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ORIGINAL PAPER

Stable integration and expression of wasabi defensin gene in "Egusi" melon (*Colocynthis citrullus* L.) confers resistance to Fusarium wilt and Alternaria leaf spot

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Abstract Production of "Egusi" melon (*Colocynthis citrullus* L.) in West Africa is limited by fungal diseases, such as Alternaria leaf spot and Fusarium wilt. In order to engineer "Egusi" resistant to these diseases, cotyledonary explants of two "Egusi" genotypes, 'Ejagham' and NHC1-130, were transformed with *Agrobacterium tumefaciens* strain EHA101 harbouring wasabi defensin gene (isolated from *Wasabia japonica* L.) in a binary vector pEKH1. After co-cultivation for 3 days, infected explants were transferred to MS medium containing 100 mgl^{-1} kanamycin to select transformed tissues. After 3 weeks of culture, adventitious shoots appeared directly along the edges of the explants. As much as 19 out of 52 (36.5%) and 25 out of 71 (35.2%) of the explants in genotype NHC1-130 and 'Ejagham', respectively, formed shoots after 6 weeks of culture. As much as 74% (14 out of 19) of the shoots regenerated in genotype NHC1-130 and 72% (18 out of 25) of those produced in genotype 'Ejagham' were transgenic. A DNA fragment corresponding to the wasabi defensin gene or the selection marker *npt*II was amplified by PCR from the genomic DNA of all regenerated plant clones rooted on hormone-free MS medium under the same selection pressure, suggesting their transgenic nature. Southern blot analysis confirmed successful integration of 1–5 copies of the transgene. RT-PCR, northern and western blot analyses revealed that wasabi defensin gene was expressed in transgenic lines. Transgenic lines showed increased levels of resistance to *Alternaria solani*, which causes Alternaria leaf spot and *Fusarium oxysporum*, which causes Fusarium wilt, as compared to that of untransformed plants.

Keywords *Colocynthis citrullus* · Cotyledon · Genetic transformation · Antifungal resistance · Wasabi defensin

Abbreviations

BA	Benzylaminopurine
Km	Kanamycin
M	Meropenem
MS	Murashige and Skoog
NPTII	Neomycin phosphotransferase
PCR	Polymerase chain reaction
WD	Wasabi defensin

Introduction

Diseases caused by fungi are the most damaging to most cucurbits. Application of effective microbicidal chemicals as a countervailing approach has only resulted in containing plant diseases to a minimal level, and many virulent strains that are less sensitive to microbicides have emerged (Ali and Reddy 2000). Furthermore, the use of chemicals to combat diseases has drawn criticism from environmental groups. Therefore, the use of disease-resistant varieties would not only significantly reduce disease spread, but

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would be appreciated by consumers as well as environmentalists.

Fusarium wilt, caused by *Fusarium oxysporum* f.sp. *melonis* occurs world wide (Mohamed et al. 1994; Zuniga and Zitter 1995), and is very devastating to fields it infests. It causes general yellowing of foliage which precedes wilting. In some cases, sudden wilting occurs without any yellowing of foliage (Zitter 1999). Fusarium wilt has adverse effects on 'Egusi' production either during early cultivation (March–August) or later in the season (September–January). The severity of the disease increases under certain environmental conditions. In Nigeria, it is common and most severe in sandy soils during late planting (September–January) especially under monoculture conditions, causing a rapid wilt of plants bearing mature fruits and may result in a total loss of the crop. Planting disease-resistant varieties is the only control measure as there are no chemical treatments available for control. Although 4 races of Fusarium wilt (Races 0, 1, 2, and 1–2) have been proposed (Risser et al. 1976), the issue of race identification is not yet concluded.

Alternaria leaf spot is caused by *Alternaria* sp. which causes small, circular, tan spots to appear on the leaves, later enlarging to 37.5 mm or more in diameter. If it is severe, leaf curling, defoliation, premature ripening, lower yields and fruit deformity may occur (Evans et al. 1992). Alternaria leaf spot mostly affects older plants of 'Egusi', from flowering to harvest time. The disease is severe in the peak of rainy season (June–August), causing considerable yield reductions in a situation in which control measures are ineffective or absent. In Nigeria and Cameroon, the disease usually occurs in southern states and in high-land areas of the central regions. In these regions, yield loses up to 60% has been reported. It has also been reported that Alternaria leaf spot can colonize and reduce the economic and nutritional value of 'Egusi' seeds during storage (Ekundayo and Idzi 1990).

Development of melon resistant to these diseases by conventional breeding is deterred by interspecific hybridization barriers which prevent the transfer of desirable agronomic traits into the genus. Moreover, introduction of multiple fungus- or virus-resistant genes into a crop may cause considerable yield reduction (Staub and Grumet 1993) or intensify other agriculturally undesirable traits, because of genetic linkage (Wu et al. 2009). The pathogen-mediated resistance approach (Sanford and Johnson 1985) is a simple and functionally reliable method of introducing disease-resistant genes into plants. Therefore, engineering melon with fungal resistance genes is considered a better alternative to conventional breeding (Gaba et al. 2004).

Plant defensins are cysteine-rich polypeptides of 5 kDa in size and are considered to play a defensive role against fungi (Thevissen et al. 1999; Broekaert et al. 1995; Florack and Stiekema 1994; Commue et al. 1992) and bacteria (Bohlmann 1994). Wasabi (*Wasabia japonica*), a Japanese horseradish widely used in Japan as a foodstuff and as wrapping material to protect food from putrefaction, has been a potential source of antimicrobial proteins. WjAMP-1 gene, isolated from the leaves of wasabi, inhibited fungal and bacterial growth when expressed in *Nicotiana benthamiana* (Saitoh et al. 2001). Transgenic potato plants expressing wasabi defensin gene were also shown to have antifungal activity against *Botrytis cinerea* (Khan et al. 2006). Similar results have been obtained in *Phalaenopsis* (Sjahril et al. 2006) and rice (Kanzaki et al. 2002).

'Egusi' melon (*Colocynthis citrullus* L.) is a highly drought-tolerant annual plant widely cultivated in West Africa. It is very common within the savannah and forest vegetation belts stretching from Cote d'Ivoire to Cameroon. 'Egusi' species are non-climbing creepers cultivated in the field or in home gardens, either in monoculture or in association with other crops, such as maize, cassava and yam at the onset of the rainy season. When intercropped, it serves as a weed control plant (Vodouhe et al. 2001; Schippers 2000, 2004). The seeds, which are a good source of high-quality edible oils (53%), protein (28%) and some other important mineral nutrients (Oyolu 1977) are a major soup ingredient in West Africa (Purseglove 1991), where they are highly relished. The oil extracted from the decoated seeds is composed mainly of unsaturated fatty acids, which gives a unique flavour to foods during cooking. It is equally used for the production of pastries, margarines and soaps (Ajibola et al. 1990), and has recently been found to have enormous potential in the production of biodiesel (Giwa et al. 2009). Despite the enormous economic potentials of 'Egusi' in West Africa and the availability of several melon cultivars as genetic materials for generating transgenic plants, development of resistant varieties through gene transfer technology has not yet been done in this crop (Ntui et al. 2009; van der Vossen et al. 2004).

In this study, we report the integration and expression of wasabi defensin gene in 'Egusi' via *Agrobacterium tumefaciens*-mediated gene transfer. Transgenic lines used for disease resistance bioassay exhibited higher degrees of resistance to *Fusarium oxysporum* and *Alternaria solani*.

Materials and methods

Plant material and culture condition

Two cultivars of "Egusi", 'Ejagham' and NHC1-130 were used in this study. After removal of seed coats, seeds were surface sterilized by treating successively with 70% alcohol for 1 min and 1% (v/v) sodium hypochlorite for 20 min, and rinsed three times with sterile distilled water.

Seeds were then germinated on MS (Murashige and Skoog 1962) medium, with 10 g/l sucrose and 8 g/l agar (Wako Pure Chemical Industry Ltd, Osaka, Japan) in a growth room at 25 ± 1°C, 16-h photoperiod with cool white fluorescent light at 30–40 μmol m^{-2} s^{-1}. The pH of the medium was adjusted to 5.8 prior to the addition of agar and autoclaving at 121°C for 15 min.

Agrobacterium strain and binary vector

The *Agrobacterium tumefaciens* strain EHA101 was used for this study. The strain harbours the binary vector, pEKH1-WD that contains the chimeric defensin gene (approximately 500 bp), isolated from *Wasabia japonica* (Saitoh et al. 2001; Kanzaki et al. 2002), as well as kanamycin and hygromycin resistance genes (Fig. 1). The binary vector was kindly provided by Iwate Biotechnology Research Centre, Kitakami, Japan. The coding region of wasabi defensin gene (500 bp) was integrated between the cauliflower mosaic virus 35S promoter and the nonpaline synthase terminator in a binary vector pEKH1 and were transferred to *A. tumefaciens* strain EHA101.

Agrobacterium tumefaciens was cultured overnight in a reciprocal shaker (120 cycles min^{-1}) at 28°C in 50 ml liquid LB medium (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, pH 7.2) containing 50 mg/l kanamycin (Wako Pure Chemical Industries, Osaka, Japan), 25 mg/l chloramphenicol and 100 mg/l spectinomycin (Sigma-Aldrich, St Louis, MO, USA). The bacterial suspension was centrifuged and then re-suspended to a final density of OD600 = 0.6 in hormone-free MS medium containing 100 μM acetosyringone (3,5-dimethoxy-4-hydroxy-acetophenone; Sigma-Aldrich, St Louis, MO, USA) and 30 g/l sucrose.

Plant transformation

Cotyledons were carefully extricated from 4-day-old in vitro grown seedlings and portions of 1 mm width along the edges were removed to maximize wounding. Then each cotyledon was cut transversely into two halves, dipped into the bacterial suspension for 10 min with gentle shaking. After inoculation, the explants were blotted dry and co-cultivated for 3 days in the dark on MS medium containing 100 μM acetosyringone, supplemented with 5 mg/l BA and solidified with 8 g/l agar.

Selection and regeneration of transformed tissues

After co-cultivation, the explants were washed three times with sterilized distilled water and transferred to the selection medium (MS medium with 30 g/l of sucrose, 8 g/l agar, 2 mg/l BA, 20 mg/l meropenem trihydrate (Meropen; Dainippon Sumitomo Pharma, Osaka, Japan; Ogawa and Mii 2007) to eliminate bacterial carry over, and 100 mg/l kanamycin to select for transformed tissues. The explants that developed shoot buds were sub-cultured at 2-week intervals to the same medium. After 6 weeks of culture on the shoot induction medium, adventitious shoots were counted, excised and transferred to the elongation medium (MS medium containing 0.1 mg/l BA, 10 mg/l meropenem trihydrate and 100 mg/l kanamycin). After 3 weeks, the propagated shoots were excised and rooted on hormone-free MS medium containing the same antibiotic concentrations. All cultures were maintained at 25 ± 1°C, 16-h photoperiod with cool white fluorescent light at 30–40 μmol m^{-2} s^{-1} and 8 h of darkness.

Polymerase chain reaction

Polymerase chain reaction (PCR) was employed to screen transformants carrying wasabi defensin and *npt*II genes. Genomic DNAs were extracted from young leaves of kanamycin-resistant and control plants using a modified cetyltrimethylammonium bromide (CTAB) method (Rogers and Bendich 1988). For the wasabi defensin gene, PCR amplifications were performed as follows: 30 cycles of 94°C for 30 s (denaturation), 62°C for 30 s (annealing) and 72°C for 1 min (elongation).The pair of primers for the detection of the wasabi defensin gene were: 5′-TGTTTCTTTTGT

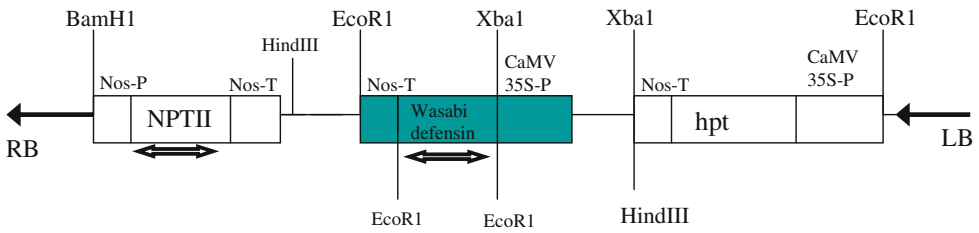

Fig. 1 T-DNA region of the binary vector pEKH1-WD used for transformation. The genes for wasabi defensin and hygromycin phosphotransferase are driven by CaMV 35S promoter, and the gene for neomycin phosphotransferase (*npt*II) by nopaline synthase promoter (nos-p). *Double arrows* represent PCR-amplified region used to confirm the existence of the defensin gene and *npt*II gene in transgenic plants. *RB* and *LB*, right and left border sequences of the T-DNA region, respectively. CaMV 35S-P cauliflower mosaic virus 35S promoter, nos-T terminator of the nopaline synthase gene. Recognition sites of restriction enzymes are also indicated

CGATGCTCACCCTGTTGTTTGGT-3′/5′-GATTGAATC CTGT-TGCCGGTCTTGCGATGATTATC-3′. For the *npt*II gene, PCR amplifications were performed as follows: 30 cycles of 94°C for 1 min (denaturation), 59°C for 1 min (annealing) and 72°C for 1 min (elongation), using one pair of primers specific to the regions of the CaMV 35 S promoter (5′-GATGTGATATCTCCACTGAC-3′) and the NOS terminator (5′-CGCAAGACCGGCAACAGGAT-3′). After amplification, 4 µl of PCR product was loaded on 1% agarose gel, electrophoresed at 100 V for 30 min and detected by ethidium bromide staining.

Southern hybridization analysis

Genomic DNA was extracted from leaves of transgenic and control plants as described by Rogers and Bendich (1988). A 15 µg of the DNA was digested overnight with *Xba*1, separated on a 0.8% agarose gel, and subsequently transferred to a nylon membrane (Immobilon-Ny+ Transfer Membrane; Millipore Co, Billerica, MA, USA). The probe DNA fragment, generated from wasabi defensin gene plasmid DNA pEKH1-WD was labelled by PCR using DIG-2′-deoxyuridine 5′-triphosphate (DIG-dUTP) according to the supplier's instruction (Boehringer Mannheim). PCR primers *WD1* and *WD2* (5′-TGTTTCTTTTGTCG ATGCTCACCCTGTTGTTTGGT-3′/5′-GATTGAATCCT GT-TGCCGGTCTTGCGATGATTATC-3′) were used to amplify the fragment of the wasabi defensin gene. Prehybridization (3 h) and hybridization (overnight) were carried out at 41 and 67°C, respectively, using high-SDS hybridization buffer containing 50% deionized formamide, 5× SSC, 50 mM sodium phosphate (pH 7.0), 2% blocking solution, 0.1% *N*-lauroylsarcosine and 10% SDS. Washing and detection were performed according to the instruction manual of the DIG labelling and Detection System (Roche Diagnostics, Mannheim, Germany). For detection of hybridization signals, membrane was exposed to a detection film (Lumi-Film Chemiluminescent Detection Film; Roche Diagnostics, Mannheim, Germany) for 1 h.

Total RNA isolation

Total RNA from untransformed control and transgenic T_0 melon plants was extracted as described by Krapp et al. (1993), with slight modification. Briefly, 200 mg of leaf from each sample was ground in liquid nitrogen in a pre-cooled mortar. The samples were then homogenized in RNA extraction buffer (4 M guanidium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl and 7.2 µl β-mercaptoethanol). After adding phenol and chloroform, the mixture was centrifuged at 12,000 rpm for 20 min; the supernatant was washed with equal volume of isopropanol and incubated at −20°C for 1 h. Total nucleic acid was precipitated with 150 µl extraction buffer and 150 µl isopropanol at −20°C overnight. The pellet after centrifugation (1,200 rpm, 10 min) was washed with 500 µl 70% ethanol and centrifuged again at 10,000 rpm for 10 min at 4°C. The pellet was then dissolved in 20 µl DEPC water.

Complementary DNA synthesis and reverse transcription (RT-PCR)

The RNA, extracted as described above, was treated with DNase at 37°C for 30 min to get rid of DNA contamination before the first-strand synthesis. RT-PCR was conducted using the first-strand cDNA synthesis kit to synthesize the first-strand cDNA according to the manufacturer's instruction (Invitrogen, USA), with minor modifications. Briefly, 5 µg of DNase-treated total RNA was mixed with 1 µl 3′ wasabi defensin primer, 4 µl dNTP mix and 7 µl diethylpyrocarbonate (DEPC) treated water. The reaction was heated at 65°C for 5 min and incubated on ice for 1 min. Then 4 µl 5× first-strand buffer, 1 µl 0.1 M DTT and 1 µl SuperScript™ III RT was added. Finally, the reaction was incubated for 1 h at 50°C and for 15 min at 70°C. These templates were then used for amplification of the wasabi defensin gene transcript using the same set of primers as those used in genomic PCR. The PCR products were then analysed by 1% agarose gel electrophoresis.

Northern blot analysis

Total RNA was extracted from "Egusi" tissues as described above. To detect the expression of the wasabi defensin gene at the messenger RNA (mRNA) level, 10 µl of denatured total RNA of each sample was resolved by electrophoresis on a 1.5% (w/v) agarose gel containing formaldehyde in 1× morphpropane sulphonic acid (MOPS) buffer and transferred onto a nylon membrane (Immobilon-Ny+ Transfer Membrane; Millipore Co, Billerica, MA, USA) in 20× SSC buffer. The membrane was dipped in 2× SSC and cross-linked by UVC 500 Cross-linker (Amersham Bioscience, UK). Northern hybridization was carried out using the DIG-labelled DNA probe of the wasabi defensin gene following the same procedure used in Southern blot analysis, with slight modification where necessary. Pre-hybridization (2 h), hybridization (overnight) and washing were performed at 50°C.

Western blot analysis

Leaf samples (50–60 g) from non-transgenic and transgenic plants were ground in liquid nitrogen and each sample was mixed with 200 µl of sample buffer (62.5 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol and 2% β-mercaptoethanol. Extracts were heated at 100°C for

3 min and cleared by centrifuging at 10,000*g* for 5 min. A 25 µl of soluble protein from each sample was resolved on a 15% SDS-PAGE (Laemmli 1970), and electro-blotted onto PVDF membrane (Bio-Rad, Bio- Active Co. Hercules, CA, USA). Detection of the wasabi defensin protein was performed using ECL Advance Western Blotting Detection Kit (Amersham Bioscience, Buckinghamshire, UK). Antiserum was raised against a synthetic peptide corresponding to part of the wasabi defensin protein (LEGARHGSCNYIFPYHRCICYFPC). Detection of the 5 kDa wasabi defensin protein was carried out with anti-rabbit IgG immunoglobulin (Boehringer, Germany) as the secondary antibody and HRP colour reagent (Kanzaki et al. 2002).

Disease resistance assay of transgenic plants

Detached leaf bioassay

The fungi *Fusarium oxysporum* and *Alternaria solani* were propagated in Petri dishes containing potato-dextrose agar (PDA, Difco) at 25°C in the dark for 7 days. Spore suspension of each of the fungi was prepared by flooding the Petri dish containing the fungal mycelia with 5 ml of sterile distilled water then rubbing the mycelia gently with a sterile loop to release spores. The spores were collected with a sterile Pasteur pipette, and their concentration was adjusted to 2×10^6 spores/ml with sterile distilled water. For leaf bioassay, detached leaves from mature transgenic and non-transgenic control plants were placed in a Petri dish containing wet filter papers. The leaves were wounded at the middle on both sides of the midrib by gently pricking the abaxial side of the leaves several times with a sterile needle. Then 20 µl of each of the fungal suspensions was introduced to the wounded area and incubated at room temperature for 10 days. Disease severity, the amount of leaf area affected by the disease, was expressed on a scale of 0–4 grades (0, 0–20% leaf area affected; 1, 21–40%; 2, 41–60%; 3, 61–80% and 4, 81–100%). The infection index was calculated following the method provided by Krishnamoorthy et al. (2004). All experiments were repeated three times.

$$\mathrm{II} = \frac{\sum n^b}{(N-1)T}$$

where n is the number of leaves in each grade, b is the grade, N is the number of grades used in the scale and T total number of leaves scored.

In vitro bioassay of whole plant and mock-inoculation

As an alternative inoculation method, melon plants were sub-cultured onto fresh MS medium without antibiotics in culture bottles and allowed to root. At rooting, 1 ml each of the fungal suspension was sprayed on the surface of the medium with a sterile syringe so that the fungus touched some of the roots of the plants. Mock-inoculation was done by spraying sterile water onto the plants and on the surface of the medium. The plants were incubated at room temperature for 5 weeks. The plants were observed daily for disease development; pictures were taken at the third week after incubation.

Statistical analysis

The experiments were setup on a completely randomized design with 3 replications. Data collected were subjected to analysis of variance test. Mean values were compared using least significant different test. The data expressed as percentage were subjected to arc sine transformation before statistical analysis.

Results

Transformation and plant regeneration

Four-day-old cotyledonary explants of cultivars ‘Ejagham’ and NHC1-130 were inoculated with *Agrobacterium* EHA101pEKH1-WD. After 6 weeks of culture on the selection medium, 19 out of 52 (36.5%) and 25 out of 71 (35.2%) of the explants in genotype NHC1-130 and ‘Ejagham’, respectively, formed adventitious shoots (Fig. 2a; Table 1). However, despite such a significant decrease in the number of explants with shoots, 73.68% (14 out of 19) of the shoots regenerated in genotype NHC1-130 and 72% (18 out of 25) of those inducted in genotype ‘Ejagham’ were PCR positive (Table 1). Well-developed shoots were excised from the explants and elongated on the medium containing 0.1 mg/l BA, 10 mg/l meropenem trihydrate and 100 mg/l kanamycin (Fig. 2b). As much as 80% (20 out of 25 in genotype ‘Ejagham’ and 15 out of 19 in genotype NHC1-130) of the elongated shoots rooted on kanamycin containing medium within 3 weeks of culture (Fig. 2c; Table 1). Three well-rooted shoots, each representing an independent clone, were selected from each cultivar for further analysis.

Integration of transgene in host genome

Kanamycin-resistant clones were subjected to PCR analysis to confirm the integration of the transgenes, wasabi defensin and *npt*II. PCR analysis detected the presence of the expected 500 bp for the wasabi defensin (Fig. 3a) and 800 bp for *npt*II (Fig. 3b) genes, indicating that the T-DNA of the binary vector was present in the transgenic lines. No

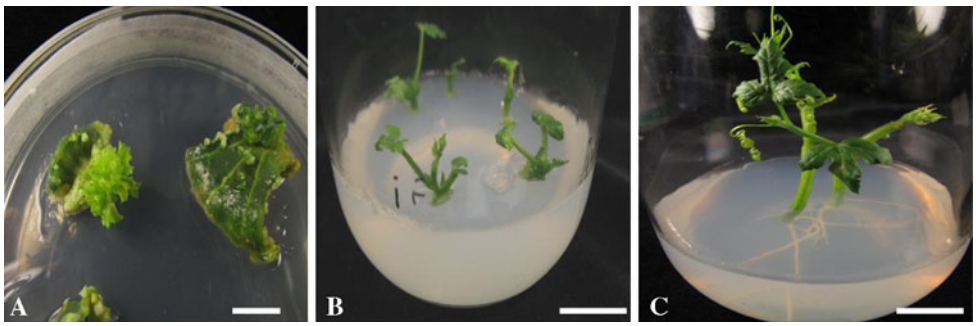

Fig. 2 Plant regeneration from "Egusi" cotyledons after treatment with *A. tumefaciens* EHA101 harbouring pEKH1-WD. **a** Shoot formation along the edges of the explant 3 weeks after co-cultivation (*bar* 0.25 cm). **b** Shoot elongation (*bar* 2 cm). **c** Transgenic melon plant rooted on MS medium containing kanamycin (*bar* 2 cm)

Table 1 Regeneration and transformation frequencies of cotyledonary explants of 'Egusi' melon treated with wasabi defensin gene

Cultivar	No. of explants treated	No. of explants with shoots	PCR + shoots	Transformation efficiency
NHC1-130	52	19 (36.54)*a	14 (73.68)a	26.92a
Ejagham	71	25 (35.21)a	18 (72)a	25.35a

Values with the same case letter on a given vertical array indicate no significant difference at 5% level of significant using least significant different test (LSD)

Transformation efficiency was evaluated as the number of independent transgenic lines with respect to the initial number of explants cultured

*Values in parentheses are percentages of regeneration or PCR + shoots

amplification was detected in the untransformed control plants (Fig. 3a, b, lane C).

Progenies of T_0-independent transgenic lines were further screened by Southern analysis to confirm the integration of wasabi defensin gene. Southern hybridization of *Xba*I digested DNA of the transgenic clones confirmed the integration of the transgene in the host genome (Fig. 3c). Dissimilar hybridization profiles of the analysed transgenic lines revealed random insertion of the transgene in the genome of the tested lines. The copy number of the transgene incorporated in different transgenic lines ranged from one to five. No transgene insertion was detected in untransformed control (Fig. 3c, lane C).

Expression of the transgene

RT-PCR analysis was performed to confirm the expression of the transcript in transgenic plants. The result showed that the transgenic lines contained the transcript of wasabi defensin gene (Fig. 4a). Furthermore, Northern hybridization of transgenic plants revealed the accumulation of the transgene transcript in different transgenic lines (Fig. 4b). The DIG-labelled probe did not detect any transcript from non-transgenic plants (Fig. 4b, lane C).

Protein extracts of leaves of transgenic and untransformed control plants were separated on SDS-PAGE, electro-blotted onto PVDF membrane and incubated with the antiserum raised against wasabi defensin gene. The 5 kDa peptide which corresponds to the translated form of wasabi defensin gene was detected in the leaves of

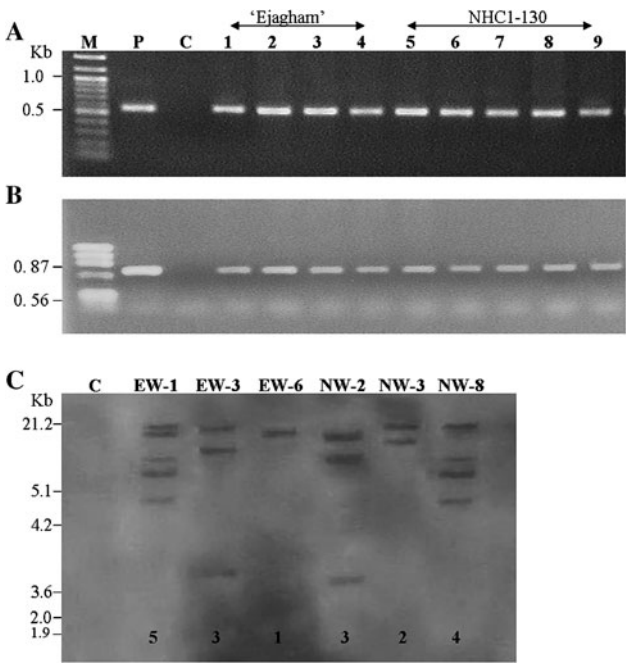

Fig. 3 Integration of wasabi defensin gene in "Egusi" melon. PCR amplification of wasabi (**a**) and *npt*II (**b**) genes. *Lane M* molecular size marker (λDNA/*Hin*dIII, øX174/*Hae*III). *Lane P* plasmid pEKH1-WD as positive control. *Lane C* untransformed control plant. *Lanes 1–4* transgenic lines of cultivar 'Ejagham'. *Lanes 5–9* transgenic lines of cultivar NHC1-130. **c** Southern blot analysis of genomic DNA digested with *Xba*I. *Lane M* digoxigenin (DIG)-labelled molecular marker. *Lane C* untransformed control plant. *Lanes EW-1–EW-6* transgenic lines of cultivar 'Ejagham'. *Lanes NW-2–NW-8* transgenic lines of cultivar NHC1-130. The copy number of the transgene is indicated in the bottom of each transgenic line

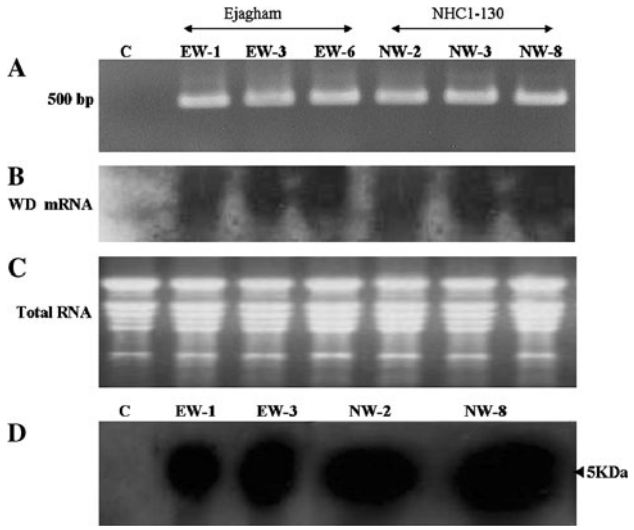

Fig. 4 Expression of wasabi defensin gene in transgenic 'Egusi' melon. **a** RT-PCR analysis of wasabi defensin transcript in transgenic lines. **b** Northern blot analysis. **c** Total RNA stained by ethidine bromide. Total RNA of each sample was resolved by electrophoresis on a 1.5% (w/v) agarose gel containing formaldehyde in 1× morphpropane sulphonic acid (MOPS) buffer, transferred and fixed onto a nylon membrane (Immobilon-Ny+ Transfer Membrane; Millipore Co, Billerica, MA, USA). Northern hybridization was carried out using the DIG-labelled DNA probe of the wasabi defensin gene. *Lanes EW-1–NW-8* wasabi defensin transcript in transgenic 'Egusi' lines. **d** Western blot analysis of the expression of wasabi defensin protein in leaves of transgenic 'Egusi' lines. *Lane C* untransformed control plant. *Lanes EW-1* and *EW-3* transgenic lines of cultivar 'Ejagham', *lanes NW-2* and *NW-8* transgenic lines of cultivar NHC1-130

transgenic lines, but not in the untransformed control plant (Fig. 4c). The level of expression varied in different transgenic lines.

Resistance of transgenic plants to *Fusarium oxysporum* and *Alternaria solani*

Detached leaf bioassay

Detached leaves inoculated with either *Fusarium oxysporum* or *Alternaria solani* were incubated at room temperature and observed daily for disease development. On the untransformed plants, disease symptoms caused either by *Fusarium oxysporum* or *Alternaria solani* appeared in the form of lesions 2–3 days after inoculation, consequently leading to the appearance of yellowish and then blackish necrotic lesions 7 days after inoculation (Fig. 5a, d). Lesion sizes in the leaves of the untransformed plants were greater than 60–90% of the total leaf area (Fig. 5a, d). On the contrary, disease symptoms on leaves of transgenic lines developed much slower (7 days after inoculation) and lesions, less than 20% of the of the total leaf area, were mainly restricted to small necrotic spots at the site of inoculation both for *F. oxysporum* (Fig. 5b, c), and *A. solani* (Fig. 5e, f).

Based on the infection indices, the plants were graded into four categories: highly susceptible (HS), when the infection index is greater than 10 (II > 10); susceptible (S), II of 5.1–10; tolerant (T), II 2.6–5; resistant (R), when the infection index is less than 2.5 (II < 2.5). No complete resistance was observed in the transgenic lines expressing the wasabi defensin gene. On the detached leaves inoculated with *F. oxysporum*, the leaves of the untransformed plants were highly susceptible because of the high infection index. On the other hand, detached leaves of transgenic lines EW-1, EW-3, EW-6, NW-2, NW-3 and NW-8 were classified as resistant because of their low infection indices (Table 2). Similarly, detached leaves of transgenic lines EW-1, EW-3, NW-2, NW-3 and NW-8 inoculated with *A. solani* were classified in the resistant category because of their low infection indices (Table 3). However, one line, EW-6, belonging to cultivar 'Ejagham' was classified as moderately resistant (tolerant) since its infection index was greater than 2.5. The detached leaves of the untransformed plants inoculated with *A. solani* were graded as being highly susceptible (Table 3).

In vitro bioassay of whole plant

To further screen transgenic lines for resistance to pathogens, 3-week-old rooted in vitro plants expressing the wasabi defensin gene as well as the untransformed control plants were challenged with 1 ml each of the fungal suspension. Since the plant medium contained a relatively high level of sucrose (30 g/l) it promoted a high rate of continuous mycelial growth, thereby creating an extremely harsh environment for plant survival. Plants were assessed based on the extent of symptom development and days of survival after fungal infection.

Three to four days after inoculation, extensive mycelia growth was observed in all the culture bottles containing the plants. In plants inoculated with *F. oxysporum*, the untransformed plants displayed browning and necrosis of the entire vascular system, resulting in severe wilt and death within 2 weeks of inoculation (Fig. 6a, b). In contrast, transgenic lines EW-1 and NW-2 expressing the *wasabi defensin* gene survived more than 2 weeks, indicating higher levels of resistance to Fusarium infection. The mock-inoculated plants showed no morphological or developmental abnormalities under the same period of study (Fig. 6g, h); suggesting that the wilting observed in the untransformed plants was as a result of the pathogenic effect of the fungus. Between the two transgenic lines tested, line NW-2 of cultivar NHC1-130 exhibited higher levels of resistance to *F. oxysporum* (Fig. 6e, f) than line EW-1 of cultivar 'Ejagham' which developed necrotic

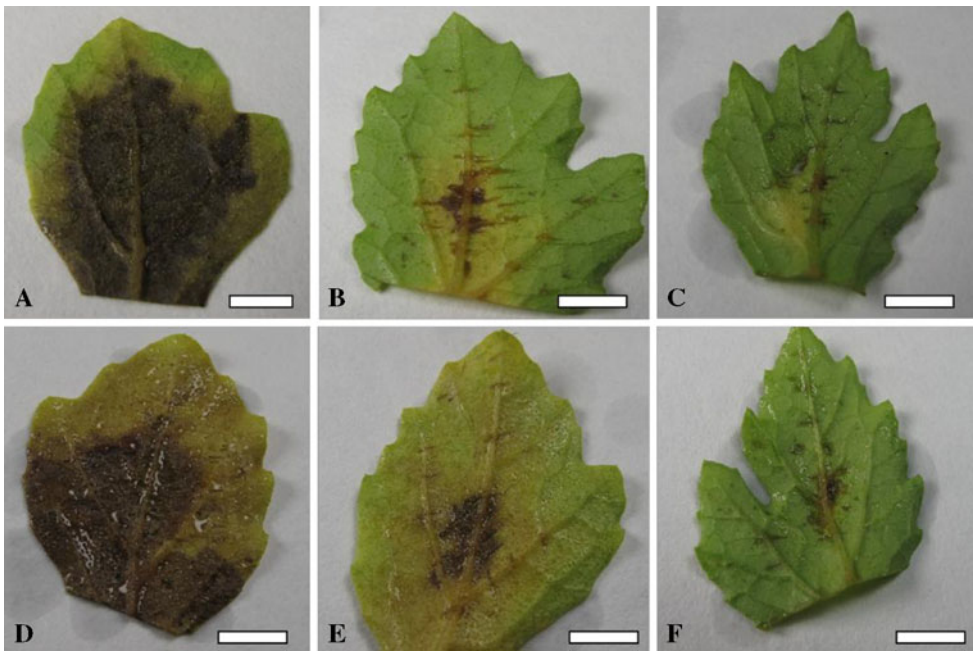

Fig. 5 Fungal resistance test of detached leaves of the untransformed and transgenic "Egusi" melon plants expressing wasabi defensin gene. The results were photographed one week after inoculation with *F. oxysporum* (*upper row*) or *A. solani* (*lower row*). **a**, **d** Untransformed control plant. **b**, **e** Transgenic line EW-1. **c**, **f** Transgenic line NW-2. *Bar* 2 mm

Table 2 Infection indices of leaves of the control and transgenic 'Egusi' melon plants inoculated with *Fusarium oxysporum*

Transgenic lines	Infection index	Disease rating
Control	16.35b	Highly susceptible
EW-1	0.83a	Resistant
EW-3	0.52a	Resistant
EW-6	1.16a	Resistant
NW-2	0.27a	Resistant
NW-3	0.22a	Resistant
NW-8	0.30a	Resistant

Mean values with the same case letters are not significantly different ($P > 0.05$) using least significant different (LSD) test

EW Transgenic lines "Ejagham" expressing the *wasabi defensin gene*. *NW* Transgenic lines NHC1-130 expressing the *wasabi defensin gene*

Table 3 Infection indices of leaves of the control and transgenic 'Egusi' melon plants inoculated with *Alternaria solani*

Transgenic lines	Infection index	Disease rating
Control	10.5c	Susceptible
EW-1	0.88a	Resistant
EW-3	0.72a	Resistant
EW-6	3.08b	Tolerant
NW-2	0.17a	Resistant
NW-3	0.81a	Resistant
NW-8	0.44a	Resistant

Mean values with the same case letters are not significantly different ($P > 0.05$) using least significant different (LSD) test

EW Transgenic lines "Ejagham" expressing the *wasabi defensin gene*. *NW* Transgenic lines NHC1-130 expressing the *wasabi defensin gene*

symptoms after 2 weeks of co-cultivation with the fungus (Fig. 6c, d). However, the necrotic lesions did not merge together and thus did not cause extensive tissue damage (Fig. 6d). Similar results were observed in plants inoculated with *A. solani.* Disease symptoms with yellowish necrotic lesions were observed on the stems of the untransformed plants within 1 week of co-cultivation with *A. solani* and extensive tissue damage was noted 10 days after inoculation. Generally, tissue damage caused by *A. solani* infection resulted in wilting, bending/twisting of the stem and death of the untransformed plants 2 weeks after infection with the fungus (Fig. 7a, b). In contrast, transgenic line EW-1 (Fig. 7c, d) and NW-2 (Fig. 7e, f) remained green with little signs of infection. This result suggests that the presence of wasabi defensin gene in transgenic lines suppresses growth of *Fusarium oxysporum* and *Alternaria solani* and thus confers resistance against these fungi.

Discussion

Plants have developed an elaborate array of inducible immune responses that become activated upon the perception of pathogens. This form of response, generally referred to as systemic induced resistance (Van Wees et al. 2008; Grant and Lamb 2006; Durrant and Dong 2004) often confer a broad-spectrum resistance against a wide range of unrelated pathogens. A large number of synthetic chemicals and several antimicrobial proteins capable of

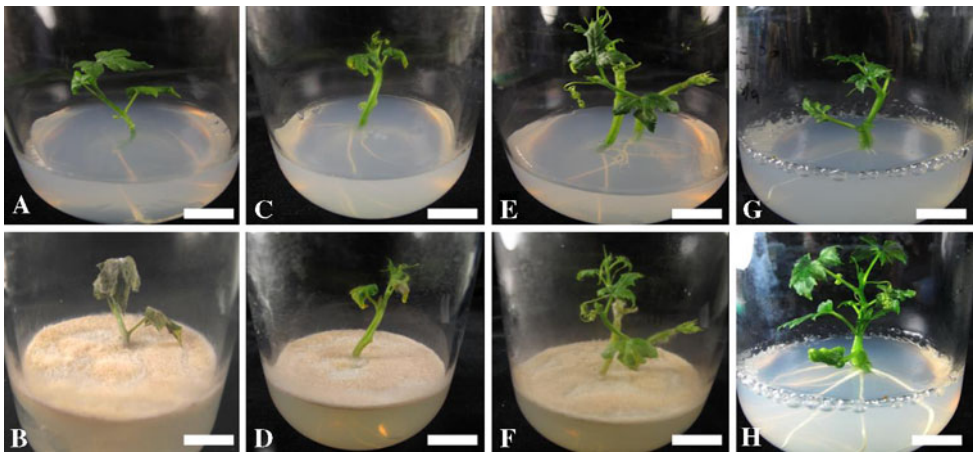

Fig. 6 In vitro evaluation of resistance against *F. oxysporum*, conferred by transgenic "Egusi" melon expressing wasabi defensin gene. The *upper row* represents start of co-cultivation with *F. oxysporum* (**a**, **c** and **e**) or with sterile water (**g**); the *lower row* represents 3 weeks after inoculation. **a**, **b** Untransformed control plant. **c**, **d** Transgenic line EW-1. **e**, **f** Transgenic line NW-2. **g**, **h** Mock-inoculated control. *Bar* 1 cm

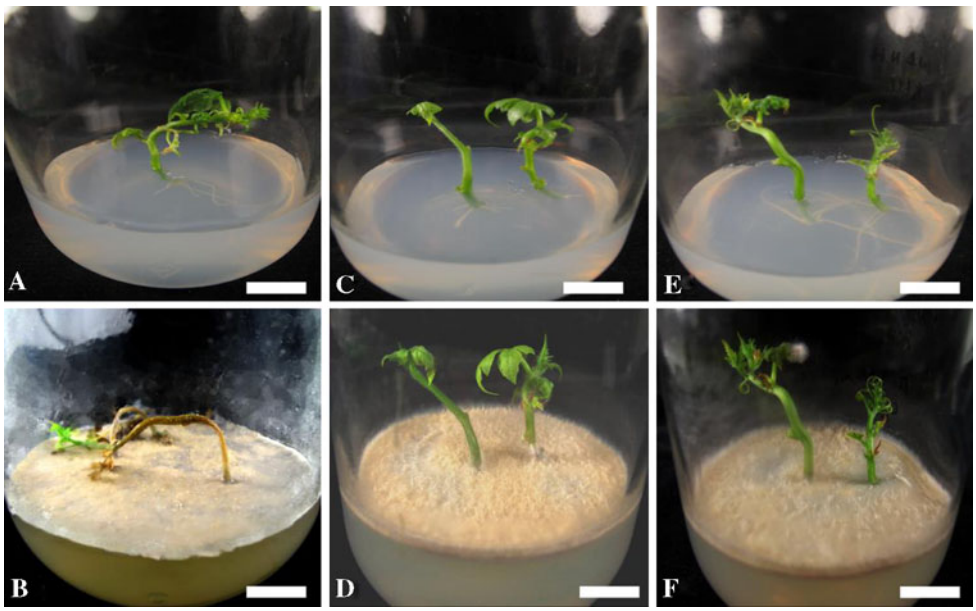

Fig. 7 In vitro evaluation of resistance against *A. solani*, conferred by transgenic "Egusi" melon expressing wasabi defensin gene. The *upper row* represents start of co-cultivation with *A. solani*; the *lower row* represents 3 weeks after inoculation. **a**, **b** Untransformed control plant. **c**, **d** Transgenic line EW-1. **e**, **f** Transgenic line NW-2. *Bar* 1 cm

inducing immune responses in plants have been identified. Wasabi defensin, a protein belonging to the defensin family, is one of such antimicrobial proteins. Defensins have been shown to cause permeabilization of fungal membranes, leading to inhibition of fungal growth (Thevissen et al. 1999; Florack and Stiekema 1994).

In this study, wasabi defensin gene was expressed in leaves of transgenic lines. All the transgenic lines challenged with either *F. oxysporum* or *A. solani* exhibited similar levels of resistance except one line, EW-6, which showed moderate-resistance (tolerance) to *A. solani*. RT-PCR and Northern hybridization analyses of the tolerance and resistance lines revealed high-level accumulation of the transgene transcripts. In most transformation experiments, expression of the transgene is dependent upon the copy number and abnormal morphology has often been reported (Matzke et al. 1994; Finnegan and McElroy 1994). In some cases, transgene copy number produced positive (Yevtushenko et al. 2005; Elomaa et al. 1995), negative (Finnegan and McElroy 1994) or no effect (Peach and Velten 1991) on transgene expression. In this study, the expression of the transcript in the transgenic lines was independent of transgene insertion. Although an increased copy number has often been associated with transgene silencing (Finnegan and McElroy 1994), the highest number of transgene insertion observed in lines EW-1, EW-3, NW2 and NW-8 had no inhibitory effect on the accumulation of wasabi defensin transcript. Moreover, all transgenic lines were healthy and showed no morphological or developmental abnormalities even in lines with the highest wasabi defensin mRNA and protein expression. Furthermore, the degree of RNA-mediated resistance of transgenic plants is often correlated to the insert numbers of transgene (Sijen et al. 1996). Southern hybridization analysis of wasabi defensin in transgenic lines suggests such correlation. For example, transgenic line EW-6 with a single copy

number of the transgene was found to be less tolerant to *A. solani* compared to the resistant lines (EW-1, EW-3, NW-2 and NW-8) with multiple copy number of the transgene. It is most likely that the level of fungal resistance is associated with the level of protein expression which is related to the copy number of the transgene. Similar correlation was observed by Yevtushenko et al. (2005).

Antimicrobial peptides may display specificity in their activity against organisms from different taxonomic groups (Ali and Reddy 2000). Some are effective only against bacteria and fungi, but show no or little toxicity to mammalian cells, e.g., magainin (Helmerhorts et al. 1999). Others, e.g., melittin and its analogues, are equally toxic to bacteria, fungi, plants and animals (Blondelle and Houghten 1991). Wasabi defensin reported here is effective against fungi and show no toxicity against "Egusi" melon plants. It has also been reported that wasabi defensin gene is effective against bacteria (Saitoh et al. 2001).

Three transgenic lines each of cultivars 'Ejagham' and NHC1-130 were selected for disease assay. Initially, disease assay was carried out on detached leaves of the untransformed and transgenic lines (Tables 2, 3; Fig. 5). Disease symptoms were more pronounced in untransformed plants than in transgenic lines after 7 days post inoculation (Fig. 5). This indicates that transgenic lines expressing wasabi defensin gene exhibited a significant level of resistance to Fusarium wilt and Alternaria leaf spot compared to untransformed plants. Further observation of disease development for long period could not be done in excised leaf tissues because of discolouration that might be due to physiological stress experienced by detached leaf tissues. Therefore, further evaluation of disease symptoms development was carried out on 3-week-old rooted whole plants for long periods. Results obtained were similar to those observed in the detached leaf assay, indicating further, the resistant nature of the transgenic lines.

Based on the calculated infection indices, no complete resistance was observed in the transgenic lines expressing wasabi defensin gene. To obtain complete resistance, overexpression of multiple antifungal proteins with different functions may be necessary. However, antimicrobial peptides when combined may interact synergistically, additively or antagonistically. Therefore, before candidate genes are transferred to plants, it is essential to know the nature of their end products as this will help find potentially useful (additive and synergistic) combinations of genes and avoid antagonistically interacting combinations (Ali and Reddy 2000). Jach et al. (1995) reported a synergistic interaction in tobacco in which transgenic plants co-expressing the barley transgenes, a class-II chitinase, a class-II β-1,3-glucanase and a type-I ribosome-inactivating protein, in a *Rhizoctonia solani* infection assay conferred significantly enhanced resistance against fungal attack compared to resistance levels obtained with corresponding isogenic lines expressing a single barley transgene at a similar level. Therefore, generation of transgenic 'Egusi' melon plants with multiple antifungal genes may confer complete resistance to fungal pathogens.

The results presented in Table 1 indicate that regeneration and transformation efficiencies of genotype NHC1-130 were higher than those of genotype 'Ejagham'. However, the values were not significantly different from each other. The concept of plant transformation combines components of both plant regeneration and *Agrobacterium*-related parameters, such as virulence induction, T-DNA activation, transfer and integration. Considering these factors, establishment of optimal conditions during induction of transgenic plants is necessary. Therefore, the protocol reported here could be used for genetic transformation of a wide number of varieties of 'Egusi' melon.

A final point worth mentioning is that, the transgenic lines obtained in this study showed higher degrees of resistance to Fusarium wilt and Alternaria leaf spot in a non-specific manner. This suggests that the resistance gained would last for a long period and would be effective under field conditions. Furthermore, it is possible that these transgenic lines may display higher levels of resistance against other types of fungal disease in addition to the ones mentioned above, since Kanzaki et al. (2002) reported that growth of blast fungus was inhibited in transgenic rice plants overexpressing wasabi defensin gene. Also, Khan et al. (2006) reported that transgenic potato plants expressing wasabi defensin gene exhibited significant resistance to gray mold (*Botrytis cinerea*). Therefore, wasabi defensin gene used in this study is expected to resist a wide range of fungal pathogens as well as bacterial diseases as shown in Sjahril et al. (2006).

The seeds produced from transgenic "Egusi" lines expressing Wasabi defensin gene could be consumed by humans or animals and may, therefore, be a biosafety issue in terms of toxicology and/or allergenicity. Wasabi defensin gene was extracted from wasabi (*Wasabia japaonica*), a staple condiment in Japanese cuisine. Since wasabi is used as a foodstuff, it is suggested that the seeds of these transgenic 'Egusi' lines may be consumed without any side effect with respect to biosafety. Although allergenic or toxicological effects are unlikely, such effects cannot be ruled out completely, therefore, biosafety of the transgenic lines obtained in the present study has to be assessed prior to any field testing and/or recommendation for consumption.

Conclusion

These results demonstrate that transgenic "Egusi" lines expressing wasabi defensin gene exhibited enhanced

resistance to the fungal pathogens tested under in vitro conditions. However, further experiments are needed to evaluate fungal resistance of transgenic lines under field conditions and to test for resistance against other pathogens. To the best of our knowledge, this report is the first to demonstrate the transformation of 'Egusi' for resistance to fungal pathogens. The approach described here has considerable utility for enhancing the breeding of new varieties of "Egusi" melon with antifungal genes.

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