
BCs	T_1 (line 22) X BU21/3	83:0	All resistant	–	–	–

S=sensitive phenotype: 10–15 mm chlorotic and/or necrotic lesions occupying≥50% of the leaf surface area. **R**=resistant phenotype: 1–2 mm water-soaked chlorotic lesions occupying <10% of the leaf surface area. BCs=backcross progeny.

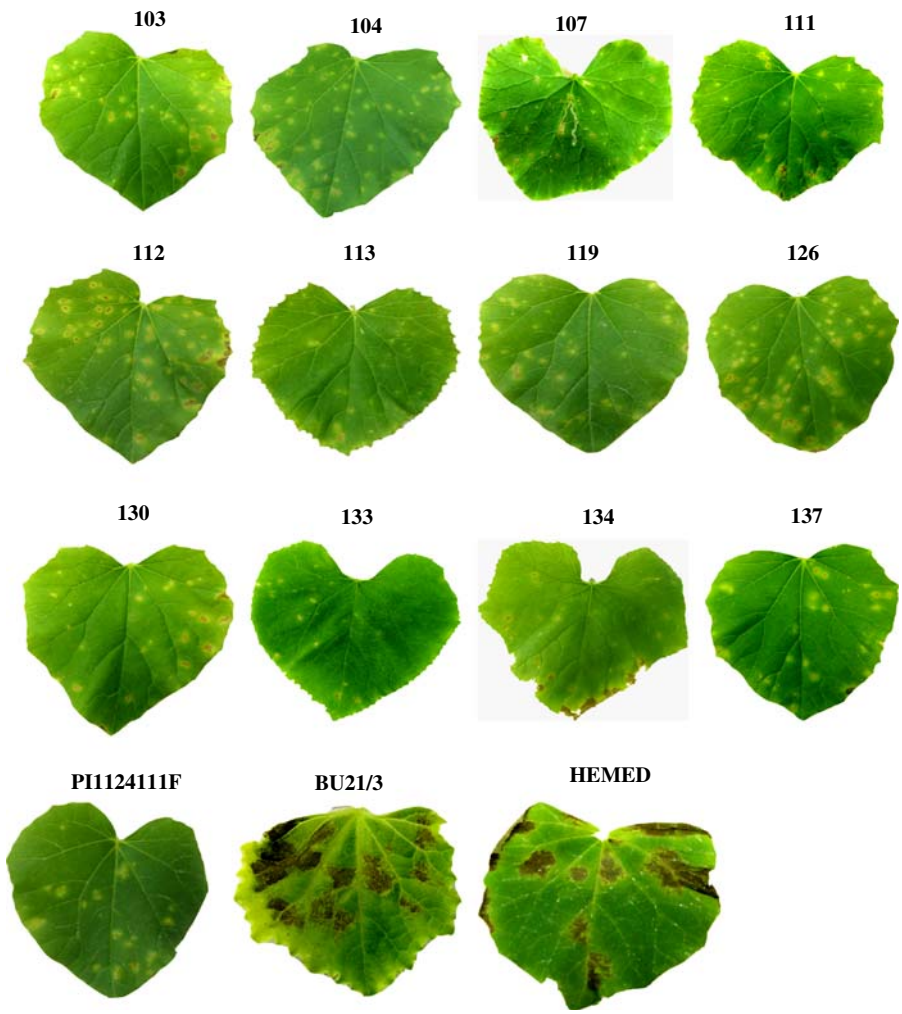

Fig. 1 Appearance of downy mildew in cisgenic T_1 BU21/3 melon leaves over expressing *At1* from the susceptible Hemed. Photos were taken at 7 days post-artificial inoculation with downy mildew (*Pseudoperonospora cubensis*). Lines 103–137 are cisgenic T1 plants over expressing *At1* from the susceptible Hemed. BU21/3 and Hemed: susceptible; PI 12411F resistant

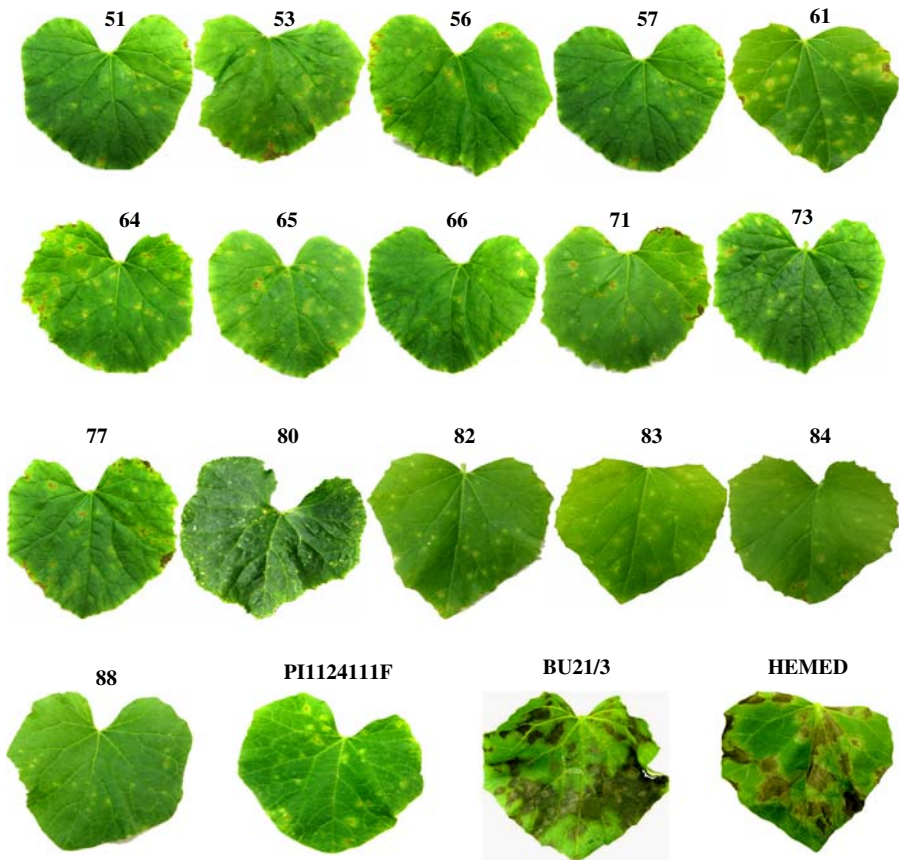

Fig. 2 Appearance of downy mildew in cisgenic T_1 BU21/3 melon leaves over expressing *At2* from the susceptible Hemed. Photos were taken at 7 days post-artificial inoculation with downy mildew (*Pseudoperonospora cubensis*). Lines 51–88 are cisgenic T1 plants over expressing *At2* from the susceptible Hemed. BU21/3 and Hemed: susceptible; PI 12411F resistant

show that, as expected, the transcription level of the transformed *At1* and *At2* in the T_1 progeny plants was significantly higher than in the susceptible Hemed or BU21/3. The transcription level of these genes in T1 plants was similar to that of the resistant PI. In some cisgenic lines transcription level was even higher than in PI. These findings indicate that both genes *At1* and *At2* from Hemed were transformed and transcribed in the cisgenic T_1 plants.

Translation of *At1* and *At2*

To test the level of translation, a polyclonal antigen against the AT1 protein was used. Because AT1 and AT2 share 93% homology in amino acid composition (Taler et al. 2004), the antigen was efficient in identifying both proteins in T_1 progeny plants. Western blot analysis revealed variable magnitudes of translation of the transformed *At1* and *At2* genes in T_1 progeny. Translation in most progeny plants was stronger compared with the susceptible lines Hemed or BU21/3, and weaker compared with the resistant line PI. In some progeny plants translation was stronger than in the resistant PI (Figs. 3c and 4c). These findings show that *At1* and *At2* genes were successfully translated in the cisgenic T1 progeny plants.

GAT activity

At1 and *At2* encode for glyoxylate aminotransferase (GAT) (Taler et al. 2004). The activity of GAT in our cisgenic lines (tested as alanine-glyoxylate aminotransferase, AGT activity) is shown in Figs. 3d and 4d. GAT activity in resistant-cisgenic plants was much higher than in the susceptible Hemed or BU21/3 and in some, even higher than in the resistant PI, probably due to the CaMV 35s promoter used. This indicates that resistance against downy mildew in melon is conferred by enhanced activity of glyoxylate aminotransferase.

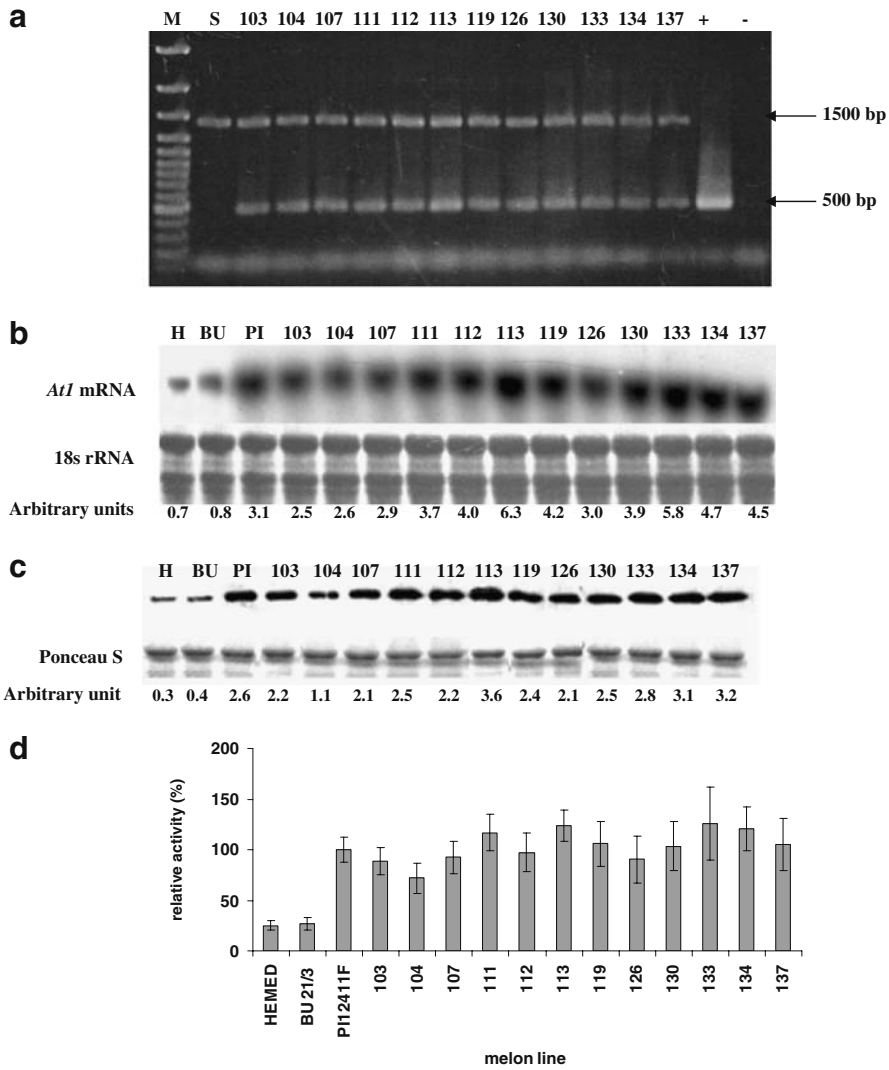

Fig. 3 Genetic and biochemical analyses of *At1* in leaves of T1 cisgenic melon plants (lines 103–137) as compared with the resistant PI124111F (PI) and the susceptible Hemed (H) and BU 21/3 (BU). (**a**) PCR-based scanning detecting the *At1* gene. The 1500 bp bands represent the endogenous *At1* and the 500 bp bands represent the cisgenic *At1* with no introns. In the susceptible melon (**S**=Hemed or BU21/3) only the endogenous *At1* appears. In the cisgenic melons (103–137) both the endogenous and the cisgenic *At1* bands appear. The positive control (+) contains *At1* cDNA and the negative control (**-**) contains no DNA. **M**=DNA ladder. (**b**) RNA transcripts of the *At1* gene. The RNA gel blot was hybridised with specific probe for *At1*. 18S RNA was used as a standard for equal loading. (**c**) Western blot analysis of the AT1 protein. Ponceau S was used as a standard for equal loading. (**d**) GAT enzymatic activity in leaves of resistant (PI), susceptible (H, BU), and cisgenic melon plants expressing *At1*. Enzyme activity was measured spectrophotometrically at 340 nm (Rehfeld and Tolbert 1972). GAT 100% activity=1.43 μmole $min^{-1} mg^{-1}$ protein

Discussion

Our finding that overexpressed *At1* and *At2* genes from susceptible Hemed melon confer resistance to downy mildew in the susceptible BU23/1 line, indicates that susceptibility results from transcriptional inhibition of the At genes

A previous study by Balass et al. (1993) revealed a 45 kDa protein associated with resistance against downy mildew in PI. This protein was not detected in the susceptible Hemed (Balass et al. 1993; Taler et al. 2004). Taler et al. (2004) later showed that P45 was encoded by the glyoxylate aminotransferase (GAT) genes, *At1* and *At2*. GAT, a peroxisomal photo-

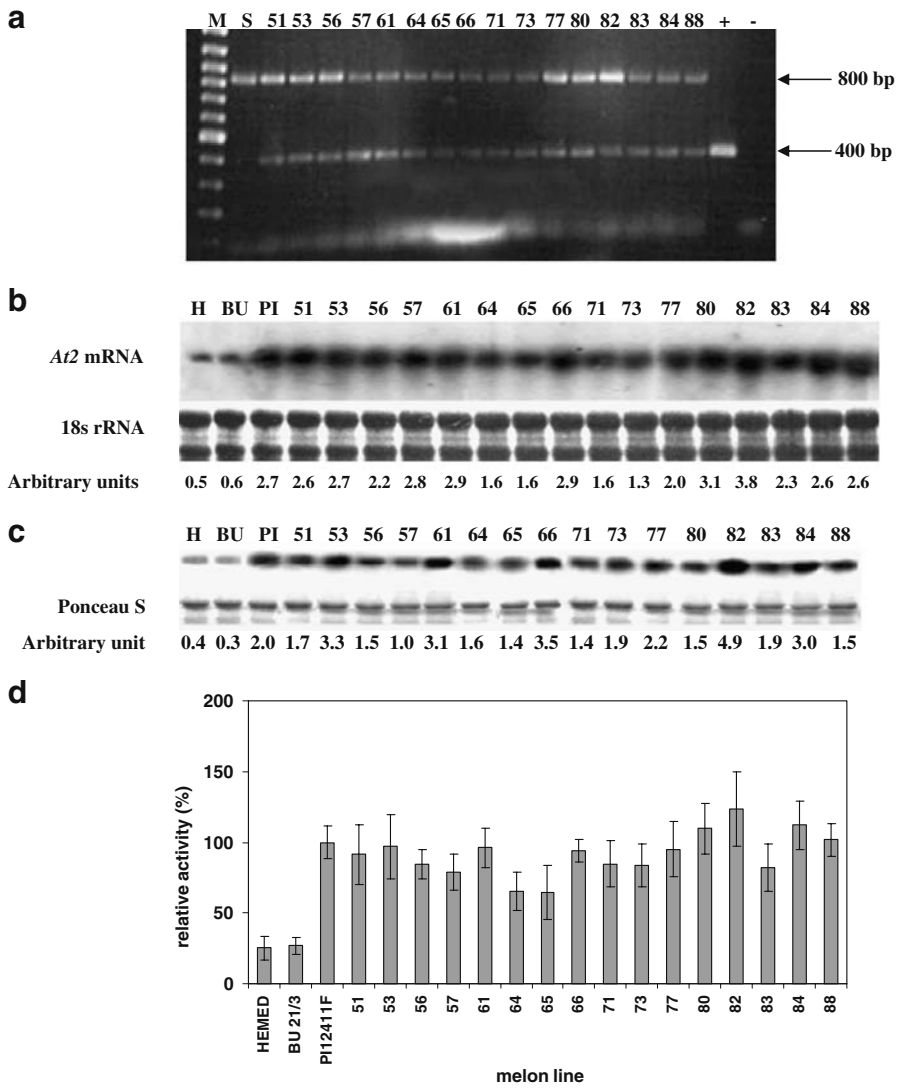

Fig. 4 Genetic and biochemical analyses of *At2* in leaves of the T1 cisgenic melon plants (lines 51–88) as compared with the resistant PI124111F (PI) and the susceptible Hemed (H) and BU 21/3 (BU). (**a**) PCR-based scanning detecting the *At2* gene. The 800 bp bands represent the endogenous *At1* and the 400 bp bands represent the cisgenic *At2* with no introns. In the susceptible melon (**S**=Hemed or BU21/3) only the endogenous *At2* appears. In the cisgenic melons (51–88) both the endogenous and the cisgenic *At2* bands appear. The positive control (+) contains *At2* cDNA and the negative control (-) contains no DNA. **M**=DNA ladder. (**b**) RNA transcripts of the *At2* gene. The RNA gel blot was hybridised with specific probe for *At2*. 18S RNA was used as a standard for equal loading. (**c**) Western blot analysis of the AT2 protein. Ponceau S was used as a standard for equal loading. (**d**) GAT enzymatic activity in leaves of resistant (PI), susceptible (H, BU), and cisgenic melon plants expressing *At2*. Enzyme activity was measured spectrophotometrically at 340 nm (Rehefeld and Tolbert, 1972). GAT 100% activity=1.43 μmole $min^{-1} mg^{-1}$ protein

respiratory enzyme (Somerville and Ogren 1980), was shown to be constitutively and strongly translated in the resistant PI but not in two susceptible lines, despite the presence, with slight modification, of the *At1* and *At2* genes in the plant genome (Taler et al. 2004). Transformation of any one of the two genes from the resistant PI into the susceptible Hemed resulted in resistance against downy mildew. Since transformation of the *At* genes from resistant or susceptible melons into bacteria resulted in equally high GAT activity, it was suggested that the slight differences between base pairs are not the cause for

the lack of resistance in the susceptible lines (Taler et al. 2004).

In the present work we cloned *At1* and *At2* from the downy mildew susceptible Hemed and introduced them into susceptible BU23/1 under control of a strong promoter, CAMV 35S. This was done to determine whether or not these genes confer resistance to downy mildew, in spite of their origin from a susceptible line of melon. A positive answer would indicate that susceptibility in Hemed and resistance in PI are controlled by down-regulation and up-regulation respectively, of P45 production at the transcriptional level. When *At1* and *At2* were cloned from the susceptible Hemed and introduced into the susceptible line BU21/3 under the strong promoter CaMV 35S, T_0 and T_1 plants were resistant against *P. cubensis*. The resistant reaction was characterised by minute chlorotic or necrotic, water-soaked lesions embedded with callose and lignin depositions, which is typically associated with *At1* and *At2* defence gene activation (e.g., cell-wall strengthening, callose deposition and lignification (Cohen et al. 1989)) which culminates in cell death and arrest of the pathogen (Taler et al. 2004).

PCR analysis of PI, Hemed, BU21/3, T_0 and T_1 plants showed that the transformation of *At1* or *At2* was successful. Furthermore, the data showed that only one copy of either *At1* or *At2* gene was inserted into the plant genome via *A. tumefaciens*. Northern blot analyses revealed a strong transcription of the genes in T_1 and PI plants compared with a weak transcription in the susceptible lines Hemed and BU21/3. Western blot analysis, using rabbit AT1 polyclonal antigen, showed that translation of these genes in PI and cisgenic plants was markedly higher than in the susceptible Hemed or BU21/3. This supports the observation of Balass et al. (1992) who failed to detect P45 in protein gels of the susceptible lines Hemed and AY. GAT enzymatic activity in the present study was 2.5–5 fold higher in the resistant PI or T_1 lines compared with the susceptible lines Hemed and BU21/3. This suggests that enhanced GAT activity conferred resistance against downy mildew regardless of whether *At1* and *At2* originated from PI or Hemed.

There is evidence in the literature showing that over expression of an endogenous gene in a susceptible line confers resistance to respective pathogens. Rice tungro disease is a significant yield constraint in rice caused by the rice tungro bacilliform virus (RTBV). Two host transcription factors, RF2a and RF2b, regulate expression of the RTBV promoter and are important for plant development. RF2a and RF2b were overxpressed in transgenic rice. Lines with elevated expression of RF2a or RF2b showed weak or no symptoms of infection after *Agrobacterium* inoculation of RTBV, whereas control plants showed severe stunting and leaf discolouration. Furthermore, transgenic plants exhibited reduced accumulation of RTBV RNA and viral DNA compared with non-transgenic plants. Similar results were obtained in studies after virus inoculation by green leafhoppers (Dai et al. 2008).

Snakin-1 (SN1), a cysteine-rich peptide with broad-spectrum antimicrobial activity *in vitro*, was evaluated for its ability to confer resistance to pathogens in transgenic potatoes. Potato plants were transformed via *A. tumefaciens* with a construct encoding the SN1 gene under the CaMV 35S promoter. Transgenic lines were molecularly characterised and challenged with either *Rhizoctonia solani* or *Erwinia carotovora* to analyse whether constitutive *in vivo* overexpression of the SN1 gene may lead to disease resistance. Only transgenic lines that accumulated high levels of SN1 mRNA exhibited significant symptom reductions (Almasia et al. 2008).

Over expression of SNF1 (Sucrose non-fermenting 1) kinase in susceptible tobacco plants confers resistance to the Gemini viruses TGMV (*Tomato golden mosaic virus*) and BCTV (*Beet curly top virus*). It is assumed that SNF1 takes part in resistance against these viruses and that their proteins (AL2, and L2 respectively), which have been found to inhibit SNF1, are a viral counter measure for infection (Hao et al. 2003). Similarly, over expression of NHO1 (a glycerol kinase) in *Arabidopsis* leads to resistance against *Pseudomonas syringae* pv. *tomato* DC 3000, which in turn inhibits NHO1 (Kang et al. 2003).

The peroxisome is thought to have an integral role in removal of H_2O_2 and O_2^- using catalase and superoxide dismutase (SOD) (Bowler et al. 1992). In certain stress conditions protein synthesis is inhibited while the photorespiration increases, leading to the accumulation of reactive oxygen species (ROS) such as H_2O_2, O_2^-, and nitric oxide (NO) in the cytosol which in turn leads to various signal transduction pathways (Alscher et al. 1997; Apel and Hirt 2004; Wingler et al. 2000). Production of ROS is one of the

first reactions encountered with pathogen attack in plants and may be involved in resistance to infection (Dangl and Jones 2001; Jackson and Taylor 1996). NO is produced by the mitochondrial photorespiratory glycine decarboxylase complex (GDC) which is one step downstream of GAT and has been shown to be involved in plant resistance against pathogenic attack (Chandok et al. 2004; de Pinto et al. 2002; Delledonne 2005; Tada et al. 2004; Zhang et al. 2003). It is possible that over expression of the GAT in the resistant PI increases the activity of GDC, thereby causing the accumulation of NO, which may be involved in signal transduction pathways leading to resistance.

In conclusion, over expression of *At1* or *At2* from the susceptible melon Hemed in the susceptible melon BU21/3 increases the activity of GAT and confers resistance against downy mildew. GAT seems to have a pivotal role in conferring resistance against *P. cubensis* in melon. Susceptibility of Hemed results from down regulation of transcription and therefore low translation of this enzyme, while resistance in PI is conferred by over expression of GAT. Analysis of *At1* and *At2* promoter linked to a reporter gene would highlight transcriptional control regions that up-regulate expression.

References

Almasia, N. I., Bazzini, A. A., Hopp, H. E., & Vazquez-Rovere, C. (2008). Overexpression of snakin-1 gene enhances resistance to *Rhizoctonia solani* and *Erwinia carotovora* in transgenic potato plants. *Molecular Plant Pathology, 9*, 329–338. doi:10.1111/j.1364-3703.2008.00469.x.

Alscher, R. G., Donahue, J. L., & Cramer, C. L. (1997). Reactive oxygen species and antioxidants: Relationships in green cells. *Physiologia Plantarum, 100*, 224–233. doi:10.1111/j.1399-3054.1997.tb04778.x.

Apel, K., & Hirt, H. (2004). Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. *Annual Review of Plant Biology, 55*, 373–399. doi:10.1146/annurev.arplant.55.031903.141701.

Balass, M., Cohen, Y., & Bar-Joseph, M. (1992). Identification of a constitutive 45 kD soluble protein associated with resistance to downy mildew in muskmelon (*Cucumis melo* L.) line PI 124111F. *Physiological and Molecular Plant Pathology, 41*, 387–396. doi:10.1016/0885-5765(92)90051-V.

Balass, M., Cohen, Y., & Bar-Joseph, M. (1993). Temperature-dependent resistance to downy mildew in muskmelon — structural responses. *Physiological and Molecular Plant Pathology, 43*, 11–20. doi:10.1006/pmpp.1993.1036.

Bowler, C., VanMontagu, M., & Inze, D. (1992). Superoxide-dismutase and stress tolerance. *Annual Review of Plant Physiology and Plant Molecular Biology, 43*, 83–116. doi:10.1146/annurev.pp.43.060192.000503.

Chandok, M. R., Ekengren, S. K., Martin, G. B., & Klessig, D. F. (2004). Suppression of pathogen-inducible NO synthase (iNOS) activity in tomato increases susceptibility to *Pseudomonas syringae*. *Proceedings of the National Academy of Sciences of the United States of America, 101*, 8239–8244. doi:10.1073/pnas.0402344101.

Cohen, Y. (1981). Downy mildew of cucurbits. In D. M. Spencer (Ed.), *The downy mildews*, pp. 341–354. London, New York and San-Francisco: Academic.

Cohen, Y., & Eyal, H. (1987). Downy mildew-resistant, powdery mildew-resistant and fusarium wilt-resistant muskmelon breeding line-PI-124111f. *Phytoparasitica, 15*, 187–195. doi:10.1007/BF02979582.

Cohen, Y., Eyal, H., Hanania, J., & Malik, Z. (1989). Ultrastructure of *Pseudoperonospora cubensis* in muskmelon genotype susceptible and resistant to downy mildew. *Physiological and Molecular Plant Pathology, 34*, 27–40. doi:10.1016/0885-5765(89)90014-3.

Cohen, Y., Meron, I., Mor, N., & Zuriel, S. (2003). A new pathotype of *Pseudoperonospora cubensis* causing downy mildew in cucurbits in Israel. *Phytoparasitica, 31*, 458–466. doi:10.1007/BF02979739.

Dai, S., Wei, X., Alfonso, A. A., Pei, L., Duque, U. G., Zhang, Z., et al. (2008). Transgenic rice plants that overexpress transcription factors RF2a and RF2b are tolerant to *Rice tungro virus* replication and disease. *Proceedings of the National Academy of Sciences, 105*, 21012–21016. doi:10.1073/pnas.0810303105.

Dangl, J. L., & Jones, J. D. G. (2001). Plant pathogens and integrated defence responses to infection. *Nature, 411*, 826–833. doi:10.1038/35081161.

de Pinto, M. C., Tommasi, F., & De Gara, L. (2002). Changes in the antioxidant systems as part of the signaling pathway responsible for the programmed cell death activated by nitric oxide and reactive oxygen species in tobacco Bright-Yellow 2 cells. *Plant Physiology, 130*, 698–708. doi:10.1104/pp.005629.

Delledonne, M. (2005). NO news is good news for plants. *Current Opinion in Plant Biology, 8*, 390–396. doi:10.1016/j.pbi.2005.05.002.

Galperin, M., Patlis, L., Ovadia, A., Wolf, D., Zelcer, A., & Kenigsbuch, D. (2003). A melon genotype with superior competence for regeneration and transformation. *Plant Breeding, 122*, 66–69. doi:10.1046/j.1439-0523.2003.00764.x.

Hao, L. H., Wang, H., Sunter, G., & Bisaro, D. M. (2003). Geminivirus AL2 and L2 proteins interact with and inactivate SNF1 kinase. *The Plant Cell, 15*, 1034–1048. doi:10.1105/tpc.009530.

Jackson, A. O., & Taylor, C. B. (1996). Plant-microbe interactions: Life and death at the interface. *The Plant Cell, 8*, 1651–1668.

Kang, L., Li, J., Zhao, T., Xiao, F., Tang, X., Thilmony, R., et al. (2003). Interplay of the *Arabidopsis* nonhost resistance gene *NHO*1 with bacterial virulence. *Proceedings of the National Academy of Sciences of the United States of America, 100*, 3519–3524. doi:10.1073/pnas.0637377100.

Kenigsbuch, D., & Cohen, Y. (1992). Inheritance of resistance to downy mildew in *Cucumis melo* PI 124112 and commonality of resistance genes with *C. melo* PI 124111F. *Plant Disease, 76*, 615–617.

Liepman, A. H., & Olsen, L. J. (1998). Sequence analysis of a cDNA encoding alanine:glyoxylate aminotransferase from *Arabidopsis thaliana* (Accession No. AF063901). *Plant Physiology, 117*, 1125. doi:10.1104/pp.117.3.1125.

Rehfeld, D. W., & Tolbert, N. E. (1972). Aminotransferases in peroxisomes from spinach leaves. *Journal of Biological Chemistry, 247*, 4803–4811.

Somerville, C. R., & Ogren, W. L. (1980). Photorespiration mutants of *Arabidopsis thaliana* deficient in serine-glyoxylate aminotrasnferase activity. *Proceedings of the National Academy of Sciences of the United States of America, 77*, 2684. doi:10.1073/pnas.77.5.2684.

Tada, Y., Mori, T., Shinogi, T., Yao, N., Takahashi, S., Betsuyaku, S., et al. (2004). Nitric oxide and reactive oxygen species do not elicit hypersensitive cell death but induce apoptosis in the adjacent cells during the defense response of oat. *Molecular Plant-Microbe Interactions, 17*, 245–253. doi:10.1094/MPMI.2004.17.3.245.

Taler, D., Galperin, M., Benjamin, I., Cohen, Y., & Kenigsbuch, D. (2004). Plant eR genes that encode photorespiratory enzymes confer resistance against disease. *The Plant Cell, 16*, 172–184. doi:10.1105/tpc.016352.

Thomas, C. E., Cohen, Y., McCreight, Y. D., Jourdion, E. L., & Cohen, S. (1987). Inheritance of resistance to downy mildew in *Cucumis melo*. *Plant Disease, 72*, 33–35. doi:10.1094/PD-72-0033.

Wingler, A., Lea, P. J., Quick, W. P., & Leegood, R. C. (2000). Photorespiration: Metabolic pathways and their role in stress protection. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences, 355*, 1517–1529. doi:10.1098/rstb.2000.0712.

Zhang, C., Czymmek, K. J., & Shapiro, A. D. (2003). Nitric oxide does not trigger early programmed cell death events but may contribute to cell-to-cell signaling governing progression of the *Arabidopsis* hypersensitive response. *Molecular Plant-Microbe Interactions, 16*, 962–972.