

The antifungal Dm-AMP1 protein from *Dahlia merckii* expressed in *Solanum melongena* is released in root exudates and differentially affects pathogenic fungi and mycorrhizal symbiosis

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Summary

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- Transformed aubergine plants constitutively expressing the Dm-AMP1 antimicrobial defensin (from *Dahlia merckii*) were generated and characterized.
- Transgenic plants were selected on kanamycin and screened by polymerase chain reaction analysis. The expression of Dm-AMP1 in plant tissues and its release in root exudates were detected by Western blot analyses. Dm-AMP1 localization was performed by immunohistochemical experiments.
- Dm-AMP1 expression ranged from 0.2% to 0.48% of total soluble proteins in primary transformants and from 0.16% to 0.66% in F₂ plants. Transformed clones showed resistance to the pathogenic fungus *Botrytis cinerea*, whose development on leaves was reduced by 36–100%, with respect to controls. The protein was released in root exudates of the transformed plants and was active in reducing the growth of the co-cultured pathogenic fungus *Verticillium albo-atrum*, whereas it did not interfere with recognition responses and symbiosis establishment by the arbuscular mycorrhizal fungus *Glomus mosseae*.
- Dm-AMP1 transformants may represent a useful model to study the interactions between genetically modified plants and pathogenic fungi or beneficial nontarget microorganisms.

Key words: arbuscular mycorrhizal fungi, *Solanum melongena*, *Botrytis cinerea*, defensin, transformed plants, *Verticillium albo-atrum*.

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Introduction

Plants protect themselves from pathogenic organisms by activating different defense strategies such as cell wall thickening, localized cell necrosis, production of phytoalexins. Moreover, plants can synthesize a wide variety of antimicrobial proteins, such as zeamatin, osmotin, ribosomal inhibitor proteins (Broekaert *et al.*, 1997; Shewry & Lucas, 1997) and enzymes able to hydrolyse fungal hyphae (Mauch *et al.*, 1988; Melchers *et al.*, 1994). Another group of plant antimicrobial compounds is represented by cysteine-rich peptides (Broekaert *et al.*, 1995; Rao, 1995) that includes thionins (Bohlmann & Apel, 1991),

lipid-transfer proteins (LTPs) (García-Olmedo *et al.*, 1995) and defensins (Broekaert *et al.*, 1995). Plant defensins are small peptides (45–54 amino acids) with a characteristic three-dimensional folding pattern stabilized by disulfide-linked cysteines. They share common characters with those described in insects and mammals, suggesting that these defense molecules predate the evolutionary divergence of plants and animals (Broekaert *et al.*, 1995). So far, over 80 defensin sequences from different plant species have been identified (Thomma *et al.*, 2002). Plant defensins were first isolated from barley and wheat seeds (Mendez *et al.*, 1990) and successively from seeds and vegetative tissues of different dicotyledon and monocotyledon

plants (Terras *et al.*, 1992, 1993; Moreno *et al.*, 1994; Osborn *et al.*, 1995; Gao *et al.*, 2000).

Brassicaceae and Saxifragaceae produce 'morphogenic' defensins, which inhibit pathogenic fungal growth by reducing hyphal elongation and increasing hyphal branching. The mode of action of plant defensins from Asteraceae, including Dm-AMP1, is typical of 'nonmorphogenic' defensins and consists of slowing down hyphal extension and not inducing marked morphological changes. Molecular mechanisms of plant defensins action is not completely well-understood. *Neurospora crassa* hyphae treated with either Rs-AFP2 or Dm-AMP1 defensins showed a rapid potassium (K⁺) efflux, calcium (Ca²⁺) uptake and alkalization of the incubation medium. In addition, membrane potential changes, but not the formation of membrane pores, were observed (Thevissen *et al.*, 1996). Defensins are able to bind to hyphal membranes at specific sites and indirect evidence indicates that this binding is required for antifungal activity (Thevissen *et al.*, 1997, 2000a). Recently, it was shown that different plant defensins are able to interact with different sphingolipids. For example, Dm-AMP1 interacts with mannosylated sphingolipids occurring in the outer plasma membrane (Thevissen *et al.*, 2000b, 2003a), whereas the radish plant defensin Rs-AFP2 interacts with fungal glucosylceramides (Thevissen *et al.*, 2004).

Some plant defensins are constitutively expressed in seeds and are released during germination. It has been suggested that the defensin Rs-AFP2 from radish (*Raphanus sativus*) could have a role in protecting plantlet tissues from pathogen attack, thus enhancing the chances of seedling survival and plant reproduction (Terras *et al.*, 1995). Other plant defensins are expressed at a low level in the leaves and other vegetative plant tissues, such as roots, stems, flowers and fruits, but their concentration greatly increases after pathogen attack (Terras *et al.*, 1995).

Antimicrobial compounds synthesized by seeds or plant tissues represent a powerful tool to enhance plant resistance against fungal pathogens, as shown in tobacco, scented geranium, canola and potato plants constitutively expressing antifungal proteins cloned from *R. sativus*, *Mirabilis jalapa*, *Amaranthus caudatus*, *Allium cepa*, *Pisum sativum* and *Medicago sativa*, respectively (Terras *et al.*, 1995; de Bolle *et al.*, 1996; Bi *et al.*, 1999; Wang *et al.*, 1999; Gao *et al.*, 2000).

Transformed plants expressing antimicrobial compounds could represent a potential benefit for human health and environmental safety because their production could decrease the need for chemical pesticides. Moreover they might represent a useful tool for studying potential risks related to the release of transformed plants and their impact on nontarget organisms.

In this work, we produced transformed aubergine (*Solanum melongena*) plants expressing the gene for Dm-AMP1 protein, cloned from *Dahlia merckii* and analysed: (1) Dm-AMP1 expression and localization in different plant tissues; (2) foliar resistance to the phytopathogenic fungus *Botrytis cinerea*; (3) the release of Dm-AMP1 in root exudates; (iv) the impact of

released Dm-AMP1 on the phytopathogenic fungus *Verticillium albo-atrum*; (4) the interaction of Dm-AMP1 transformed plants with the symbiotic fungus *Glomus mosseae*.

Materials and Methods

Plant material used for genetic transformation experiments

Seeds of aubergine (*S. melongena* L.) cv. Violetta of New York were surface sterilized in 1.5% sodium hypochlorite for 20 min and then rinsed several times with sterile distilled water. Seeds were germinated in sterile Magenta culture vessels (GA7; Sigma-Aldrich, Milan, Italy) in half-strength B5 medium (Gamborg *et al.*, 1968) supplemented with 0.7% bacto-agar adjusted to pH 5.8. Seedling growth and all subsequent *in vitro* culture steps were carried out in a temperature-controlled 25°C growth chamber under cool 6–12 µmol m⁻² s⁻¹ white fluorescent light with a dark-light cycle of 16h : 8 h. Four- to five-week-old plants were used as explant source in genetic transformation experiments.

Bacterial strains and vectors

Agrobacterium strain EHA105 carrying the plasmid pDm-AMPLCC (kindly provided by Dr A. Greenland, Zeneca Agrochemical, Calgary, Alberta, Canada) was used as vector system for transformation. Plasmid pDmAMPLCC contains the neomycin phosphotransferase II (*nptII*) gene under the nopaline synthase (*nos*) promoter and the Dm-AMP1 gene under the cauliflower mosaic virus (*CaMV35S*) promoter, with duplicated enhancer region. The Dm-AMP1 defensin gene and protein sequences (Osborn *et al.*, 1995) have GenBank accession numbers AAB34972 and A26963. The bacterial strain pDmAMPLCC/EHA105 was grown on Luria Bertani broth (LB) medium with appropriate antibiotics (50 mg l⁻¹ kanamycin and 20 mg l⁻¹ rifampicin). For co-cultivation, isolated colonies of bacteria were picked up from selection plates and grown overnight in 10 ml of LB liquid medium at 28°C to the late logarithmic stage (OD₆₀₀ 0.8–1). The bacteria were centrifuged and the pellet was re-suspended 1 : 10 in MS medium (Murashige & Skoog, 1962) and used for the infection and co-cultivation procedure in the genetic transformation experiments.

Genetic transformation experiments

The transformation system described by Billings *et al.* (1997) was used with some modifications. Leaf disks 1.5 cm in diameter were excised and placed on shoot regeneration (SR) medium (MS basal salt and vitamins, 10 µM N6-[isopentyl] adenine (2iP), 0.1 mM thidiazuron (TDZ), 2% sucrose, 0.6% agar, pH 5.8), covered with a *Nicotiana glauca* feeder layer, for 48 h of preculture before co-cultivation. The *N. glauca* cell

suspensions were subcultured every 14 d. Feeder plates were prepared by plating 2 ml of exponentially growing cell suspensions on 25 ml of solidified SR medium in Petri dishes. After 1 d of incubation in the growth chamber in the dark, discs of dried sterile filter paper were placed on top of the *N. glauca* cells and the leaf explants were then deposited on the filter paper after infection (1 min) with *Agrobacterium tumefaciens* cell suspension. After 48 h of co-cultivation, explants were transferred on SR medium containing 300 mg l⁻¹ augmentin and 50 mg l⁻¹ kanamycin. Explants were subcultured every 10 d on fresh selection medium until shoots started to regenerate. Regenerated shoots were transferred on half-strength MS medium with 50 mg l⁻¹ kanamycin.

Polymerase chain reaction (PCR) analysis

For PCR analysis, DNA was isolated from kanamycin-resistant leaves and wild-type control leaves, using a cetyltrimethylammonium bromide (CTAB) procedure (Doyle & Doyle, 1990). The PCR was performed in a Hybaid Omnigene (Hybaid, Teddington, UK) thermocycler. The primers used for amplification of the 344 bp fragment of the *nptII* gene were 5'-TTCTTTTGTCAAGACCGACCT-3' (upstream) and 5'-TTCGTCCAGATCATCCT-3' (downstream). A sample of 100 ng of each DNA were amplified with the following protocol: 1 cycle at 94°C for 2 min, 30 cycles at 94°C for 1 min, 50°C for 1 min and 72°C for 1 min, 1 cycle at 72°C for 5 min.

Western blot analysis

Total soluble proteins were extracted using liquid nitrogen and resuspended in extraction buffer (15 mM Na₂HPO₄, 10 mM NaH₂PO₄, 100 mM KCl, 2 mM ethylenediaminetetraacetic acid (EDTA), pH 7). Samples were heated for 10 min at 85°C and heat-labile denatured proteins were removed by centrifugation (12 000 g). Soluble proteins were quantified using Bradford reagent (Bio-Rad, Milan, Italy) and recording solution absorbance at $\lambda = 595$ nm, with different concentrations of bovine serum albumin (BSA) as standards. Different quantities (20, 40 μ g) of total soluble proteins for each sample were separated by sodium dodecyl sulfate (SDS) gel electrophoresis (acrylamide : bisacrylamide 30 : 0.8) through a 5% stacking gel overlaid on a 15% separating gel at constant 200 V. The purified protein DmAMP1 (25, 50 and 100 ng) was used as standard. Proteins were blotted at constant 250 mA for 1 h at 4°C onto a nitrocellulose membrane (Amersham Bioscience, Milan, Italy) using a Mini Trans-blot Transfer Cell (Bio-Rad). After blotting the membrane was blocked in Tris-buffered saline (TBS), containing 0.05% Tween and 5% skimmed milk, for 1 h at room temperature, then incubated for 1 h with Dm-AMP1 antibody (1 : 1000), kindly provided by Zeneca Agrochemicals, washed three times with TBS and incubated for 1 h with 1 : 2000 peroxidase-labeled goat antirabbit

antibody (Santa Cruz Biotechnology). The membrane was then washed three times before detection carried out using the enhanced chemiluminescence (ECL) method (Amersham). Images acquired with ImageMaster VDS system (Amersham) were analysed with ImageJ software for signals quantification.

Immunohistochemical procedure

Isolated young leaves and roots from transformed (line 12) and control plants micropropagated *in vitro* in MS media were fixed with 4% paraformaldehyde + 1% glutaraldehyde mixture in 0.15 M phosphate buffer, pH 7.4, for 18 h and embedded in paraffin. Sections, 8 μ m thick, were prepared by using a manual microtome and processed for immunological detection of Dm-AMP1 protein. The slides were rehydrated and treated with H₂O₂ (3%) to inhibit endogenous peroxidase activities. After extensive washing the slides were buffered in Solution A (0.1 M phosphate-buffered saline (PBS) pH 7.4, 1% BSA, 0.1% Tween 20). All the steps described below were performed at room temperature in a moist chamber. The slides were covered for 15 min with 1.5% normal goat serum in solution A to block nonspecific sites. Incubation with the polyclonal antibodies against Dm-AMP1 was carried out for 2 h using 1 : 100 diluted serum in solution A. After three washes in solution A, the slides were exposed to the secondary antibody (1 : 50 peroxidase conjugate goat antirabbit; Santa Cruz) for 1 h. Three washes in solution A and three in PBS facilitated the removal of unbound secondary antibodies and the progression to the detection step. The slides were incubated with 0.06% 3, 3'-diaminobenzidine (DAB) solution in PBS for 10 min. After the addition of H₂O₂ to a final concentration of 0.06%, the incubation time was extended for further 10 min. The color development was stopped by washing three times in PBS and three times in distilled water. The slides were then air-dried and mounted for microscopic analysis. Images were captured under a Axioskop microscope (Zeiss, Milan, Italy) equipped with a video-camera (DC100; Leica, Milan, Italy).

Conventional controls were performed in each experiment and the specificity of the immunolabeling was tested by means of: (1) treatment with a nonimmune rabbit serum instead of the primary antibodies; (2) further dilution and omission of the primary antibody; (3) omission of the secondary peroxidase conjugated antibody; (4) inhibition of the immunoresponse by preincubating the antibodies with an excess of homologous antigens before use.

Hydroponic cultures

In vitro-transformed aubergine plants (lines 5 and 12) were placed in sterile 100 ml flasks with roots plunged into 30 ml of sterile half-strength MS basal medium with no sugar. Sterile air was continuously blown into the liquid by placing a 0.22 μ m filter upstream a pipette linked to a pump. Liquid

samples (10 ml) were collected after 4 d from the beginning of the hydroponic culture, dialysed against double-distilled water using dialysis tubing (molecular weight cut off (MWCO) 5 kDa) and concentrated by lyophilization. The concentrated proteins were resuspended in 40 µl double-distilled water and used for Western blot analysis.

Bioassay on *Verticillium albo-atrum*

In vitro-micropropagated transformed aubergine plants of lines 1, 5, 8 and 12 were placed with roots lying on the agar surface in the center of a 160 mm Petri dish, half-filled with half-strength MS agar medium. The fungus *V. albo-atrum* was grown on potato dextrose agar (PDA) at 24°C for 2–3 wk. Mycelial plugs were removed from a colony with a 5 mm diameter sterile cork borer, and placed, mycelium-side down, near the plant roots. Six transformed and six control plants were used in this bioassay, with two mycelial plugs per plant. Plants were placed on the agar simultaneously with the mycelial plugs. Petri dishes were incubated for 18 d, and at the end of this period mycelial growth was estimated by measuring fungal colony diameters and calculating corresponding areas. Results obtained were analysed by one way ANOVA.

Resistance to *Botrytis cinerea*

Plant resistance to *B. cinerea*, a phytopathogenic fungus infecting leaves, was tested as follows: the third to fifth leaf from the apical meristem of transformed aubergine plants (lines 1, 5, 8 and 12, three leaves and at least three plants for each line) and nontransformed controls were harvested. Leaves were placed in sterile Petri dishes (15 cm diameter) containing sterile moistened filter paper, and 30-µl drops of sterile water were placed on the adaxial surface of each leaf. Mycelial plugs (5 mm diameter) from PDA plates containing 1-wk-old growing mycelium of *B. cinerea* were placed on each droplet and plates were sealed with both Parafilm and polyethylene film to maintain moisture. Plates were incubated for 72 h at 21°C to allow the development of mycelium before evaluating necrotic lesions sizes. Results obtained were analysed by one-way ANOVA.

Bioassay with symbiotic fungi

The experiments were carried out on the arbuscular mycorrhizal (AM) fungus *G. mosseae* (Nicol. and Gerd.) Gerdemann & Trappe (IMA 1) obtained from pot cultures maintained in the collection of the Department of Chemistry and Agricultural Biotechnology, University of Pisa, Italy. Sporocarps were extracted from pot-culture soil by wet-sieving and decanting, down to a mesh size of 100 µm (Gerdemann & Nicolson, 1963). Fungal material retained on sieves was flushed into Petri dishes and sporocarps were manually collected with forceps under a dissecting microscope (Wild; Leica). Sporocarps were placed on cellulose ester Millipore (Milano, Italy) membranes (0.45 µm

diameter pore size) and used to assess the development of hyphal differential morphogenesis and of mycorrhizal infection in roots of micropropagated transformed aubergine plants (lines 1, 5, 8 and 12) as previously described (Giovannetti *et al.*, 1993, 1994). Briefly, plant root system was placed between two Millipore membranes, one containing 15 sporocarps of *G. mosseae*. Another membrane containing 15 sporocarps was placed over the membranes containing the roots. Five replicates were set up for both transformed and control plants. The plants were transplanted into pots containing sterile quartz grit and maintained under controlled conditions (18–24°C, 16–8 h photoperiod). Plants were watered daily and were not fertilized during the growing period (1 month).

One month after inoculation plants were removed from pots, root sandwiches were opened and membranes were stained with 0.05% Trypan blue in lactic acid to assess hyphal growth and differentiation. Plant roots were cleared and stained following Phillips & Hayman (1970), using lactic acid instead of lactophenol. Infected root length was calculated by using the grid-line intersect method (Giovannetti & Mosse, 1980). Colonized roots were mounted on microscope slides and observed under a Polyvar light microscope (Reichert-Jung, Vienna, Austria) to assess the number of entry points per cm of root.

Results

Characterization of Dm-AMP1 transformed plants

Agrobacterium tumefaciens-mediated genetic transformation experiments were performed on aubergine leaf explants to obtain the constitutive expression of the protein Dm-AMP1. The antibiotic resistance gene *nptII* was used as a selectable marker. We selected 12 putative regenerated plants which were resistant to kanamycin. They were analysed by PCR experiments using the primers corresponding to the *nptII* coding sequence. As illustrated in Fig. 1, in which six putative transformed

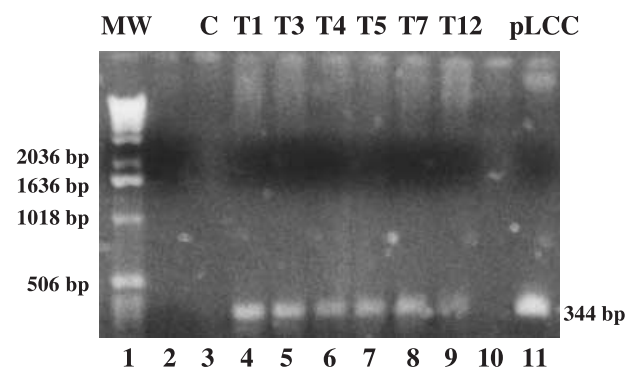


Fig. 1 Polymerase chain reaction analysis showing the presence of *nptII* gene in six Dm-AMP1 transformed aubergine (*Solanum melongena*) plants. The expected *nptII* band was 344 bp. Lanes: 1, molecular weight marker (MW); 3, DNA from nontransformed control plants (C); 4–9, DNA from six transformed plants (T1–T12); 11, positive control from pDmAMPLCC plasmid (pLCC).

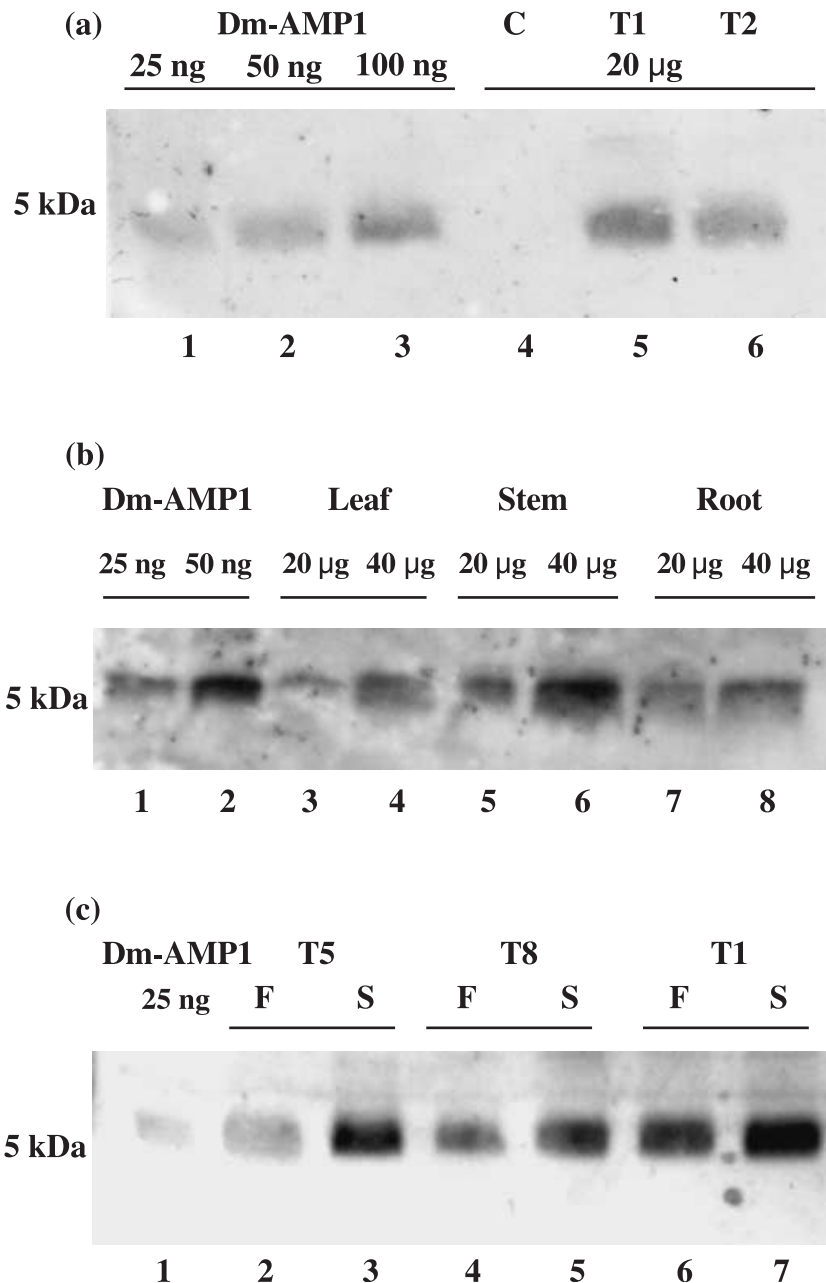


Fig. 2 Western-blot analysis showing the expression of Dm-AMP1 peptide in aubergine (*Solanum melongena*) transformed plants. (a) Analysis of Dm-AMP1 expression in primary transformants leaves. Lanes: 1–3, purified Dm-AMP1 peptide; 4, total soluble proteins of nontransformed control plants; 5–6, total soluble proteins of two different transformed aubergine plants. (b) Analysis of different plant tissues of transformed aubergine (line 12) detecting the expression of Dm-AMP1 protein. Lanes: 1–2, purified Dm-AMP1 peptide; 3–4, total soluble proteins from leaves; 5–6, total soluble proteins from stems; 7–8, total soluble proteins from roots. (c) Analysis of Dm-AMP1 expression in fruit tissues of different transformed aubergine lines. Lanes: 1, purified Dm-AMP1 peptide; 2–3, total soluble proteins of transformant 5, 40 µg; 4–5, total soluble proteins of transformant 8, 40 µg; 6–7, total soluble proteins of transformant 1, 40 µg. F, fruit flesh; S, fruit skin.

plants are shown, all the DNA extracted from the leaves of the putative transformed plants and from the positive control (pDmAMPLCC plasmid) showed the expected 344 bp band, which was absent in DNA from nontransformed plants.

To verify the expression of the protein Dm-AMP1 in the tissues of aubergine-transformed plants, Western blot experiments were performed. In Fig. 2a representative Western blot results, carried out on total soluble proteins obtained from leaves, are shown. Nonspecific bands, resulting from the use of a polyclonal antibody, were detected on Western blots of both transformed and nontransformed plants, but their molecular weights did not correspond to that of Dm-AMP1 protein.

Transformed plants produced the Dm-AMP1 protein, which was not detectable in control plants. The densitometric measures of the bands corresponding to the Dm-AMP1 protein in the different primary transformants analysed, revealed expression levels ranging from 0.20% to 0.48% of the total soluble proteins of leaf extracts. Comparable expression levels of the Dm-AMP1 protein were present in all vegetative tissues of plants (Fig. 2b) and in fruits (Fig. 2c). Transformed lines, showing bands of expected size upon PCR and immunoblot analyses, were self pollinated to obtain F₁ plants. Western-blot analyses were carried out on F₁ plants showing a 3 : 1 segregation of kanamycin resistance in segregation experiments. The

F₁ kanamycin-resistant plants were further self-pollinated to obtain homozygous F₂ plants. Sample leaves of F₂ seedlings were analysed by Western blot to reveal Dm-AMP1 signals, and plants showing Dm-AMP1 expression level ranges of 0.16–0.25% (transformed line 12), 0.38–0.66% (transformed line 5), 0.24–0.62% (transformed line 1) and 0.34–0.47% (transformed line 8) were selected for studying their interactions with pathogenic and symbiotic fungi.

Localization of the Dm-AMP1 protein in aubergine tissues

With the aim of localizing the Dm-AMP1 protein, immunohistochemical experiments were carried out using anti-Dm-AMP1 antibodies. Analysis was performed on clones of the primary transformant 12 and on control plants. This study revealed that the Dm-AMP1 defensin was detectable in the cytoplasm of leaves (Fig. 3b) and roots (Fig. 3d) cells of transformed aubergine. It can be observed in Fig. 3d that Dm-AMP1 protein was also localized in cytoplasmic globular structures in root cells. No signals or background were observed

in any of the conventional controls performed to test the specificity of the immunolabeling (nonimmune rabbit serum, omission of primary antibody, omission of secondary antibody, antibodies preincubated with homologous antigens).

Occurrence of the Dm-AMP1 protein in root exudates of transformed aubergine plants

To verify whether Dm-AMP1 was released by transformed aubergine roots, transformed plants (lines 5 and 12) were grown in hydroponic culture and root exudates, collected after 4 d, were analysed by Western blotting. A band corresponding to the Dm-AMP1 protein was present in exudates released from both transformed clones (Fig. 4). The Dm-AMP1 band was never detected in the exudates released from roots of control plants.

Verticillium albo-atrum bioassay

To study the effect of exudates released from Dm-AMP1 transformed plants on the pathogenic fungus *V. albo-atrum*,

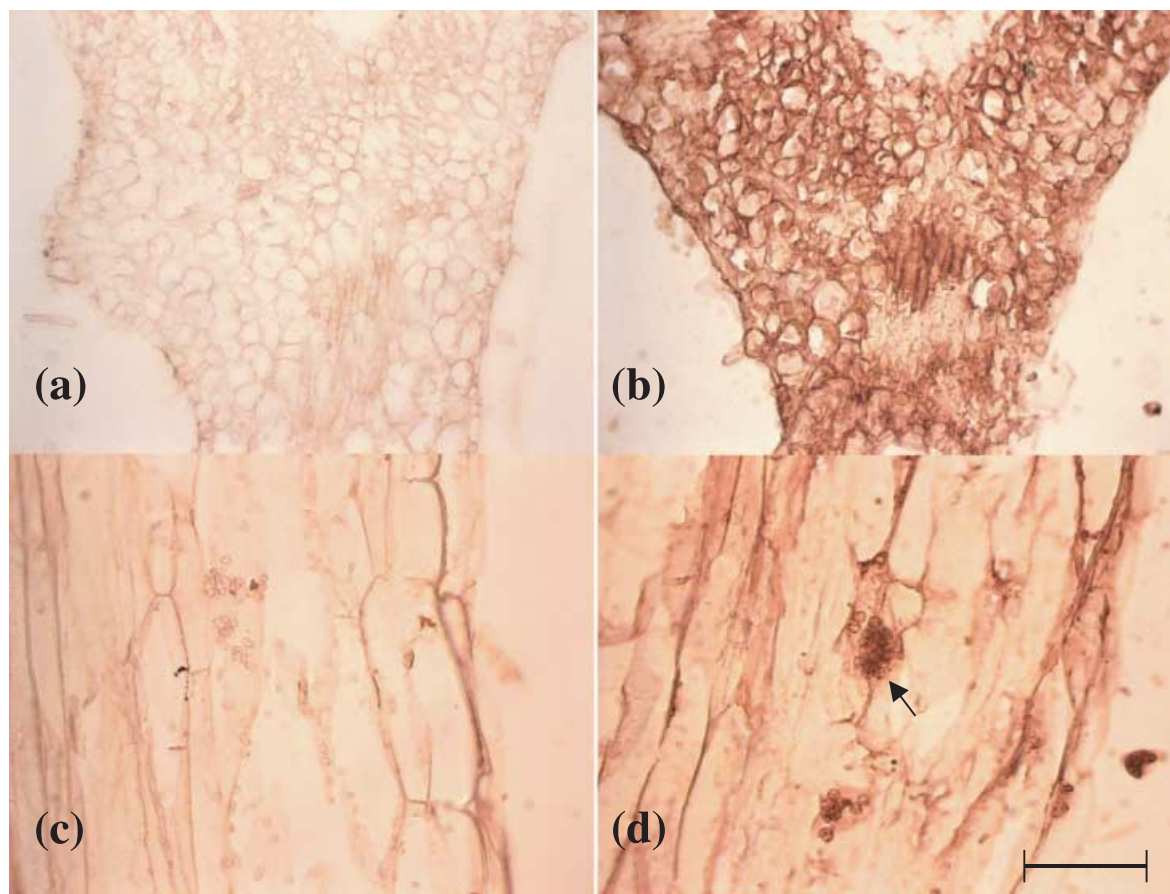
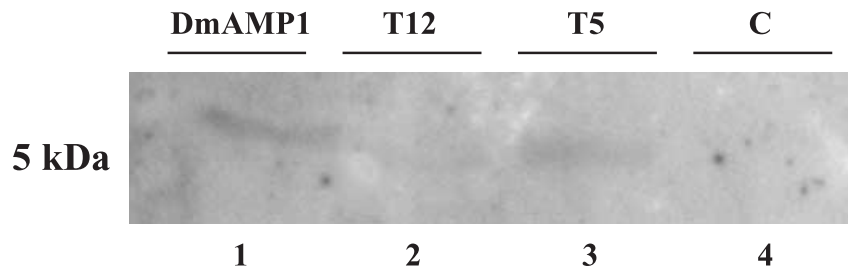


Fig. 3 Immunolocalization of transformed Dm-AMP1 in leaves and roots of transformed aubergines (*Solanum melongena*) assayed using an anti-Dm-AMP1 antibody. (a,b) Longitudinal sections of leaves of a nontransformed control (a) and transformed (b) aubergine plants. (c,d) Longitudinal sections of roots of the same nontransformed control (c) and transformed (d) aubergine plants. Arrow in (d) indicates vesicles stained after the immuno-reaction with the anti-Dm-AMP1 antibody. Bar, 100 μ m (a–d).

Fig. 4 Western blot analysis of aubergine (*Solanum melongena*) root exudates released after 4 d of hydroponic culture. Lanes: 1, purified Dm-AMP1 peptide; 2, exudates released from transformed aubergine line 12; 3, exudates released from transformed aubergine line 5; 4, exudates released from nontransformed aubergine.



we developed a bioassay in which transformed plants (lines 1, 5, 8 and 12) or controls were co-cultured together with the pathogen. Fungal development was evaluated by measuring the area of fungal colonies growing near plant roots. The results of the assay showed a significant difference ($P < 0.03$) between the areas of the colonies grown in presence of all transformed plant lines compared with control plants (Table 1). Since there was no contact between *V. albo-atrum* mycelium and plant roots, this bioassay confirmed the release of active antifungal protein from the roots of transformed aubergine plants.

Resistance to *Botrytis cinerea*

In order to test plant resistance to phytopathogenic fungi infecting leaf tissues we used *Botrytis cinerea* and monitored the development of necrotic areas on leaves of control and transformed aubergines (lines 1, 5, 8 and 12). Results indicated that transformed lines 1, 5 and 8 were highly resistant to *B. cinerea*, showing, respectively, 100%, 85% and 94% reduction of necrotic areas, compared with controls. By contrast, transformant 12 revealed a lower ability to react against this phytopathogenic fungus, showing 36% reduction of necrotic areas with respect to controls (Fig. 5).

Table 1 Mycelial growth of the phytopathogenic fungus *Verticillium albo-atrum* in the presence of Dm-AMP1 transformed *Solanum melongena* plants

Plant line	Mycelial colony area (mm ²)	Mycelial growth reduction	<i>P</i>
Transformant 1	37.58 ± 3.2	64	0.003
Control	103.10 ± 8.2		
Transformant 5	29.92 ± 2.1	71	0.003
Control	103.10 ± 8.2		
Transformant 8	39.80 ± 2.01	55	0.00001
Control	89.20 ± 5.32		
Transformant 12	68.30 ± 13.2	49	0.029
Control	133.01 ± 20.4		

Means (± SE) obtained in the presence of transformed lines are significantly different from their controls for *P* reported in the table.

Bioassay with the AM symbiont *G. mosseae*

With the aim of assessing whether Dm-AMP1 protein affected the nontarget AM fungus *G. mosseae*, two steps of its life cycle were analysed (i.e. host recognition responses and the

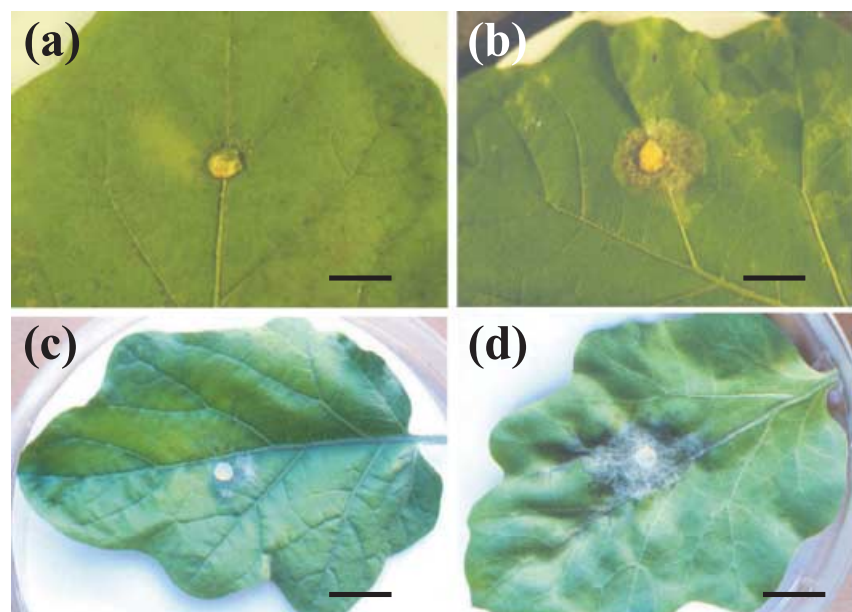


Fig. 5 Differential development of necrotic areas on aubergine (*Solanum melongena*) leaves inoculated with the phytopathogenic fungus *Botrytis cinerea* after 72 h of incubation. (a) Transformed aubergine line 1; (b) nontransformed aubergine; (c) transformed aubergine line 12; (d) nontransformed aubergine. Bar, 1 cm in (a,b); and 1.43 cm in (c,d).

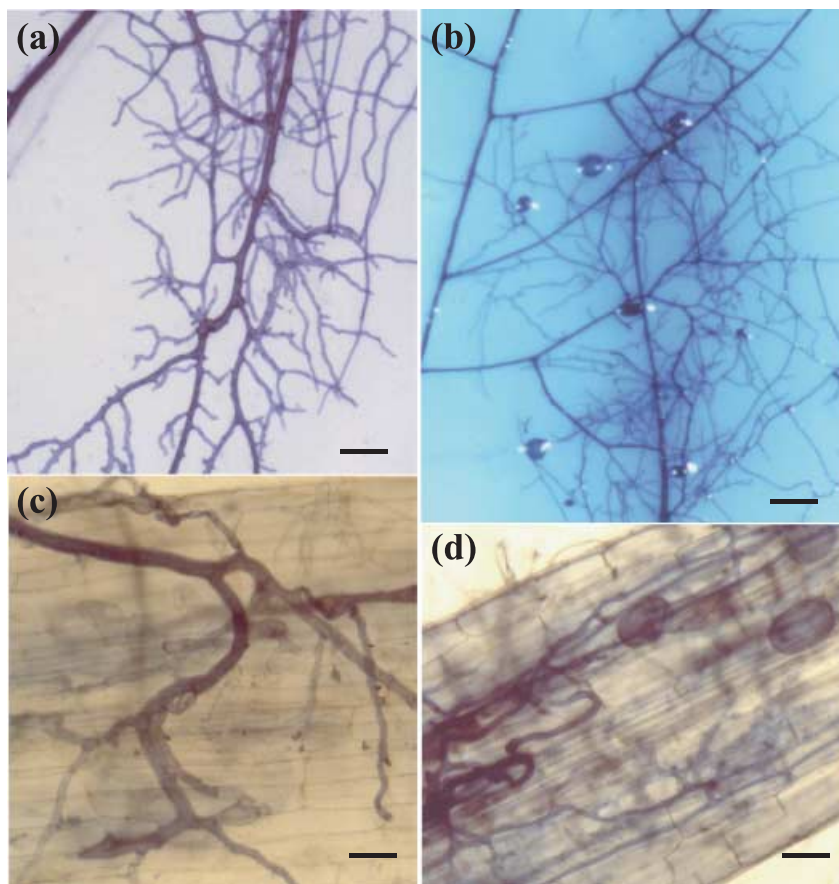


Fig. 6 Light photomicrographs showing hyphal morphogenesis and root infection structures of the arbuscular mycorrhizal fungus *Glomus mosseae* in the presence of aubergine (*Solanum melongena*) plants. (a,b) Differential morphogenesis developed on membranes overlying nontransformed (a) and transformed (b) roots. Bar, 50 μ m in (a) and 90 μ m in (b). (c,d) Development of appressoria (c) and symbiotic structures (vesicles, arbuscules) (d) in transformed roots. Bar, 25 μ m in (c) and 20 μ m in (d).

establishment of symbiosis). *Glomus mosseae* hyphal branching enhancement elicited by the presence of host-derived signals were not affected by released defensins. In fact, both control and transformed aubergine (lines 1, 5, 8 and 12) roots, growing underneath the membranes, elicited differential hyphal morphogenesis in *G. mosseae* mycelium (Fig. 6a,b), showing that the antifungal protein Dm-AMP1 released in root exudates did not interfere with the host recognition system of AM fungi. The establishment of mycorrhizal symbiosis in transformed plants did not differ from controls. Neither the infected root length percentages, ranging between 30% and 60% in different plants and lines, nor the number of entry points, between 2.5 and 3.5 per root cm, showed statistically significant differences. Characteristic structures of arbuscular mycorrhizas, namely appressoria, arbuscules and vesicles, were detected in root systems of both transformed and control plants (Fig. 6c,d). Transformed plants inoculated with *G. mosseae* showed larger growth than uninoculated controls, a general sign of mycorrhizal functioning (Fig. 7).

Discussion

In this work, we obtained transformed aubergines expressing Dm-AMP1 defensin from *D. merckii* in all plant tissues

and showing resistance against the phytopathogenic fungus *B. cinerea*. Transformants were also able to release the antimicrobial Dm-AMP1 protein in root exudates, which reduced the growth of the phytopathogenic fungus *V. albo-atrum*, but did not interfere with recognition events and symbiosis establishment by the AM fungus *G. mosseae*, considered as a nontarget organism.

The important role played by antimicrobial compounds in plant defense strategies has been shown by many authors (Broekaert *et al.*, 1995; Terras *et al.*, 1995; de Bolle *et al.*, 1996). The use of genes expressing antimicrobial compounds to obtain transformed phenotypes more resistant to pathogens was successful in the case of tobacco, geranium and potato plants (Terras *et al.*, 1995; de Bolle *et al.*, 1996; Bi *et al.*, 1999; Gao *et al.*, 2000). The expression level of Dm-AMP1 in aubergine lines ranged between 0.16% and 0.66% of total soluble protein content and it was higher than those reported for tobacco plants transformed with different antimicrobial protein gene constructs cloned from *M. jalapa* and *A. caudatus* (de Bolle *et al.*, 1996), which showed expression levels of 0.1% of total soluble protein content. The difference between the expression values of Dm-AMP1 in aubergine compared with those reported in literature might be due to the translational enhancer Ω derived from tobacco mosaic virus (Gallie



Fig. 7 Transformed aubergine (*Solanum melongena*) plants (line 12) inoculated with the arbuscular mycorrhizal fungus *Glomus mosseae* (left), showing growth enhancements compared with controls (right).

& Walbot, 1992) used in our construct. The expression level of genes for antimicrobial proteins is generally correlated with plant resistance in transformed plants, although resistance also depends on the kind of antimicrobial activity and on the subcellular localization of peptides. Defensins expressed in seeds, such as Rs-AFPs from *R. sativus*, were shown to occur in the outer cell wall layers of the seeds and to be released in the growth medium (Terras *et al.*, 1995). By means of immunolocalization, the authors showed that Rs-AFPs were expressed in the middle lamellae of cell walls throughout the different seed tissues (endosperm, cotyledons and hypocotyl) where the first contacts with the invading fungi occurred. In this work we observed that Dm-AMP1, whose amino acid sequence is highly homologous to Rs-AFPs (Osborn *et al.*, 1995), is detectable in leaves and root cells of transformed aubergines. Although polyclonal antibodies show a reduced specificity, the immunolocalization experiment provides further support to evidence of Dm-AMP1 expression in the different plant tissues. It is interesting to note that in root cells the Dm-AMP1 protein is localized in cytoplasmic globular structures resembling either protein bodies, which might have an accumulation function, or vesicles, which might be involved in transferring the antimicrobial protein outside root cells.

Our findings provide the first evidence of the release of Dm-AMP1 in transformed aubergine root exudates. This is an important trait of transformed plants to be taken into account since exudates containing antimicrobial proteins might affect nontarget microbial soil communities (Siciliano & Germida, 1999; Griffiths *et al.*, 2000). Recent researches, carried out on maize plants expressing the insecticidal toxin Cry1Ab from *Bacillus thuringiensis*, showed that *Bt* plants released the active toxin in root exudates (Saxena *et al.*, 1999, 2002; Saxena & Stotzky, 2000) and that these did not affect saprophytic soil fungi (Saxena & Stotzky, 2001). However, the effect of root exudates from transgenic plants releasing insecticidal toxins or antimicrobial compounds on the

complete range of soil microorganisms is still controversial and should be studied in detail.

Results obtained from *V. albo-atrum* bioassay showed that the presence of Dm-AMP1 transformed roots in the culture medium reduced the growth of nearby colonies of the pathogenic fungus by 49–71% with respect to controls, suggesting that released defensin, consistent with previous work (Osborn *et al.*, 1995), was still active.

Plants expressing Dm-AMP1 protein showed reduced necrotic areas development, with respect to nontransformed plants, when leaves were inoculated with the phytopathogenic fungus *B. cinerea*. Infection assays performed with leaves or leaf discs represent a useful tool to evaluate plant resistance against foliar pathogens through the assessment of necrotic lesions development or localized sporulation density (Laemmlen & Sink, 1978; Bi *et al.*, 1999).

In this work, transformed line 12, showing lower Dm-AMP1 expression with respect to lines 1, 5 and 8, also displayed the largest *B. cinerea*-induced lesions on leaf tissues whereas no, or highly reduced, necrotic lesions occurred on leaves of the other transformants. These results suggest a correlation between Dm-AMP1 protein expression in leaves and plant resistance to foliar pathogens.

From these results, which might be the effects of transformed plants and their exudates on beneficial symbiotic fungi? Although Dm-AMP1 was highly expressed in all transformed plant tissues, roots included, and was released in exudates, it did not interfere with the host recognition system of AM fungi and with the following steps leading to the establishment of mycorrhizal symbiosis by *G. mosseae*. Moreover, the typical growth enhancement due to the presence of the symbiosis was observed in all the transformed lines tested, suggesting that the established symbiosis was functional.

Previous studies showed that plants of *Nicotiana sylvestris* and *Nicotiana tabacum* constitutively expressing tobacco chitinases and different kinds of pathogenesis-related proteins

(PRs), respectively, were colonized by *G. mosseae* to the same extent as control plants (Vierheilig *et al.*, 1993, 1995). The reasons why mycorrhizal fungi were not affected by plant defense compounds remain to be investigated. Some studies reported that AM fungal colonization induced transient enhancement or accumulation of antimicrobial compounds such as chitinases, glucanases, phytoalexins and phenolics (Volpin *et al.*, 1994; Dumas-Gaudot *et al.*, 1996; Gianinazzi-Pearson *et al.*, 1996; Morandi, 1996). Accordingly, the hypothesis that AM fungi have developed a general tolerance to plant defense compounds is also supported by our results on plant defensins. Since recent works reported that Dm-AMP1 induced membrane destabilization after preferential binding to membrane patches containing sphingolipids (Thevissen *et al.*, 2000b, 2003a), we could hypothesize that the AM fungus *G. mosseae* does not have suitable binding sites for Dm-AMP1.

The occurrence of Dm-AMP1 defensin in root exudates of transformed aubergines allowed us to show that the mycorrhizal symbiont *G. mosseae* was still able to recognize host-derived signals inducing differential morphogenesis and infection structures formation (Giovannetti *et al.*, 1993, 1994, 1996; Giovannetti, 2000). The experimental model system used in this work may represent a reliable biotest for assessing the impact of transformed plants and their root exudates on important nontarget soil microorganisms such as AM fungi, which are fundamental to soil fertility and plant nutrition (Smith & Read, 1997).

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