

## Ectopic Expression of an Oat Thionin Gene in Carnation Plants Confers Enhanced Resistance to Bacterial Wilt Disease

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### Abstract

Under the control of *Arabidopsis* tryptophan synthase  $\beta$  subunit promoter (*PTSBI*), the oat thionin gene (*Asthi1*) was introduced into carnation (*Dianthus caryophyllus* L.) via *Agrobacterium*-mediated gene transfer. Leaf bases of three cultivars of carnation ('Scania', 'Percian Pink-Sim' and 'U. Conn. White Sim') were infected with *Agrobacterium* harboring the binary vector with *PTSBI::Asthi1* and hygromycin genes, and cultured on the medium containing hygromycin. Independent 99 lines resistant to hygromycin were regenerated and the introduction of the transgene was confirmed in 11 lines by polymerase chain reaction. Transgenic carnation plants exhibited the resistance against infection with *Burkholderia caryophylli* which caused bacterial wilt, and the levels of resistance correlated with those of the thionin gene transcript.

**Key words:** bacterial wilt, disease resistance, thionin, transgenic carnation.

### Abbreviations

*Asthi1*, the oat leaf thionin gene; Hyg<sup>R</sup>, hygromycin resistant; Km<sup>R</sup>, kanamycin resistant; *P35S*, the 35S RNA promoter of *Cauliflower mosaic virus*; *PTSBI*, the promoter of the *Arabidopsis* tryptophan synthase protein  $\beta$  subunit gene.

### Introduction

Carnation (*Dianthus caryophyllus* L.) is one of the major cut flower in floriculture market and an important target for breeding of new varieties in the world. In Japan, carnation plants are cultured through the year in the greenhouse. Such a continuous planting frequently causes the outbreak of soil-borne diseases. Bacterial wilt caused by *Burkholderia caryophylli* is one of the most serious diseases of carnation plants. The bacteria prefer high temperatures for multiplication displaying severe systemic symptoms in infected plants, especially during summer in Japan. Finding and elimination of infected plants are not so easy because the pathogen infects the roots and colonizes the vascular system of plants without external symptoms. Instead of the impor-

tance of breeding of resistant carnation to bacterial disease, the breeding by conventional crossing has just begun to study recently (Onozaki *et al.*, 2002). Thus, we attempted to generate resistant carnation plants to the disease by genetic manipulation, which is a promising strategy to improve crops in a short period without the need for time-consuming crosses.

As the genes available for this purpose, thionin genes are of particular interest because they have been reported to have antimicrobial activity; 1) a barley leaf thionin induced by *Septoria nodorum* (Stevens *et al.*, 1996) and *Drechslera graminea* (Vale *et al.*, 1994) was toxic to phytopathogenic bacteria and fungi *in vitro* (Bohlmann *et al.*, 1988; Molina *et al.*, 1993), 2)  $\alpha$ -holdothionin from barley seed, conferred enhanced resistance to *Pseudomonas syringae* when overproduced in tobacco plants (Carmona *et al.*, 1993), and 3) over-expression of the *Arabidopsis thi2.1* gene in *Arabidopsis* plants enhanced resistance to *Fusarium oxysporum* (Epple *et al.*, 1997), 4) over-expression of the oat leaf thionin (*Asthi1*) conferred resistance against the bacterial attack in transgenic rice plants (Iwai *et al.*, 2002). Among these thionin genes, we selected

*Asthi1* gene for our experimental use because *Asthi1* was useful for soil-borne bacterial attack.

For the expression of transgene, the choice of a promoter suitable for the experimental purpose is critical to generate useful transformants with desirable characteristics. The promoter of *Arabidopsis* tryptophan synthase protein  $\beta$  subunit gene (*PTSBI*) was shown to be a powerful promoter comparable to the 35S RNA promoter (*P35S*) of the *Cauliflower mosaic virus*, and *PTSBI* expressed at a high level in the vascular system (Shirasawa-Seo *et al.*, 2002) in which *B. caryophylli* preferentially multiplies and accumulates.

We described here that an oat thionin gene *Asthi1* under the control of *PTSBI* conferred enhanced resistance against bacterial wilt in an expressed gene product-dependent manner in transgenic carnation.

## Materials and Methods

### Construction of the introduced gene

The E7 $\Omega$ I promoter in the binary vector pMLH7133-thionin (Iwai *et al.*, 2002) was exchanged by *PTSBI* (Shirasawa-Seo *et al.*, 2002) at the sites of *Hind*III and *Bam*HI. The resulting construct pMLH-TSB1-thionin (*PTSBI::Asthi1*) (Fig. 1), which contains kanamycin-resistance gene (*Km<sup>R</sup>*) driven by the promoter of the nopaline synthase gene from *Agrobacterium tumefaciens*, *Asthi1* gene from oat driven by *PTSBI* and hygromycin-resistance gene (*Hyg<sup>R</sup>*) driven by a modified promoter of the *Cauliflower mosaic virus* 35S RNA (Mochizuki *et al.*, 1999), was introduced into *A. tumefaciens* EHA101 (gift from Dr. Elizabeth Hood, VP Technology Prodigene) as described (Holsters *et al.*, 1978).

### Generation of transgenic carnation plants

As plant materials, three cultivars of carnation

(*Dianthus caryophyllus* L.), 'Scania (syn. Red Sim)', 'Percian Pink-Sim' and 'U. Conn. White-Sim' were used. Transformation was performed as described (Firoozabady *et al.*, 1995) with some modifications as follows. *A. tumefaciens* EHA101 containing the binary vector pMLH-TSB1-thionin was cultured for 2 days (at 28 °C in dark) on solid LB medium containing 50 mg l<sup>-1</sup> hygromycin and 50 mg l<sup>-1</sup> kanamycin. After the overnight-culture in the liquid LB medium containing 50 mg l<sup>-1</sup> hygromycin and 50 mg l<sup>-1</sup> kanamycin, the bacterial suspension was incubated with the leaflets from the shoots cultured *in vitro* with 100  $\mu$ M acetosyringon for a few minutes. The infected leaflets were transferred on the IT medium (Firoozabady *et al.*, 1995) and incubated further seven days at 20 °C in dark. The following culture was done at 24 °C under 16 h light/day (67.2  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>). After co-culture, the infected leaflets were transferred on selection medium ITCH (IT supplemented with 500 mg l<sup>-1</sup> carbenicillin and 50 mg l<sup>-1</sup> hygromycin) and incubated for one month. After further incubation on MS medium (Murashige and Skoog, 1962) containing 500 mg l<sup>-1</sup> carbenicillin for one month, regenerated shoots were excised and their leaflets placed on fresh ITCH medium for secondary selection for one month. Then regenerated shoots were transferred on MS medium to allow rooting. We confirmed the integration of the transgene in hygromycin-resistant plants by detecting the *PTSBI* region and the thionin coding region by polymerase chain reaction (PCR). For the former region (1.5 kb in size) a sense primer 5'-CAGTAAGCTTGAATTCCTTCATATCTCCTGCAAAGT-3' (corresponding to nucleotide positions 1 to 26; accession number M23872) and an antisense primer 5'-TAGCGGATCCTACTGAATGAATCTCTCTCTGA-3' (1571 to 1550) were used. For the latter region (0.5 kb in size), a sense primer 5'-CCAAGGATCCATGGGAAGTATC-3' containing *Bam*HI site in the 5' flanking

pMLH-TSB1-thionin

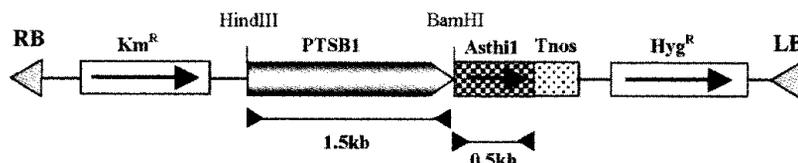


Fig. 1. Structure of the introduced gene.

pMLH, the binary vector which is a derivative from pBI121; RB and LB, right and left borders of T-DNA of *Agrobacterium tumefaciens* Ti plasmid, respectively; *PTSBI*, the promoter sequence of the *Arabidopsis* *TSBI* gene; *Asthi1*, the coding sequence of oat thionin; *Tnos*, the terminator sequence of the nopaline synthase gene; *Km<sup>R</sup>*, kanamycin-resistance gene driven by *Pnos*; *Hyg<sup>R</sup>*, hygromycin-resistance gene driven by a modified *P35S*. Bi-directional arrow shows the region amplified by PCR.

region and an antisense primer 5'-GCATGAGCT-CTTAGGCTGCAA-3' containing *SacI* site in the 5' flanking region were used (Fig. 1). The PCR reaction was run at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min for 30 cycles.

#### RNA and DNA gel blot analyses

Total RNA was extracted from leaves using RNeasy Plant Mini Kit (Qiagen). Genomic DNA was isolated from leaves as described (Murray and Thompson, 1980). RNA and DNA gel blot analyses were performed using the DIG non-radioactive nucleic acid labeling and detection system (Roshe) according to the manufacturer's instructions. The thionin coding region as described above was used as the probe.

#### Protein extraction and immunoblot analysis

Thionin protein was extracted from leaves according to the method by Carmona *et al.* (1993) with modifications as follows. Leaves were ground in liquid nitrogen, treated with 10% (v/v) trichloroacetic acid in acetone at -20 °C for 45 min, washed with cold acetone, and dried in air. The acetone powder was extracted with 0.05 M H<sub>2</sub>SO<sub>4</sub> (3 ml per gram fresh weight) for 1 h. The extract was washed twice with cold acetone, resolved in 1 x SDS-PAGE sample buffer (0.05 mM Tris-HCl, pH 6.8, 2% SDS, and 1% mercaptoethanol, 15% sucrose, 0.1% bromophenol blue), boiled for 5 min, and loaded to PAGEL SPU-15S gel (ATTO). Immunoblot analysis was performed as described (Mitsuhashi *et al.*, 2000). As a primary antibody, anti-thionin antibody (Iwai *et al.*, 2002) was used at a dilution rate of 1:1000. As a secondary antibody, anti-rabbit IgG (SIGMA) was used at a dilution rate of 1:10,000.

#### Bacterial infection assays and evaluation of resistance

Bacterial infection was performed by the method of Onozaki *et al.* (1999) with some modifications as follows. Pathogen bacteria *Burkholderia caryophylli* (MAFF302795), which were from MAFF

collection (Tsukuba, Japan), were stored in skim milk medium at -80 °C. The defrosted *B. caryophylli* that had been multiplied on potato dextrose agar (PDA) medium for 3 days at 28 °C was cultured in potato dextrose (PD) liquid medium on a rotary shaker for 2 days at 28 °C. For infection of carnation plants with *B. caryophylli*, a cut-root soaking method was used. Scions of each cultivar were transferred to the rooting medium (vermiculite) and induced roots from the cut stem end. After about 30 days, the tips of the formed roots were cut out. Then, the cut end of roots in plants were dipped into the bacterial suspension containing 0.5 to 5 x 10<sup>6</sup> colony forming units (cfu) ml<sup>-1</sup> in plastic cases for 30 min. Bacterial population in the inoculum was measured by the dilution plate method. Inoculated plants were planted in sterilized soil in 7.5 cm plastic pots in a growth chamber maintained at 28 °C. The number of wilted plants was counted up to 90 days after inoculation, and the disease index was recorded with the scale 0 to 1. Scale 0, no visible symptoms; scale 0.5, wilted; scale 1, dead.

## Results

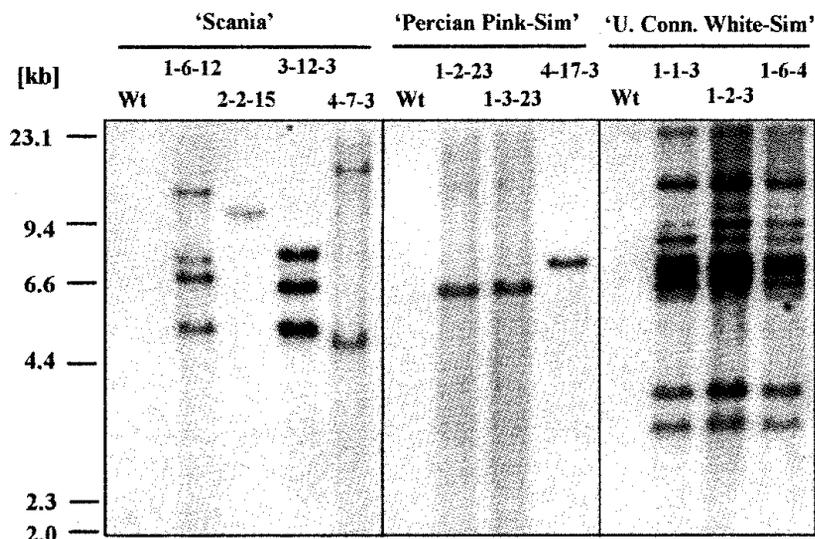
#### Generation of transgenic carnation plants

We constructed a binary vector, pMLH-TSB1-thionin (Fig. 1), which contains *PTSBI* (Shirasawa-Seo *et al.*, 2002) as a strong promoter for expression of the oat leaf thionin gene *Asthi1* and a hygromycin resistance gene (*Hyg<sup>R</sup>*) as a selectable marker (Mochizuki *et al.*, 1999). This vector was introduced into 3 cultivars of carnation plants by the *Agrobacterium*-mediated transformation. Total 99 independent hygromycin-resistant shoots were generated. The PCR analysis revealed that 11 out of the 99 shoots had the transgene and thus the transformation efficiency is 0.2 to 0.6% (Table 1). The 11 independent shoots were further subjected to secondary selection to eliminate non-transformed cells. As the consequence, 3 to 18 regenerated shoots containing the transgene per each independent shoot were obtained from all of 11 shoots. These regenerated shoots were cultured on MS

**Table 1.** Transformation of three cultivars of carnation.

Cultivar	No. of infected leaf sections (A)	No. of hygromycin resistant shoots	No. of PCR positive plants (B)	Efficiency of transformation <sup>1)</sup> (%)
'Scania'	630	20	4	0.6
'Percian Pink - Sim'	594	73	6	1.0
'U. Conn. White - Sim'	594	6	1	0.2

<sup>1)</sup> Efficiency of transformation = {(B) / (A)} x 100



**Fig. 2.** DNA gel blot analysis of the *Asthi1* gene in transgenic carnation lines.

Digested genomic DNA (10  $\mu$ g) with *Bam*HI was hybridized with the thionin region of *Asthi1* as a probe under high stringency conditions (two washes with 0.1 x SSC [1 x SSC is 0.15 M NaCl plus 0.015 M sodium citrate] and 0.1% sodium dodecyl sulfate at 65 °C for 20 min). Numbers under cultivar names indicates that (No. of transformants)–(No. of regenerated shoots after secondary selection)–(No. of rooted plants from the regenerated shoots)

medium and rooted plants from the shoots were planted on soil. DNA blot analysis showed a single or multiple inserts in the transgenic plants (Fig. 2). All transgenic carnation plants showed normal phenotype.

#### *Expression of thionin gene construct in carnation plants*

RNA gel blot analysis showed that the *Asthi1* transcripts were detected in the leaves of all *PTSBI::Asthi1* carnation plants tested. The data of cv. 'Scania' was shown in Fig. 3A. The level of *Asthi1* transcripts was 16 to 95% of that in a positive control transgenic tobacco line T5, with *Asthi1* gene under the control of *PE7 $\Omega$ I*, which is a modified *P35S* promoter conferring 20-fold higher activity than *P35S* (Mitsuhara *et al.*, 1996). 'Scania'2-1-45, 2-2-15, 2-3-12, 2-3-13 and 2-6-13, which are the clone and have one copy of the transgene (Fig. 2), showed lower level of *Asthi1* transcripts (28 to 63%) than other 'Scania' transformants which have two or more copies of the transgene (62 to 95%) (Fig. 3A). Thus, the copy number of the transgene is considered to have effect on the level of transcript. Furthermore, thionin protein was detected in leaves of the transgenic carnation line 'Scania'3-11-12 (S3-11-12) which contained the highest level of *Asthi1* transcript among regenerated *PTSBI::Asthi1* carnation plants (Fig. 3A, B).

#### *Evaluation of resistance to Burkholderia caryophylli*

We evaluated the resistance of the *Asthi1* expressers against infection of *B. caryophylli*. At 40 days after inoculation of cut roots originated from carnation scions with bacteria, disease symptoms were determined. The examples of disease symptoms of *PTSBI::Asthi1* carnation scions were shown in Fig. 4A. The progression of the disease phenotypes was monitored in 10 to 90 days after inoculation using 25 wild-type plants as control. When 6 to 7 plants were used for each *PTSBI::Asthi1* lines, appearance of symptom was delayed 10 to 20 days in some lines within 50 days. In P1-1-32 and P1-2-46 line, 28 and 18% of the inoculated plants showed resistance even at 90 days after bacterial inoculation, while all wild type plants were wilted at 70 days (Fig. 4B). The level of *Asthi1* transcripts was correlated to that of the average of disease index ( $r = -0.783$ ,  $n = 20$ ), indicating expression of the oat leaf thionin gene confers resistance to the bacterial attack (Fig. 4C). The transgenic carnation line S3-11-12, which contained the highest level of *Asthi1* transcript among regenerated *PTSBI::Asthi1* carnation plants (Fig. 3A, B), showed lowest disease index 0.5.

#### **Discussion**

We describe here that generation of transgenic carnation plants resistant to carnation bacterial wilt disease by overproduction of an oat leaf thionin

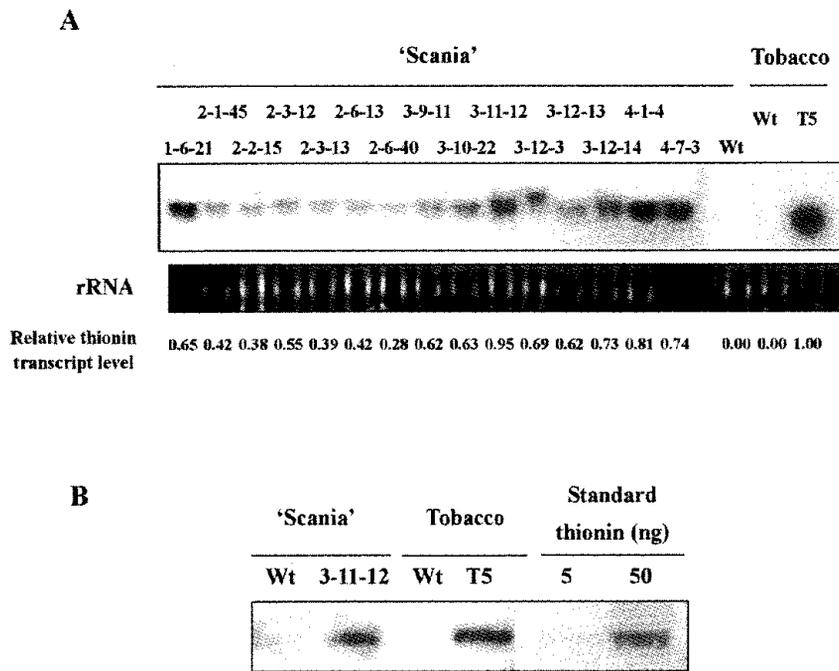


Fig. 3

(A) RNA gel blot analysis.

Total RNA (20  $\mu$ g) from the leaf was hybridized with the thionin region of *Asthi1* as a probe under high stringency conditions [two washes with 0.1 x SSC (1 x SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate at 65 °C for 20 min]. Numbers under cultivar names indicates that (No. of transformants)–(No. of regenerated shoots after secondary selection)–(No. of rooted plants from the regenerated shoots). Relative thionin transcript levels are shown at the bottom of the figure. Wt, wild type; T5, transgenic tobacco line 5 introduced *Asthi1* under the modified *P35S*. Control (lower panel); rRNA stained with ethidium bromide.

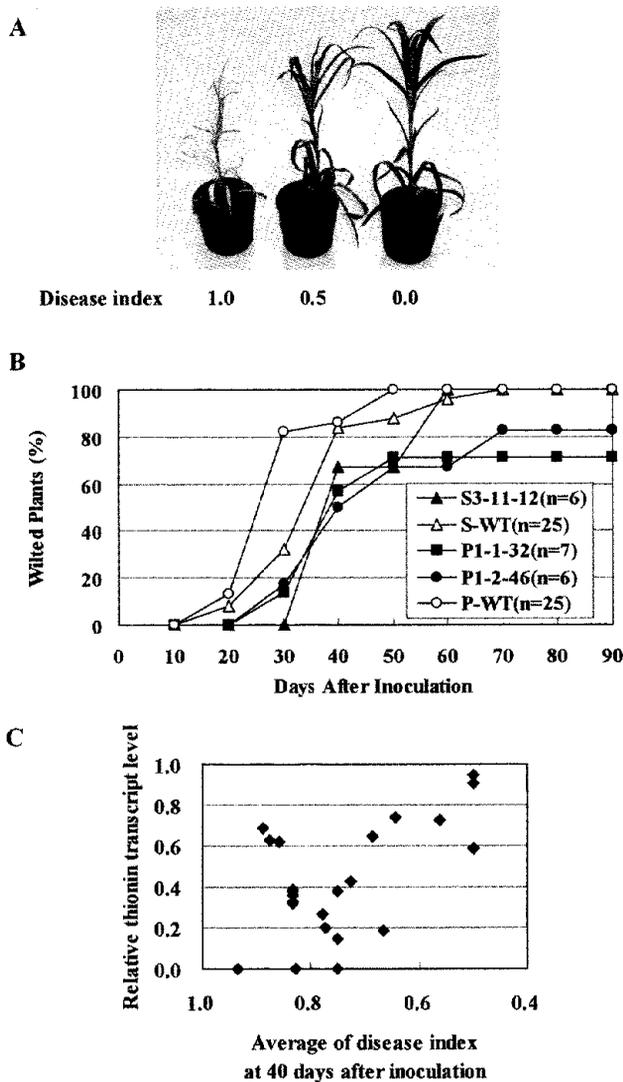
(B) Immunoblot analysis.

Accumulation of *Asthi1* protein in the leaf of transgenic carnation lines 'Scania'3–11–12. Synthetic *Asthi1* was used as the standard. Polyclonal antibody against synthetic *Asthi1* was prepared in rabbits and used as the primary antibody. Crude protein extract corresponding to 20 mg of fresh leaf was used in each lane. Wt, wild type; T5, transgenic tobacco line 5 introduced *Asthi1* under the modified *P35S*.

*Asthi1*. Iwai *et al.* (2002) showed overproduction of *Asthi1* in rice plants conferred enhanced resistance to soil-borne bacterial diseases. Together the *Asthi1* would be effective to provide resistance against a wide-rang of soil-borne bacterial disease in both monocotyledonous and dicotyledonous plants.

For the expression of the *Asthi1* gene, we used *Arabidopsis* promoter *PTSBI* but not *P35S* to avoid the repeated use of *P35S* sequence in the binary vector, in which *P35S* is used for driving the *Hyg<sup>R</sup>* gene. Introduction of repeated sequences into plant genome has potential to trigger the sequence homology-dependent gene silencing (Vaucheret, 1993; Park *et al.*, 1996). Furthermore, *PTSBI* is patent free and would confer a high level of the thionin gene expression in the vascular system (Shirasawa-Seo *et al.*, 2002), in which the pathogenic bacteria,

*B. caryophylli* preferentially proliferate. We could detect delayed appearance of wilting symptom in transgenic carnation. The level of resistance is at a considerable level, but not enough for practical use. The transgenic rice plants introduced *Asthi1* gene driven by the modified *P35S*, which was 20 and 70-fold stronger than *P35S* in tobacco and rice cells, respectively (Mitsuhara *et al.*, 1996) showed high resistance to bacterial disease (Iwai *et al.*, 2002) with 40-fold higher accumulation of thionin protein compared with our transgenic carnation plants [2.5  $\mu$ g (g fresh leaf)<sup>-1</sup>]. We think that the use of *PTSBI* itself was useful for specific expression in vascular system in which bacteria multiply. Another reason for the middle level of resistance would be the sensitivity of thionin protein to plant proteases in apoplast. The thionin is originally secreted out-



**Fig. 4.** Response of *PTSBI::Asthi1* carnation scions to *Burkholderia caryophylli*.

A. Disease symptoms of *PTSBI::Asthi1* carnation scions at 40 days after inoculation.

B. Disease progression curves of transgenic carnation plants.

S, 'Scania'; P, 'Percian Pink-Sim'; WT, Wild type. Numbers after cultivar names indicates that (No. of transformants)–(No. of regenerated shoots after secondary selection)–(No. of rooted plants from the regenerated shoots).

C. Correlation between the relative thionin transcript level and the average of disease index at 40 days after inoculation. The thionin transcript level was calculated as the relative value compared to that in transgenic tobacco line 5 which contains *PE7Q1::Asthi1* gene.

side cells and ionically bound to the cell wall in oat plant and in transgenic rice plant (Iwai *et al.*, 2002). Because the signal peptide for secretion to apoplast was contained the *PTSBI::Asthi1* construct, the expressed thionin in carnation plants should be

transferred outside cells. The stability of antimicrobial peptide in apoplast depends on the plant species (our unpublished data). In the apoplast of carnation plants, *Asthi1* protein would be not so stable. Anyway, the middle level of resistance to *B. caryophylli* was clearly found by over-expression of *Asthi1* gene in this study. Improvement of the promoter by such as the use of an enhancer sequence would give an enough expression level of the thionin gene with enhanced resistance to bacterial disease.

In this study, we got only eleven independent transgenic carnation plants from 1,818 *Agrobacterium*-infected leaf sections from 3 cultivars. Previously we tried to introduce the transgene to other carnation cultivars, for example, 'Coral', 'Izu-pink', 'Izu-rose', 'Kurenainotsubasa' and 'Yukihi', but regenerated shoots did not carry the transgene (data not shown). The low transformation efficiency is a hurdle to overcome for generation of transgenic carnation. Further improvement of transformation system is also required.

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