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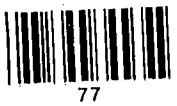

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FINAL REPORT

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Osmotin and Osmotin-like Proteins as a Novel Source for Phytopathogenic Fungal Resistance in Transgenic Carnation and Tomato Plants

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Osmotin and Osmotin-like Proteins as a Novel Source of Phytopathogenic Fungal Resistance in Transgenic Carnation and Tomato Plants

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The goal of this project is to enhance fungal resistance of carnation and tomato through the ectopic expression of osmotin and other pathogenesis-related (PR) proteins. The research objectives were to evaluate *in vitro* antifungal activity of osmotin and osmotin and other PR protein combinations against phytopathogens (including *Fusarium oxysporum*, *Verticillium dahliae*, *Botrytus cinerea* or *Phytophthora infestans*), develop protocols for efficient transformation of carnation and tomato, express PR proteins in transgenic carnation and tomato and evaluate fungal resistance of transgenic plants. Protocols for microprojectile bombardment and *Agrobacterium*-mediated transformation of carnation were developed that are applicable for the biotechnology of numerous commercial cultivars. Research established an efficient organogenetic regeneration system, optimized gene delivery and transgene expression and defined parameters requisite to the high frequency recovery of transgenic plants. Additionally, an efficient *Agrobacterium*-mediated transformation protocol was developed for tomato that is applicable for use with numerous commercial varieties. Rigorous selection and reducing the cytokinin level in medium immediately after shoot induction resulted in substantially greater frequency of adventitious shoots that developed defined stems suitable for rooting and reconstitution of transgenic plants. Transformation vectors were constructed for co-expression of genes encoding osmotin and tobacco chitinase Ia or PR-1b. Expression of osmotin, PR-1 and/or chitinase in transgenic carnation mediated a high level resistance of cv. White Sim (susceptible variety) to *F. oxysporum* f. sp. *dianthi*, race 2 in greenhouse assays. These plants are being evaluated in field tests. Comprehensive analysis (12 to 17 experiments) indicated that germination of *B. cinerea* conidia was unaffected by PR protein expression but germ tube elongation was reduced substantially. The disease severity was significantly attenuated by PR protein expression. Constitutive expression of osmotin in transgenic tomato increased resistance to *B. cinerea*, and *P. infestans*. Grey mold and late blight resistance was stable through the third selfed generation.

The research accomplished in this project will have profound effects on the use of biotechnology to improve carnation and tomato. Transformation protocols that are applicaple for efficient stable gene transfer to numerous commercial varieties of carnation and tomato are the foundation for the capacity to bioengineer these crops. The research further establishes that PR proteins provide a measure of enhanced disease resistance. However, considerations of PR protein combinations and conditional regulation and targeting are likely required to achieve a sustained level of resistance.

BARD SCIENTIFIC REPORT

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Osmotin and osmotin-like proteins as a novel source for phytopathogenic fungal resistance in transgenic carnation and tomato plants.

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Report Submitted to BARD

by Alexander Vainstein

The relationship between mankind and flowers has a very long and romantic history. In more modern times, flowers have become a highly important economic commodity and today, they are sold worldwide, with a market value of over US $30 billion. Among the ca. 20 types of major cut flowers, carnation, rose, lily, tulip and chrysanthemum are the top sellers and account for most international sales.

Carnation, native to the Mediterranean coastal region, is one of the most important flower crops worldwide, with a value of over $200 million annually (VBN, 1995). Carnation export from Israel is estimated at $25 million per year (representing one-fifth of all exported flowers). In this economically important agricultural area, the market demand for flowers with improved traits (such as new colors, new flower forms, better fragrance, disease resistance and longer vase life) constitutes the main driving force for breeders to create new and more attractive varieties annually.

Carnation is a member of the Caryophyllaceae and belongs to the genus *Dianthus*, which contains over 300 species. Commercial carnations, grouped into the phenotypical categories "standard" and "spray", result from crosses within *Dianthus caryophyllus.* Pot carnations, resulting from crosses involving *D. chinensis* and *D. barbatus*, are also becoming popular among consumers. As one of the major contributors to the cut-flower market and a commercial leader in terms of the number of stems sold worldwide (Jensen and Malter, 1995), carnation is an important target for the breeding of new varieties with novel characteristics.

Every year, breeders create new and attractive carnation varieties. Novel traits such as new colors, altered flower forms, flowers with better fragrance and longer vase life are in high demand by the consumer, who is continually searching for new products. From the grower's

point of view, carnations with improved agronomic performance are no less important. Thus, production yield and resistance to diseases and insects contribute significantly to the establishment of a commercially successful cultivar. To date, new vegetatively propagated carnation varieties have been produced mainly via classical breeding (Zuker et al., 1998). This route of new flower variety production, which involves a great deal of time and space, makes use of two main breeding methods:

1) New varieties are produced by selecting individuals with the most desirable decorative characteristics from a cross between two usually very heterozygous parents. The selected plants are then propagated by cuttings. The heterozygosity of the parent plants ensures high variability in the progeny, but it also necessitates the screening of thousands of plants to obtain a single genotype with desirable characteristics. Subsequent testing of selected genotypes usually takes another 3 to 5 years, to ascertain the suitability of their other horticulturally important traits for marketing.

2) New varieties are produced by mutagenesis, either chemically or by gamma-irradiation. The former is less frequently used because of its lack of precision. Even when successful, the mutagenesis approach usually results in the disruption of non-sporogenic tissues and the formation of chimera.

These two approaches have produced great achievements in the flower industry. Nevertheless they have severe limitations, chief among them being the limited gene pool of a given species, high heterozygocity and the inability to alter single, specific traits. This prevents breeders, for example, from producing plants with disease resistance, novel morphology and flowers in a full spectrum of colors. To partially circumvent this problem they must resort to interspecific crosses, which often result in unwanted changes in many of the characteristics of the originally desired genotype.

New tools for the introduction of foreign genes into plants and the growing knowledge and technology related to gene identification and isolation have enabled the specific alteration of single traits in an otherwise successful cultivar (for review, see Zuker et al., 1998). Furthermore, such developments have enabled a broadening of the available gene pool of a given species. The application of biotechnological approaches, such as genetic engineering, to cut flowers has clearly become instrumental for the floriculture industry, particularly in carnation. However, despite the great progress and interest in gene transfer to these crops, their transformation is considered routine in only a few laboratories. For the most part, its application is still an "art form" (Zuker et al., 1998).

Wilt disease, caused by *Fusarium oxysporum* f. sp. *dianthi*, is a major problem in carnation, and the methods currently used to control this soilborne fungus are very hazardous, as well as ineffective and costly. Although *Dianthus* does exhibit *Fusarium* resistance, selection for that resistance based on phenotype is proving difficult, if not impossible. To date, only resistant genotypes, obtained following extensive and costly screening in infected soil, can successfully compete in the flower market (Ben-Yefet et al., 1993). In the course of this project, we established and fully characterized a highly efficient procedure for carnation transformation and for the regeneration of transgenic plants. The main features of this procedure, which has enabled the generation, from different cultivars, of dozens of transgenic carnation lines expressing a variety of target genes (see attached manuscripts), are its effectiveness, simplicity and suitability to theoretically any gene/genotype. It should be noted that low transformation efficiency is a very serious bottleneck in the production of plants, carnation in particular, with novel traits of interest, since a large number of transgenes must be generated and screened to enable selection of the target genotype. The established transformation procedure has been successfully used by us to generate transgenic carnation with increased *Fusarium* resistance.

Materials and methods

Plant material. Carnation (*Dianthus caryophyllus* L.) plants were grown under standard greenhouse conditions. Cultivars White Sim, Eilat, Darling, Visa and Lior were grown at the Faculty of Agriculture (The Hebrew University of Jerusalem) and Shemi Ltd., and cultivar Desio was received from Mizpor Ltd. (Tquma, Israel). Cultivars White Sim, Desio and Visa belong to the standard category while cv. Eilat, Darling and Lior belong to the spray category. Stem cuttings with six or eight fully mature leaves (not counting the apical leaves which were not fully expanded), harvested from greenhouse-grown plants and stored for up to 1 month at 4°C, were used to prepare stem explants (Zuker et al., 1995).

Media composition and tissue-culture conditions. MS basal medium (Murashige and Skoog, 1962) with sucrose (30 g/l) and solidified with agar (8 g/l) (basic medium), was supplemented with growth regulators and antibiotics for cocultivation with *Agrobacterium*, regeneration and selection of adventitious shoots, and elongation and rooting of transgenic plants. All media were adjusted to pH 5.8 prior to autoclaving (121°C for 20 min). For cocultivation with *Agrobacterium*, the basic medium was supplemented with 5 mg/l α-naphthalene acetic acid (NAA) and 100 μM acetosyringone (cocultivation medium). For shoot regeneration and two-step selection of transformants, the basic medium was supplemented with 0.1 mg/l NAA and 1 mg/l 1-phenyl-3(1,2,3-thiadiazol-5-yl)-urea (TDZ) (SI-T1, first selection cycle), or with 0.1 mg/l NAA and 1 mg/l 6-benzylaminopurine (BAP) (SI-B1, second selection cycle). Both media were also supplemented with 300 mg/l carbenicillin and, unless otherwise stated, 100 mg/l kanamycin. Elongation and rooting of transgenic shoots, following the second selection cycle, were performed on the basic medium containing 0.1 mg/l NAA, 0.1 mg/l gibberellic acid (GA), 200

mg/l carbenicillin and 100 mg/l kanamycin. All cultures were maintained in a growth room at 25±1°C under a 16-h photoperiod using cool white light (60 μmol/m^2s) unless otherwise indicated.

Bacterial strains. *Agrobacterium tumefaciens* strains AGLO (Lazo et al., 1991) and EHA105 (Hood et al., 1993) carrying the binary plasmids pCGN7001 (Comai et al., 1990) and pKIWI105 (Janssen and Gardner, 1989), respectively, were used for transient and stable transformation of carnation, respectively. Both plasmids carried the *uidA* gene coding for β-glucuronidase (GUS) (Stomp, 1992) driven by either a CaMV 35S promoter (pKIWI105) or a mannopine synthetase (MAS) promoter (pCGN7001) (Comai et al., 1990), and the *nptII* gene coding for neomycin phosphotransferase II (NPTII) (Beck et al., 1982) driven by either a nopaline synthase (NOS) promoter (pKIWI105) or the CaMV 35S promoter (pCGN7001). The GUS-encoding gene is not expressed in *Agrobacterium* cells carrying pKIWI105 due to the lack of a bacterial ribosome-binding site, making this plasmid suitable for transient transformation studies (Janssen and Gardner, 1989). Digestion of pCGN7001 with *Eco*RI releases a 3.8-kb fragment containing *uidA* and part of *nptII*, whereas *Hind*III is a unique restriction site within the T-DNA fragment (Comai et al., 1990).

A. tumefaciens strain EHA105, carrying binary plasmids pXBOC or pXBOP which contain a combination of tobacco antifungal genes encoding osmotin and chitinase (pXBOC) or osmotin and PR-1b (pXBOP), was used to generate transgenic carnation with *Fusarium* resistance. Vacuolar targeting sequences were deleted from the antifungal genes driven by the CaMV 35S promoter. The plasmids also contained 35S-driven *nptII* to allow selection of the carnation transgenes.

Bacteria from a single colony were grown at 28°C for ca. 20 h in liquid LB medium (10 g/l bacto-tryptone, 5 g/l bacto-yeast extract, 5 g/l NaCl, 2 g/l glucose, pH 7.5) on a rotary shaker (250 rpm). The medium was supplemented with 100 μM acetosyringone, 50 mg/l rifampicin, and 25 mg/l gentamycin or 50 mg/l kanamycin for pCGN7001 or pKIWI105/pXBOC/pXBOP, respectively. Bacteria (OD_{550} = 0.5) was harvested by centrifugation at 10,000*g* for 2 min; the pellet was resuspended in liquid cocultivation medium (OD_{550} = 0.5 or 1.0), and the suspension was used for inoculation.

Microprojectile bombardment. Leaves and shoot apices were removed from sterilized cuttings and the three primary nodes were wounded prior to inoculation in a Biolistics PDS 1000/He system using tungsten M-25, 1.7 μm in diameter (Bio-Rad) (Zuker et al., 1995). Explants placed on MS basal medium solidified with 1% agar (30 explants per petri dish) were bombarded twice. Each bombardment consisted of 100 μl of tungsten particles in an aqueous suspension (15 mg/ml). The particles were finely dispersed with ultrasonic cleaner (Sonicor Instrument Corporation) before bombardment. Particle acceleration parameters were: a bombardment pressure of 1500 psi and a distance of 9 cm from the launching plate to the tissue.

Optimization of transient transformation. Stem explants with or without prior wounding by microprojectile bombardment were immersed for 10 min in a bacterial (*A. tumefaciens* EHA105/pKIWI105) suspension (OD_{550} = 1). Inoculated stem explants were then blotted dry and cultured in an upright position on the cocultivation medium under various light regimes for a period of up to 5 days. Following cocultivation, stem explants were histochemically evaluated for transient GUS expression by counting the number of GUS-expressing stem explants, as well as the number of blue spots per explant, under a stereo-microscope.

Transformation and regeneration of transgenic plants. Following bombardment-mediated wounding, stem explants were inoculated with bacterial (AGLO/pCGN7001 or EHA/pXBOC or EHA/pXBOP) suspension (OD_{550} = 0.5). During cocultivation and all consecutive steps, explants were cultured in an upright position. After 5 days of culture on the cocultivation medium (3 days in the dark followed by 2 days in THE light), three primary nodes were sectioned into ca. 3-mm slices and transferred to SI-T1 medium for shoot regeneration and the first selection cycle. It should be noted that apical meristem breakage was considered undesirable. Hence, to prevent the development of non-transformed axillary shoots, all identifiable shoot apices were removed from the stem explants prior to inoculation with bacteria. After 10 days of culture, the explants were cleaned again, as needed, of the occasionally developing shoots, cross-sectioned into halves, and transferred to fresh SI-T1 medium. After ca. 2 additional weeks, clusters of regenerated adventitious shoots were excised from the primary stem explants. Leaves from all of the shoots of each independent cluster were pulled off and cultured on SI-B1 medium for adventitious shoot regeneration and selection of transgenes (second selection cycle). After 10 to 12 days, new adventitious shoots emerged from the leaf basal area. These shoots were transferred to elongation and then rooting media and evaluated as to their transgenic nature. Following hardening (Vainstein et al., 1992), transgenic plants were transferred to the greenhouse where they developed and flowered normally. Since cv. White Sim, like most commercial carnation varieties, is male-sterile and can only be out-crossed, GUS-expressing T_0 White Sim plants were crossed with wild-type (non-transformed) red (b.l. 13261), yellow (b.l. 13262) or white (b.l. 13263) male plants to generate T_1 progeny. All crosses were carried out in the fall/winter and seeds were collected and sown in the summer. Seedlings were evaluated for GUS expression and

were subjected to *uidA* and *nptII* PCR analysis. All transformation experiments were repeated at least five times.

Evaluation of transformants. A histochemical assay of GUS activity was performed according to Stomp (1992). Tissue samples were incubated for a few hours to overnight at 37°C in a 0.1% (w/v) X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronic acid sodium salt, Biosynth Inc., Staad, Switzerland) solution containing 0.1 M sodium phosphate buffer (pH 7.0), 10 mM EDTA, and 0.1% (w/v) Triton X-100. When necessary, green tissues were bleached after staining by immersion in 50% (v/v) EtOH for a few hours, followed by several washes with 70% EtOH. It should be noted that no background GUS activity was detectable in any of the analyzed—intact or wounded—tissues of control plants.

DNA extraction, primers for *uidA*, and PCR conditions were as previously described (Tzfira et al., 1997). The primers for *nptII* amplification were 5'-GAGGCTATTCGGCTATGACT-3' and 5'-AATCTCGTGATGGCAGGTTG-3'; for osmotin 5'-ATGGGCAACTTGAGATC-3' and -5'-CTAAGGACAAAAAGATAA CCC-3'; for chitinase 5'-AATGAGGCTTTGTAAATTCACAGC-3' and 5'-TTAATTT CCAAAAGACCTCTGGTT-3'; for PR-1b 5'-CATGGGATTCTTAACAACAAT-3' and 5'-TTAAAAGGGACGTTGTCCTATAAA-3'. Amplified DNA was electrophoresed on a 1.5% (w/v) agarose gel, using Tris-borate buffer (1.3 M Tris, 0.7 M boric acid and 24.5 mM EDTA, pH 8.4). Gels were stained with ethidium bromide, photographed under ultraviolet light, and analyzed by Southern blotting.

DNA was extracted from leaves by the CTAB (cetyltrimethylammonium bromide) procedure described previously (Tzuri et al., 1991). DNA (10 μg) was digested with *Hind*III or *Eco*RI and electrophoresed in 1% (w/v) agarose gels. DNA was transferred to a nylon membrane (Hybond

N^+, Amersham) by capillary blotting as previously described (Maniatis et al., 1982). *Pst*I fragments of 2.6 kb and 1.9 kb from the binary plasmid p35SGUSINT (Vancanneyt et al., 1990) served as probes for *uidA* and *nptII*, respectively. The probes were ^{32}P-labeled by random priming (Feinberg and Vogelstein 1984), denatured and added to the hybridization mixture. Pre-hybridization and hybridization were performed as previously described (Ben-Meir and Vainstein, 1994) at 65°C for 3 h and 18 h, respectively. Post-hybridization washes consisted of two high-stringency washes in 0.45 M NaCl, 0.045 mM sodium citrate, 0.1% (w/v) SDS, 65°C, for 20 min each, followed by one wash in 0.15 M NaCl, 0.015 mM sodium citrate, 0.1% SDS, 65°C, for 20 min. The blots were exposed to an imaging plate (Fujix Bas 1000, Fuji, Japan) for 2-7 h. The plate was then read in an imaging plate reader (Fujix Bio Imaging Analyzer Bas 1000). Two washes of 0.6 M NaCl, 0.06 mM sodium citrate, 0.1% SDS, 65°C, for 20 min each, were added prior to the three post-hybridization washes when Southern blots were performed with the PCR products.

To determine the resistance of control and transgenic carnation lines to *Fusarium oxysporum* f. sp. *dianthi*, race 2, greenhouse tests were performed (20 plants per line) and disease incidence (% of diseased plants out of total analyzed) and disease severity (evaluated on a scale of 0 to 4, 0 = healthy plants, 4 = completely wilted plants) evaluated as described previously (Ben-Yefet et al., 1993). Total protein isolation from carnation leaves and western blot analyses of osmotin were performed as in Vishnevetsky et al. (1999).

Results

Optimization of transient transformation

uidA (GUS) reporter gene expression was used to monitor early transformation events in carnation stem explants. Preliminary experiments testing different wounding methods (vortexing of stem explants in the presence of glass beads, sand or carborundum particles, or poking and scratching with a needle or scalpel) yielded neither efficient nor reliable transient transformation following inoculation (with or without vacuum infiltration) with *Agrobacterium* (Ahroni, 1996). In contrast, when explants were wounded by microprojectile bombardment, efficient and highly reproducible transient transformation was obtained, based on both the percentage of GUS-expressing inoculated explants and the frequency of transformation events per explant. Following a 5-day cocultivation with EHA105/pKIWI105 under constant light, 90% of the cv. White Sim stem explants expressed GUS when they had been wounded by bombardment, as compared to only 20% GUS-expressing explants when wounding had been effected by poking or scratching. The transient frequency events (the number of blue spots per explant) increased 7.5-fold when bombardment-mediated wounding of explants was employed (Fig. 1). It should be noted that cocultivation with *Agrobacterium* for less than 5 days yielded lower transformation efficiencies (data not shown).

Different light conditions during cocultivation also strongly affected the efficiency of transient transformation. An almost threefold increase in the number of transformation events per explant was obtained when non-bombarded explants were cocultivated in the dark instead of under constant light (Fig. 1). When bombardment-mediated wounding was combined with etiolation during cocultivation, the frequency of transformation events per explant increased to over 10-fold that of explants wounded by other means and cocultivated in the light (Fig. 1). However, the relatively long etiolation period led to decreased regeneration from stem explants in both *Agrobacterium*-inoculated and noninoculated (control) stem explants. To restore these explants' high regeneration potential (Zuker et al., 1995) while preserving the positive effect of

etiolation on transformation, the effect of different dark/light regimes on transient GUS transformation and regeneration efficiencies was assessed. When stem explants were cocultivated with *Agrobacterium* for 3 days in the dark then 2 days in the light, the high regeneration efficiency of the explants was restored, reaching the level of those cocultivated for 5 days in the light. Moreover, transformation efficiency was not affected relative to explants cocultivated for 5 days in the dark (cv. White Sim in Figs. 1 and 2).

To assess the suitability of the combined wounding and dark/light regime to other carnation cultivars, transient transformation frequencies in five varieties representing both standard and spray categories were analyzed. All five were susceptible to transformation with EHA105/pKIWI105 under the aforementioned conditions. For all cultivars, 80-90% of the inoculated explants expressed GUS, albeit with some variation in the frequency of transformation events per stem explant (Fig. 2). Further experiments were performed using this combination of wounding and inoculation/cocultivation conditions.

Stable transformation and regeneration of transgenic plants

To improve the effectiveness of kanamycin for transgene selection (Zuker et al., 1998), stable transformation of cv. White Sim was performed with pCGN7001 which carries 35S-driven *nptII*, rather than the NOS-driven *nptII* of pKIWI105 used in the transient transformation experiments. When inoculation was performed with a high concentration of bacteria ($OD_{550} \geq 1$), their extensive growth prevented the establishment of aseptic cultures, even at high concentrations of antibiotics, and negatively affected adventitious shoot regeneration. Inoculation of explants with bacteria at an OD_{550} of 0.5 was optimal, allowing controlled bacterial growth with no adverse effect on the further tissue culture and regeneration of plantlets following transfer to the regeneration/selection SI-T1 medium.

After ca. 1 month in culture following inoculation, adventitious shoot clusters, regenerated directly from sectioned stem explants, were easily scorable (Fig. 3). Interestingly, while all three internodes showed high regeneration ability in aseptic tissue culture (Zuker et al., 1995), only the two top internodes retained this potential following inoculation and selection. Histochemical evaluation of regenerated clusters revealed a chimeric GUS expression pattern in most of the adventitious shoots (Fig. 3), despite high selection pressure (100 mg/l kanamycin) during regeneration; only 1 to 3% of all regenerated shoots expressed GUS in and throughout all analyzed tissues. Since the application of a higher kanamycin concentration (120 and 150 mg/l) in the first selection cycle led to reduced shoot regeneration while transformed plants remained mostly chimeric in nature (data not shown), a second selection cycle was performed to eliminate the chimeric plants. Leaves originating from individual clusters were cultured, separately for each cluster, on SI-B1 medium to eliminate the possibility of generating transgenes representing a single transformation event. After ca. 2 weeks of the second selection cycle, two-thirds of the independent clusters selected on 100 mg/l kanamycin yielded scorable shoots (Table 1). These adventitious shoots regenerated directly from the basal part of the leaves, and only from an area which remained green under kanamycin selection (Fig. 3D). In almost all of these shoots, histochemical assay revealed GUS expression in all organs and throughout the tissues, with no observable chimerism (Table 1, Fig. 3E,F). To assess the overall efficiency of the two cycles of selection, only one GUS-expressing shoot per individual cluster was counted, even though 5 to 20 GUS-expressing shoots were usually generated from leaves of each cluster. Based on this consideration, which allows an estimation of independent transformation events, the overall yield of the procedure was 19 GUS-expressing shoots generated per 100 *Agrobacterium*-inoculated stem explants.

The possibility of further increasing the overall yield by lowering the selection pressure during the first selection cycle was assessed (Table 1). Following cocultivation, stem explants were cultured on SI-T1 medium containing 80 and 90 mg/l kanamycin instead of 100 mg/l. The lowest kanamycin concentration yielded ca. three times more and 90 mg/l kanamycin ca. two times more shoot clusters relative to the number regenerated on 100 mg/l kanamycin. However, only ca. one-third of the clusters generated at both 80 and 90 mg/l kanamycin yielded adventitious shoots from leaves following the second selection cycle. Thus, while the overall number of independent GUS-expressing shoots was essentially the same for the three levels of kanamycin analyzed, 100 mg/l was considered optimal because it almost completely prevented the generation of escapees (Table 1).

Forty randomly selected independent, kanamycin-resistant, GUS-expressing T_0 cv. White Sim plants exhibited a normal phenotype when, following hardening, they were grown to flowering in the greenhouse. Progeny generated from an outcross of the T_0 White Sim lines also expressed GUS and flowered normally (Fig. 3G,H).

To further confirm the transgenic nature of the generated kanamycin-resistant GUS-expressing plants, Southern blot analysis was performed. Hybridization of *Eco*RI-digested genomic DNA from T_0 lines with *uidA* probe yielded the expected 3.8 kb fragment (Fig. 4, lanes 1-4); this fragment was not detectable in the non-transformed control line (Fig. 4, lane C). Two to four fragments of different sizes were revealed following digestion of T_0 genomic DNA with *Hind*III (Fig. 4), thus confirming integration of the GUS-encoding gene construct in the plant genome. Integration of the *nptII* gene into the T_0 plant genome was also confirmed by Southern blotting using *nptII* as a probe (data not shown). The molecular analysis of seedlings derived from one of the crosses between T_0 plants and non-transgenic breeding line (b.l.) 13261 is shown in Fig. 5. *nptII* PCR amplification followed by Southern blotting using *nptII* as a probe yielded a

DNA fragment of the expected size (0.8 kb) in all analyzed kanamycin-resistant T_1 seedlings (Fig. 5) and not in controls (Fig. 5, lane C). Similarly, *uidA* PCR amplification of these T_1 seedlings followed by hybridization with *uidA* probe yielded a DNA fragment of the expected 0.53 kb in all cases (data not shown).

The applicability of the transformation procedure was also assessed with another two, genetically unrelated, commercially highly successful cultivars—Desio and Eilat, which had been used in transient transformation experiments. The efficiency of selection for these varieties was essentially identical to that detailed for cv. White Sim and overall transformation efficiencies of 13% and 18% (transgenes out of total inoculated explants) were obtained for cvs. Desio and Eilat, respectively. The transgenic nature of these plants was confirmed by Southern blot analysis (data not shown). Interestingly, the overall efficiencies of stable transformation for the three analyzed varieties were in good agreement with the frequency of transient transformation events (see Fig. 2).

With the aim of generating *Fusarium*-resistant carnation, cvs. White Sim and Eilat were transformed with pXBOC (osmotin+chitinase) and pXBOP (osmotin+PR-1b). Cv. White Sim is highly susceptible to *Fusarium* while cv. Eilat is moderately resistant (Ben-Yefet et al., 1993). Putative transgenes were selected and following hardening were propagated in the greenhouse. The transgenic nature of the kanamycin-resistant plants was assessed by PCR analyses using primers specific to osmotin, chitinase and PR-1b genes. Both osmotin+chitinase and osmotin+PR-1b plants (outcome of transformation with pXBOC and pXBOP, respectively) yielded amplified DNA fragments not present in control carnation plants (Figs. 6,7). Western blot analyses of osmotin in osmotin+chitinase plants (Fig. 8) further confirmed their transgenic origin.

Carnations transgenic for osmotin+chitinase were propagated in the greenhouse via cuttings and evaluated for resistance to *Fusarium*. Selected transgenic lines of cv. White Sim showed a high level of resistance to a major carnation pathogen (*Fusarium oxysporum* f. sp. *dianthi*, race 2) following greenhouse testing (Figs. 9,10). As early as 4 weeks after inoculation, over 90% of the control plants showed strong symptoms, whereas disease symptoms in the transgenic lines were apparent in only 16% of the plants. Furthermore, relative to control plants, disease symptoms in the transgenic lines were markedly less severe. Following further growth in the contaminated soil, disease resistance in the transgenes was broken down. In cv. Eilat transgenic for osmotin+chitinase no increase in resistance was observed following greenhouse tests (not shown). Selected lines transgenic for osmotin+chitinase are currently being evaluated for *Fusarium* resistance under field conditions and those transgenic for osmotin+PR-1b are being evaluated under greenhouse conditions.

Conclusions

Three experimental conditions were combined in the present study to produce an efficient carnation transformation procedure: (1) the use of microprojectile bombardment to wound stem explants, (2) a specific dark/light regime to optimize transformation, and (3) an optimized two-cycle selection procedure. Microprojectile bombardment, known mainly as a tool for direct gene transfer (Klein and Fitzpatrick-McElligott, 1993), has been shown in a few recent reports to also be an efficient tool for wounding plant tissue prior to *Agrobacterium*-mediated transformation (Bidney et al., 1992). We found microprojectile bombardment to be very important for the effective generation of transgenes from stem explants. In fact, following microprojectile wounding, almost 90% of the inoculated stem explants were transformed at high frequency. The observed efficiency and reproducibility may be due mainly to the microprojectiles' high velocity

and small size, as well as their relatively uniform spreading in the bombarded tissues (Klein and Fitzpatrick-McElligott, 1993).

Cocultivation of microprojectile-wounded explants with *Agrobacterium* in the dark vs. light led to a further increase (ca. 150%) in the frequency of transient transformation. Etiolation may improve the transformation efficiency of a number of plant systems (lime, *Phaseolus*, *Gladiolus*, *Anthurium*) via *Agrobacterium* activation and/or by increasing the susceptibility of the plant tissue to infection (Chapel and Glimelius, 1990; Chen and Kuehnle, 1996; Kamo, 1997; Pena et al., 1997; Zhang et al., 1997). However, prolonged (over 3 days) etiolation of carnation stem explants led to a severe reduction in their regeneration potential and did not enhance transformation frequency.

Direct regeneration is known to lead to reductions in both chimeric plant production and somaclonal variation, as compared to organogenesis via callus. Nevertheless, even with direct shoot regeneration, undesired chimeric plant production remains a common problem in transformation procedures (Dong and McHughen, 1993; Laparra et al., 1995; Mathews et al., 1995; Yao et al., 1995). On the other hand, Firoozabady et al. (1995) have suggested actually aiming for chimeric plant production in order to improve the yield of solid transgenes. The authors used 2,4-D to induce callus formation and to delay adventitious shoot regeneration from primary leaf explants, in order to allow additional cell division prior to the organization of new meristem. Moreover, they selected their plantlets first using sublethal levels of chlorosulfuron; this was followed by a second selection cycle to generate solid transgenes from the chimeric plantlets. Chlorosulfuron was used instead of the more common kanamycin because selection on the latter yielded mostly escapees (Firoozabady et al., 1995). In contrast, using different primary explants—stem explants—and a direct regeneration approach, we found kanamycin to be highly suitable as a selection agent, yielding—under optimal conditions—90% GUS-expressing

plantlets of the total generated following the second selection cycle. Moreover, reducing kanamycin concentration to sublethal levels during the first selection cycle was not beneficial, because it led to a higher number of escapees while not affecting the final yield of transgenes.

An overall transformation efficiency of 1-2 transgenes per 10 explants was obtained for the three varieties analyzed, when optimal conditions (for wounding of the explants, light regime during cocultivation, and selection) were employed. It should be noted that to date, several dozen transgenes have been produced following this procedure (data not shown). Since almost no escapees were generated (9 out of 10 kanamycin-selected plantlets expressed GUS), this procedure relies almost exclusively on the selectable marker gene's activity and not on that of the scorable reporter gene. As such, this procedure is instrumental for introducing new traits of interest into carnation, since a relatively high number of transgenes needs to be generated and screened to obtain a few desirable features.

The established transformation procedure was used to improve an agronomic trait, i.e. fungal resistance in carnations. To generate fungal resistance, transgenic carnation with osmotin, PR-1 and/or chitinase genes were produced. A high level of resistance in these transgenes to a major carnation pathogen (*Fusarium oxysporum* f. sp. *dianthi*, race 2) was demonstrated in greenhouse tests. It should be noted that enhancement of resistance was successful in the susceptible variety (cv. White Sim), while no further increase in resistance was obtained in the moderately resistant cv. Eilat. Pending satisfactory results from the field test, which is currently under way, it may be possible in the future to introduce resistance into commercially promising but wilt-disease susceptible carnation cultivars.

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Table 1. Efficiency of selection of transformed carnation plants. Stem explants of cv. White Sim were transformed with *Agrobacterium tumefaciens* AGLO/pCGN7001 as described in the experimental protocol.

	First selection cycle	Second selection cycle (100 mg/l kanamycin)	
Kanamycin (mg/l)	No. of selected clusters *	No. of clusters generating shoots **	No. of GUS-expressing shoots ***
80	92 ± 35	29 ± 1 (32)	17 ± 1 (59)
90	65 ± 4	25 ± 2 (38)	20 ± 1 (80)
100	32 ± 4	21 ± 1 (66)	19 ± 1 (90)

* The number of harvested independent shoot clusters regenerated from a total of 100 stem explants, following the first selection cycle.

** The number of independent clusters that generated shoots from leaves following the second selection cycle. The numbers in brackets represent percentages of independent clusters generating shoots from leaves following the second selection cycle out of the total number of independent clusters selected in the first selection cycle.

*** The number of independent clusters generating GUS-positive shoots from leaves following the second selection cycle. The numbers in brackets represent percentages of independent clusters generating GUS-positive shoots from leaves out of the total number of independent clusters generating shoots following the second selection cycle.

Figure 1. Effect of wounding and cocultivation on the efficiency of transient transformation of cv. White Sim stem explants. Explants were wounded by bombardment (+) or just poking/scratching and cocultivated with EHA105/pKIWI105 for 5 days under constant light or in the dark. GUS expression is presented as the number of blue spots per stem explant. S.E. of the mean ($p = 0.05$) is indicated ($n = 5$).

Figure 2. Transient transformation frequencies of different carnation cultivars. Following bombardment, stem explants were cocultivated with EHA105/pKIWI105 for 3 days in the dark and 2 days under constant light as described in Materials and Methods. GUS expression is presented as the number of blue spots per stem explant. S.E. of the mean ($p = 0.05$) is indicated ($n = 5$).

Figure 3. Transformation and regeneration of transgenic carnation plants. (A) GUS expression on the cut surface of a stem explant 14 days after inoculation. (B) Shoot regeneration from a stem explant. (C) The chimeric pattern of GUS expression following the first selection cycle. (D) Second selection cycle of adventitious shoots. Shoots developed from the leaf area which showed resistance to kanamycin, as reflected by its green color. The white, non-resistant area remained non-regenerative (arrow). (E,F) Solid, non-chimeric GUS expression in adventitious shoots regenerated from leaves following the second selection cycle. (G) Transgenic flowers. (H) GUS-expressing seedlings (T_1) obtained from crosses of transgenic cv. White Sim with non-transformed (b.l. 13261/2/3) male plants.

Figure 4. Southern blot analysis of DNA from four independent transgenic (1-4) and one non-transformed (C) cv. White Sim carnation plants. Total DNA (10 μg) was digested with *Eco*RI

(left) or *Hind*III (right) and hybridized with a *uidA* probe. (P) Plasmid pCGN7001 digested with *Eco*RI.

Figure 5. Southern blot of PCR analysis of offspring (T_1). DNA for *nptII* PCR analyses was prepared from GUS-expressing kanamycin-resistant T_1 progeny plants (T_0 cv. White Sim x b.l. 13261); T_0 plants; control, non-transformed plants (C); and plasmid pCGN7001 (P). Following *nptII*-PCR amplification and gel electrophoresis, the products were hybridized with *nptII* probe.

Figure 6. PCR analyses of genes coding for osmotin (A) and chitinase (B) in transgenic carnation. DNA was isolated from leaves of osmotin+chitinase transgenic and control carnation lines and analyzed by PCR using specific primers as described in Materials and Methods. M – molecular weight marker (*Hind*III+*Eco*RI-digested λ DNA), C – control carnation plant, P – pXBOC, and numbered lanes indicate independent selected lines transgenic for osmotin+chitinase.

Figure 7. PCR analyses of genes coding for osmotin (A) and PR-1b (B) in transgenic carnation. DNA was isolated from leaves of osmotin+PR-1b transgenic and control carnation lines and analyzed by PCR using specific primers as described in Materials and Methods. M – molecular weight marker (*Hind*III+*Eco*RI-digested λ DNA), C – control carnation plant, P – pXBOP, and numbered lanes indicate independent selected lines transgenic for osmotin+PR-1b.

Figure 8. Western blot analysis of osmotin expression in transgenic carnation. Total protein was isolated from leaves of osmotin+chitinase transgenic and control carnation lines, separated by SDS-PAGE and analyzed using osmotin antisera as described in Materials and Methods. C –

control carnation plant; numbered lanes indicate independent selected lines transgenic for osmotin+chitinase.

Figure 9. Fusarium (*Fusarium oxysporum* f. sp. *dianthi*, race 2) resistance in transgenic vs. control carnation plants. Disease incidence and severity in two independent selected carnation lines transgenic for osmotin+chitinase (1,2) and control carnation (C) were assessed in the greenhouse as described in Materials and Methods.

Figure 10. Resistance of carnation transgenic for osmotin+chitinase to *Fusarium oxysporum* f. sp. *dianthi*, race 2. Greenhouse evaluation of resistance was performed as described in Materials and Methods. From left: two independent osmotin+chitinase transgenic lines and control carnation.

Figure 1

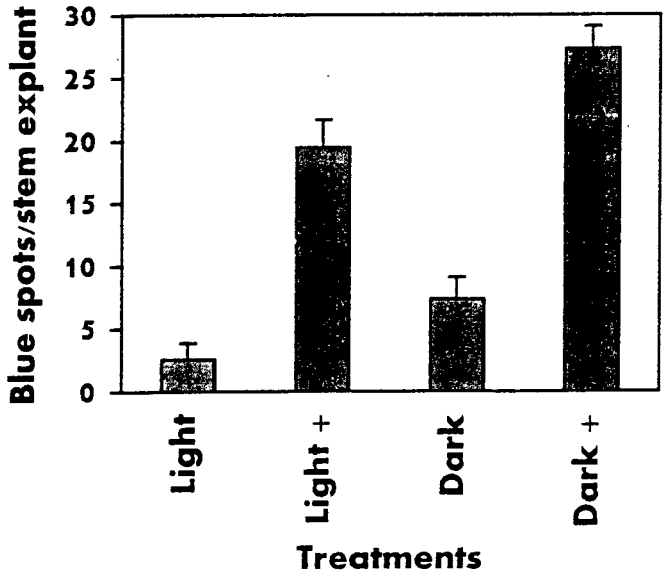

Blue spots/stem explant
30
25
20
15
10
5
0
Light
Light +
Dark
Dark +
Treatments

Figure 2

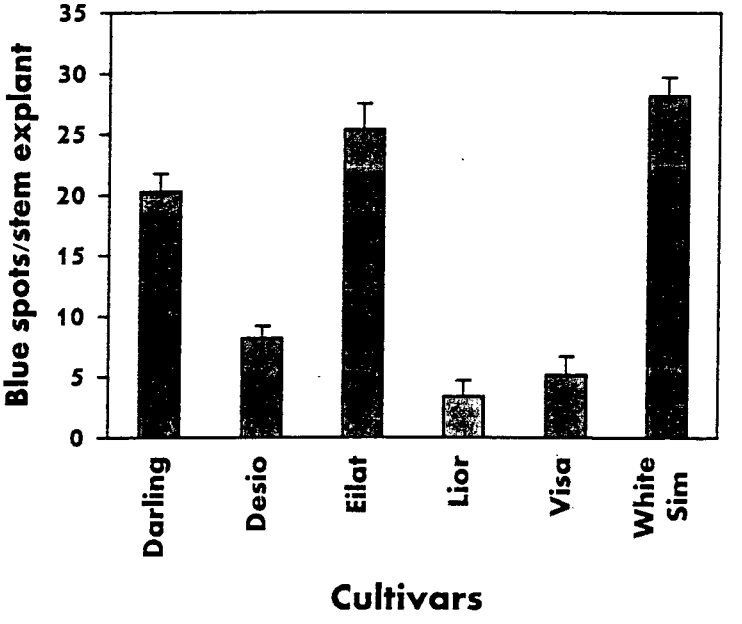

Blue spots/stem explant
35
30
25
20
15
10
5
0
Darling
Desio
Eilat
Lior
Visa
White Sim
Cultivars

Figure 3

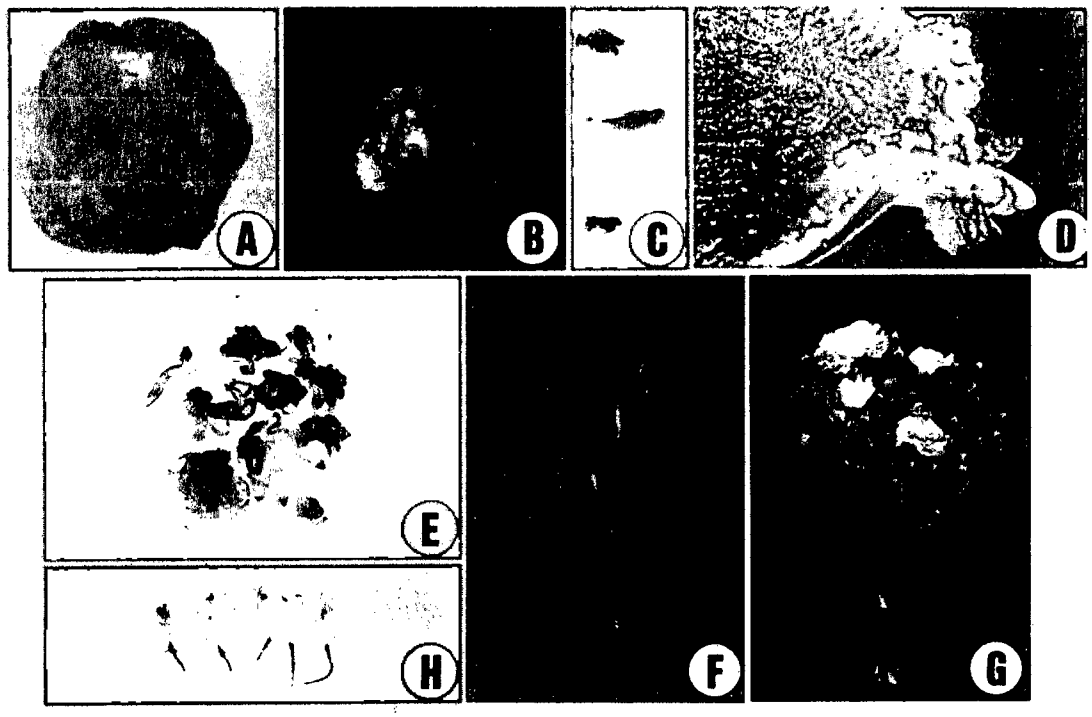


Figure 4

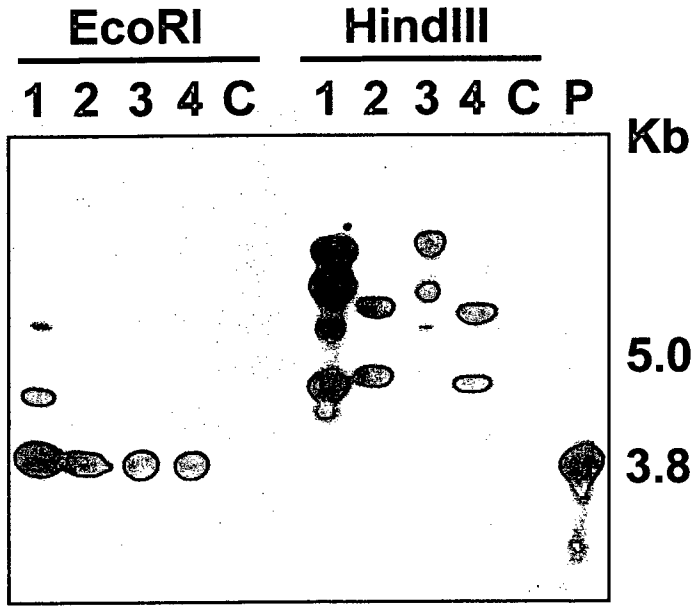


Figure 5

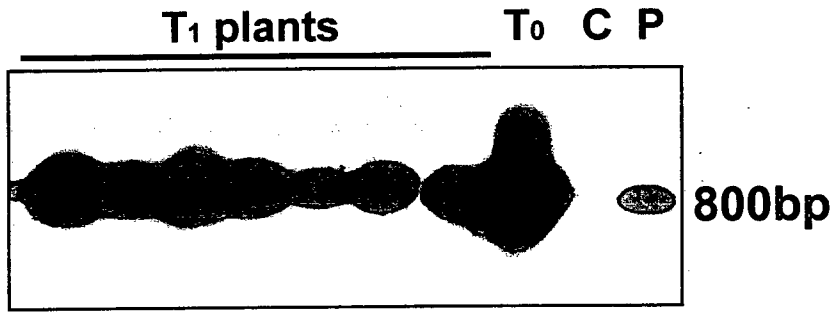


Figure 6

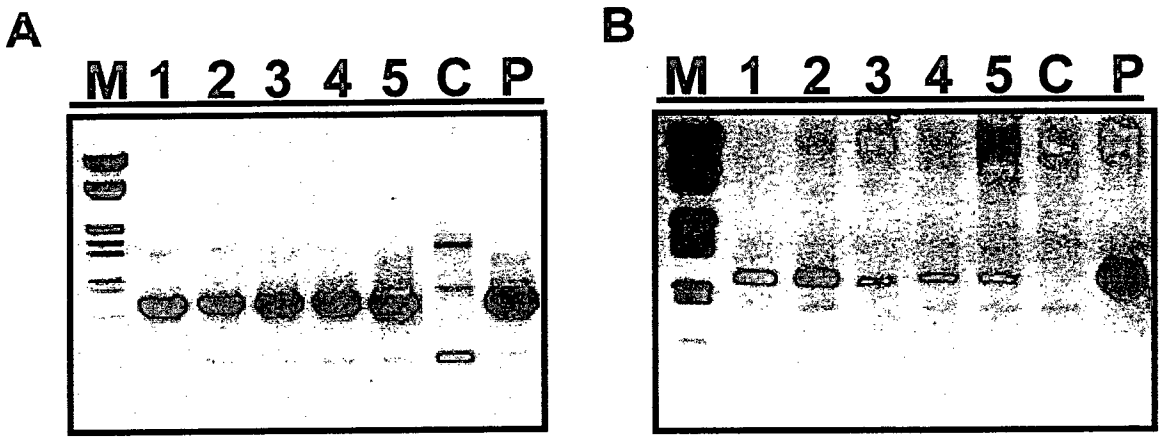


Figure 7

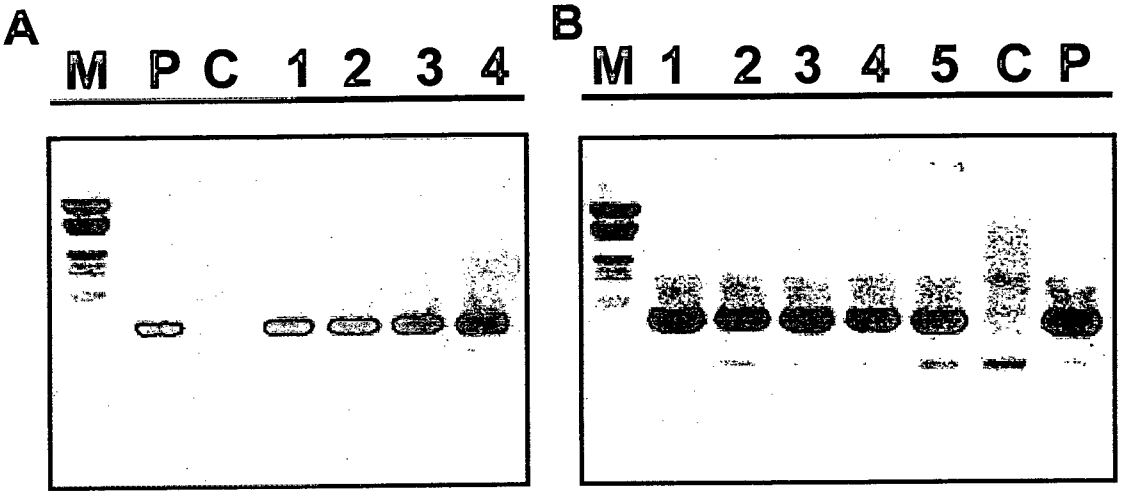


Figure 8

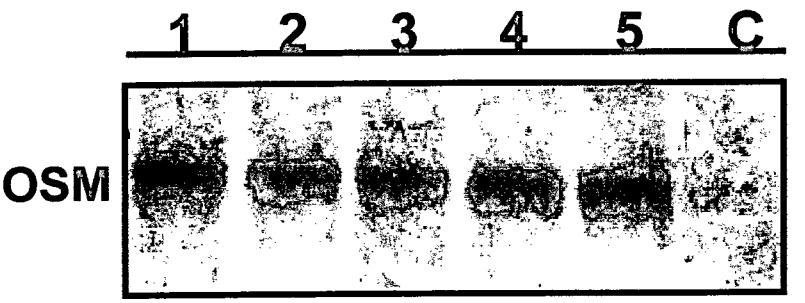
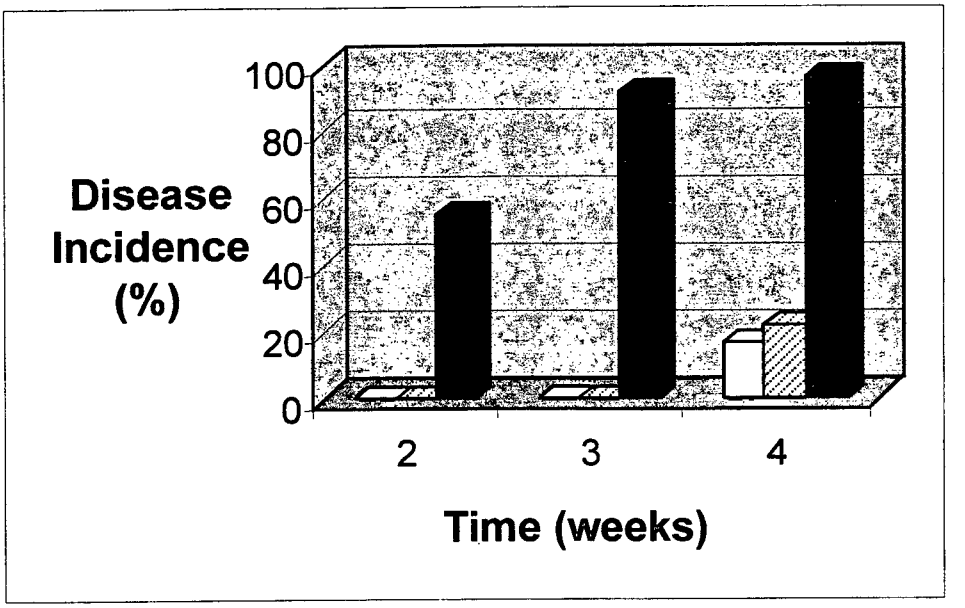
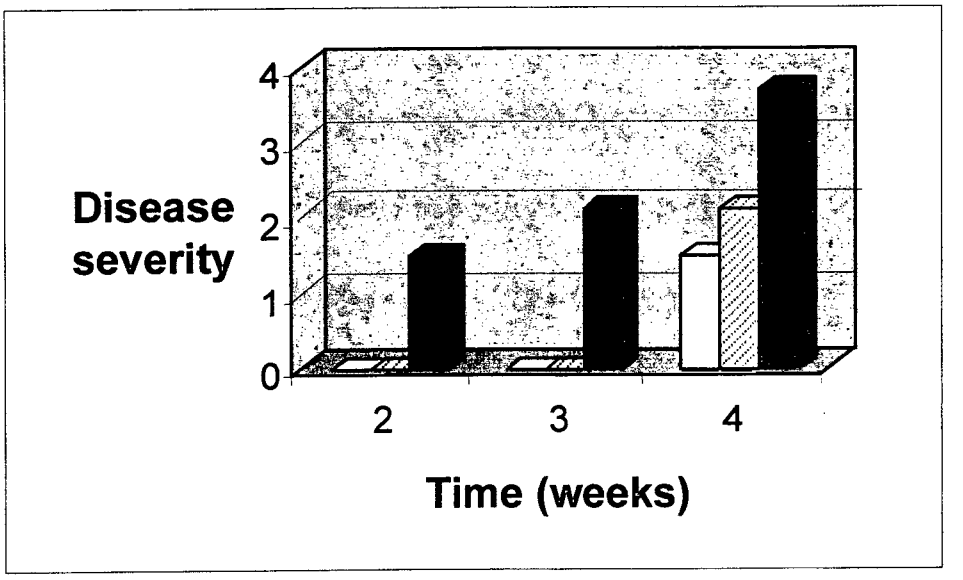


Figure 9


Disease Incidence (%)
100
80
60
40
20
0
2
3
4
Time (weeks)


Disease severity
4
3
2
1
0
2
3
4
Time (weeks)

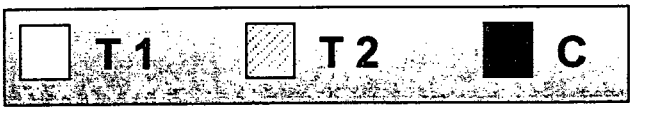

T 1
T 2
C

Figure 10

Resistance of transformed carnation lines towards *Botrytis cinerea*

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Abstract

Flowers of transformant carnation lines from two cultivars were tested for their susceptibility to *Botrytis cinerea* by challenging flower petals with conidial suspensions of the pathogen. Eilat lines showed resistance to *B. cinerea* although not in all cases. White Sim lines did not show consistent performance of resistance and in some cases showed increased susceptibility. The rate of germination of *B. cinerea* conidia on the petals was not affected by the transformation but the germ-tube elongation of these conidia was markedly affected by the transformation. Therefore, the resistance although not always manifested is probably associated with an inhibition of the growth of the pathogen.

Introduction

Carnation plant material was transformed to carry chitinase and osmotin genes. The transformants were grown and their flowers were tested for resistance to infection by the pathogenic fungus *Botrytis cinerea. B. cinerea* is a pathogen of many crops including several ornamentals. On carnation it causes Botrytis flower blight and gray mold. The life cycle of this pathogen includes the landing of conidia on plant surfaces, germination of conidia which readily occur in a film of water that contains nutrients, penetration into the host tissue and its maceration and the formation of conidia on conidiophores over the rotted plant organ. The present research

focussed on the resistance against germination of conidia, penetration into the host tissue and formation of rot lesion on carnation flowers.

Materials and Methods

Plants

Plants of carnation (*Dianthus caryophyllus*) were grown in pots. During 1998-1999 flowers were harvested with 30-50 long stem and immediately placed in a vase containing water and maintained in room temperature. Wild type plants of cultivars Eilat and White Sim were always sampled when the transformants of these cultivars were sampled. Petals of the flowers were detached in order to test their susceptibility to *B. cinerea.*

Pathogen and infection

Botrytis cinerea Pers.:Fr. Isolate BC I 16 was grown on potato dextrose agar at 20 °C. Conidia from 12-14 day-old cultures were suspended in water containing 0.01% Tween 80 to give 10^5 cells / l. Inoculum for infection consisted of the conidial suspension supplemented with 0.05% glucose and 0.05% KH_2PO_4. Suspension drops of 20 µl were used for inoculation of carnation detached petals. The petals were placed on a plastic grid at the bottom of a box (15x25x40 cm) that was placed in a transparent polyethylene bag to ensure high relative humidity (>95% at 20 °C). Disease was evaluated according to a scale of 0-100% where 100% stems for a fully developed lesion under the inoculation drop.

In some experiments inner petals were compared with outer petals or petals from older flowers were compared with petals from young flowers. However, if not otherwise mentioned, outer petals from flowers of harvesting stage were used throughout. Severity of symptoms on inoculated petals was determined according to percentage out of the area of a fully developed lesion (25 mm^2). Disease development was followed 5-10 days post inoculation and disease progress curves were drawn.

The germination of conidia of *B. cinerea* in the water drops on the surface of carnation petals was determined microscopically after 20 h of incubation. Petals bearing the suspension were placed on glass slides, dyed with aniline blue, incubated in room temperature for 5 min. and

observed under the microscope. Percent of germination and germ tube length were recorded for samples of 50 conidia each.

Inoculation of whole flower buds was carried out with a suspension similar to the mentioned above by spraying fine droplets at a volume of 0.3 ml/ flower. Flowers were placed in a vase and incubated in a humidity chamber at 20 °C for up to 10 days. Disease severity was evaluated according to the percentage of rot that developed on the flower buds.

Calculations

Ten to twenty petals were used as replicates for each flower type. Data from *B. cinerea* susceptibility testing were analyzed using the standardized area under the disease progress curve (AUDPC); i.e. the calculated area divided by the number of days the tests lasted. Percent of disease severity of a certain transformant out of disease severity of a wild type plant was calculated using the standardized AUDPC. Similar comparison was performed for the rate of germination after arcsin transformation of the data and for the germ-tube length. Treatments (carnation line) were compared statistically using Fisher's protected least significant differences.

Results and conclusions

Experiments with flowers of carnation were carried out 17 times. For each experiment flowers of at least one cultivar and of at least one transformant were tested. The petals were evaluated for their susceptibility to *B. cinerea*. In most cases disease in the wild type petals developed to a severity of 50-100% within 5-7 days. The results obtained with flowers of cultivar Eilat are described in table 1 and those obtained with cultivar White Sim are described in Table 2. Examples of disease development and calculations of AUDPC and AUDPC/day are given in Figs. 1-3. Table 3 describes the germination of *B. cinerea* conidia on the carnation petals of the different lines of both carnation cultivars.

Four lines transformed Eilat lines were tested for resistance. Out of these lines E-1 and E-4 were tested 12-13 times. These lines showed resistance in 58-69% of the tests performed on outer petals. Resistance was found in 50% and less cases when inner petals were used for the bioassay. The two other lines were tested only 1-2 times and showed resistance (Table 1). Cases of resistance of lines E-1 and E-4 are presented in Figs. 1-3.

Two lines of transformed White Sim lines were tested for resistance. Outer petals of line WS-5 were tested in 18 cases and results were confusing. In 8 cases significant higher susceptibility was found and in 3 cases resistance was found. Similar situation was found on inner petals. Line WS-6 was tested in 20 cases out of which outer petals showed higher susceptibility in 9 cases and resistance in 3 cases (Table 2).

Germination of *B. cinerea* conidia on petals of flowers that were also used for disease resistance tests in experiment 12 was at the rate between 75-100% on wild type petals and 50-100% on petals of the transformed lines. In spite of the fact that the Eilat lines showed resistance in the parallel test (Experiment 12, Table 1), this was not associated with a decrease of conidial germination (except for one case out of the four cases). No significant increase or decrease in percent of germination was observed on the White Sim lines (Table 3) in spite of the fact that disease was significantly higher in this cultivar (experiment 12, Table 2). Germ-tube length was decreased in most cases and on lines of both cultivars (Table 3).

It may be concluded that Eilat lines show resistance to *B. cinerea* although not in all cases. White Sim lines did not show consistent performance of resistance. The rate of germination of *B. cinerea* conidia on the petals was not affected by the transformation but the germ-tube elongation of these conidia was markedly affected by the transformation. Therefore, the resistance although not always manifested is probably associated with an inhibition of the growth of the pathogen.

Table 1: Disease level on transformed Eilat flowers as compared with wild type flowers

Exp. No.	Flower age[a]	Petals position[b]	WT disease (AUDPC/days)[c]	Transfor-mant No.	Percentage (Transformant/WT)[d]
1	Young	Outer	34.6	E-1	58.6 **
2	Young	Outer	26.3	E-1	57.4**
3	Young	Outer	51.5	E-2	74.3*
				E-4	98.1
5	Young	Outer	25.2	E-4	106.6
6	Young	Outer	7.5	E-4	65.3*
	Mature	Outer	45.3	E-4	95.4
	Old	Outer	25.4	E-4	62.3*
7	Young	Outer	14.94	E-1	123.4
		Inner	29.8	E-1	35.7**
7	Mature	Outer	17.4	E-1	111.4
		Inner	11.0	E-1	111.3
8	Mature	Outer	55.8	E-1	55.8*
		Inner	75.7	E-1	75.7
9	Young	Outer	19.3	E-1	64.7*
	Mature	Outer	26.6	E-1	57.89*
				E-4	104.5
	Mature	Inner	38.0	E-1	76.8*
				E-4	121.1
11	Young	Outer	28.6	E-1	101.7
				E-4	46.7**
		Inner	47.7	E-1	95.2
				E-4	27.3**
12	Young	Outer	23.9	E-1	76.1*
				E-4	71.0*
		Inner	21.6	E-1	135.1*
				E-4	75.17
13	Young	Outer	19.3	E-1	80.5

				E-4	110.4
		Inner	28.3	E-1	85.7
				E-4	96.8
14	Young	Outer	28.1	E-1	52.9*
				E-4	46.3**
	Mature	Outer	25.3	E-1	75.0*
				E-4	76.3*
15	Young	Outer	12.7	E-1	114.1
				E-4	71.7*
	Mature	Outer	15.9	E-1	53.4*
				E-4	38.4**
16	Young	Whole flower	34.1	E-1	129.3*
				E-4	124.2
	Mature	Whole flower	49.8	E-1	121.5
				E-4	106.1
17	Young	Outer	29.7	E-4	45.5**
				E-4	20.6**
				E-6	6.5**
				E-6	69.7*

[a] Flowers sampled at age of commercial harvest (young) or at more mature stage (open flowers).

[b] Petals were sampled from the outer, more mature petals or from the inner, younger petals.

[c] Disease severity of wild type flowers expressed as standardized AUDPC (Area Under Disease Progress Curve derived by the experiment incubation time in days)

[d] Percentage of severity level obtained on transformed flowers out of the disease on WT flowers (standardized AUDPC of transformant divided by standardized AUDPC of WT and multiplied by 100. * = transformant is significantly different from the WT, $P \leq 0.05$; ** = transformant is significantly different from the WT, $P \leq 0.01$.

Table 2: Disease level on transformed White Sim flowers as compared with wild type flowers

Exp. No.	Flower age[a]	Petals position[b]	WT disease (AUDPC/days)[c]	Transfor-mant No.	Percentage (Transformant/WT)[d]
2	Young	Outer	5.0	WS-5	141.8*
				WS-6	141.1*
4	Young	Outer	7.9	WS-5	127.1
				WS-6	162.8*
5	Young	Outer	30.5	WS-5	69.0*
				WS-6	93.3
6	Young	Outer	78.6	WS-6	3.3**
7	Young	Outer	5.2	WS-5	121.3
				WS-6	213.8**
		Inner	39.9	WS-5	74.0*
				WS-6	130.2
7	Mature	Outer	25.9	WS-5	168.5*
		Inner	33.7	WS-5	97.5
8	Mature	Outer	13.4	WS-5	76.7
				WS-6	109.1
		Inner	14.0	WS-5	123.5
				WS-6	124.7
9	Young	Outer	32.4	WS-5	44.4**
				WS-6	32.7**
	Mature	Outer	17.6	WS-5	161.4*
10	Young	Outer	22.0	WS-6	160.5**
	Mature	Outer	31.6	WS-6	119.6*
				WS-6	147.5*

11	Young	Outer	25.6	WS-5	113.2
					92.7
				WS-6	94.2
					167.8*
		Inner	32.0	WS-5	160.9*
					84.0
				WS-6	95.3
					157.8*
12	Young	Outer	14.1	WS-5	182.8**
				WS-6	114.6
		Inner	20.9	WS-5	164.5*
				WS-6	111.9
	Mature	Outer	13.8	WS-5	185.9**
				WS-6	132.3
		Inner	14.6	WS-5	147.8*
				WS-6	195.6**
13	Young	Outer	8.5	WS-5	194.1*
				WS-6	123.5
		Inner	12.2	WS-5	122.4
				WS-6	153.1*
14	Young	Outer	31.7	WS-5	47.4*
				WS-6	36.8*
	Mature	Outer	20.6	WS-5	82.7
				WS-6	118.3
15	Young	Outer	4.1	WS-5	184.8**
				WS-6	172.7*
	Mature	Outer	3.2	WS-5	123.1
				WS-6	151.5*

16	Young	Whole flower	3.3	WS-5	131.0
				WS-6	0**
	Mature	Whole flower	10.0	WS-5	147.5
				WS-6	75
17	Young	Outer	28.3	WS-6	108.6

[a] Flowers sampled at age of commercial harvest (young) or at more mature stage (open flowers).

[b] Petals were sampled from the outer, more mature petals or from the inner, younger petals.

[c] Disease severity of wild type flowers expressed as standardized AUDPC (Area Under Disease Progress Curve derived by the experiment incubation time in days)

[d] Percentage of severity level obtained on transformed flowers out of the disease on WT flowers (standardized AUDPC of transformant divided by standardized AUDPC of WT and multiplied by 100. * = transformant is significantly different from the WT, $P \leq 0.05$; ** = transformant is significantly different from the WT, $P \leq 0.01$.

Table 3: Germination of *Botrytis cinerea* conidia on petals of transformed carnation flowers as compared with wild type flowers. Experiment 12

A. Germination rate (%)

Cultivar	Flower age[a]	Petals position[b]	WT % germination	Transformant No.	Transformant % germination	Percentage effect (Transformant/WT)[c]
Eilat	Young	Outer	90	E-1	85	94.4
				E-4	95	105.6
		Inner	100	E-1	50	50.0*
				E-4	85	85.0
White Sim	Young	Outer	95	WS-5	80	84.2
				WS-6	90	94.7
		Inner	75	WS-5	75	100.0
				WS-6	95	126.7
	Mature	Outer	85	WS-5	100	117.6
				WS-6	95	111.7
		Inner	75	WS-5	90	120.0
				WS-6	100	133.3

B. Germ-tube length (μm)

Cultivar	Flower age[a]	Petals position[b]	WT % germination	Transformant No.	μm Germ-tube	Percentage effect (Transformant/WT)[c]
Eilat	Young	Outer	131.5	E-1	82.0	62.3*
				E-4	137.5	104.5
		Inner	129.5	E-1	67.5	52.1**
				E-4	207.0	159.8
White Sim	Young	Outer	105.0	WS-5	103.0	98.1
				WS-6	70.5	67.1*
		Inner	135.0	WS-5	34.0	25.2**
				WS-6	97.0	71.8*
	Mature	Outer	62.5	WS-5	57.5	92.0
				WS-6	47.5	76.0*
		Inner	57.5	WS-5	108.5	188.7*
				WS-6	247.5	430.4**

[a] Flowers sampled at age of commercial harvest (young) or at more mature stage (open flowers).

[b] Petals were sampled from the outer, more mature petals or from the inner, younger petals.

[c] Germination obtained on transformed flowers out of the germination on WT flowers (germination on transformant divided by germination on WT and multiplied by 100. * = transformant is significantly different from the WT, $P \leq 0.05$; ** = transformant is significantly different from the WT, $P \leq 0.01$.

Legends to Figures

1. Development of disease cause by *Botrytis cinerea* on young (commercial harvest stage) 'Eilat' flowers, AUDPC and standardized AUDPC. Experiment 1.
2. Development of disease cause by *Botrytis cinerea* on mature and young (commercial harvest stage) 'Eilat' flowers, AUDPC and standardized AUDPC. Experiment 8.
3. Development of disease cause by *Botrytis cinerea* on mature and young (commercial harvest stage) 'Eilat' flowers, AUDPC and standardized AUDPC. Experiment 14.

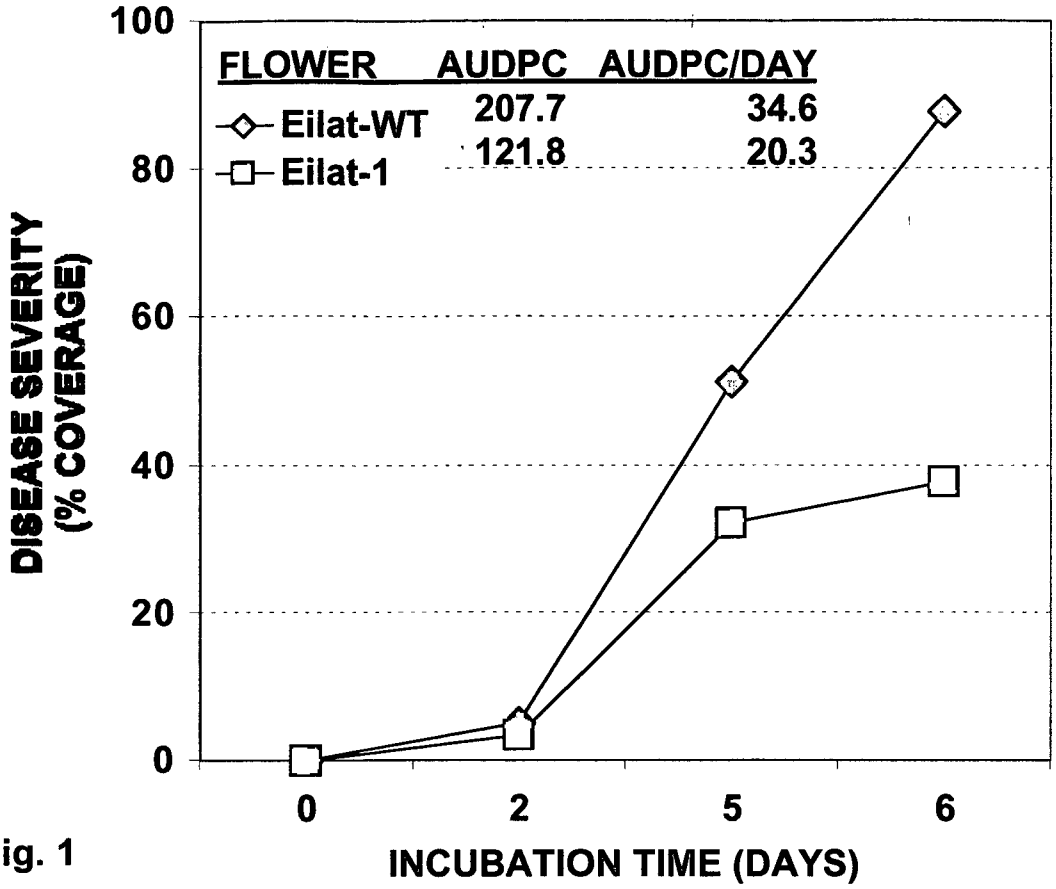
FLOWER AUDPC AUDPC/DAY
Eilat-WT 207.7 34.6
Eilat-1 121.8 20.3
DISEASE SEVERITY (% COVERAGE)
100
80
60
40
20
0
0
2
5
6
INCUBATION TIME (DAYS)

Fig. 1

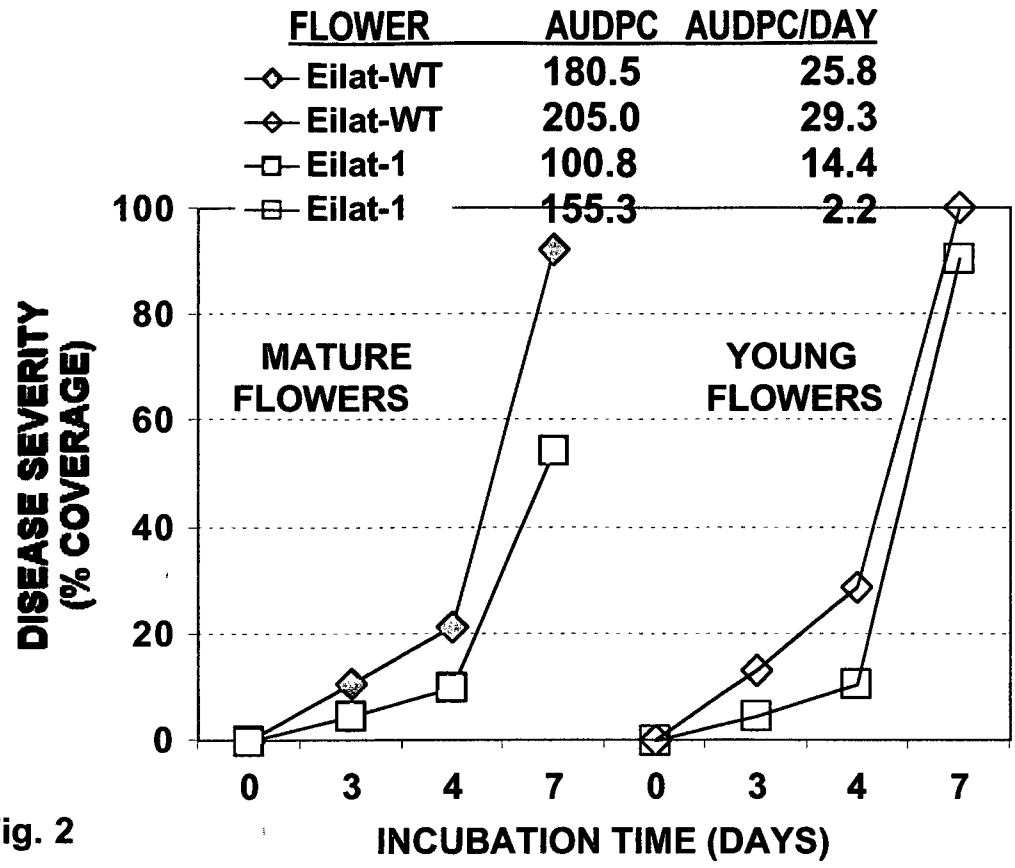
FLOWER
AUDPC
AUDPC/DAY
Eilat-WT 180.5 25.8
Eilat-WT 205.0 29.3
Eilat-1 100.8 14.4
Eilat-1 155.3 2.2
DISEASE SEVERITY (% COVERAGE)
100
80
60
40
20
0
MATURE FLOWERS
YOUNG FLOWERS
0 3 4 7 0 3 4 7
INCUBATION TIME (DAYS)

Fig. 2

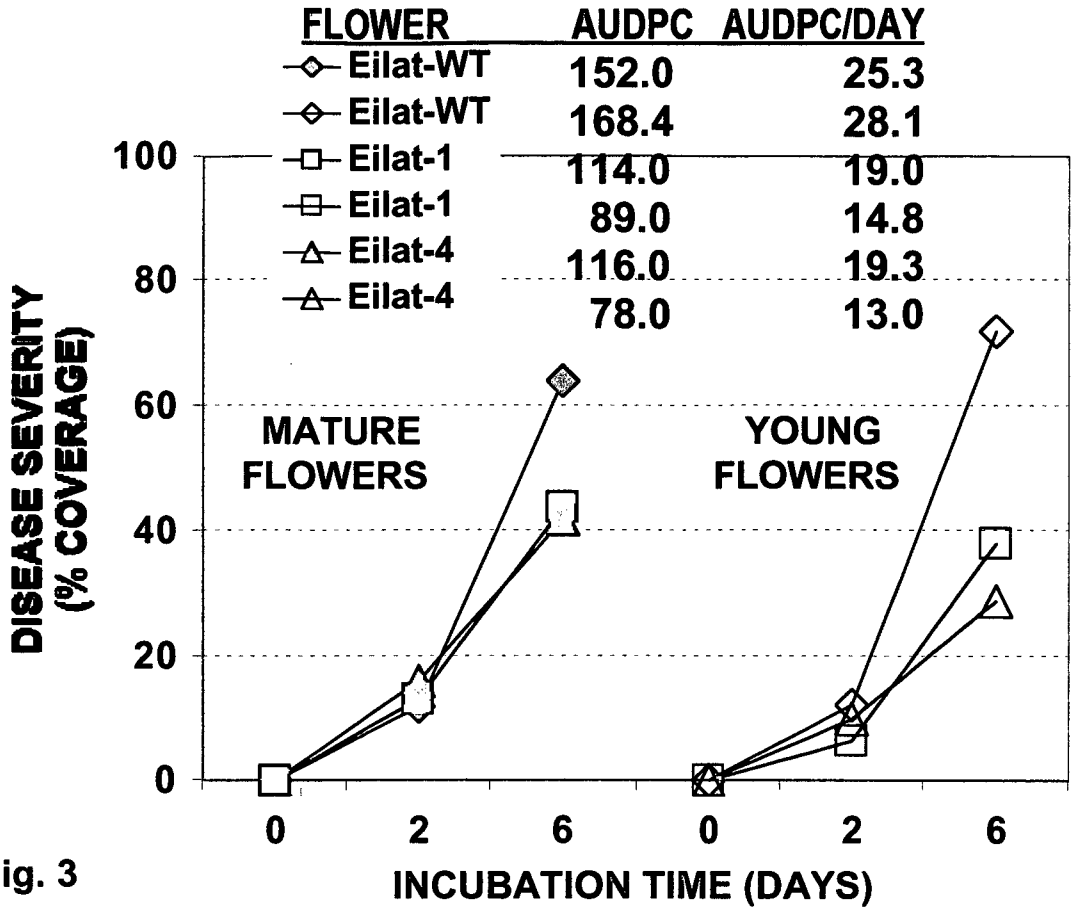
FLOWER
AUDPC
AUDPC/DAY
Eilat-WT 152.0 25.3
Eilat-WT 168.4 28.1
Eilat-1 114.0 19.0
Eilat-1 89.0 14.8
Eilat-4 116.0 19.3
Eilat-4 78.0 13.0
DISEASE SEVERITY (% COVERAGE)
100
80
60
40
20
0
MATURE FLOWERS
YOUNG FLOWERS
0
2
6
0
2
6
INCUBATION TIME (DAYS)

Fig. 3

Osmotin and Osmotin-like Proteins as a Novel Source for Phytopathogenic Fungal Resistance in Transgenic Carnation and Tomato Plants
BARD Project No. IS-2610-95CR/USDA No. 96-34339-3484
Paul M. Hasegawa and Ray A. Bressan

Scientific Report

I. Characterization of *In Vitro* Antifungal Activity of Osmotin, and Combinations of Osmotin and Other Antifungal Proteins

The *in vitro* antifungal activity of osmotin was evaluated against 31 fungal isolates representing 18 fungal genera (Abad *et al.*, 1996). This evaluation indicated that sensitivity to osmotin is genus specific. Hyphal growth of *Bipolaris*, *Fusarium*, and *Phytophthora* species and *Trichoderma longibrachiatum* was very sensitive to osmotin. *Botrytus* and *Verticillium* species were also inhibited by the PR protein but to a lesser degree than isolates of the genera indicated previously. Osmotin induced spore lysis of *V. dahliae* and *F. oxysporum* f. sp. *dianthi* and *lycopersici* (24.6, 10.6 and 4.6 μg/ml, respectively). A combination of osmotin and chitinase had a synergistic inhibitory effect on *Botrytus cinerea* and *F. moniliforme* spore germination (Figures 1A and 1B).

II. Transgenic Tomato Plants that Are Overproducing Osmotin, and Osmotin and Chitinase Combinations

A. Tomato regeneration and transformation protocol – An efficient *Agrobacterium*-mediated tissue culture-based transformation has been developed that involves adventitious organogenesis from cotyledonary explants (Figure 2). The regeneration protocol described by Meissner *et al.*, 1997 was modified to enhance adventitious shoot initiation, shoot elongation, and rooting. Succinctly, cotyledon explants (2.5 x 3 mm) from 7- to 9-day-old seedlings are most responsive morphogenetically, and little difference in caulogenesis occurs from explants isolated from proximal or distal portions of the cotyledon. Shoot meristem induction and shoot formation is achieved on a medium with 2 mg/L zeatin. The principal constraint to transformation of tomato is the low frequency of adventitious shoots that develop defined stems required for successful rooting *in vitro*. Reducing exposure to cytokinin, immediately after shoot induction and initiation, resulted in stem development from a much greater number of primordial

shoots. Propagules with small shoots are transferred to medium with 1 mg/L zeatin to stimulate complete development. Shoots, with defined stems are transferred to medium w/o growth regulators for additional elongation and then to medium with 2 mg/L indole butyric acid (IBA) for rooting, prior to soil transplantation and acclimation for growth in the greenhouse.

Transformation of several tomato cultivars (UC82B, Moneymaker, Ohio 7870, Micro-Tom and Pera) is achieved using LBA4404 or other common laboratory *Agrobacterium tumefaciens* strains (Figure 2). About 85% of the explants survive kanamycin selection and proliferate into propagules on shoot induction/initiation/regeneration medium. All plantlets transferred to rooting medium initiated roots and these were transferred successfully to soil in the greenhouse. Greater than 95% of the plants in the greenhouse were determined to be transformed. A transformation frequency of greater than 30% (explants that produce transgenic plants) was achieved.

B. Tomato transformation for expression of tobacco osmotin and chitinase – The *Agrobacterium tumefaciens* binary vector (pDJ) used for co-expression of osmotin and class I chitinase of tobacco in transgenic carnation and tomato plants is illustrated in Figure 3. The expression cassettes are derivatives of pUC19 that were modified to have unique flanking linker sites of either *EcoR* I or *Hind* III (Figure 3A). The vectors were constructed for insertion into the *EcoR* I and *Hind* III sites, respectively of the binary vector pDJ (Figure 3B), a derivative of pBI121. All the expression cassettes were modified to include unique multiple cloning sites between 35S promoter and the Ocs terminator (Figure 3). From 1 to 3 target genes can be expressed using the pDJ binary vector.

Osmotin was constructed into pDJ by inserting a *Sam* I/*BamH* I osmotin open reading frame fragment (deletion of the vacuolar targetting sequence) into the complementary sites in the binary vector (pDJ). Tobacco chitinase I encoding (minus vacuolar targettting sequence) cDNA was obtained by PCR using RNA from NaCl adapted cells as template. The cDNA was cloned into the *Sma* I/*Sal* I site of pRM(E) the expression cassette was in turn subcloned into pDJ. The binary vector was mobilized into either LBA4404 or EHA105.

Cotyledons of tomato seedlings were co-cultivated with *Agrobacterium* harboring the binary vector and subjected to kanamycin selection during shoot regeneration and rooting (Veronese *et al.*, 1999). The antibiotic was an effective selection agent both during regeneration and rooting. Kanamycin resistant shoots and plants were obtained (Veronese *et al.*, 1999; Figure 4). Several of the transgenic plants expressed osmotin and chitinase in abundant quantities, from 0.5 to 2% of total protein (Figure 5). T_3 progeny retained high level of PR protein expression.

C. Evaluation of disease resistance – Constitutive expression of osmotin in transgenic tomato increased resistance to *Botrytis cinerea* (gray mold), *Leveillula taurica* (powdery mildew), *Oidium lycopersici* (powdery mildew), and *Phytophthora infestans* (late blight) (Veronese *et al.*, 1999; Tables 1, 2 and 3). Artificial inoculations were made onto detached leaves of pre-flowering plants. Lesion area and sporulation indexes were determined after 100% of the leaves from wild type plants were infected. The level of resistance amongst these transgenic lines was correlated with the accumulation of the antifungal protein and was retained through the T_3 generation. Resistance to grey mold and late blight was similar in osmotin and osmotin and chitinase expressing transgenic plants (Veronese *et al.*, 1999).

This research was supported in part by BARD project No. IS-2610-95CR/USDA No. 96-34339-3484

References

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Narasimhan ML, Damsz B, Coca MA, Ibeas JI, Pardo JM, Yun DJ, Hasegawa PM, Bressan RA. Osmotin, a plant pathogenesis-related protein, induces programmed cell death in yeast. (in preparation)

Veronese P, Crino P, Tucci M, Colucci F, Yun DJ, Hasegawa PM, Bressan RA, Saccardo F (1999) Pathogenesis-related proteins for the control of fungal disease in tomato. *In* GT Scarascia Mugnozza, E Porceddu, MA Pagnotta (eds), Genetics and Breeding for Crop Quality and Resistance. pp. 15-24, Kluwer Academic Publishers, London

Ibeas JI, Yun D-J, Damsz B, Narasimhan ML, Uesono Y, Ribas JC, Lee H, Hasegawa PM, Bressan RA, Pardo JM (2000) Resistance to the plant PR-5 protein osmotin in the model fungus *Saccharomyces cerevisiae* is mediated by the regulatory effects of SSD1 on cell wall composition. Plant J. (in press)

Table 1. Powdery mildew (*L. taurica* and *O. lycopersici*) resistance of osmotin over-producing tomato plants. Detached leaves were inoculated with a leaf disk obtained from active sporulating lesions of infected tomato plants. Illustrated are data of wild type (untransformed) plants, independent primary regenerants (OsmA, E, F, H, and M) over-producing tobacco osmotin, and T_2 progeny of OsmA, F, H, and M. Data were analyzed using the Duncan's multiple range test, P = 0.05).

	Causal agent: *Oidium lycopersici*			Causal agent: *Leivellula taurica*		
T_0 genotypes	% Infection	Lesion area (cm^2)	N° spores/ disk lesion	% Infection	Lesion area (cm^2)	N° spores/ disk lesion
Wild type	100	0,5 a	21750 a	100	0,7 a	22500 a
OsmA	100	0,6 a	32190 a	100	0,6 a	21250 a
OsmE	100	0,6 a	35310 a	100	0,8 a	18960 b
OsmH	100	0,2 c	4250 c	50	0,1 b	5208 c
OsmM	100	0,3 b	16880 b	100	0,5 a	17920 b
OsmF	100	0,1 c	10077cd	-	-	-

	Causal agent: *Oidium lycopersici*		
T_2 genotypes	% Infection	Lesion area (cm^2)	Mycelium development (scale 0 to 3)
Wild type	100	1,0 a	2,0 a
OsmA	75	0,6 a	2,0 a
Osm F	75	0,2 b	1,0 b
OsmH	89	0,4 b	0,5 c
OsmM	67	0,4 b	1,0 b

Table 2. Gray mold (*B. cinerea*) resistance of osmotin over-producing tomato plants. Detached leaves were inoculated with mycelial plugs (0.6 cm in diameter). Illustrated are data of untransformed (wild type) plants, independent primary regenerants (OsmA, E, F, H, and M) over-producing tobacco osmotin, and T_2 progeny of OsmA, F, H, and M. Data were analyzed using the Duncan's multiple range test, P = 0.05).

		Lesion area (cm^2)	
T_0 genotypes	% Infection	4 days	8 days
Wild type	100	1,6 a	5,7 b
OsmA	100	2,2 a	6,3 a
OsmE	100	2,2 a	6,8 a
OsmF	100	0,7 b	1,9 d
OsmH	100	0,8 b	4,1 c
OsmM	100	1,0 b	3,9 c
T_2 genotypes			
Wild type	100	0,7 a	1,5 a
OsmA	100	0,7 a	1,6 a
OsmF	100	0,3 c	0,7 b
OsmH	100	0,4 b	0,8 b
OsmM	93	0,6 b	1,0 b

Table 3. Late blight (*P. infestans*) resistance of osmotin over-producing tomato plants. Detached leaves were inoculated with 30 µl of *P. infestans* spore suspension (2 x 10^4 spores/ml). Illustrated are data of untransformed (wild type) plants and primary regenerants (OsmA, E, and M) over-producing tomato osmotin. Data were analyzed using the Duncan's multiple range test, P = 0.05).

T_0 genotypes	% Infection	Lesion area (cm^2)	Mycelium development (scale 0 to 3)
Wild type	100	2,6 a	2,2 a
OsmA	100	1,1 b	1,2 b
OsmE	93		0,8 b
OsmM	90	0,2 c	0,2 c

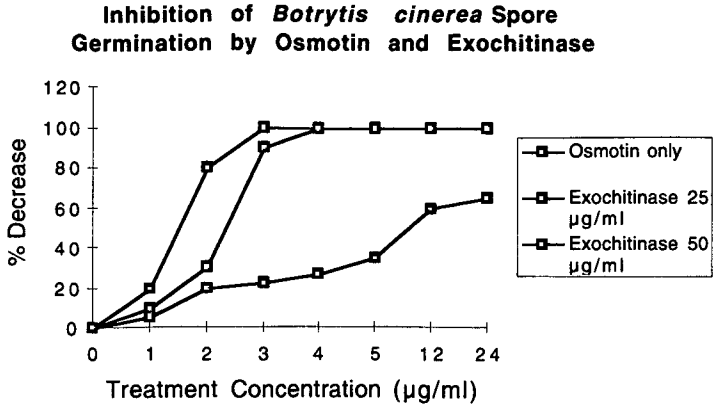
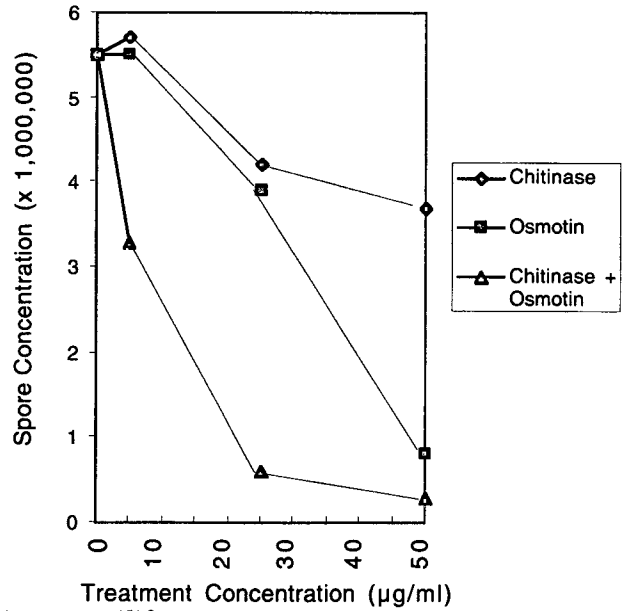


Figure 1. A. A combination of osmotin and chitinase has synergistic *in vitro* antifungal activity. Inhibition of *Botrytis cinerea* spore germination by the combination of tobacco osmotin and exochitinase. Tobacco osmotin, exochitinase derived from *Trichoderma harzianum* (Lorito *et al.*, 1994a; 1994b) or water was added to a known concentration of fungal spores suspended in a growth medium and incubated at 25 C. After 24-30 h, the percentage of germinating conidia was determined. Each experiment contained three treatment replicates and was repeated twice. These data suggest that there is substantial synergistic inhibitory effect of osmotin and *Trichoderma* derived-exochitinase on the germination of *B. cinerea*. (Data kindly provided by Dr. Matteo Lorito, Universita degli Studi di Napoli and Centro CNR di Studio delle Tecniche di Lotta Biologica, Napoli, Italy as part of a collaborative research interaction.)



B. Inhibition of *Fusarium moniliforme* spore germination by the combination of tobacco osmotin and chitinase. Tobacco osmotin, recombinant tobacco chitinase Ia, osmotin + chitinase in combination or water was added to a known concentration of fungal spores suspended in growth medium and incubated at 25 C. After 17-18 h, samples were removed and spore concentration and germination were determined. Chitinase was obtained as bacterial recombinant protein using the vector pGEX-KG (Guan and Dixon, 1991; Chen *et al.*, 1994). The protein was used in *in vitro* assays after thrombin cleavage of glutathione-S-transferases. The recombinant protein exhibited chitinase activity and comparable antifungal activity as the native chitinase isolated from tobacco leaves. Three samples of each of two replicates per treatment were evaluated. The treatments composed of a combination of osmotin and chitinase contained fewer spores when compared to either water controls or to treatments containing osmotin or chitinase alone. The additive effect of the individual osmotin and chitinase treatments was substantially less than the inhibitory effect mediated by the combined treatment, indicating that osmotin and chitinase act synergistically to cause spore lysis.

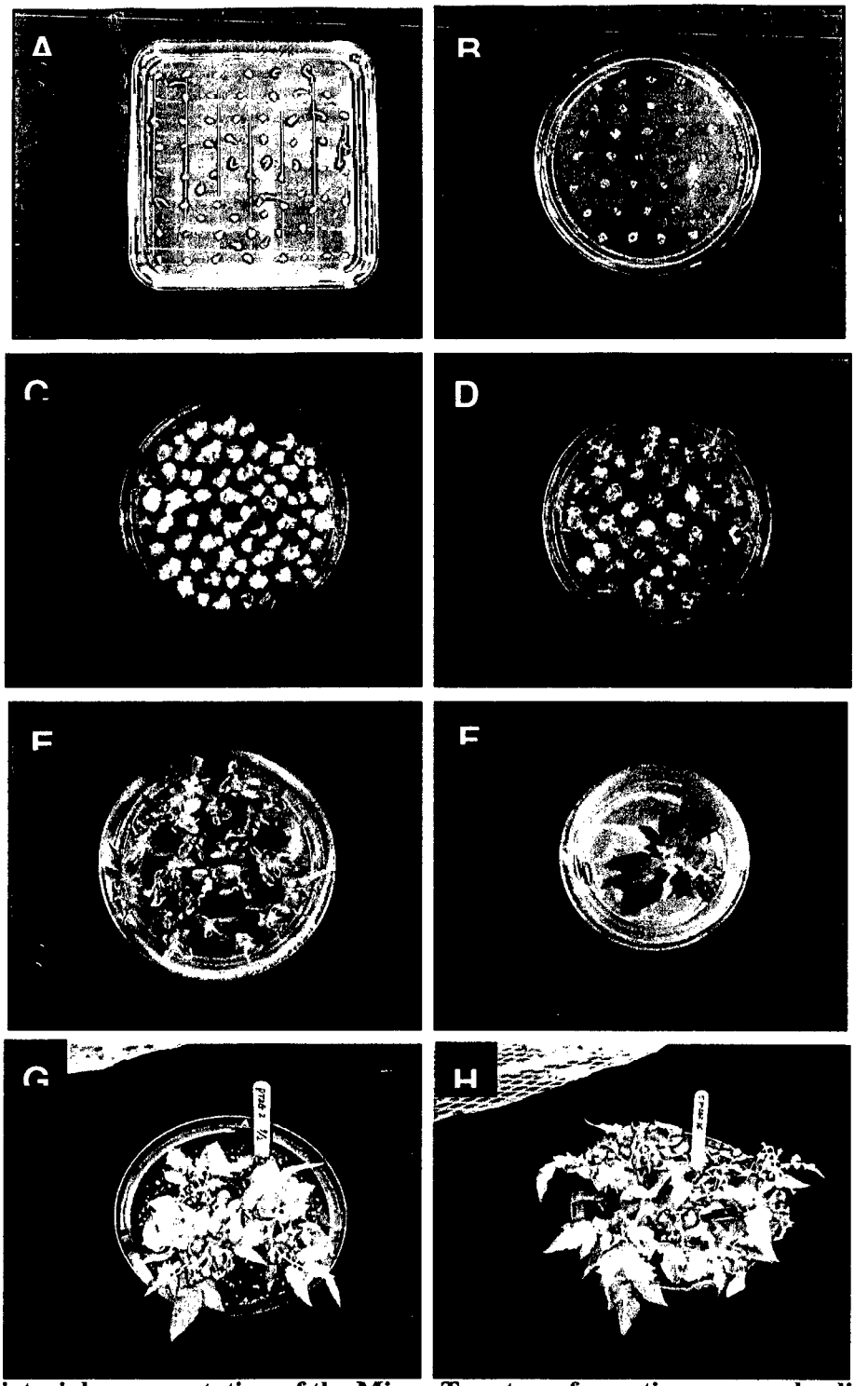


Figure 2. Pictorial representation of the Micro-Tom transformation process leading to production of transgenic plants. Illustrated are **A.** seedlings after germination *in vitro*, **B.** cotyledon explants on shoot induction/regeneration medium after 48-hr co-cultivation w/*Agrobacterium*, **C.** calli and small shoots on propagules after 2 weeks on shoot induction/regeneration medium, **D.** propagules w/shoots 2 weeks after reculture onto shoot induction/regeneration medium, **E.** shoots after 2 weeks on elongation medium, **F.** plantlet after 2 weeks on rooting medium, and **G.-H.** transgenic plants.

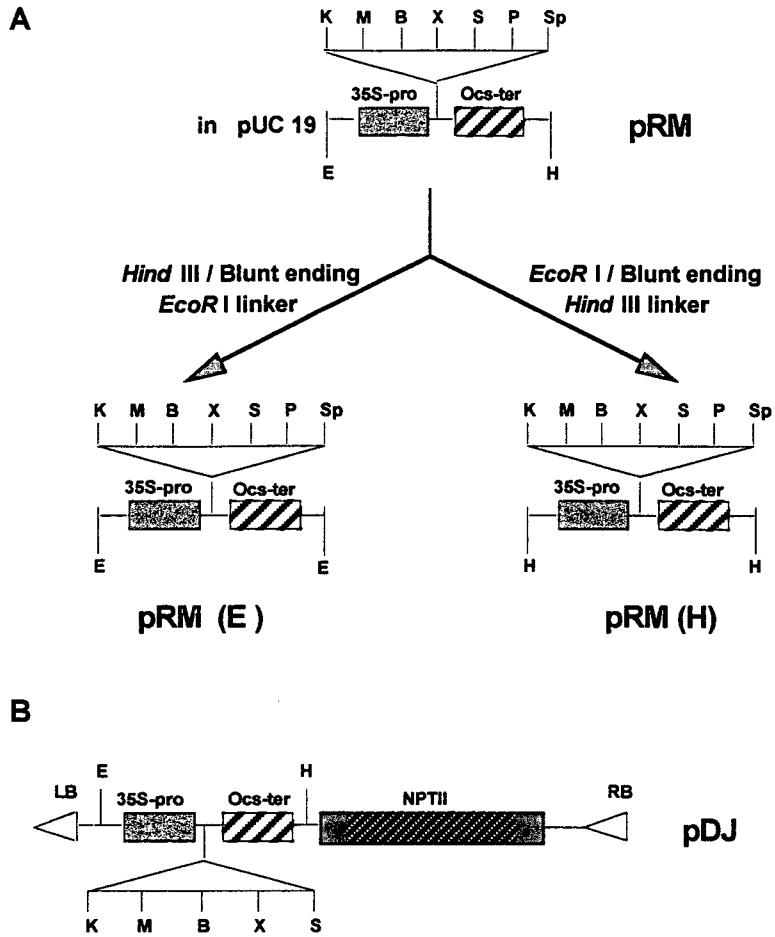


Figure 3. ***Agrobacterium*** **binary vector construction for co-expression of osmotin and tobacco chitinase I in transgenic plants. A.** Two plant expression cassette vectors were derived from pUC 19 (pRM) by modifying the linker sites to be either both *EcoR* I, pRM(E) or *Hind* III, pRM(H). **B.** The binary vector pBI121 was modified to create unique *EcoR* I and *Hind* III sites for insertion into pDJ of the pRM(E) and pRM(H) expression cassettes, respectively. The restriction sites in the vector are: **B**.B, *BamH* I, K, *Kpn* I; M, *Sma* I; P, *Pst* I; S, *Sal* I; Sp, *Sph*; and X, *Xba* I.

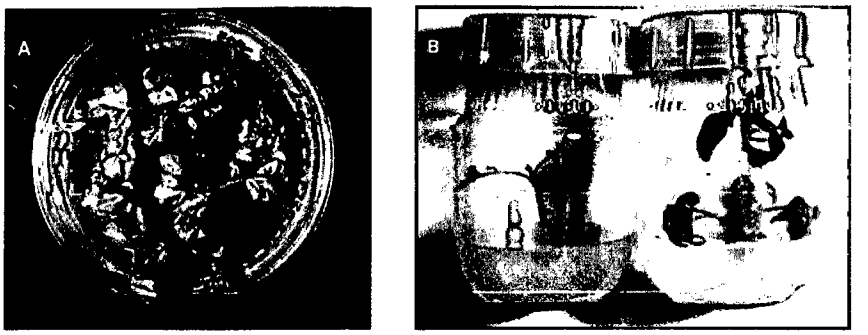


Figure 4. Tomato regeneration after co-cultivation with *Agrobacterium* EHA105 harboring pDJ containing osmotin and chitinase I ORF cDNAs, and kanamycin selection. A. Adventitious shoots and **B.** plantlets in rooting medium (left, negative control and right, putative transgenic) derived from cotyledon explants.

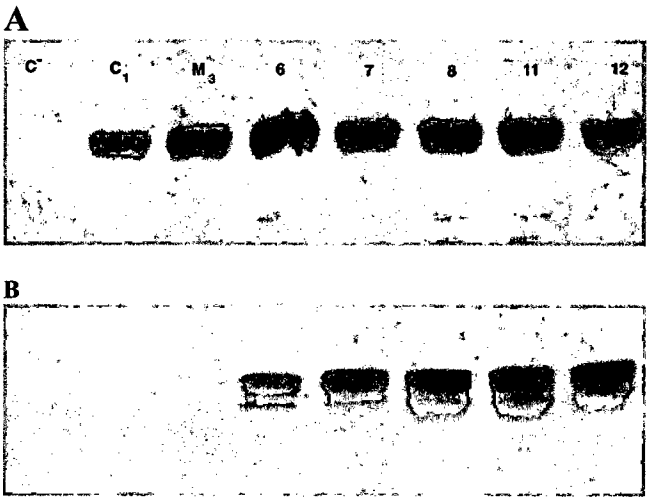


Figure 5. Transgenic tomato plants expressing antifungal proteins. Immunoblots reacted with **A.** anti-osmotin and **B.** tobacco anti-chitinase I. Illustrated are immunoblots of total protein separated by SDS-PAGE from the following plants: C^-, untransformed control; C_1 and M_3, osmotin expressing plants (osmotin positive control); and 6, 7, 8, 11 and 12 tomato putative transgenic plants.