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 (Sobczewski 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 **symptoms caused by** *Erwinia amylovora*. In addition, T4L has been reported to increase the access of lysozyme to the peptidoglycan layer of bacteria. Attacin increased sensitivity of *Erwinia amylovora* to the peptidoglycan layer of bacteria. 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 increases the 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* (Mourgue et al., 1998b).

T4L shows greater in vitro inhibition of *E. amylovora*, although the reasons for the greater activity of T4L against *E. amylovora* are not known (Mourgue et al., 1998b).

The main goals of this research were to 1) genetically transform ‘Galaxy’ apple with both *attE* and *T4L* genes, 2) study the expression of *attE* under the control of the potato protease 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 NcoI, and cloned into the NcoI site of pBIS525 (Datla et al., 1993) to create pBIS525T4. The HindIII and EcoRI fragment of pBIS52T4 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 HindIII and EcoRI sites on pBI121 (representing CaMV35S promoter/gus/NOST) to produce p35SAMVT4. pLDB15 (Fig. 1B) (Ko et al., 2000; Norelli et al., 1994) contains the *attE* gene under the control of the *Pin2* promoter (Pin2Att).

To construct pPin2Att35SAMVT4 (Fig. 1C) the HindIII frag-
ment of pLDB15 containing Pin2 promoter (Pin2p)/attE_Pin2 terminator (Pin2t) (Destefano-Beltran et al., 1991) was cloned into the HindIII 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 attE 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 attE, the AMV/T4L gene fragment and the Pin2/attE35S/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 µg·mL⁻¹), 6-benzylaminopurine (BAP) (1 µg·mL⁻¹), and kinetin (3 µg·mL⁻¹). To produce leaves for transformation, shoots in proliferation medium were tangentially transferred to leaf expansion medium, which differed in PGR [NAA (1 µg·mL⁻¹) and 6-(α-dimethylallyl)amino purine (2µP)] (8.0 µg·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 µg·mL⁻¹) and cefotaxime (250 µg·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 virG as described by Ko et al. (1998). The same shoot was anchored tangentially into proliferation medium containing paromomycin (100 µg·mL⁻¹) for further propagation. Genomic DNA was extracted for PCR as described by Cheung et al. (1993). An attE primer set (Ko et al., 2000) and a T4L primer set [T4L forward (T4LF): 5’-GCT CTA GAA TGG GGA AGA ACCGGCAAGCGCT-3’ and T4L reverse (T4LR): 5’-CGG CAT CCT TAG AAG TTT TTA TAC CGG TCC CAA-3’] were used for PCR amplification of attE (453 bp) and T4L (590 bp) sequences, respectively (Fig. 1). The amplified T4L sequence contained the EcoRI 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 attE gene under the Pin2 promoter, the leaves and stems of the tissue cultured plant were crushed with tissue forceps (Aesculus 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. AttE, T4L, and EF1-α PCR reactions were conducted with attE primers (Ko et al., 2000), T4L primers (T4F and T4R), and EF1-α primers (Ko et al., 2000), respectively. PCR programs for attE, T4L, and EF1-α were 1) 40 cycles of 1.5 min at 94 °C, 2 min at 55 °C and 2 min at 72 °C; 2) 30 min at 94 °C, 35 cycles (30 s at 94 °C, 1 min at 65 °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 rootstock, 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 × 10⁸ 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. AttE 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 (Ko et al., 1987); AMV: untranslated leader sequence of alfalfa mosaic virus RNAα (Datia 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 (Destefano-Beltrán, 1991); attE: cDNA of attE from H. cecropia (Destefano-Beltrán, 1991; Kockum et al., 1984). All binary vectors contain the nptII 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 attE 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/attE/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 Lys29 and Gly35 being replaced by Met29 and Arg35, 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 attE 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 μg·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 attE 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’, attE 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 attE gene expression increased 1 h after wounding and decreased 24 h after wounding in a Pin2Att transgenic apple (Fig. 2). However, the attE 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 attE 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 Pin2 promoter in transgenic tobacco (Thorung et al., 1987). Keinonen-Mettälä et al. (1998) reported that the Pin2 promoter resulted in greater constitutive gus gene expression than ubi, rolC, act1, 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·mg–1) was similar to the 35S promoter (47 ng·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 attE and T4L gene expression, respectively. A strong, constitutive promoter, such as the 35S promoter could cause a greater incidence of post-transcriptional
nation in the RNA samples. These results indicate the treated with reverse transcriptase (data not presented), indicating transgenic lines, western blots with a detection limit of 2 ng·mg–1 amylovora, ax'y (67 %LL) and also, these transgenic lines were not significantly had significant disease reduction compared to nontransgenic 'Gal-

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 Pin2 promoter suggests that the Pin2 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 attE (Fig. 3A), although line T818 showed weak amplification of T4LcDNA. All 11 of the 35SAMVT4 lines also showed amplification of T4L by RT-PCR but gave no attE amplification (Fig. 3B). Neither T4L nor attE 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 attE 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 ‘Gal-

**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.

This indicates 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 (Durinig 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 35SAMVT4 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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