Combined overexpression of chitinase and defensin genes in 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 T1 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 inflict 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
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 (Sridive 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 *aalfAFP* 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°C and light intensity of 50-60 µmol/m² 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 *aalfAFP* and pEAFP-Chi plasmid with *aalfAFP* and *Chi* gene driven by the 35S promoter and *A. tumefaciens* nopaline synthase (*nptII*), transcriptional terminator sequence was used as the vector system for transformation. The neomycin phosphotransferase II (*nptII*) 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°C 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°C 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 M₁ at 26°C 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 M₁ for a day. After co-cultivation, the infected cotyledons were transferred to selective regeneration medium M₂ 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 (M₃). 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 *aalfAFP* specific primer sequences (5′...3′) GAAT-GAGGCTTT GAA GAA GTC TCT TG and AAC ATC TTT TGA GAC ACC AG and *Chi* gene specific primer sequences (5′...3′) GAAT-GAGGCTTT GTAAATTTCAC and CGTTAAATTTCAAAGACC TCTG GTT were performed in 25 µl (total volume) of reaction mixture consisting of 10 × PCR reaction buffer including 1.5 µM Mg²⁺, 50 ng template DNA, 0.2 mmol/l deoxynucleotide triphosphates, 1.5 mM MgCl₂, 0.2 µM of each primer and 1 unit of Taq DNA polymerase. After the initial denaturing for 2.5 min at 94°C, PCR was performed during 35 cycles (denaturing at 94°C for 1 min, annealing at 56°C for 1 min, synthesis at 72°C for 1.5 min). Thereafter the program was terminated by an extension at 72°C for 10 min. The amplification was analyzed by electrophoresis in 1% agarose ethidium bromide gels.

For genomic Southern blot analysis, 20 µg of T₂ plant DNA from transgenic plants and untransformed control plant was digested with *HindIII* and then fractionated by electrophoresis in 1% agarose gel. For Northern blot analysis, total RNA was extracted from leaves of T₂ 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 32P-labeled probe containing *nptII* gene according to the

**Figure 1.** Schematic diagram of the whole binary vectors pAFP-Chi.
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 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).

Evaluation of disease resistance

Leaves from T1 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) x 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. tumefaciens inoculation for 2-day co-cultivation, followed by two–step selective culture. Calli were formed at the wounded cotyledon petioles during selection on M2 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 (M3). Most of the shoots rooted within 1 week, while no rooting occurred in control plants under selective regeneration medium (M2). 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 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.

The frequency of single inserts was higher (Figure 4). Five of the nine plants contained a single T-locus. The number of inserted loci of the transgenes in the plant genome bands that varied from one to four reflected the number of hybridizing genes were detected in most T0 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 T0 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.

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

<table>
<thead>
<tr>
<th>Media</th>
<th>Composition (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>MS + 2 mg/l ZT + 0.2 mg/l IAA + 50µM AS + sucrose 15 g/l + 0.5% agar</td>
</tr>
<tr>
<td>M2</td>
<td>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</td>
</tr>
<tr>
<td>M3</td>
<td>MS + IAA2.0 mg/l + Km 100 mg/l + Cef 300 mg/l + sucrose 20 g/l + 0.5% agar</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Foreign gene</th>
<th>No. of explants</th>
<th>No. regenerated explants</th>
<th>Regeneration frequency (%)</th>
<th>No. transplanted GFP-positive plants</th>
<th>Transformation frequency(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHI – AFP</td>
<td>139</td>
<td>67</td>
<td>48.2</td>
<td>32</td>
<td>23.0</td>
</tr>
<tr>
<td>alfAFP</td>
<td>124</td>
<td>54</td>
<td>43.6</td>
<td>28</td>
<td>22.6</td>
</tr>
<tr>
<td>CHI</td>
<td>93</td>
<td>55</td>
<td>59.1</td>
<td>23</td>
<td>24.7</td>
</tr>
<tr>
<td>Vacant vector</td>
<td>110</td>
<td>81</td>
<td>73.6</td>
<td></td>
<td></td>
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</tbody>
</table>
Figure 2. Expression of GFP in different parts of T₀ 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 T₀ 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 T₀ 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 T₀ transformed plants (T₀ lines: S₁, S₃, S₄, S₈, S₁₂, S₁₅, S₁₆, S₂₀, S₂₄).

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 T₀ 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 S₁₂, 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
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 T1 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 differences of resistance did not reach a significant level in the single gene group. The result suggested that there was a synergism of allAFP 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 S1 and S3, 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 T0 plants possessed several copies of the gene, whereas others contained only one copy. Northern blotting demonstrated that transcription levels differed among T0 plants. Tomato disease degree caused by B. cinerea was tested with T1 lines transgenic plants, different lines in T1 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 (S4, S8) showed large differences between CHI and allAFP expression level. It may be that multiple genes could
Figure 6. Resistance to *Botrytis cinerea* of T<sub>1</sub> 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<sub>1</sub>, T<sub>1</sub> transformed line with CHI gene. P<sub>1</sub>, T<sub>1</sub> transformed line with alfAFP gene.

Figure 7. Bioassay of homozygous T<sub>3</sub> 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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