Generation and Characterization of Transgenic Plum Lines Expressing the *Gastrodia* Antifungal Protein

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Abstract. The *Gastrodia* antifungal protein (GAFP) is a monocot mannose-binding lectin isolated from the Asiatic orchid *Gastrodia elata*. This lectin has previously been shown to provide increased resistance in transgenic *Nicotiana tabacum* against taxonomically unrelated root pathogens *Phytophthora nicotianae*, *Rhizoctonia solani*, and *Meloidogyne incognita*, but its potential to confer disease resistance in tree species is not known. *Agrobacterium tumefaciens*-mediated transformation yielded three *gafp-1* expressing plum lines (*Prunus domestica*) designated 4J, 4I, and 5D. These lines possessed one, two, and four copies of the *gafp-1* gene, respectively, as demonstrated by DNA blotting. Lines 4J and 4I were not phenotypically different from the nontransformed control line, but line 5D showed significant divergence in leaf morphology and growth habit. Compared with the inoculated control line, lines 4J and 4I exhibited increased tolerance to *Phytophthora* root rot (PRR) caused by *P. cinnamomi*. When inoculated with the root-knot nematode, *Meloidogyne incognita*, the 4J and 4I lines showed a significantly lower degree of root galling than the inoculated control line. Nematode reproduction, as measured by the presence of egg masses and the number of eggs produced per gram fresh root, was significantly reduced in line 4J compared with the inoculated control line. The results of this study suggest that the expression of *gafp-1* in the roots of a woody plant may confer some level of resistance to PRR and root-knot nematode. Long-term field trials will be necessary to confirm this hypothesis.

The peach industry is of considerable economic importance in the southeastern United States. South Carolina and Georgia are the main peach-producing states in this

region, generating ≈\$60 million worth of fruit per year [Anonymous, 2004; Integrated Pest Management (IPM), 2004]. The root-knot nematode (RKN) and *Phytophthora* root rot (PRR) are two important diseases associated with premature tree decline in stone fruit orchards. Root-knot nematodes belonging to the genus *Meloidogyne* are sedentary endoparasites that are ubiquitous in soils of the southeastern region. *M. incognita*, for example, was found in 95% of the peach orchards sampled in South Carolina (Nyczepir et al., 1997). *Meloidogyne* sp. parasitize root systems of a number of herbaceous and woody hosts and infestation by RKN can cause significant damage to *Prunus* in the form of stunted growth, loss of vigor, and early defoliation of 1- to 2-year-old trees when recommended management practices are not followed. Present management techniques for *M. incognita* include preplant fumigation (i.e., Tclone II[®], Dow

Agrosciences, Indianapolis), which improves tree stand establishment and gives control for 2 to 5 years. The efficacy of the control procedure depends, however, on the quality of the fumigation, the nematode involved, and the use of resistant or tolerant rootstocks (when available) (Nyczepir, 1991; Sharpe et al., 1993). Current research efforts in the southeast and California have shifted toward various forms of nonchemical nematode control. Emphasis on nonchemical control is partially the result of concerns surrounding the environmental problems associated with soil fumigation reflected in the recent ban on methyl bromide use.

PRR disease is caused by various members of the genus *Phytophthora*, and is favored when conditions such as excessive soil moisture and warm temperatures persist. The host range of *Phytophthora* encompasses a wide variety of plant species, including peach, plum, apricot, nectarine, and cherry (IPM, 1999). *Phytophthora* sp. can infect and damage the roots as well as the scion of infected plants. The pathogen attacks the tissues at the soil line, producing an area of necrotic tissue that eventually rings and girdles the tree. Symptoms of PRR infestation include stunted growth, stem necrosis, wilting, and chlorosis of leaf tissues, which dry up and remain attached to the plant. Although younger trees are generally more susceptible to infection, disease onset can occur at any age of the tree (Brown and Mircetich, 1995). Root and crown rots typically result in the eventual death of the plant; therefore, effective management practices for *Phytophthora* are vital. Fungicides such as fosetyl-al (Aliette[®], Bayer CropScience, Research Triangle Park, NC) and Ridomil[®] (Syngenta Crop Protection, Greenboro, NC) are available as postdisease-onset rescue treatments, but more sustainable management tools include selection of tolerant rootstocks and effective water management practices (IPM, 1999).

Engineering disease resistance in agricultural crops is now becoming a viable alternative to traditional disease management methods. The first transgenic crop, the ripening-delayed FLAVR SAVR[™] tomato, was deregulated in the United States in 1992 (Medley, 1992), and since then, the total geographic area devoted to growing genetically modified (GM) crops has seen a significant increase in the United States. Many of the deregulated transgenic crops grown in the United States have been engineered for tolerance to viruses, insects, or herbicides (Schahczenski and Adam, 2006). Currently, the main transgenic crops to be cultivated globally are soybean, maize, and cotton (James, 2003). Woody species are now being targeted for genetic modification of traits controlling resistance to various pathogens as well (Hoenicka and Fladung, 2006). Resistance to harmful insect pests has been demonstrated in walnut (Dandekar et al., 1998) and persimmon (Tao et al., 1997). GM citrus, plum, and papaya have shown resistance to the citrus tristeza virus

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(Dominguez et al., 2002), plum pox virus (Malinowski et al., 2006), and papaya ring-spot virus (Lius et al., 1997), respectively.

Plant defense lectins have become a promising class of molecules for engineering resistance to root-associated pathogens. Homologs of the hevein-binding lectin from the rubber tree (*Hevea brasiliensis*) strengthened resistance in transgenic tomato against *Fusarium oxysporum* and *Phytophthora capsici* (Lee et al., 2003) and in tobacco against *Phytophthora parasitica* (Koo et al., 2002). Expression of the monocot mannose-binding garlic lectin, GNA (*Galanthus nivalis* agglutinin), was able to combat RKN infestation in *Arabidopsis* (Ripoll et al., 2003). The *gafp-1-vnf* sequence, designated *gafp-1* in this study, encodes an isoform of another monocot mannose-binding lectin, the *Gastrodia* antifungal protein (GAFP-1) (Wang et al., 2001). The *gafp-1* sequence may be a promising genetic element for enhancing resistance to root-associated pathogens in *Prunus* species.

The GAFP lectin comes from the Asiatic orchid *Gastrodia elata* and has been shown to inhibit a wide range of pathogenic fungal species in vitro, including *Trichoderma viride*, *Rhizoctonia solani*, *Fusarium oxysporum*, *Valsa ambiens*, *Gibberella zeae*, *Ganoderma lucidum*, *Botrytis cinerea*, and *Pyricularia oryzae* (Hu and Huang, 1994). Additionally, GAFP was able to provide resistance to the ascomycete fungus *Verticillium dahliae* in field tests on transgenic cotton (Wang et al., 2004). In tobacco, GAFP-1 conferred resistance against a variety of pathogens, including the stramenopile *Phytophthora nicotianae*, the phytoparasitic root-knot nematode *Meloidogyne incognita*, and the basidiomycete fungus *Rhizoctonia solani* (Cox et al., 2006). These data were the first to indicate that GAFP-1 is active against nonfungal organisms. The objective of this study was to generate transgenic plum lines expressing *gafp-1* and characterize these lines for resistance to PRR and the RKN. We chose plum as our model woody plant system for a *Prunus* species as a result of the availability of a reliable transformation protocol.

Materials and Methods

Transformation of plum. The nucleotide sequence of the *gafp-1-vnf* isoform was inserted into the multiple cloning site of the binary vector pAVAT1, a derivative of the pTHW136 expression vector (Plant Genetic Systems N.V., Gent, Belgium). The resulting chimeric vector was designated pAVNFbin (Wang et al., 2001). The *gafp-1-vnf* gene (from here on referred to as *gafp-1*) was placed under the control of the 35S promoter and omega leader sequence (Wang et al., 2001). *Agrobacterium tumefaciens* strain EHA 101 was transformed with the pAVNF vector using the freeze-thaw protocol (Chen et al., 1994). Bacterial colonies were selected on Luria broth agar (10 g·L⁻¹ tryptone, 5 g·L⁻¹ yeast extract, 10 g·L⁻¹ NaCl, 18 g·L⁻¹ agar; pH 7) amended with 50 mg·L⁻¹ kanamycin and 150 mg·L⁻¹ spectinomycin. Presence of *gafp-1*

was confirmed in selected colonies by polymerase chain reaction (PCR) with *GAFP-1* specific primers (Wang et al., 2001). Plum (*Prunus domestica*) hypocotyls from seeds of open-pollinated ‘Stanley’ were transformed following the protocols of Padilla et al. (2003). After transformation, genomic DNA was isolated from root and leaf tissue of transformed plums using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA) and amplified by PCR as described previously to initially verify the presence of *gafp-1*. Four transgenic lines containing the *gafp-1* gene were obtained through *A. tumefaciens*-mediated transformation. Three lines were chosen for further experimentation and were named 4J, 4I, and 5D. The fourth line exhibited weak growth under normal growing conditions in the greenhouse and propagation success with this line was inadequate. Consequently, this line was excluded from subsequent experiments.

DNA blotting. DNA was isolated from young, fully expanded plum leaves of transgenic and nontransformed plum plants following the procedures of Kobayashi et al. (1998). Briefly, DNA (13 µg) was digested with the restriction enzyme *Bam*HI (New England Biolabs, Ipswich, MA), separated on a 1% (w/v) agarose gel, and blotted to a positively charged nylon membrane (Roche Diagnostics Corporation, Indianapolis, IN). The filter was hybridized with a Digoxigenin-11-dUTP alkali-labile labeled probe (Roche Diagnostics Corporation) coding for *gafp-1* cDNA. The probe was generated by PCR using the *gafp-1* specific primers described previously.

Detection of *Gastrodia antifungal protein* in transgenic lines. GAFP-1 protein synthesis was confirmed in transgenic lines using immunoblot analysis of root tissue. Total cellular protein was extracted from young root tissue of 1-year-old trees according to the TRIzol method (Chomczynski, 1993) using TRIzol Reagent® (Invitrogen Corporation, Carlsbad, CA). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was performed on total cellular protein using a Mini-Protein® 3 with 18% Tris-HCl Ready Gels (Bio-Rad Laboratories, Hercules, CA). Proteins were transferred to an immunoblot polyvinylidene difluoride membrane using a Mini-Trans-Blot® electrophoretic transfer cell (Bio-Rad Laboratories). Membranes were rinsed in deionized water and air-dried for 1 h to fix peptides to the membrane. Before immunoblotting, the membranes were rewet in methanol and rinsed in deionized water. Immunoblotting was accomplished using rabbit anti-GAFP-1 polyclonal antisera (1:10⁴ dilution) developed by Zymed® Laboratories (Invitrogen Corporation) and goat antirabbit alkaline phosphatase conjugated antibodies (1:10⁵ dilution) (Promega Corp., Madison, WI) according to standard methods (Gallagher et al., 1997). GAFP-1 bands were detected by soaking the membrane in a detection solution of 1 Sigmafast™ BCIP/NBT tablet (Sigma Aldrich, St. Louis) dissolved in 10 mL deionized water. Recombi-

nant, purified GAFP-1 was used as a standard in all immunoblot assays. Detection assays were performed twice for each tissue sample.

Phenotypic data. Two-year-old trees from the transgenic lines 4J, 4I, and 5D and the nontransformed control line were analyzed for morphological and growth characteristics. Being roughly elliptical in shape, leaf areas were estimated by the formula $A = \pi LW$, where L and W are the length and width of the leaf, respectively. Leaf area was measured for leaves at positions 1, 3, 5, 7, and 9 on a single branch. Leaf position 1 corresponds to the first fully expanded leaf at the apical meristem exhibiting noncurled edges. To determine the average internode number per branch, internodes were counted on a 30-cm branch section starting at leaf position 1. Nine branches were analyzed per line for leaf area and internode characteristics.

Plant materials and treatments. Plants for the experimental assays were propagated from the original, transformed plum lines (T₀ lines). T₀ lines were pruned every 3 months to encourage shoot growth. When shoots were ≈12 to 15 cm long, they were pruned to a length of 10 cm and gently scored (5 mm in length) along the base of the shoot with a razor blade. The scored area was dipped briefly in Miracle-Gro “Fast Root” rooting hormone [10% indole 3-butyric acid (IBA); Miracle-Gro Lawn Products, Marysville, OH]. The fragile shoots were then placed ≈3 cm deep in 15-cm³ sterile vermiculite (≈15 to 20 shoots per line) and incubated in humidity chambers to prevent dehydration. Humidity chambers were removed ≈1 to 2 weeks after transplanting. At the end of 90 d, only the healthiest rooted cuttings were selected for disease screening.

All experiments were performed twice with five to 10 single-tree replicates per disease treatment. Inoculated, nontransformed plum lines were used as positive controls for decline as a result of pathogen infection. An additional five to 10 tree replicates from each transgenic line and the control line were left uninoculated during disease assays and served as the negative control groups. Inoculated and uninoculated experimental plants were arranged in randomized complete blocks on benches in an air-conditioned, biosafety level 2 greenhouse (27 ± 5 °C). Plants were watered, fertilized, and pruned as necessary.

Disease resistance screening. Experimental plum lines were inoculated with *Phytophthora cinnamomi* (isolate 05-1127) to investigate resistance to infection by a stramenopile. This isolate was obtained from naturally infected peach (*Prunus persica*) in South Carolina in 2005. To prepare the inoculum, *P. cinnamomi* was grown on PARP (per liter H₂O:5% by volume clarified V8® juice (Campbell’s, Camden, NJ) concentrate, 15 g agar, 10 mg pimarcin, 250 mg ampicillin, 10 mg rifmycin, 66.7 mg pentachloronitrobenzene) selective medium (Jeffers and Martin, 1986) in petri dishes (90 mm in diameter) in the dark at 22 °C for 3 d. Five plugs (6 mm in diameter) were taken from the

periphery of actively growing colonies and added to 500-mL Erlenmeyer flasks containing 300 mL of a sterile, V8 juice:vermiculite (1:2 v/v) mixture. The flasks were incubated in the dark at 22 °C for 8 weeks. At the end of the incubation period, small pieces of infested vermiculite were plated on potato dextrose agar and incubated for 5 d in the dark to confirm the purity of the inoculum.

Experimental plum lines were transplanted to 5-cm diameter plastic torpedo pots containing $\approx 400 \text{ cm}^3$ sterile potting soil mixed with 2% of the infested V8 juice:vermiculite. Negative control plants were transplanted into 400-cm³ sterile potting soil mixed with 2% uninfested, sterile V8 juice:vermiculite. Pots were suspended in plastic racks (10 pots per rack) and placed in 10-gallon plastic bins. Plants were watered as needed and after 1 week were flooded for 48 h to promote infection. Disease symptoms were evaluated every other day. Shoot symptoms were rated as: 0 = healthy plant with no affected tissues, 1 = wilting and less than 25% of the plant exhibiting symptoms of chlorosis and necrosis, 2 = between 26% and 50% of the plant exhibiting symptoms of chlorosis and necrosis, 3 = between 51% and 75% of the plant exhibiting symptoms of chlorosis and necrosis, or 4 = greater than 75% of the plant exhibiting symptoms of chlorosis and necrosis. The experiments were concluded after 20 d, when the majority of inoculated control plants had died. Fresh root weight was recorded for inoculated seedlings. Random root pieces were sampled from inoculated seedlings and were plated on PARPH [PARP + 50 mg 5-methylisoxazol-3-ol (hymexazol)] selective medium to confirm the presence of the *P. cinnamomi*.

Experimental plum plants were challenged with *Meloidogyne incognita* (Kofoid & White) Chitwood to investigate levels of resistance to this root-knot nematode species. *M. incognita* populations were originally isolated from infected peach (*Prunus persica*) in Georgia. Plum plants were transplanted into 20-cm diameter plastic pots containing 1000 cm³ of sterile sand:vermiculite (1:1 by volume). Root-knot nematode egg inoculum was extracted from infected tomato roots (cv. Rutgers) using a 10% NaOCl solution diluted in tap water (Hussey and Barker, 1973). Twelve-week-old plum plants were inoculated with 6000 eggs per pot by creating three holes ($\approx 1 \text{ cm}$ deep) in the medium around the base of each plant. The egg solution was mixed thoroughly before pipetting 2 mL (1000 eggs/mL) into each of the three holes. Negative control plants were inoculated by pipetting 2 mL tap water into each of the three holes, or a total volume of 6 mL water added to each plant. Five 60-day-old tomato seedlings (cv. Rutgers) were inoculated at the same time, in a similar manner, to confirm inoculum viability.

The study was ended 60 d after inoculation and the following data were collected for inoculated seedlings: number of egg masses per root system, number of eggs per root system, and fresh root weight. Root systems were also rated for the percentage of root

galls present. Briefly, experimental root systems were harvested, washed with tap water, and dried to a damp condition. Root systems were then stained with Phloxine B for 20 min to detect the presence of egg masses (Dickson and Struble, 1965). When determining egg mass numbers, counting time was limited to 1 min to standardize the measurement for all root systems. Root systems were then rated for percentage of galling according to the

following scale: 0 = no detectable infection, 1 = trace infection or less than 10% of the root system galled, 2 = between 10% and 25% of the root system galled, 3 = between 26% and 50% of the root system galled, 4 = between 51% and 75% of the root system galled, or 5 = greater than 75% of the root system galled (Barker, 1985). Individual root systems were then dampened dried with a paper towel and their fresh root weight determined. Eggs

Fig. 1. Phenotypic variation among 2-year-old trees of the nontransformed control line and transgenic plum lines 4J, 4I, and 5D. (A) Representative leaves from position 8 on actively growing plum branches. Leaf position 1 corresponded to the first expanded leaf at the apical meristem. (B) Average leaf areas for leaf positions 1, 3, 5, 7, and 9. (C) Average number of internodes on 30-cm branch sections. For B–C, error bars represent the sds for results from nine branches per line.

were extracted from the plum roots as described previously for the infected tomato roots and quantified with a hemacytometer. Egg concentration for each root system was normalized against fresh root weight to obtain eggs produced per gram of fresh root.

Statistical analysis. All calculations were performed using SAS (SAS version 9.1; SAS Institute, Cary, NC) and a significance level of $\alpha = 0.05$. Observational data from the *P. cinnamomi* and *M. incognita* (gall ratings) assays were analyzed as follows. Symptom severity scores of all plants were noted from the last day of each experiment. The percentage of plants in a scored category was determined for each line by dividing the number of plants assigned a discrete score by the total number of inoculated plants. The percentages were used to create frequency distributions for the three inoculated transgenic lines and the inoculated, nontransformed control line for each performance of an experiment. The replication effect of the two independent experiments was tested using Cochran-Mantel-Haenszel (CHM) statistics. The frequency distributions between independent experiments were combined for each line if replication effects were not found to be significant. Overall line effect and pairwise line differences were tested using χ^2 analysis within the PROC FREQ procedure in SAS incorporating Fisher's exact test for low sample sizes.

Egg data from the *M. incognita* assays were analyzed differently. For each inoculated line, mean values were calculated for the number of egg masses present on the root systems. Values for eggs produced per gram fresh root were $\log_{10}(x + 1)$ transformed to account for the highly skewed frequency distribution generally observed with nematode egg counts. Line means were then determined for the transformed egg count data. Replication effects among the two experiments were tested for mean egg mass numbers and eggs produced per gram fresh root using analysis of variance (ANOVA). When replication effects were found to be insignificant, the data means were combined. Overall line effects were tested using ANOVA and Tukey's studentized range test was used for post hoc multiple comparison analysis.

Results

Transformation of plum. Transgenic lines 4J, 4I, and 5D were analyzed for any variation in phenotypic characters compared with the nontransformed control line. Transgenic lines 4J and 4I were indistinguishable from the nontransformed control line in terms of their vigor and growth habit. Line 5D displayed pronounced phenotypic variation compared with the other lines. Line 5D plants had stunted leaves, as shown in Figure 1A–B, and shorter internodes, as shown in Figure 1C. Plants also exhibited increased lateral branching (data not shown). The three transgenic lines used in the study were siblings because they originated from 'Stanley' seeds,

Fig. 2. (A) Schematic representation of the pAVNF T-DNA insert. Restriction sites for *Bam*HI and the position of the *GFP-1* probe are indicated. (B) Southern analysis of *GFP-1* in transgenic plum lines. Lane 1: nontransformed plum; lanes 2–4: transgenic lines 4J, 4I, and 5D, respectively; MII = DIG-labeled DNA molecular weight marker II; MIII = DIG-labeled DNA molecular weight marker III (Roche Diagnostics Corporation).

but each was genotypically unique. Nontransformed controls were also 'Stanley' seedlings and consisted of 12 seedling lines. These controls were expected to represent the potential range in genetic variation for inherent disease resistance in 'Stanley' seedlings.

DNA and immunoblot analysis. Digestion of transgenic plum DNA with *Bam*HI and subsequent hybridization with a *gfp-1* specific probe, as shown in Figure 2A, revealed varying copy numbers of *gfp-1* gene among transgenic lines. Transgenic line 4J contained one copy of the *gfp-1* insertion, whereas lines 4I and 5D contained two and four copies, respectively, as shown in Figure 2B. All experimental lines produced the GFP-1 protein in root tissues as shown in Figure 3.

Disease resistance screening. In both independent experiments, the nontransformed control line and transgenic lines 4J and 4I developed characteristic symptoms associated with PRR 8 to 10 d after inoculation with *P. cinnamomi*. Line 5D showed an accelerated onset of disease symptoms compared with the inoculated control with symptoms becoming apparent as early as day 2 (data not shown). Symptoms that are diagnostic of *Phytophthora* infection such as wilting, chlorosis, and necrosis of the leaves and stem were observed in diseased

Fig. 3. Immunoblot analysis showing GFP-1 protein synthesis in root tissue of transgenic plum lines. Lane 1: purified GFP (≈ 12 kDa); Lanes 2–4: transgenic lines 4J, 4I, and 5D, respectively; Lane 5: nontransformed control line.

plants as shown in Figure 4. A small number of plants from the uninoculated, negative control groups displayed symptoms of water logging. Leaves from these affected plants underwent chlorosis and eventual abscission after the pots were flooded. However, these water-logged plants recovered before the end of the experiment, flushing new leaves and displaying healthy growth, whereas inoculated plants continued to decline over the entire period. Additionally, symptomatic leaves persisted on *Phytophthora*-infested plants, whereas leaves abscised from water-logged seedlings. In neither experimental trial did the average final root mass of the inoculated control line significantly differ from the average root mass of any of the transgenic lines (data not shown).

Replication effects were not found to be significant between experiments by CHM

Fig. 4. Disease symptoms representative of the scoring system for plum plants infected with *Phytophthora cinnamomi*. Plants were assigned discrete scores based on their symptom severity: score 0 = healthy plant with no affected tissues, score 1 = wilting and less than 25% plant affected, score 2 = 26% to 50% of the plant affected, score 3 = 51% to 75% of the plant affected, score 4 = greater than 75% of the plant affected.

analysis ($P = 0.97$). By the end of the 20-day incubation period, most plants from the inoculated control and 5D lines were severely diseased and primarily distributed in scored categories 3 and 4, as shown in Table 1. In contrast, most transgenic plants from lines 4J and 4I developed weaker symptoms, resulting in frequency distributions that were

significantly shifted to mean scores of 1 and 2 compared with the inoculated control line distribution (4J and 4I, $P = 0.02$). The 4J and 4I distributions did not differ significantly from each other by χ^2 analysis ($P = 0.77$). The 5D line distribution was not significantly different from the inoculated control line ($P = 0.20$).

Initial seedling size of inoculated and uninoculated plum lines varied as a result of differences in propagation and planting dates. Therefore, parameters related to seedling size such as root mass were not used as measures of disease susceptibility. Rather, evaluations were based on the percentage of root system affected or measurements were normalized against root mass to reduce the effects of root size variability. Percentage of root galling was used as a measure of *M. incognita* infection. Replication effects between the independent experiments were not found to be significant in the nematode assay with respect to gall ratings (CHM; $P = 0.16$). Lines 4J and 4I showed a lesser degree of root-galling severity than the 5D and nontransformed control lines with the majority of plants distributed in lower score categories for gall ratings, as shown in Table 2. Distributions for the inoculated 4J and 4I lines were found to be significantly different from the inoculated control line ($P = 0.004$ and $P = 0.0003$, respectively) but not significantly different from each other by pairwise χ^2 analysis ($P = 0.17$). The distribution for the 5D line was not found to be significantly different from the inoculated control line by χ^2 analysis ($P = 0.06$).

Indications of increased resistance were not quite as strong when lines were evaluated for *M. incognita* reproduction. Reproduction was determined by evaluating lines for the presence of egg masses as well as counting the number of eggs produced per gram of fresh root (egg count). Inoculated tomato seedlings served as positive controls for infection and exhibited severe root galling and reduced plant vigor overall, verifying the inoculum viability (data not shown). Lines 4J and 4I showed a significant reduction in number of egg masses compared with the inoculated control line as shown in Figure 5A. This was correlated with the decrease in the percentage of the root system galled in these lines. Additionally, there was a significant reduction in the egg count of the 4J line over the inoculated control line as shown in Figure 5B. Replication effects between the independent experiments were not found to be significant with respect to number of egg masses per plant (ANOVA; $P = 0.27$) or transformed egg counts (ANOVA; $P = 0.92$). The 5D line was not significantly different from the inoculated control with respect to either egg mass numbers or egg counts. In neither experimental trial did the average final root mass of the inoculated control line significantly differ from the average final root mass of any of the transgenic lines (data not shown). The uninoculated, negative control groups for each line remained healthy for the duration of the experiments and exhibited no onset of disease symptoms.

Discussion

GAFP was first discovered in the Asiatic orchid *Gastrodia elata* (Hu et al., 1988). This achlorophyllous orchid depends completely

Table 1. Disease symptom development in 3-month-old plum seedlings 20 d after inoculation with *Phytophthora cinnamomi*.

Line	Symptom severity score ^z					Observations ^y
	0	1	2	3	4	
Control a ^x	5.26	0.00	15.79	31.58	47.37	19
4J b	7.69	30.77	38.46	15.38	7.69	13
4I b	0.00	31.25	31.25	25.00	12.50	16
5D a	0.00	0.00	0.00	21.43	78.57	14

^zPlants representative of the scoring system are shown in Figure 4.

^yThe total number of inoculated plants analyzed for each line.

^xFrequency distributions followed by the same letter are not significantly different according to χ^2 analysis ($P \leq 0.05$), incorporating Fisher's exact test for low sample sizes.

Table 2. Disease symptom development in 3-month-old plum seedlings 60 d after inoculation with *Meloidogyne incognita*.

Line	Symptom severity score ^z							Observations ^y
	0	1	2	3	4	5	D	
Control a ^x	0.0	25.0	30.0	35.0	5.0	0.0	5.0	20
4J bc	15.0	70.0	15.0	0.0	0.0	0.0	0.0	20
4I b	30.0	65.0	0.0	0.0	0.0	0.0	5.0	20
5D ac	5.0	40.0	50.0	0.0	0.0	0.0	5.0	20

^zGall ratings were assigned to root systems according to the following symptom severity scale: 0 = no galls detectable, 1 = less than 10% of roots galled, 2 = 10% to 25% of roots galled, 3 = 26% to 50% galled, 4 = 51% to 75% galled, 5 = greater than 75% galled. Plants that died (D) during the infection process are also represented.

^yThe total number of inoculated plants analyzed for each line.

^xFrequency distributions followed by the same letter are not significantly different according to χ^2 analysis ($P \leq 0.05$), incorporating Fisher's exact test for low sample sizes.

on the presence of an associated fungus, the basidiomycete *Armillaria mellea*, for its growth and reproduction. It has been demonstrated that this lectin is likely the primary protein responsible for inhibiting the growth of the fungal hyphae within *Gastrodia*, preventing the complete infestation of the orchid's root system (Hu and Huang, 1994; Yang and Hu, 1990). In this study, *gafp-1* expression in plum lines 4J and 4I showed promise for providing increased tolerance to the stramenopile *P. cinnamomi*. The production of GAFF-1 was associated with a significant decrease in disease symptoms in our two phenotypically normal lines when they were challenged with this pathogen. These results mirrored those obtained in previous experiments when transgenic tobacco lines expressing *gafp-1* were infested with a related pathogen, *P. nicotianae* (Cox et al., 2006).

Increased PRR resistance was not observed in the 5D line, although this line did produce GAFF-1 in root tissue. Line 5D was the most severely affected in the *P. cinnamomi* assay, displaying less tolerance and a quicker onset of disease symptoms than the other inoculated lines. DNA blotting revealed that line 5D contained multiple *gafp-1* gene copies and its growth pattern was observed to be divergent from the non-transformed control line as well as lines 4J and 4I. There are multiple hypotheses as to why GAFF-1 expression was not effective in the 5D line. We suspect that 5D's altered phenotype is indicative of a physiological defect that occurred during the transforma-

tion procedure. The occurrence of multiple transgene copies raises the possibility of a problematic insertion event, or this line may have been a somaclonal variant negatively affected by the regeneration process. Alternatively, it is possible that multiple *gafp-1* gene copies could be causing overproduction of the GAFF-1 protein, leading to direct negative physiological impacts or otherwise evoking a pleiotropic response in the host. A threshold effect is sometimes observed in transgenic studies, in which increased copy number or transcript expression levels do not necessarily correlate to an increased resistance phenotype (Dandekar et al., 1998; Ripoll et al., 2003). Lastly, all transgenic plum lines represent separate genotypes because all transformants were generated from different seeds of the Stanley cultivar. Consequently, any inherent variation in traits controlling pathogen resistance in the 5D line may also be contributing to its weaker performance.

Decreased susceptibility to *M. incognita* infestation was observed in plum lines expressing GAFF-1. The transgenic plum lines 4J and 4I revealed significantly reduced root gall severity and a significant reduction in egg mass numbers. These results are consistent with an earlier study describing increased resistance to RKN in transgenic tobacco lines producing GAFF-1 (Cox et al., 2006). Additionally, the 4J line displayed significantly reduced egg counts over the inoculated control line. Although egg counts in lines 4I and 5D revealed no significant

reduction when compared with the inoculated control, there was still a trend toward decreased reproduction within these lines. Again, the effects of GAFF-1 on nematode infection in plum line 5D were not as striking as was observed in the other two transgenic lines. Taking into account an inoculum density of 6000 eggs/plant, the degree of infection observed in the inoculated control line was comparable to other studies conducted on *Prunus* species with root-knot nematodes (Lu et al., 2000; Rubio-Cabetas et al., 2001).

The mechanism by which GAFF is able to inhibit fungal pathogen growth remains undetermined. As a member of the monoco mannose-binding lectin family, it has been postulated that GAFF may be able to bind mannose-containing glycans or glycoproteins in the fungal cell wall, structurally interfering with the growth of fungal hyphae (Wang et al., 2003). Supporting this is a recent study that showed preferential localization of GAFF in fungal cell walls at the apices and septa of actively growing hyphae of *Trichoderma viride* (Xu and Liu, 2003). In addition to mannose, the GAFF lectin is also able to bind single residues and polymers of N-acetylglucosamine in vitro (Xu et al., 1998). The ability to bind a glucose derivative is rare among the monoco mannose-binding lectins, which are diagnostic for their specificity toward mannose (Barre et al., 2001; Liu et al., 2005; Van Damme et al., 1998). GAFF's ability to accommodate chitin could theoretically contribute to its activity in fungi. The mechanism of GAFF-1's effect on stramenopile and nematode pathogens as reported by Cox et al. (2006) and this study is unknown. *Phytophthora* species are cell wall-enclosed mycelial organisms; therefore, GAFF-1 may act on stramenopiles in a manner similar to fungi. As for the root-knot nematode, insecticidal monoco mannose-binding lectins (MMBLs) such as GNA are able to target RKN by binding mannose-type glycoconjugates within the insect (Ripoll et al., 2003). It is possible that GAFF-1 could have analogous activity within *M. incognita*, perhaps binding to surface glycans on receptors of the endoparasite to create a toxic effect or otherwise impeding hatching.

Although currently, there are no GM woody tree species in commercial production, a transgenic, woody rootstock with resistance or increased tolerance to soilborne pathogens would be of great benefit for the fruit industry, because virtually all the major tree fruit crops are propagated by grafting onto rootstocks. Additionally, if it can be shown that the gene products do not traverse the graft union, a transgenic rootstock combined with a nontransgenic scion may cause less public concern because pollen and fruit would be produced on the nontransgenic parts of the plant. GAFF-1 significantly decreased the effects of infection by *P. cinnamomi* in 4J and 4I plum lines and indicated increased tolerance to *M. incognita*. Future work will help us determine if the expression of GAFF-1 is able to control infection by *Armillaria tabescens*, the causal

Fig. 5. Reproduction of *Meloidogyne incognita* on roots of the nontransformed control line and transgenic plum lines 4J, 4I, and 5D. Egg mass counts and number of eggs per gram of fresh root were determined 60 d after inoculation with *M. incognita*. Before statistical analysis, values for eggs per gram fresh root were $\log_{10}(x + 1)$ transformed. Values for egg mass numbers (A) and $\log_{10}[(\text{eggs per gram fresh root}) + 1]$ (B) are means and ses taken across both independent experiments. The total number of plants (n) analyzed per line is represented under the x-axis. Plants that died during the infection process (Table 2) were not included in the analysis. An asterisk indicates that the line mean was significantly different from the inoculated control line according to Tukey's honestly significant difference ($P \leq 0.05$).

agent of Armillaria root rot and a closely related species to GAFF's target pathogen, *A. mellea*. Multiyear field tests are necessary to investigate this transgenic system under infection conditions.

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