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Full Length Research Paper

Combined overexpression of chitinase and defensin genesin transgenic tomato enhances resistance to *Botrytis cinerea*

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The rice chitinase gene (*CHI*), the alfalfa defensin gene (*alfAFP*) and their bivalent gene (*CHI-AFP*) were introduced into tomato line Micro-Tom via *Agrobacterium*-mediated gene transfer method. Transformants were obtained and confirmed by GFP, PCR and Southern blot hybridization. One to four copies of transgene were integrated into the tomato nuclear genome. Transcription levels of chitinase, *alfAFP* and their bivalent gene *CHI-AFP* in various transgenic lines were determined using Northern blot and Western blot analysis. Performance test of resistance analyses to *Botrytis cinerea* with T_1 generation transgenic tomato lines showed the transgenic lines exhibited higher resistance to the pathogens infected than that of the non-transgenic plants and the resistance levels were related to expression levels of the transgene, showing dosage-effect. The transgenic tomato harboring *CHI-AFP* cassette showed the highest disease resistance; it suggested that co-transformation with *alfAFP* and chitinase gene was more effective than individual transformations on the resistance to *B. cinerea*. Some independent lines with high disease resistance, low variability and stable expression of transgenes could be selected for the further studies and molecular breeding.

Key words: Transgenic tomato, rice chitinase gene, alfalfa defensin gene, *Botrytis cinerea*.

INTRODUCTION

Gray mold caused by *Botrytis cinerea* Pers. Fr. is one of the important destructive diseases throughout the world which inflicts serious losses in many crops. The disease symptoms are characterized by gray, fuzzy sporulating lesions commonly observed under humid conditions (Sutton, 1995; Jayaraj and Punja, 2007).

A wet, humid greenhouse environment provides a very favourable condition for the rapid growth and prolific sporulation of *B. cinerea*. Fungicides are commonly used to control gray mold. However, this is becoming less acceptable since it increases the potential for the build-up of resistance in *B. cinerea* to fungicides and also conflicts with the public concern for fungicide residues (Decognet et al., 2009). Biological control, on the other hand, has advantages over fungicides, but its efficacy varies depending on the timing and the environmental conditions. Moreover, *B. cinerea* still could develop resistance to biological control agents (Gentile et al., 2007). Traditional breeding for resistant cultivars has not been very successful so far, mainly because of a lack of host resistance to *B. cinerea* (Bi et al., 1999; Decognet et al., 2009).

Some strategies have been used to improve crop resistance to *B. cinerea* through transformation technologies (Punja and Raharjo, 1996; Jayaraj and Punja, 2007). Scented geranium transformed with a gene encoding the antimicrobial protein *Ace*-AMP1 could reduce the infection of *B. cinerea* compared with the wild types. Plant β-1,3-glucanases are abundant proteins widely distributed among seed plant species. The expression of many β-1,3-glucanases could be induced by fungal elicitors, wounding, salicylic acid, ethylene and other chemical inducers (Wally et al., 2009). However, these genes may

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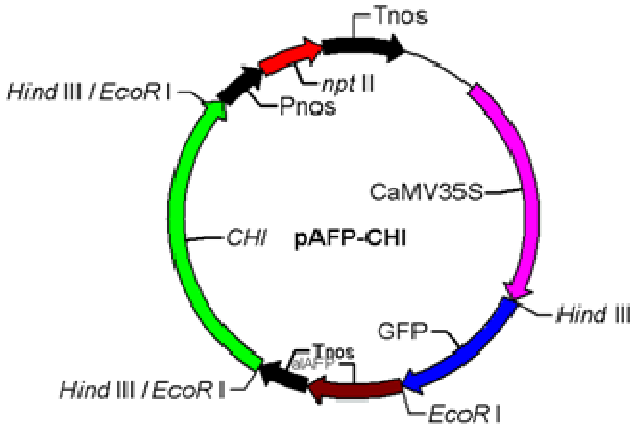


Figure 1. Schematic diagram of the whole binary vectors pAFP-CHI.

not universally provide enhanced resistance to *B. cinerea* (Bi et al., 1999). When the same basic tobacco chitinase transgene was used to genetically transform cucumber, no increased resistance to any of the test pathogens, including *B. cinerea*, could be obtained (Punja and Raharjo, 1996).

Among biological control agents, defensin and chitinase gene played important roles in practical applications. Defensin belongs to antimicrobial and malignant-toxic peptides widely existing in animals and plants. It exerts broad-spectrum poisoning effects on many bacteria, fungi and viruses (Farrokhi et al., 2007). Tomato transgenic plants containing rabbit defensin gene (*NP*-1) showed resistance to pathogen *Fusarium oxysporum in vivo* (Zhang et al., 2000). Transgenic tobacco and peanut plants expressing the mustard defensin can be deployed for deriving fungal disease resistance (Anuradha et al., 2008). Chitinase gene could activate the expression of pathogenesis-related genes, subsequently enhancing transgenic plant resistance to gray mold (Sridevi et al., 2008). It has been proved that synergistic expression of plant defense genes is a preferred approach to protect crops against pathogen infection (Carvalho and Gomes, 2009).

Cv. Micro-Tom is a miniature tomato with many advantages for molecular biology and plant physiology studies. Micro-Tom is generally susceptible to most of the important tomato pathogens and showed typical symptoms (Hideki et al., 2005).

The objective of this study was to obtain transgenic tomato material resistant to *B. cinerea*. At the same time investigation was also made to check whether there was synergistic expression in alfalfa defensin gene *alAFP* and rice chitinase gene.

MATERIALS AND METHODS

Tomato seeds (*Lycopersicon esculentum*, cv Micro-Tom supplied by Tomato Growers, United states) were surface-sterilized with 75% alcohol for 30 s and 8% sodium hypochlorite for 12 min, subsequently rinsed several times with sterile distilled water. These seeds were sown on phytohormone-free 1/2 MS medium containing 15 g/l sucrose and 8 g/l agar. The surface sterilized seeds germinated in a culture chamber at 16/8-h (light/dark) photoperiod, 25℃ and light intensity of 50-60 µmol/m^2 s. Cotyledons were excised about 10 days after sowing (Naoki et al., 2005).

Binary vector, bacterial strains and plant transformation

Agrobacterium tumefaciens strain EHA105 harboring pEChi plasmid with rice chitinase gene, pEAFP plasmid with *alfAFP* and pEAFP-Chi plasmid with *alfAFP* and *CHI* gene driven by the 35S promoter and *A. tumfaciens* nopaline synthase (*nos*), transcriptional terminator sequence was used as the vector system for transformation. The neomycin phosphotransferase II (*npt*II) as a selective marker gene was adjacent to the pathogenesis-related protein gene. All the vectors were similar to the vector pEAFP-Chi (Figure 1).

A. tumefaciens strain EHA105 was stripped on solid LB medium supplemented with 100 mg/l kanamycin and 50 mg/l rifampicin for 2-3 days at 27℃ to form colonies. A single colony with a diameter of 1 mm was picked out and cultured in liquid LB medium with 50 mg/l rifampicin and 100 mg/l kanamycin on a shaker at 27℃ for about 12 h. The bacteria were collected by centrifugation for 8 min at 1000 g and were resuspended in MS liquid medium prior to use.

Cotyledons from 10 days old seedlings were cultured on pre-culture medium M_1 at 26℃ for a day in the dark. Cotyledons were immersed in bacterial suspension for 8 min, blotted dry with sterile dry filter paper and co-cultivated on M_1 for a day. After co-cultivation, the infected cotyledons were transferred to selective regeneration medium M_2 for 2 weeks. The explants were sub-cultured every two weeks.

When adventitious shoots grown from the explants grew up to 2-3 cm in height, each shoot was excised and transferred to rooting medium (M_3). The rooted shoots were transplanted to soil after acclimation (Table 1).

Molecular analyses of transgenic plants

Total genomic DNA was isolated from leaves of the wild-type plant and putative transgenic plants by the methods of Tzfira et al., (1997). The *alfAFP* specific primer sequences (5′…3′) AAT GGA GAA GAA GTC TCT TG and AAC ATC TTT TGA GAC ACC AG and *Chi* gene specific primer sequences (5′…3′) GAAT-GAGGCTTT GTAAATTCAC and CGTTAATTTCCAAAAGACC TCTGGT were performed in 25 µl (total volume) of reaction mixture consisting of 10 × PCR reaction buffer including 1.5 µM Mg^{2+}, 50 ng template DNA, 0.2 mmol/l deoxynucleotide triphosphates, 1.5 mM $MgCl_2$, 0.2 µM of each primer and 1 unit of Taq DNA polymerase. After the initial denaturing for 2.5 min at 94℃, PCR was performed during 35 cycles (denaturing at 94℃ for 1 min, annealing at 56℃ for 1 min, synthesis at 72℃ for 1.5 min). Thereafter the program was terminated by an extension at 72℃ for 10 min. The amplification was analyzed by electrophoresis in 1% agarose ethidium bromide gels.

For genomic Southern blot analysis, 20 µg of T_0 plant DNA from transgenic plants and untransformed control plant was digested with *Hind*III and then fractionated by electrophoresis in 1% agarose gel. For Northern blot analysis, total RNA was extracted from leaves of T_0 plants and 30 µg RNA was fractionated on 1% agarose gel (Chomczynski and Sacchi, 1987). Southern and Northern blotting were performed as previously described using Hybond N membranes (Amersham Biosciences, U.K.) and hybridized with a ^{32}P-labeled probe containing *npt*II gene according to the

Table 1. Ingredients of the media for tomato transformation and regeneration.

Media	Composition (mg/l)
M_1	MS + 2 mg/l ZT + 0.2 mg/l IAA + 50μM AS+ sucrose 15 g/l + 0.5% agar
M_2	MS + 2 mg/l ZT + 0.2 mg/l IAA + Km 100 mg/ml + Cef 500 mg/ml + sucrose 20 g/l + 0.5% agar
M_3	MS + IAA2.0 mg/l + Km 100 mg/l + Cef 300 mg/l + sucrose 20 g/l + 0.5% agar

Table 2. Regeneration and transformation efficiency mediated by *A. tumefaciens.*

Foreign gene	No. of explants	No. regenerated explants	Regeneration frequency (%)	No. transplanted GFP-positive plants	Transformation frequency(%)
CHI – AFP	139	67	48.2	32	23.0
alfAFP	124	54	43.6	28	22.6
CHI	93	55	59.1	23	24.7
Vacant vector	110	81	73.6		

manufacturer's instructions (Amersham Biosciences) (Sambrook et al., 1989).

Total protein was extracted from the leaves and estimated using the method of Bradford. Twenty microgram aliquots of protein samples were separated by 10% SDS-PAGE along with a marker (Rainbow marker, Amersham Biosciences Ltd., Little Chalfont, UK) and subjected to Western blot analysis. The antibodies for chitinase and *alfAFP* were used at 1:1000 (v/v) dilution. The goat anti-rabbit IgG (H + L) alkaline phosphatase conjugate (Bangalore Genei, Bangalore, India) was used at a dilution of 1:2000 (v/v) and finally treated with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium colour reagent until the bands appeared (Jayaraj and Punja, 2007; Sridevi et al., 2008).

GFP expression was visualized using a Leica DMRA2 stereo fluorescence microscope (Leica Instruments Pty, North Ryde, Australia). This microscope is equipped with a Leica GFP plus filter (480/40 nm excitation filter) and a narrow bandpass interference filter (S550/100 NP) which blocks the red autofluorescence of chlorophyll.

Evaluation of disease resistance

Leaves from T_1 transgenic tomato lines as well as control plants were inoculated with mycelial disks (5 mm diameter) of *B. cinerea* grown on potato-dextrose agar medium for 3 days. Untransformed plants regenerated from leaf disks, which were grown under the same conditions as transformants, were used as controls. The mycelial disks were put on the upper side of leaves and kept in a moist container in the dark at 20°C. Ten inoculation sites were prepared using three to six leaves per transformant and the experiment was performed in three replicates (Bi et al., 1999).

The diameters of the necrotic lesions were measured 4 and 8 days after inoculation and their areas were calculated. CE for each treatment was calculated by using the values of the disease severity measurements in the transgenic plants and control (*Dt* and *Du*, respectively) as follows: CE (%) = 100 - (*Dt*/*Du*) × 100. The effect of disease control was evaluated based on reduction of the index of symptoms (Ruth et al., 2001).

RESULTS

Tomato cotyledons were subjected to 1-day pre-culture, then to *A. tumfaciens* inoculation for 2-day co-cultivation, followed by two–step selective culture. Calli were formed at the wounded cotyledon petioles during selection on M_2 medium. About half of the calli were green that was taken as kanamycin resistance. Shoots of 1 cm in length were excised and transferred to the rooting medium (M_3). Most of the shoots rooted within 1 week, while no rooting occurred in control plants under selective regeneration medium (M_2). The rooted plants of 4-5 cm in height were transplanted to soil and they grew, formed flower and produced the seeds. Regeneration and transformation efficiency with foreign genes were summarized in Table 2.

GFP expression in T_0 transformed plants was observed by fluorescence microscope. Leaves, stems, flowers, fruits and seeds were randomly selected to assay GFP activity (Figure 2). Green fluorescence was clearly visible in callus and the other part of transformed plants, while it expressed yellow fluorescence in the control. It proved that GFP gene was transformed into tomato genome. The rooted transformants expressing GFP activity were then transferred to soil and maintained in greenhouse to produce seeds.

To confirm the presence of the target gene in the putative transgenic plants, the T_0 plants were subjected to PCR analysis with the primers specific for *CHI* gene and *alfAFP* gene. Agarose gel electrophoresis of PCR products from the transgenic plant samples showed the expected 238 bp for *alfAFP* gene and 992 bp for *CHI* gene bands (Figure 3).

Nine randomly sampled PCR-positive plants for *CHI–AFP* gene were further subjected to Southern blot analysis (Figure 4). *CHI–AFP* genes were detected in most T_0 plants analyzed, whereas no hybridization signal was detected in the control test. The number of hybridizing bands that varied from one to four reflected the number of inserted loci of the transgenes in the plant genome (Figure 4). Five of the nine plants contained a single T-DNA locus. The frequency of single inserts was higher

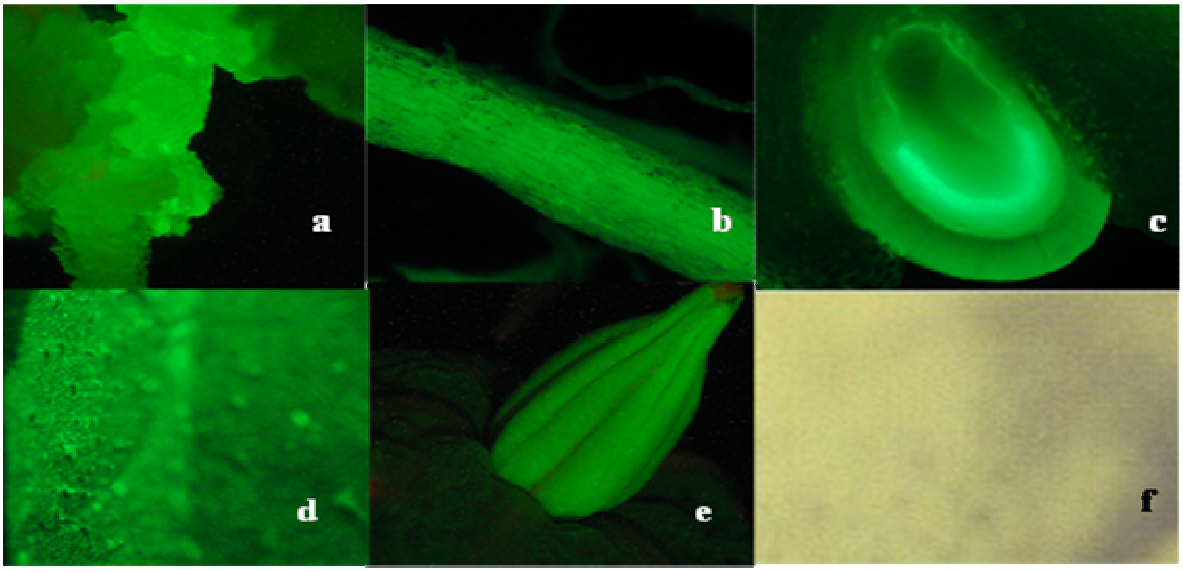
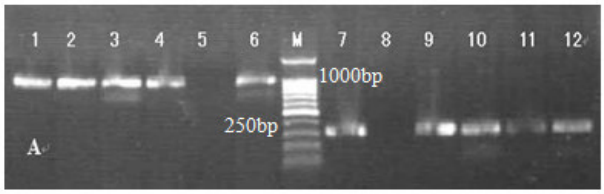
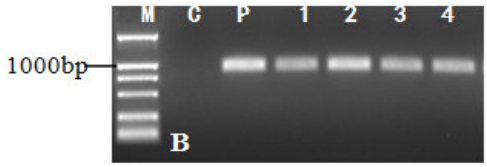


Figure 2. Expression of GFP in different parts of T_0 transgenic tomato plants: a, calli; b, root; c, seed; d, leaf; e, flower; and f, leaf of non-transgenic.





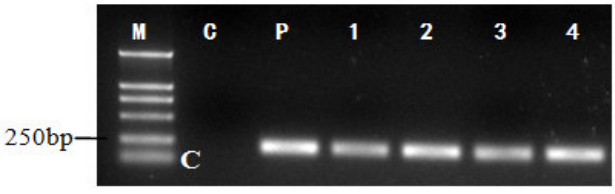


Figure 3. Detection of *alfAFP* gene, *CHI* gene and *CHI - AFP* gene in T_0 transgenic tomato by PCR. M: DNA markers. (A) Lanes 1 – 4, independent transgenic lines (detection of *CHI* gene); lanes 5 and 8 nontransformed plants; lanes 6 and 7 plasmids; lanes 9 - 12 independent transgenic line (detection of *alfAFP* gene). Note: in lanes 4 and 9, 3 and 10, 2 and 11, 1 and 12, DNA from the same lines as templates, respectively, was used. (B) Lane C, non-transformed plants; lane, P-plasmids; lanes 1 - 4, independent transgenic lines (detection of *CHI* gene). (C) Lane C, non-transformed plants; lane, P-plasmids; lanes 1 - 4, independent transgenic lines (detection of *alfAFP* gene).

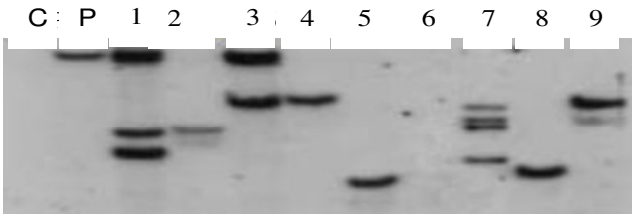


Figure 4. Southern-blot analysis of PCR-positive T_0 transformed plants with CHI - AFP gene. Lane C, DNA from nontransformed plant; lane P, plasmid pAFP-Chi as positive control; lanes 1-9, DNA from T_0 transformed plants (T_0 lines: S_1, S_3, S_4, S_8, S_{12}, S_{15}, S_{18}, S_{20}, S_{24}).

than that observed in tomato by Ouyang et al. (2003). The difference could be due to the *Agrobacterium* strain and plasmid. EHA105 was much more virulent than other strains such as LBA4404. This could lead to more frequent T-DNA transfer to a single plant cell (Gelvin, 2003).

The transcription levels of PCR-positive T_0 plants were determined by Northern blotting (Figure 5). The line number in accordance with the lines analyzed in Southern blot (Figure 4). Five of six transformants examined showed hybridization signal, which lacked in the control plant. No signal was observed for both *CHI* and *AFP* gene expression from S_{12}, which indicated something wrong (deletion, rearrangement) on the sequence of the transformed DNA.

Transcriptional silencing of transgene could be caused by position effect (Wen and Tim, 2000), enhanced promoters (Covey and Al-Kaff, 2000) or dose effect (Mao et al., 2003).

Western blotting revealed the accumulation of

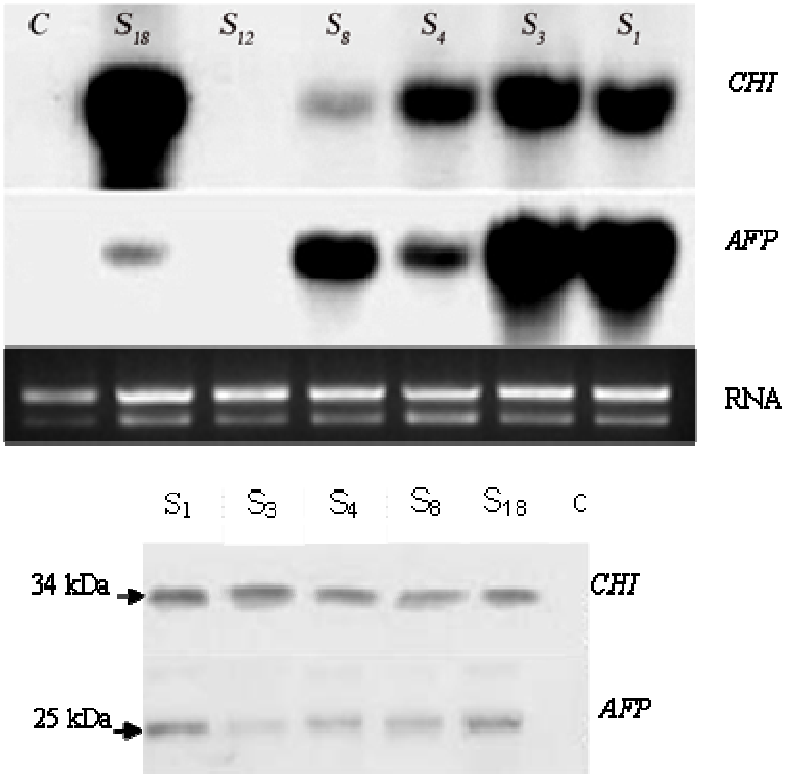


Figure 5. Northern blot and Western blot analysis with leaves of T_1 transgenic plants with *CHI- AFP* gene. T_1 transgenic plants - lines S_{18}, S_{12}, S_8, S_4, S_3, S_1 and C - nontransformed control plant. Northern blot was hybridized with ^{32}P-labeled DNA fragments encoding rice CHI gene and alfalfa defensin gene *alfAFP*.

transgenic protein in leaves. The protein bands were of the expected sizes (34 kDa for *GHI* and 25 kDa for *AFP*) (Figure 5). No bands of similar sizes were seen in nontransformed controls.

To confirm the inheritance of the transgenes, the T_1 transgenic lines grown in greenhouse were analyzed (Figure 6). Most of the transgenic plants were survived in comparison to control plants which after inoculation showed severe symptoms and all died within 2 weeks after infection (Figure 7). The transgenic plant harboring *CHI-AFP* cassette showed the highest disease resistance among all three types of transgenic plants. However, the diffe-rences of resistance did not reach a significant level in the single gene group. The result suggested that there was a synergism of *alfAFP* and *CHI* gene contributing to the resistance to *B. cinerea*.

DISCUSSION

Transgenic tomato plants carrying *CHI* and *AFP* gene were obtained, thus indicating that co-transformation with plant defense genes can be an approach to protect crops against *B. cinerea* infection than individual transformations. Some independent lines which had high disease resistance as well as high expression levels of *CHI* and *AFP* could be selected for the further studies and molecular breeding for *B. cinerea* resistance, such as S_1 and S_3, which showed higher consistency in expression levels.

Whether transcript levels are related to resistance levels, no definitive answer exists (Park et. al., 2005). Southern-blot analysis indicated that some T_0 plants possessed several copies of the gene, whereas others contained only one copy. Northern blotting demonstrated that transcription levels differed among T_0 plants. Tomato disease degree caused by *B. cinerea* was tested with T_1 lines transgenic plants, different lines in T_1 generation being variable. The higher resistance was related to expression level of transgene showing dosage effect. Apparently, low variability and stable expression of transgenes were the foundation for molecular breeding of disease resistance. Correlation analysis showed that levels of gene expression did not depend on the number of integrated transgene copies (at $P < 0.05$), as reported by other authors (Allen et al., 2000). Two transformants (S_4, S_8) showed large differences between *CHI* and *alfA FP* expression level. It may be that multiple genes could

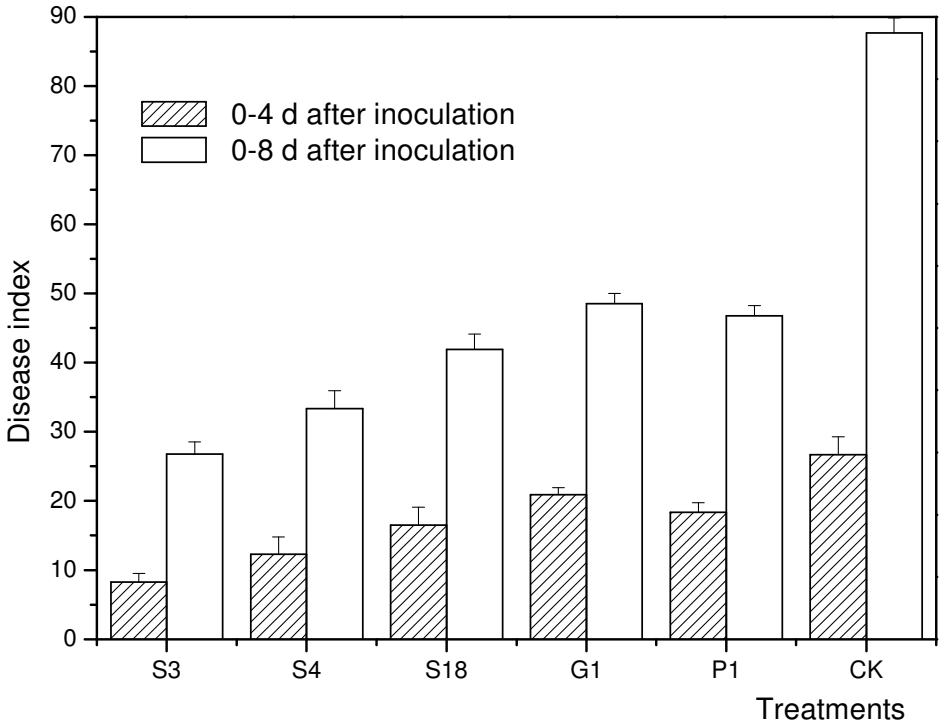


Figure 6. Resistance to *Botrytis cinerea* of T_1 transgenic tomato lines. CK, Nontransformed plant; (1) disease index increase 0-4 day(s) after inoculation; (2) lesion increase 0-8 day (s) after inoculation. G_1, T_1 transformed line with *CHI* gene. P_1, T_1 transformed line with *alfAFP* gene.

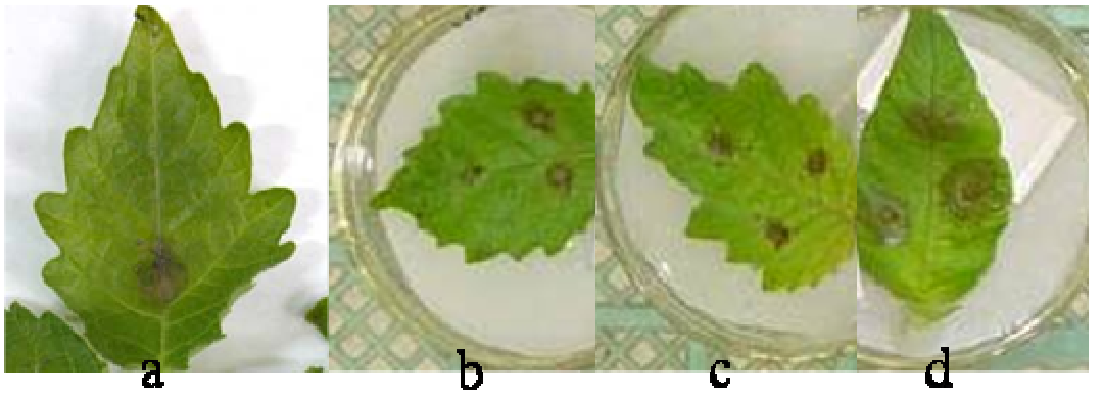


Figure 7. Bioassay of homozygous T_3 transgenic rice lines with *Botrytis cinerea* to evaluate gray mold disease resistance.
Control and transgenic plants were infected with *B. cinerea* and pictures were taken 4 days after inoculation. a - c: transgenic plants; 3: control.

influence expression levels on each other or the expression levels are highly variable and are poorly correlated among some individual transformants (Fagard and Vaucheret, 2000).

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