Enhanced Resistance to *Verticillium dahliae* in Transgenic Strawberry Plants Expressing a *Lycopersicon chilense* Chitinase Gene

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ABSTRACT. A chitinase gene (*pcht28*), isolated from *Lycopersicon chilense*, was transferred into ‘Joliette’ strawberry using a stipule regeneration method and *Agrobacterium*-mediated gene transfer technique. Stipules showed a high rate of shoot regeneration (>90%) through direct or indirect organogenesis. Stipules were cocultured with a transformation plasmid in which *pcht28* was under the control of the CAMV 35S promoter. A high tendency in production of chimaeric shoots was observed. Shoots which did not show any sign of bleaching after several subcultures in kanamycin containing medium were considered as stable transformants. These shoots were successfully rooted in the presence of 50 µg·mL⁻¹ kanamycin. Transgenic nature of the plants was confirmed by PCR as well as Southern blot analysis. Constitutive expression of the chitinase gene was demonstrated by Northern analysis. In growth chamber studies, the transgenic strawberry plants which expressed *pcht28* had significantly higher resistance to *Verticillium dahliae* as compared to nontransgenic controls. These results demonstrate that *pcht28* plays a role in defense against this fungal disease in strawberry.

Strawberry is a highly consumed fruit extensively cultivated throughout many regions of the world. Cultivated strawberry (*Fragaria xananassa* Duch.) is octoploid (2n = 8x = 56) and highly heterozygous. A key objective of all strawberry breeding programs should be to maximize the genetic diversity of this species (Sjulin and Dale 1987). The main reason is that inbreeding reduces seed germination, vigor, fertility, fruit size and yield (Galletta and Maas 1990). It has also been suggested that inbreeding might make strawberry susceptible to pests, diseases and environmental stresses (Watt et al., 1999).

Strawberry plants like other agricultural crops are susceptible to numerous pathogens such as fungi, viruses, bacteria, nematodes and insects (Maas, 1992). Among these pathogens fungi are the most important cause for severe yield loss (Schickler and Chet, 1997). Plant breeders have been successful, to some extent, in improving resistance to some of these diseases, nevertheless, disease control remains to be a significant challenge in strawberry cultivation areas. Fungicides are being used extensively and there are legitimate concerns regarding consequences of the use of these chemicals on human health and environment (Chet and Inbar, 1994). Any means that can improve resistance of crops to pest and diseases might reduce the use of chemicals.

In a previous study we isolated a chitinase gene (*pcht28*) from a wild tomato species, *Lycopersicon chilense* (Chen et al., 1994). Chitinase, a pathogenesis related protein (PR), catalyzes

the hydrolysis of chitin, a linear polymer of β-1,4 linked N-acetyl-D-glucosamine residues which is the main structural components of cell wall of most filamentous fungi (Collinge et al., 1993).

Chitinase genes have been successfully transferred into different plant species. Transgenic plants expressing chitinase genes have reacted quite differently to inoculation with various fungi, depending on the plant species, type of chitinase(s) and the species of fungus. For example, tobacco plants expressing a chitinase gene from *Rhizopus oligosporus* showed less symptoms of infection when inoculated with the dicomycete pathogens *Sclerotinia sclerotiorum* and *Botrytis cinerea* in comparison to the control plants (Terakawa et al., 1997). Transgenic cucumber plants containing an acidic chitinase gene from petunia as well as cucumber plants containing a basic chitinase gene from tobacco or bean had the same sensitivity as nontransgenic plants to inoculation with *Alternaria cucumeris*, *B. cinerea*, *Colletotrichum lagenarium*, and *Rhizoctonia solani* (Punja and Raharjo, 1996). In the same study, using carrot plants transformed with a tobacco basic chitinase gene the extent of lesion development caused by *B. cinerea*, *R. solani* and *Sclerotium rolfsii* were significantly lower as compared to nontransformed plants. Transformed tobacco and potato plants with a chitinase gene from mycoparasitic fungus *Trichoderma harzianum* demonstrated a high level of resistance to *Alternaria alternata*, *A. solani*, *B. cinerea*, and *R. solani* (Lorito et al., 1998). Transgenic tomato plants expressing *L. chilense* chitinase gene demonstrated enhanced resistance to *Verticillium dahliae* race 2 (Tabaeizadeh et al., 1999).

In the present article we describe the insertion of *L. chilense* chitinase gene, through an *Agrobacterium tumefaciens*-mediated gene transfer technique, to ‘Joliette’ strawberry. This cultivar which was developed by Khanizadeh et al. (1996) has high yield and large moderately firm fruits. It is recommended for eastern central Canada (Khanizadeh et al. 1996). Due to the fact that an efficient adventitious shoot regeneration system is necessary for

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production of transgenic plants we had to initially develop such a system for 'Joliette'. The first series of transgenic plants were evaluated for their resistance to *V. dahliae* and were shown to be resistant to the fungi as compared to nontransgenic plants.

Materials and Methods

PLANT MATERIAL. *In vitro* shoots of 'Joliette' strawberry (obtained from *in vitro* culture laboratory of Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec, MAPAQ, Ste Foy, Québec) were used. Stock plants were propagated every month on multiplication medium containing MS salts (Murashige and Skoog, 1962), B5 vitamins (Gamborg et al., 1968), 100 mg·L⁻¹ myo-inositol, 30 g·L⁻¹ sucrose, 0.1 mg·L⁻¹ IBA (indole-3-butyric Acid), 1 mg·L⁻¹ BAP (6-benzylaminopurine), and 0.1 mg l⁻¹ GA3 (gibberellic acid), pH 5.7, solidified with 0.6% agar. All of the culture medium ingredients were purchased from Sigma (St. Louis). Some *in vitro* shoots were rooted in the same medium without growth regulators. Both *in vitro* shoots and rooted plantlets were used for transformation. *In vitro* plants were maintained in an incubator under 16-h photoperiod (50 to 70 µmol·m⁻²·s⁻¹ light intensity) at a temperature of 24 °C ± 1 during the day and 20 °C at night.

PLASMID CONSTRUCT. For transformation, the *L. chilense* chitinase gene designated as *pcht28*, which is induced by drought and abscisic acid (Chen et al., 1994) was used. The construction of the transformation vector has been described elsewhere (Tabaeizadeh et al., 1999). Briefly, the coding region of *pcht28* was placed at BamHI site under the transcriptional control of cauliflower mosaic virus (CaMV) 35S. This construct was cloned in binary vector pBin19 (Bevan, 1984) at HindIII site. This vector contains neomycin phosphotransferase II gene under the control of nopaline synthase promoter conferring resistance to kanamycin. The resulting plasmid (pBScht28) was introduced in nononcogenic *A. tumefaciens* strain LABA4404 (Hoekema et al., 1983) using the method of Delauney et al. (1988).

PREPARATION OF BACTERIAL SUSPENSION. A single colony of *A. tumefaciens* containing the transformation vector was grown overnight in Luria Broth (LB) (Sambrook et al., 1989) containing 100 µg·mL⁻¹ streptomycin and 100 µg·mL⁻¹ kanamycin, on a shaker (200 rpm) at 28 °C. After culture, the optical density (OD) of the bacterial cells was measured and adjusted to 0.5 at 600 nm. The bacterial suspension was centrifuged at 2000 g_n and resuspended in fresh LB medium before being used for cocultivation.

SENSITIVITY OF STIPULES TO KANAMYCIN. Sensitivity to kanamycin was tested by exposing stipules to 0, 10, 25, 50, 100 µg·mL⁻¹ of kanamycin. Ten stipules were cultured onto 60 × 15-mm petri dishes on 15 mL of adventitious shoot regeneration medium which contained MS salts (Murashige and Skoog, 1962), B5 vitamins (Gamborg et al., 1968), 100 mg·L⁻¹ myo-inositol, 20 g·L⁻¹ glucose, 600 mg·L⁻¹ casein hydrolysate, 0.5 mg·L⁻¹ IBA, 3 mg·L⁻¹ BAP, pH 5.7. The medium was solidified with 0.6% agar. Four petri dishes were used for each treatment.

COCULTIVATION OF STIPULES WITH *A. tumefaciens*. Stipules were dissected from 4- to 5-week-old *in vitro* shoots growing on multiplication medium and *in vitro* rooted plantlets on rooting medium. After separating leaves from the crown, the lower parts of stipules were cut as well as the junction of petioles and stipules to make sure that no preformed bud primordia was left on bottom edge of stipule, where new plantlets usually emerge. Thereafter, the stipules were cocultivated with *A. tumefaciens* suspension in LB medium for 30 min on a shaker (800 rpm).

The infected explants were blotted dry on sterile Whatman filter paper and placed on top of the filter paper in nurse culture plates (Tabaeizadeh et al., 1999) containing adventitious shoot regeneration medium. After 2 d, explants were transferred to the same medium supplemented with 50 µg·mL⁻¹ kanamycin and 500 µg·mL⁻¹ carbenicillin. Noninoculated stipules were placed on the medium with and without antibiotics as controls.

PLANT REGENERATION AND ROOTING. Transformed shoots selected on kanamycin containing medium were propagated in multiplication medium containing 500 µg·mL⁻¹ carbenicillin and 50 µg·mL⁻¹ kanamycin. After one month, shoots were transferred to the rooting medium which contained half-strength MS salts and B5 vitamins without any growth regulators, and supplemented with 500 µg·mL⁻¹ carbenicillin and 25 to 50 mg·L⁻¹ kanamycin. Rooted plants were transferred to sterile soil and acclimated for 2 weeks in a growth chamber under 16 h photoperiod (150 to 200 µmol·m⁻²·s⁻¹ light intensity) at a temperature of 24 °C ± 1 during the day and 20 °C at night.

DNA ISOLATION. Due to the high content of polysaccharides and phenolic compounds in strawberry, isolation of high quality nucleic acids is not straightforward in this species. After testing a few DNA isolation methods, Jobs et al. (1995) protocol was used with minor modifications. Powder of 2 g fresh leaf tissue, ground in liquid nitrogen, was added into 20 mL of preheated extraction buffer containing 100 mM sodium acetate (pH 4.8), 100 mM EDTA (pH 8.0), 500 mM sodium chloride, 10 mM DTT, 2% (w/v) PVP, pH 5.5 and 100 µg·mL⁻¹ proteinase K which was added just before use. After incubation for 1 h in a water bath at 55 °C, 1.5% SDS (using 10% stock solution) was added to the sample, mixed vigorously well and again incubated for an additional 1 h at 55 °C. After centrifugation at 10,000 g_n for 10 min at room temperature, 0.7 volume of 3 M potassium acetate (pH 4.8) was added to the supernatant, mixed gently and incubated at -20 °C for 30 min. Salt-insoluble material was removed by centrifugation at 10,000 g_n for 10 min at 4 °C. The nucleic acids were precipitated for 30 min with 0.6 volume of isopropanol at -20 °C. After a centrifugation at 10,000 g_n the pellet was dissolved in 5 mL sterile water. It was extracted twice with an equal volume of 25 phenol : 24 chloroform : 1 isoamyl alcohol (by volume) and once with 24 chloroform : 1 isoamyl alcohol (by volume). After addition of 0.6 volume of cold isopropanol and incubation at -20 °C for 30 min, the DNA was pelleted at 10,000 g_n for 10 min at 4 °C, washed with 70% ethanol and suspended in TE (10 mM Tris-HCl, 1 mM EDTA, at pH 7.6).

PCR ANALYSIS. Primers derived from the 5' (5'-CAA GAC GAT CTA CCC GAG-3') and 3' (5'-CCT TAT ATA GAG GAA GGG-3') ends of CAMV 35S promoter were used for PCR analysis. PCR amplification was carried out in 50 µL reaction mixture containing 5 µL of 10× PCR buffer (100 mM Tris-HCl, pH 8.4, 500 mM KCl and 15 mM Mg Cl₂), 200 µM each dTTP, dCTP, dGTP and dATP, 2.5 units of Taq DNA polymerase (Amersham Pharmacia Biotech (Little Chalfont, UK) 122 pM of each primer and 1 µg of template genomic DNA. Initially the template was denatured at 95 °C for 5 min, then submitted to 35 cycles of denaturation at 95 °C for 1 min, annealing at 52 °C for 1 min, and extension at 72 °C for 3 min. Amplified DNA fragments were subjected to electrophoresis on a 1.5% agarose gel and stained with ethidium bromide.

SOUTHERN BLOT ANALYSIS. For Southern blot analysis, ~20 µg of genomic DNA from transgenic and control plants were digested using the restriction enzymes, Hind III and EcoRI to cut out the 1.7 kb 35S promoter and *pcht28* insert. The digested DNA frag-

ments were subjected to agarose gel electrophoresis and blotted to Hybond-N membrane according to Sambrook et al. (1989). The membrane was baked for 2 h at 80 °C and prehybridized at 65 °C for 2 h in 25 mL of 5× SSC, 5× Denhardt's solution, 0.5% (w/v) SDS and 100 µg·mL⁻¹ sonicated herring sperm DNA. The *pcht28* cDNA was labeled using a DNA labeling kit according to the manufacturer instructions (Amersham Pharmacia Biotech). Hybridization was carried out at 65 °C for 16 to 20 h. The membrane was washed twice in 2× SSC, 0.1% (w/v) SDS at room temperature for 10 min, and once in 1× SSC, 0.1% (w/v) SDS at 65°C for 15 min, and exposed to X-Ray film (X-Omat Blue X-B1 Kodak Co., Rochester, N.Y.).

RNA EXTRACTION. High quality RNA from transformed and control strawberry plants was isolated according to Wan and Wilkins (1994) with some modifications. Plant tissue (2g) was ground to a fine powder and transferred to a 50-mL polypropylene tube containing the preheated (85 °C) extraction buffer (5 mL·g⁻¹ tissue). The extraction buffer contained 200 mM sodium borate (Borax), pH 9.0, 30 mM EGTA (ethylene glycol bis[β-aminoethyl ether]-N,N'-tetraacetic acid), 1% (w/v) SDS, 10 mM DTT, 1% (w/v) sodium deoxycholate and 2% (w/v) PVP (M 40,000). The sample was shaken vigorously by hand for 2 min and was incubated at 42 °C in a water bath for 1.5 h, after adding 100 µg·mL⁻¹ proteinase K. Then, potassium chloride was added to the homogenized to a final concentration of 160 mM and incubated on ice for 1 h. Subsequently the suspension was centrifuged at 12000 g_n for 20 min at 4 °C and the supernatant was filtered through Miracloth. After adding potassium acetate (final concentration 200 mM, pH 5.5) the tube was incubated on ice for 15 min. Salt insoluble material was removed by centrifugation at 10,000 g_n for 10 min at 4 °C. Subsequently one volume isopropanol was added and the sample was incubated for 30 min at -20 °C. Total nucleic acids was pelleted by centrifugation at 10,000 g_n for 10 min at 4 °C, resuspended in 2 mL of DEPC-treated water and extracted twice with equal volume of phenol:chloroform and once with chloroform. Total RNA was precipitated with 1/3 volume of 8 M LiCl overnight at 4 °C. The sample was pelleted at 12,000 g_n for 20 min and the pellet was cleared from pigments by 3 times sequential suspension and centrifugation with 5 mL of cold LiCl. After third precipitation, the RNA was resuspended in 2 mL of DEPC-treated water, precipitated with 2.5 volumes of ethanol overnight at -20 °C. Finally, RNA was pelleted, washed with 70% cold ethanol and resuspended in DEPC-treated water.

NORTHERN BLOT ANALYSIS. The expression of the *L. chilense* chitinase gene (*pcht28*) was examined in transgenic strawberry plants. Total RNA (10 µg) was mixed with the loading buffer [36%(v/v) formamide, 12% (v/v) formaldehyde, 20 mM Mops, 36 µg·mL⁻¹ ethidiumbromide, 0.01% bromophenol blue]. Samples were heated at 70 °C for 10 min, cooled on ice for 2 min and electrophoresed in 18% formaldehyde-1.2% agarose gel. Separated RNA was blotted on to Hybond-N membrane (Amersham Pharmacia Biotech) in 20× SSC (Sambrook et al., 1989). The prehybridization and hybridization was performed essentially as described above for DNA blot analysis.

EVALUATION OF TRANSGENIC STRAWBERRY PLANT FOR RESISTANCE TO *Verticillium dahliae*. Well rooted in vitro transgenic clones (J_A, J_M, J_W) and untransformed in vitro plants (used as control; C⁻) were transplanted into 7.5-cm pots in PROMIX soil (Les Toubières Premier LTÉE, Rivière du Loup, QC, Canada) and acclimated for 10 days. Plants were incubated at 90% relative humidity in a growth chamber with a 14-h photoperiod at 20 °C/15 °C (light/dark) and 150 µmol·m⁻²·s⁻¹ light intensity. After acclimation, plants were

transferred to greenhouse at 22 to 16 °C (day-night) and 16-h photoperiod provided by direct sunlight or on cloudy days, by 1000-W high intensity discharge lamps, Super Metalarc model (Osram Sylvania, Ind.). Plants were fertilized by adding 50 mL of 5 g·L⁻¹ solution of 10-52-10 (N-P-K) to each pot. At 5-6 leaf stage, they were transplanted in 12.5 cm pots with the same soil type and fertilized once with 200 mL of 5 g·L⁻¹ solution of 10-52-10 and then every 2 weeks with 100 mL of 5 g·L⁻¹ solution of 20-20-20. Plants were irrigated twice daily at 8 AM and 4 PM with an automatic irrigated system.

Evaluation for disease resistance was carried out when plants were at 10-leaf stage. For each transgenic line and nontransformed line, 15 plants were inoculated with *Verticillium dahliae* and 10 plants were used as noninoculated controls. The experiment was conducted in a complete randomized design (CRD). Inoculation procedure was as follows: plants were removed from their pots, additional crowns were removed in order to keep only one crown per plant. This caused wounding of the roots which was preferable, since it promotes penetration of pathogenic fungi. Thereafter plants were replanted after pouring 25 mL of a spore suspension (10⁵/mL) of *Verticillium dahliae* in the planting hole. To promote the severity of *Verticillium* wilt, three weeks before harvest a water stress was applied by withholding water up to wilting point and then rewatering. Three months after inoculation, plants were evaluated for their visual symptoms and then harvested and examined for the presence of *Verticillium* in the crown tissues. Visual symptoms were rated on a scale of 1 to 5 as follows: 1 = healthy plants with no wilting symptoms; 2 = start of wilting; 3 = 25% to 50% of leaves were wilted; 4 = more than 50% of leaves were wilted and 5 = dead plant.

To assess the presence of *Verticillium dahliae* in crown tissues, crown of each plant was washed under running tap water, dipped in 70% ethanol and flamed. Then, the crown was cut longitudinally with a flamed scalpel and 3 pieces of vascular tissue were taken off from each half of the crown and put on synthetic nutrient agar (SNA) medium (Nirenberg, 1981). Cultures were incubated in the dark at 22 °C for 4 to 5 d and subsequently *Verticillium dahliae* growth and sporulation were detected using a dissecting microscope.

ANALYSIS OF DATA. All data were expressed as the mean ± standard error of mean (SEM). Crown infection and leaf wilting symptoms were analyzed using a one-way ANOVA. When the main effect was significant Duncan's new post hoc tests were used to find the treatment effect. Test for associations between crown infection and leaf wilting symptoms was also performed using Pearson Correlation Coefficient analyses. A level of statistical significance at *p* < 0.05 was used in all analyses. The data were analyzed using Procedure-General Linear Model of SAS (version 6.12, 1997) software.

Results

ADVENTITIOUS SHOOT REGENERATION. In a series of experiments, different explant organs (leaf disks, petioles and stipules), different plant growth regulators and growth conditions such as photoperiod, were tested for shoot regeneration of 'Joliette' strawberry. In spite of using many different combinations of growth regulators (IBA and IAA, with concentration of 0.1 to 2 mg·L⁻¹ in combination with BAP, BA, Zeatin and TDZ, with concentration of 1 to 5 mg·L⁻¹) and culture conditions, shoot regeneration from leaf disk and petiole was not successful. Except occasional regeneration of few shoots, almost all of these explants produced few or no cal-

Fig. 1. Polymerase chain reaction (PCR) amplification of CAMV 35S promoter. C⁺, DNA from PBI-121 vector, containing 35S-*pcht28*; J_A, J_M, and J_W, DNA from three transgenic strawberry plants; C⁻, DNA from nontransformed strawberry plant. The DNA size standards are shown on the extreme left and right.

lus; most of them turned yellow and degenerated after 2 weeks.

A high rate of shoot regeneration was produced when stipules were used as explants. Stipules were isolated from either plantlets growing on rooting medium (without growth regulators) or plantlets growing on multiplication medium (with growth regulators). The excision of stipules from rooted plantlets was much easier, due to their bigger size and less compact structure, as compared to those from shoots in multiplication medium. On the other hand shoot regeneration capacity of stipules dissected from plantlets on multiplication medium was 98% while regeneration from rooted plantlets growing on the rooting medium was 12.5%.

EVALUATION OF STIPULES FOR RESISTANCE TO KANAMYCIN. Green shoots emerged from >90% of stipules cultured on regeneration medium without kanamycin (control) in 2 to 4 weeks. When 10 $\mu\text{g}\cdot\text{mL}^{-1}$ kanamycin was added to the medium some stipules produced bleached shoots or calli. Shoot regeneration from stipules was completely inhibited at 25 and 50 $\mu\text{g}\cdot\text{mL}^{-1}$ kanamycin. On medium containing 100 $\mu\text{g}\cdot\text{mL}^{-1}$ kanamycin all stipules became necrotic within 2 to 3 weeks. Therefore in transformation experiments 50 $\mu\text{g}\cdot\text{mL}^{-1}$ of kanamycin was used in the selection medium.

PRODUCTION OF TRANSGENIC PLANTS. About 1500 stipules were inoculated with *Agrobacterium tumefaciens* containing the transformation vector, in fifteen separate transformation experiments. During our preliminary

Fig. 2. Genomic DNA blot analysis of three transgenic lines (J_A, J_M, and J_W) and nontransformed strawberry plants (C⁻) using *pcht28* cDNA probe. The DNA size standards are shown on the left.

experiments we determined that 25 $\mu\text{g}\cdot\text{mL}^{-1}$ of kanamycin is sufficient for completely bleaching noninoculated green tissues in 3 to 4 weeks or for inhibiting proliferation of green tissues from noninoculated stipules. The transformation experiments, initially, resulted in 60 putative transgenic shoots which regenerated on the medium containing 50 $\mu\text{g}\cdot\text{mL}^{-1}$ kanamycin. However, after several subcultures, only three clones were considered to be stable transformants; the remainder were assumed to be chimaeric. Chimaeric shoots sustained resistance to kanamycin much better than control (nontransgenic) shoots for a while; however, eventually after 3 to 4 subcultures (4 to 5 months) they became partially bleached. Moreover, they failed to root in the presence of kanamycin. Transgenic shoots were micropropagated on medium containing 50 $\mu\text{g}\cdot\text{mL}^{-1}$ kanamycin. No sign of bleaching was observed on this medium, even after 18 months. Transgenic clones were successfully rooted in the presence of 25 and 50 $\mu\text{g}\cdot\text{mL}^{-1}$ of kanamycin, whereas nontransformed shoots failed to root and died in less than a month. Rooted transgenic plants were transferred to soil. With regard to phenotypic appearance, there were no discernible differences between transgenic and nontransgenic plants.

PCR ANALYSIS. The result of PCR amplification with primers from 5' and 3' ends of the CAMV 35S promoter confirmed the transgenic nature of the three selected clones. A fragment of 750 bp was observed in the three transgenic lines (Fig. 1). An extra nonspecific band of 550 bp in transgenic line J_M was also detected. No amplification was observed for nontransformed strawberry plants (Fig. 1).

SOUTHERN BLOT ANALYSIS. The integration of the *pcht28* gene into transformed plants was demonstrated by Southern blot analysis using *pcht28* cDNA as the probe (Fig. 2). All three independent transgenic lines showed multiple copy of the insert with a unique hybridization pattern in each line. Transgenic line J_A yielded two bands of 12 and 5 kb. Transgenic line J_M showed three bands of 8, 2.5 and 1.8 kb. The yielded bands in transgenic line J_W were four with the size of 12, 9, 3, and 1.7 kb. Transgenic lines J_W and J_M showed bands of about 1.7 kb liberated fragment of the 35S promoter and *pcht28* insert by restriction enzymes Hind III and EcoRI. All other hybridizing bands were bigger than 1.7 kb

Fig. 3. (A) Expression of *pcht28* in transgenic and control plants. J_A, J_M, and J_W, three independent transgenic strawberry plants; C⁺, a transgenic tomato plant expressing *pcht28*; C⁻, nontransformed strawberry plant. (B) Ethidium bromide stained gel.

Fig. 4. Susceptibility of transgenic (JA, JM and JW) and nontransgenic (C⁻) strawberry plants to *V. dahliae* infection (%). Different letters show significant differences ($p < 0.05$). Bars represent standard error of mean ($n = 20$).

and might suggest that the inserted gene was rearranged. In addition to the intense hybridization bands, extra faint bands were present for the transgenic lines J_M and J_W. As shown in figure 2, no hybridization band was detected with the DNA isolated from nontransformed plant. These results and those of PCR analysis confirmed that both the CAMV 35S promoter and the *pcht28* genes were integrated into the genome of the transgenic strawberry plants. Moreover, the results of genomic DNA blot hybridization matched the kanamycin resistance phenotype of these plants, demonstrating that both the neomycin phosphotransferase II gene, and chitinase gene (*pcht28*) are cointegrated into the strawberry genome.

EXPRESSION OF *pcht28* IN TRANSGENIC STRAWBERRY PLANTS. Northern blot analysis of total RNA was carried out to assess the expression of chitinase gene (*pcht28*) in transgenic plants. A transgenic tomato plant expressing a high level of chitinase mRNA was used as positive control. As can be seen in Fig. 3, a high level of *pcht28* expression was observed in transgenic strawberry plants, while no expression was detected in nontransgenic plants. The analysis was repeated two more times with several months interval (data not shown). The same results were obtained; different level of expression among the three transgenic lines and no expression in nontransgenic plants.

RESISTANCE TO *VERTICILLIUM DAHLIAE* OF TRANSGENIC STRAWBERRY PLANTS. The response of transgenic lines to inoculation with

Fig. 5. Transgenic (JA, JM, and JW) and nontransgenic (C⁻) strawberry plants leaf wilting symptoms (mean), rated as follow: 1 = healthy plants with no wilting symptoms; 2 = start of wilting; 3 = 25% to 50% of leaves were wilted; 4 = >50% of leaves were wilted, and 5 = dead plant. Means followed by the same letter are not significantly different at ($p < 0.05$). Bars represent standard error of mean ($n = 20$).

V. dahliae was assessed by a method that measures resistance to fungal penetration. For each transgenic line and nontransformed control plants 25 identical plants were propagated from *in vitro* shoots with the same age and size. These plants were rooted *in vitro* and after acclimatization they were transferred into the green house. Among these 25 plants, 15 plants were inoculated with *V. dahliae* and 10 plants were not inoculated and kept as control. At 10-leaf stage, all of the noninoculated control plants were tested negative for crown infection. However, a strong difference between nontransformed and transgenic strawberry plant was observed. The effect of inoculation of *V. dahliae* on crown infection was highly significant ($p < 0.0001$). Transgenic plants showed a low rate of infection (10%, 5%, and 10% for J_A, J_M, and J_W respectively). They were significantly different from control plants which showed 65% infection rate (Fig. 4). When scoring resistance by measuring wilting symptoms, again nontransformed plants were more susceptible to wilting than transgenic plants and significantly different from J_A and J_W, although not statistically different from J_M (Fig. 5). When wilting symptoms distribution (Table 1) within each clone was considered, most transgenic plants did not show serious wilting symptoms, while 30% of control plants were seriously wilted (symptoms 3 and 4). When association between leaf symptoms and crown contamination was considered, a low ($r = 0.40$) but highly significant ($p < 0.01$) and positive correlation was found.

Discussion

In this study we developed an efficient regeneration and transformation procedure for 'Joliette' strawberry. Adventitious shoot regeneration of this cultivar was made possible by using stipules as explants. There are a few reports of direct (Jemmali et al., 1994; Liu and Sanford, 1988; Nehra et al., 1989; Sorvari et al., 1993) and indirect (callus mediated) (Rugini and Orlando, 1992; Nehra et al., 1990a) shoot regeneration of some strawberry cultivars. When leaf discs, petioles, runners and roots are used as explants, different strawberry cultivars demonstrate different shoot regeneration capacity (Rugini and Orlando, 1992; Finstad and Martin, 1995). However, such variation is less or it does not exist in case of stipules. The high shoot regeneration capacity of stipules seems natural, since in strawberry new shoots normally emerge from this part of the plant.

In spite of our efforts, the transformation rate was low. There are reports on enhancement effect of preculturing of explants (before transformation) on transformation efficiency (Tabaeizadeh et al., 1999; Sunilkumar et al., 1999). However, in our study we did not observe any positive effect, when the explants were submitted to a 2-d preculture, before the inoculation. Moreover, due to the small size of the explants, it was difficult to harvest them from the preculture medium and transfer them to *Agrobacterium* suspension.

Table 1. Distribution of wilting symptoms (1 = healthy plants with no wilting symptoms; 2 = start of wilting; 3 = 25% to 50% of leaves were wilted; 4 = >50% of leaves were wilted, and 5 = dead plant) in transgenic (JA, JM, JW) and nontransgenic (C⁻) plants inoculated with *V. dahliae*.

Clone	Wilting symptoms (%)				
	1	2	3	4	5
JA	90	10	---	-	---
JM	75	20	---	5	---
JW	90	5	5	---	---
C ⁻	60	10	15	15	---

Other attempts to improve transformation efficiency consisted in prolonging the inoculation period and adding of acetosyringone either to the bacterial growth medium before cocultivation or to the coculture medium (Matsumoto and Fukui, 1998; Nishibayashi et al., 1996). In this study most putative transformants and all 3 true transgenic lines were produced when the coculture medium was supplemented with 250 μM acetosyringone.

During the production of transgenic strawberry plants, we observed a numerous of chimaeric shoots. In presence of 50 $\mu\text{g}\cdot\text{mL}^{-1}$ kanamycin, inoculated stipules were able to regenerate shoots or produce calli. Green shoots which were regenerated on kanamycin containing medium, were considered putative transformants. After growth this medium for 4 to 5 months, most shoots gradually bleached. At this point they were considered chimaeric. Nehra et al. (1990b) observed reduced rooting ability for their transgenic clones on kanamycin containing medium. Mathews et al. (1995) suggested that a lower rooting ability in the presence of kanamycin is a probable evidence for chimaeras (Mathews et al. 1995). Our results are in agreement with these authors; all clones which were not able to produce adequate roots on kanamycin containing medium were later found to be chimaeric. According to James et al. (1990) 14 to 21 d of exposure to kanamycin would be sufficient for selection of strawberry transgenic plants. Our studies revealed that some chimaeric shoots can survive on medium with 50 $\mu\text{g}\cdot\text{mL}^{-1}$ kanamycin even for few months without showing any sign of bleaching. We found, that absolute tolerance to kanamycin (no sign of bleaching after 18 months exposure to kanamycin) and normal rooting ability on medium containing 50 $\mu\text{g}\cdot\text{mL}^{-1}$ kanamycin can be considered as reliable criteria for selection of true transformants in strawberry, which was further confirmed by molecular analysis. Mathews et al. (1995) suggested a selection protocol for recovering uniform transgenic plants from chimaeric transformants. In this method they recultured explants from putative transformants on regeneration medium with increased level of kanamycin. We do not find this method reliable for eliminating all nontransformed cells since new transformed shoots regenerated from mosaics of transformed and nontransformed cells.

Kanamycin resistance phenotype combined with PCR analysis, Southern blot hybridization and Northern blot analysis confirmed the stable integration of insert genes into strawberry genome. Data of DNA blot hybridization revealed a unique pattern of *pcht28* integration for each transgenic line. Since all three transgenic lines were derived from independent transformation events and strawberry is an octoploid plant, these results were not unexpected and reported earlier (Mathews et al., 1995). The expression level of the *pcht28* was different among transgenic lines (Fig. 3). The results of three Northern blot analyses which were performed with a few months interval after transformation were consistent (data not shown). In all of those RNA analyses, transgenic line J_M always showed lower expression level as compared to the one of the other two transgenic lines (Fig 3). However, according to Southern blot analysis data, line J_M does not have the lowest copy number of the gene among three transgenic lines. Therefore it would be difficult to correlate the expression levels of *pcht28* gene with the number of gene copies. Moreover, other factors such as the position of the insert can also have an effect on the expression level of an insert gene (Liu and Tabe, 1998).

The three true transgenic clones were evaluated for their resistance to *Verticillium* wilt caused by *V. dahliae*. *Verticillium* disease symptoms include browning of vascular tissues, stunting, foliar chlorosis, wilting and eventually the plant death (Robb et

al., 1994). It should be noted that visual symptoms do not always correspond to the presence of pathogens inside plant tissues and only isolation and culturing of pathogen can confirm the visual symptoms. In this study we performed a laboratory culture of infected crowns which is the most reliable method for evaluation of resistance to *V. dahliae*. Crown infection incidence data demonstrate that transgenic plants were significantly more resistant to *V. dahliae* as compared to nontransgenic plants. We believe that this significant resistance is due to the potential antifungal activity of chitinase which is present in the transgenic plants. The accumulation of chitinase enzyme in transgenic plants may inhibit hyphal growth or spore germination of *V. dahliae*.

Chitinase genes isolated from different sources have been used to produce transgenic plants with enhanced resistance to fungal diseases (Marchant et al., 1998; Tabaeizadeh et al., 1999; Terakawa et al., 1997). *Verticillium* wilt is a widespread disease and a major limiting factor in most strawberry producing areas (Maas, 1992). To our knowledge, no sources of resistance to *V. dahliae* have been identified in strawberry germplasms. After the University of California strawberry germplasms were evaluated for resistance to *Verticillium*, most of the 41 strawberry genotypes and 1000 hybrids of 23 of these genotypes were identified as highly susceptible to *V. dahliae* (Shaw et al., 1996). Joliette is a high quality, high yield strawberry cultivar (Khanizadeh et al., 1996) which is resistant to leaf spot (*Mycosphaerella fragariae* Tul.) (Delhomez et al., 1995) and 6 races of red stele (*Phytophthora fragariae* Hickman) (Khanizadeh, 1994), but it is susceptible to *Verticillium* wilt. The constitutive expression of *L. chilense* chitinase gene in transgenic Joliette plants has improved resistance of this cultivar to *Verticillium* wilt.

This is the first report of successful genetic transformation of strawberry using stipule regeneration system. The transgenic strawberry lines produced in this study can be considered as a valuable genetic source for resistance to *V. dahliae*. However, more effort should be directed towards improving further the techniques to increase the efficiency of the transformation rate. In such a condition strawberry plants transformed with a control vector can be easily produced. These plants can be used as transgenic control and ultimately along with nontransformed plants as well as transgenic plants harboring the chitinase gene will be subjected to a field trial for disease resistance.

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