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# T4 Lysozyme and Attacin Genes Enhance Resistance of Transgenic 'Galaxy' Apple against *Erwinia amylovora*

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**Abstract. Genes encoding lysozyme (*T4L*) from T4 bacteriophage and attacin E (*att*E) from *Hyalophora cecropia* were used, either singly or in combination, to construct plant binary vectors, pLDB15, p35SAMVT4, and pPin2Att35SAMVT4, respectively, for *Agrobacterium*-mediated transformation of 'Galaxy' apple, to enhance resistance to *Erwinia amylovora*. In these plasmids, the *T4L* gene was controlled by the cauliflower mosaic virus 35S promoter with duplicated upstream domain and the untranslated leader sequence of alfalfa mosaic virus RNA 4, and the *att*E gene was controlled by the potato proteinase inhibitor II (*Pin2*) promoter. All transgenic lines were screened by polymerase chain reaction (PCR) for *T4L* and *att*E genes, and a double-antibody sandwich enzyme-linked immunosorbent assay for neomycin phosphotransferase II. Amplification of *T4L* and *att*E genes was observed in reverse transcriptase-PCR, indicating that these genes were transcribed in all tested transgenic lines containing each gene. The attacin protein was detected in all *att*E transgenic lines. The expression of *att*E under the *Pin2* promoter was constitutive but higher levels of expression were observed after mechanical wounding. Some *T4L* or *att*E transgenic lines had significant disease reduction compared to nontransgenic 'Galaxy'. However, transgenic lines containing both *att*E and *T4L* genes were not significantly more resistant than nontransgenic 'Galaxy', indicating that there was no in planta synergy between *att*E and *T4L* with respect to resistance to *E. amylovora*.**

Fire blight, caused by *Erwinia amylovora* (Burr.) Winslow et al., is one of the most destructive diseases of apple (*Malus* sp.) and pear (*Pyrus* sp.), and many Rosaceous ornamental plants in many production regions (Sobiczewski et al., 1997). Many commercial apple scion cultivars including 'Gala', 'Idared', 'Jonathan', 'Fuji', and 'Gingergold' and the rootstocks, 'M.9' and 'M.26' are highly susceptible to *E. amylovora* (Aldwinckle et al., 1998). Introduction of antibacterial protein genes using *Agrobacterium*-mediated transformation could be an effective way to enhance resistance to phytopathogenic bacteria (Destéfano-Beltrán et al., 1993; Montanelli and Nascari, 1990; Mourgues et al., 1998a; Norelli et al., 1994; Reynoird et al., 1999).

Attacin is an antimicrobial protein that is induced in *Hyalophora cecropia* (giant silk moth) pupae in response to bacterial infection. The gene encoding attacin E (*att*E) has been transferred to tobacco (*Nicotiana tabacum* var. *Xanthi*) (Destéfano-Beltrán, 1991), apple rootstocks and scion cultivars (Borejsza-Wysocka et al., 1999; Ko et al., 2000; Norelli et al., 1994), pear (*P. communis* L.) (Reynoird et al., 1999) and anthurium (*Anthurium andraeanum*) (Chen and Keuhnle, 1996), to enhance resistance to phytopathogenic bacteria. The insertion, transcription, and translation of *att*E were confirmed in a transgenic line of 'M.7' apple rootstock, and fire blight resistance was enhanced (Norelli et al., 1994; Ko et al., 2000). Similarly, transgenic pear expressing *att*E showed a reduction of symptoms caused by *E. amylovora* in in vitro stem tissue (Reynoird et al., 1999).

T4L hydrolyzes *N*-acetylmuramyl-(1-4)-β-linkages in the peptidoglycan of bacteria (Düring, 1996). In addition, T4L has been reported to have microbicidal activity that is independent of its hydrolase activity (Düring et al., 1999). Bacteriophage T4 lysozyme (T4L) inhibits the growth of *E. amylovora* in vitro (Mourgues et al., 1998b). Transgenic potato plants expressing the *T4L* gene showed increased resistance to *E. carotovora* sp. *atroseptica*, causing potato soft rot (Düring et al., 1993).

Attacin increases permeability of the bacterial outer membrane and inhibits synthesis of some outer membrane proteins (Carlsson et al., 1991, 1998; Ourth et al., 1994). Carlsson et al. (1998) and Engström et al. (1984) hypothesized that in *H. cecropia* pupae attacin functions to increase the access of lysozyme to the peptidoglycan layer of bacteria. Attacin increased sensitivity of *Escherichia coli* to hen egg white lysozyme (HEWL) in vitro (Engström et al., 1984). In vitro synergy between cecropin and HEWL has been reported in the inhibition of *E. amylovora* (Mourgues et al., 1998b). The mode of action of HEWL is similar to that of T4L, but T4L shows greater in vitro inhibition of *E. amylovora*, although the reasons for the greater activity of T4L against *E. amylovora* are not known (Mourgues et al., 1998b).

The main goals of this research were to 1) genetically transform 'Galaxy' apple with both *att*E and *T4L* genes, 2) study the expression of *att*E under the control of the potato proteinase inhibitor (*Pin2*) promoter, 3) determine the effect of the *T4L* gene on fire blight resistance, and 4) investigate whether there is synergy between attacin and T4L with respect to resistance to fire blight in transgenic apple lines.

## Materials and Methods

**Plant transformation vectors.** To construct p35SAMVT4 (Fig. 1A) the *T4L* gene was restriction digested from pSR8-36 (Porsch et al., 1998) with *Nco*I, and cloned into the *Nco*I site of pBI525 (Datla et al., 1993) to create pBI525T4. The *Hin*dIII and *Eco*RI fragment of pBI525T4 containing the cauliflower mosaic virus 35S promoter with duplicated upstream B domain (35S) (Kay et al., 1987)/AMV/*T4L*/NOST was then cloned between the *Hin*dIII and *Eco*RI sites on pBI121 (replacing CaMV35S promoter/*gus*/NOST) to produce p35SAMVT4. pLDB15 (Fig. 1B) (Ko et al., 2000; Norelli et al., 1994) contains the *att*E gene under the control of the *Pin2* promoter (Pin2Att).

To construct pPin2Att35SAMVT4 (Fig. 1C) the *Hin*dIII frag-

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ment of pLDB15 containing *Pin2* promoter (Pin2p)/*att*E/*Pin2* terminator (Pin2t) (Destéfano-Beltrán et al., 1991) was cloned into the *Hin*dIII site in p35SAMVT4 to produce pPin2Att35SAMVT4.

DNA restriction, DNA ligation, and gel electrophoresis were performed using standard procedures (Sambrook et al., 1989). *T4L* of pSR8-36 and AMV/*T4L* of p35SAMVT4 were sequenced as described by Sanger et al. (1977) using a 373 DNA sequencer (Applied Biosystems, Foster City, Calif.) (Fig. 1B). The *att*E coding region was not sequenced in this study but had been sequenced previously (Ko et al., 1999). Each plasmid binary vector was transferred to *Agrobacterium tumefaciens* EHA105 using electroporation transformation (Dower et al., 1988). Insertion of *att*E, the AMV/*T4L* gene fragment and the Pin2/*att*E/35S/AMV/*T4L* gene fragment in each plasmid was confirmed using PCR and enzyme restriction analyses.

**Plant materials.** Tissue cultures of 'Galaxy' and 'Liberty' apple were obtained from stock cultures maintained in the Department of Plant Pathology, Cornell University, New York State Agricultural Experiment Station, Geneva. 'Galaxy' shoots were subcultured on an in vitro proliferation medium described by Norelli et al. (1988), but with altered plant growth regulator (PGR) concentrations of naphthaleneacetic acid (NAA) (1 $\mu g \cdot mL^{-1}$), 6-benzylaminopurine (BAP) (1 $\mu g \cdot mL^{-1}$), and kinetin (3 $\mu g \cdot mL^{-1}$). To produce leaves for transformation, shoots in proliferation medium were tangentially transplanted to leaf expansion medium, which differed in PGR [NAA (1 $\mu g \cdot mL^{-1}$) and 6-($\gamma\gamma$-dimethylallylamino) purine (2iP) (8.3 $\mu g \cdot mL^{-1}$)]. Young leaves from leaf expansion medium were used for *Agrobacterium*-mediated transformation as described by Norelli et al. (1996).

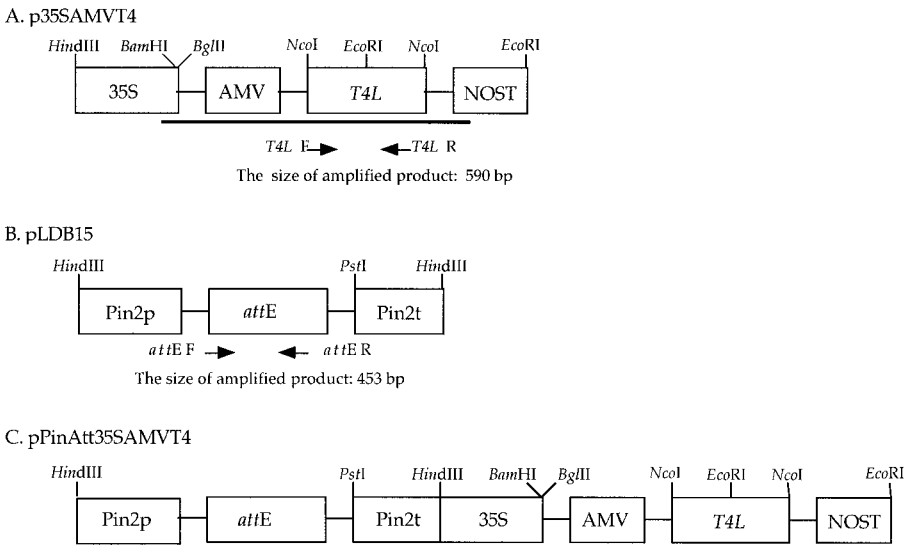
**Confirmation of transformation.** Regenerants produced from leaf segments in regeneration culture with selection were transferred to proliferation medium containing paromomycin (100 $\mu g \cdot mL^{-1}$) and cefotaxime (250 $\mu g \cdot mL^{-1}$). After 4 or 5 weeks on proliferation medium, leaves produced from one regenerated shoot were sampled for polymerase chain reaction (PCR) and a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) to detect neomycin phosphotransferase II (NPTII) to confirm transformation as described by Ko et al. (1998). To verify that the PCR bands obtained were not due to *A. tumefaciens* contamination, genomic DNA samples were amplified with primers for *vir*G as described by Ko et al. (1998). The same shoot was anchored tangentially into proliferation medium containing paromomycin (100 $\mu g \cdot mL^{-1}$) for further propagation. Genomic DNA was extracted for PCR as described by Cheung et al. (1993). An *att*E primer set (Ko et al., 2000) and a *T4L* primer set [*T4L* forward (*T4L*F): 5'-GCT CTA GAA TGG GGA AGA ACG GCA GCC T-3' and *T4L* reverse (*T4L*R): 5'-CGG GAT CCT TAT AGA TTT TTA TAC GCG TCC CAA-3'] were used for PCR amplification of *att*E (453 bp) and *T4L* (590 bp) sequences, respectively (Fig. 1). The amplified *T4L* sequence contained the *Eco*RI site. The PCR program was 40 cycles of 1.5 min at 94 °C, 2 min at 55 °C, and 2 min at 72 °C.

**Total RNA extraction and RT-PCR.** Total RNA was isolated from leaves of tissue cultured plants according to Verwoerd et al. (1989) with some modification (Ko et al., 2000). Northern blot analysis was conducted as described by Ko et al. (2000). To investigate the expression of the *att*E gene under the *Pin2* promoter, the leaves and stems of the tissue cultured plant were crushed with tissue forceps (Aesculap BD-591, Burlingame, Calif.). Leaves were harvested before wounding and 1, 4, and 24 h after wounding, frozen in liquid nitrogen, and stored at –80 °C until used for total RNA extraction.

Reverse transcription (RT) was conducted with 1 µg of total RNA as described by Rosati et al. (1997). PCR reaction buffer solutions were prepared as described by Ko et al. (1998) and mixed with 2 µL of cDNA sample. *Att*E, *T4L*, and *EF1*-a PCR reactions were conducted with *att*E primers (Ko et al., 2000), *T4L* primers (T4F and T4R), and *EF1*-a primers (Ko et al., 2000), respectively. PCR programs for *att*E, *T4L*, and *EF1*-a were 1) 40 cycles of 1.5 min at 94 °C, 2 min at 55 °C and 2 min at 72 °C; 2) 30 s at 94 °C, 35 cycles (30 s at 94 °C, 1 min at 65 °C, and 1 min at 72 °C), and 1 min at 72 °C; and 3) 5 min at 94 °C, 30 cycles (30 s at 94 °C, 30 s at 58 °C and 1 min at 72 °C), and 1 min 72 °C, respectively.

**Evaluation of resistance to *E. amylovora*.** Transgenic lines and controls were rooted, acclimated in vitro (Bolar et al., 1998), and grown in a growth chamber at 25 °C, 80% RH, conditions favorable for the development of fire blight. The two youngest actively growing leaves on 20- to 30-cm-tall potted plants with a single shoot were inoculated by cutting the leaves transversely with scissors dipped in an inoculum of $7.5 \times 10^7$ cfu of *E. amylovora* Ea273/mL (Ko et al., 2000; Norelli et al., 1994). The necrotic lesion length of the shoot was measured at 30 d after inoculation and divided by total shoot length to calculate percentage lesion length (%LL), which was used to evaluate disease resistance. The replicate unit was a single shoot potted plant, and there were two to 18 replicates of each transgenic line for resistance evaluation. Evaluations were conducted twice, but not all lines were included in each test.

Fig. 1. *Att*E and *T4L* genes in three different binary vectors for *Agrobacterium*-mediated apple transformation. The T-DNA regions were transferred to 'Galaxy' using *A. tumefaciens* EHA105. 35S: cauliflower mosaic virus 35S promoter with duplicated upstream B domain (Kay et al., 1987); AMV: untranslated leader sequence of alfalfa mosaic virus RNA4 (Datla et al., 1993); NOST: terminator of nopaline synthase gene (Bevan, 1984); *T4L*: T4 lysozyme gene from T4 bacteriophage (Düring et al., 1993; Owen et al., 1983). This gene is fused to signal peptide of α-amylase from barley (Rothstein et al., 1984). Pin2p and Pin2t: promoter and terminator of potato proteinase inhibitor II (*Pin2*) gene from potato, respectively (Destéfano-Beltrán, 1991); *att*E: cDNA of *att*E from *H. cecropia* (Destéfano-Beltrán, 1991; Kockum et al., 1984). All binary vectors contain the *npt*II gene under the control of the NOS promoter for a selectable marker to confer resistance to the antibiotics kanamycin and paromomycin (Bevan, 1984). Arrow sets and a bar indicate the PCR primer sets for *att*E and *T4L* gene, and the sequenced region of AMV/*T4L*, respectively.



Since no significant interaction occurred between the tests, the data were combined using General Linear Model analysis (SAS Institute Inc., Cary, N.C.). 'Liberty' was included as a relatively resistant control cultivar. To test if there was synergy between attacin and T4L with respect to resistance to *E. amylovora*, a single degree of freedom contrast was tested with the null hypothesis [%LL of Pin2Att35SAMVT4 lines = (%LL of Pin2Att lines + %LL of 35SAMVT4 lines)/2].

## Results and Discussion

**Construction of p35SAMVT4 and pPin2Att35SAMVT4.** Nucleotide sequence analysis indicated that both p35SAMVT4 and pSR8-36 contained the same *T4L* gene sequence. PCR and enzyme restriction analyses indicated that the AMV/*T4L* and Pin2/*att*E/35S/AMV/*T4L* gene fragments were inserted into each p35SAMVT4 and pPin2Att35SAMVT4 plasmid (Fig. 1). The *T4L* sequence in newly constructed plasmids and pSR8-36 differed from the *T4L* sequence reported previously in that the nucleotides T (86) and C (103) of *T4L* gene (Owen et al., 1983) were mutated to A (86) and G (103), resulting in $Lys^{29}$ and $Gly^{35}$ being replaced by $Met^{29}$ and $Arg^{35}$, respectively. The potato transgenic lines transformed with pSR8-36 contain the same T4L mutations and are resistant to *E. carotovora*, indicating that the antibacterial activity of the mutant T4L is not altered in planta (de Vries et al., 1999; Düring et al., 1993; Düring, personal communication). The *T4L* gene in pSR8-36, p35SAMVT4 and pPin2Att35SAMVT4 is fused to α-amylase signal peptide sequences from barley (Porsch et al., 1998). The *att*E gene sequences in pPin2Att35SAMVT4 had two silent mutations present in pLDB15 as described by Ko et al. (1999).

**Transformation of 'Galaxy'.** Transformation of 'Galaxy' apple was conducted three to six times for each construct. Most regenerants produced shoots after being transferred to proliferation medium containing paromomycin (100 $\mu g \cdot mL^{-1}$). However, some regenerants turned chlorotic or necrotic, and died after transfer, indicating that they were escapes (Ko et al., 1998). All transformants that survived on the proliferation medium were able to amplify *att*E or full length *T4L* and had significantly higher NPTII levels than nontransgenic 'Galaxy'. Transformation with pLDB15, p35SAMVT4, and pPin2Att35SAMVT4 produced nine, 22, and 20 transformants, for a transformation rate per inoculated leaf of 1.3, 7.4, and 2.4%, respectively.

**Attacin expression under the Pin2 promoter.** To investigate whether the *Pin2* promoter is induced by mechanical wounding, western and northern blots were conducted in Pin2Att 'Galaxy'. With potted plants of Pin2Att 'Galaxy', *att*E gene expression was constitutive but increased after mechanical wounding (data not presented). The attacin amount between tissue cultured and potted plants was positively correlated (data not shown). Northern blot analysis indicated that the *att*E gene expression increased 1 h after wounding and decreased 24 h after wounding in a Pin2Att transgenic apple (Fig. 2). However, the *att*E mRNA gene was detected without wounding, supporting the results of western blot analyses where attacin protein was detected without wounding. These results indicate that the expression of *att*E gene under the *Pin2* promoter is constitutive but mechanical wounding induced higher levels of expression. The *Pin2* promoter from potato is wound-inducible in potato and tobacco (Keil et al., 1989; Peña-Cortés et al., 1988; Sanchez-Serrano et al., 1987). Constitutive gene expression was also observed with the *gus* gene under the *Pin*2 promoter in transgenic tobacco (Thornburg et al., 1987). Keinonen-Mettälä et al. (1998) reported that the *Pin*2 promoter resulted in greater constitutive *gus* gene expression than *ubi*, *rol*C, *act*1, and CaMV 35S promoters in birch. In previous studies (Ko et al., 2000), the mean value of attacin expression under the control of the *Pin2* promoter (40 $ng \cdot mg^{-1}$) was similar to the 35S promoter (47 $ng \cdot mg^{-1}$) in transgenic apple. To avoid transcriptional gene silencing, which can be caused by homology in promoter regions (Matzke et al., 1994), *Pin2* and 35S promoters were used for *att*E and *T4L* gene expression, respectively. A strong, constitutive promoter, such as the 35S promoter could cause a greater incidence of post-transcriptional

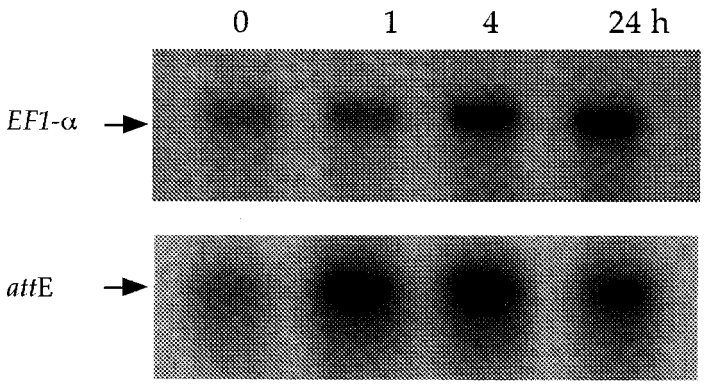



Fig. 2. Attacin expression under the control of *Pin2* promoter in transgenic apple. Northern blot to investigate transcription level of *att*E gene at 0 (before wounding), 1, 4, and 24 h after mechanical wounding. Leaves and stems of tissue cultured plant were crushed with tissue forceps (Aesculap BD-591, Burlingame, Calif.). To verify equal amounts of RNA were loaded in all lanes, a northern blot membrane was stripped and rehybridized with a cDNA fragment probe from *EF1*-a.

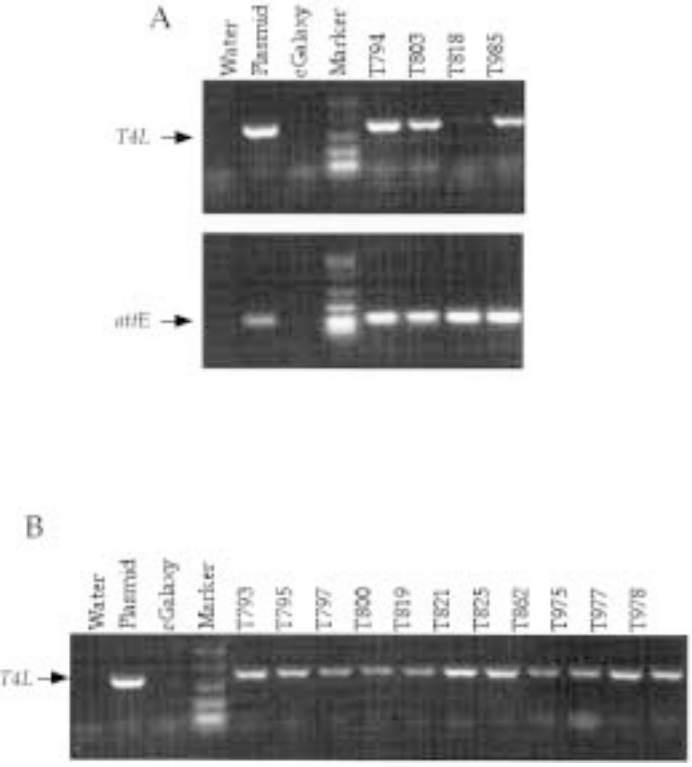



Fig. 3. RT-PCR to detect *T4L* and *att*E transcripts in (**A**) Pin2Att35SAMVT4 and (**B**) 35SAMVT4 transgenic lines of 'Galaxy' apple, that were evaluated for fire blight resistance. (**A**) Plasmid: pPin2Att35SAMVT4 binary vector; cGalaxy: reverse-transcribed cDNA sample from nontransgenic 'Galaxy'; Marker: PCR markers (Promega, Madison, Wis.). *T4L* amplified band size was 590 bp. *Att*E amplified band size was 453 bp. (**B**) Plasmid: p35SAMVT4 binary vector; cGalaxy: reverse-transcribed cDNA sample from nontransgenic 'Galaxy'; Marker: PCR markers (Promega). *T4L* amplified band size was 590 bp.

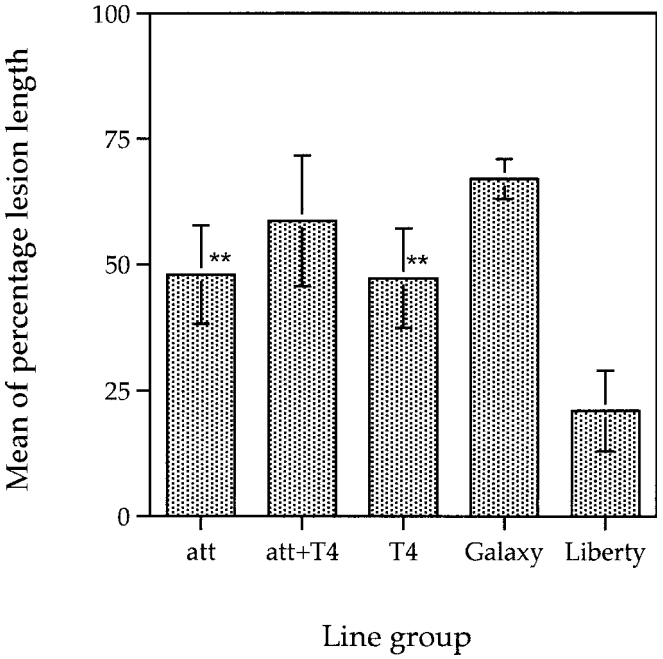



Fig. 4. The effect of T4L and attacin on disease resistance to *E. amylovora*. att, att+T4, and T4 indicate Pin2Att, Pin2Att35SAMVT4, and 35SAMVT4 transgenic line groups, respectively. 'Liberty' is a relatively resistant apple scion cultivar. The percentage lesion length was measured 30 days after *E. amylovora* Ea273 inoculation. The mean of percentage lesion length was obtained from two different disease evaluation events. Each group of Pin2Att, Pin2Att35SAMVT4, and 35SAMVT4 transgenic line includes seven, seven, and 14 transgenic lines, respectively. Each transgenic line had two to 18 replicate plants. Nontransgenic line group had 39 replicate plants. **Significantly less percentage lesion length of transgenic lines compared to nontransgenic line ($P = 0.05$).

gene silencing (PTGS) than a weaker promoter. In this respect, a nonconstitutive promoter might be better suited to avoid PTGS (Que et al., 1997). The moderately constitutive expression of attacin under the *Pin*2 promoter suggests that the *Pin*2 promoter could be a promising alternative promoter for high-level transgene expression in apple.

**Transcription of T4L and attE genes in transgenic 'Galaxy' lines.** Eleven out of 22 transgenic lines for 35SAMVT4 and four out of 20 transgenic lines for Pin2Att35SAMVT4 were tested by RT-PCR. The Pin2Att35SAMVT4 lines tested by RT-PCR showed amplification of both *T4L* and *att*E (Fig. 3A), although line T818 showed weak amplification of *T4L* cDNA. All 11 of the 35SAMVT4 lines also showed amplification of *T4L* by RT-PCR but gave no *att*E amplification (Fig. 3B). Neither *T4L* nor *att*E amplification was detected by PCR of tested lines when the RNA sample was not treated with reverse transcriptase (data not presented), indicating that the *T4L* and *att*E amplification was not due to DNA contamination in the RNA samples. These results indicate the *T4L* gene was transcribed in transgenic 'Galaxy' lines.

**Resistance of T4L transgenic lines of 'Galaxy' apple to *E. amylovora*.** Although RT-PCR indicated *T4L* gene transcription in transgenic lines, western blots with a detection limit of 2 ng·mg$^{-1}$ (leaf fresh weight) failed to detect T4L protein (data not presented), suggesting that lysozyme concentration in these lines may be below the detection limit. However, five 35SAMVT4 lines [T793 (37.8 %LL), T795 (39.9 %LL), T797 (41.0 %LL), and T975 (43.3 %LL)] had significant disease reduction compared to nontransgenic 'Galaxy' (67 %LL) and also, these transgenic lines were not significantly different from the relatively resistant 'Liberty' control (21.0 %LL), suggesting that even a low level of T4L might be sufficient to enhance resistance to *E. amylovora* in planta (Fig. 3B). A low level of T4L also enhanced resistance to *E. carotovora* in transgenic potato (Düring et al., 1993). Somaclonal variation also could play a role in the resistance to *E. amylovora* observed in some of these transgenic apple lines. After the lines have flowered, the effect of somaclonal variation on resistance will be evaluated in progeny of *T4L* transgenic lines by determining the cosegregation of the transgene and resistance.

Pin2Att35SAMVT4 transgenic line group (58.6 %LL) tested were not significantly more resistant to *E. amylovora* than nontransgenic 'Galaxy' (67.0 %LL), although Pin2Att transgenic line group (47.9 %LL) and 35SAMVT4L transgenic line group (47.2 %LL) showed significant increases in resistance (Fig. 4). A single degree of freedom contrast test showed that the null hypothesis [%LL of Pin2Att35SAMVT4 lines = (%LL of Pin2Att lines + %LL of 35SAMVT4 lines)/2] was not rejected ($P = 0.1217$), indicating no evidence of a synergistic or additive effect of attacin and T4L in enhancing resistance.

These data suggested that although both attacin E and T4 lysozyme enhanced resistance to *E. amylovora* they were not synergistic in planta. The enhanced resistance of two Pin2Att lines [T606 (39.1%LL) and T656 (33.0%LL)] with the highest amounts of attacin among seven lines in this study is in agreement with previously published results showing a significant correlation between attacin content and disease resistance (Ko et al., 2000). Also, several 35SAMVT4 lines had significantly less infection than control lines as described above and expression of T4L in these lines appeared to be adequate to reduce infection. Therefore, the failure to detect additivity or synergy between attacin and T4L is unlikely to be due to lack of *T4L* gene expression. Perhaps, the expression of both transgenes could have negative effects on the plant that might result in increased susceptibility to *E. amylovora*. High expression of attacin in pear was reported to cause reduced growth in vitro (Reynoird et al., 1999). However, in this study a reduction of plant growth was not observed in any Pin2Att35SAMVT4, Pin2Att, or 35SAMVT4 lines. Because biologically active, purified attacin E was not available, we were unable to evaluate the interaction of attacin E and T4L in vitro. To understand the interaction between attacin and T4L in planta, more information about the mode of the action of attacin and T4L is required. In addition, correlation between disease resistance and *T4L* transcript level or T4L protein content in planta is required to understand the effect of T4L.

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