The *Gastrodia* Antifungal Protein (GAFP-1) and Its Transcript Are Absent from Scions of Chimeric-grafted Plum

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Abstract. The *Gastrodia* antifungal protein (GAFP-1) is a monocolonmanose-binding lectin found in the Asiatic orchid *Gastrodia elata*. Transgenic plum (*Prunus domestica* var. ‘Stanley’) lines (4J and 4I) expressing GAFP-1 exhibit enhanced disease resistance to the stramenopile pathogen *Phytophthora cinnamomi* and the root-knot nematode *Meloidogyne incognita*. Rootstocks created from such transgenic lines might be more readily accepted by consumers if it can be shown that foreign gene products are not migrating into a grafted, nontransgenic scion on which fruit is produced. In this study, wild-type (WT) plum tissue was budded onto transgenic plum lines 4J and 4I to create chimeric-grafted trees. Tissues from chimeric-grafted trees were analyzed for *gafp-1* transcripts (leaf and root) and protein (leaf, soft shoot, and root) by reverse transcription–polymerase chain reaction and immunodetection, respectively. Transcripts of *gafp-1* were detected consistently in the root tissues but not within the leaves of the grafted, WT scions. Similarly, the GAFP-1 lectin was identified within the roots, but not in the soft shoot or leaf tissues of the grafted, WT scions. These results suggest that *gafp-1* mRNA and protein are not moving into the WT scion tissues of chimeric-grafted plum trees.

Development of genetically modified (GM) agricultural crops has given producers new options to combat pests and diseases. These transgenic options can be limited, however, depending on the type of crop and the nature of the affliction. For instance, despite the important economic impacts that root diseases can have on fruit production, only a few GM fruit tree species have been engineered for resistance to root-associated pathogens (Petri and Burgos, 2005). Transgenic plum (*Prunus domestica* var. ‘Stanley’) lines (4J and 4I) expressing GAFP-1 are a factor in the consumer’s willingness to purchase such items (Boccaletti and Moro, 2000; Bkenya and Wright, 2007; Burton et al., 2001). Grafting cultivar scions to rootstocks with desirable attributes is already common practice in fruit tree propagation; therefore, a potentially more consumer-friendly way to use GM technology would be to combine a transgenic, disease-resistant rootstock with a nontransgenic scion. Ideally, foreign gene products expressed in the root tissues would remain in the rootstock and not enter the fruit produced on a grafted scion. Whether the GAFP-1 lectin or its transcripts can move across a graft union into nontransformed scion tissues is not known.

The presence of foreign gene products in consumables is a controversial issue. Perceptions about the safety of GM foods are a factor in the consumer’s willingness to purchase such items. Untransformed (UG) trees were kept as additional controls for the detection of *gafp-1* mRNA and protein. Three tree replicates received two buds each for a total of six budding attempts per line. Buds were wrapped in Parafilm® (Pechiney Plastic Packaging Company, Chicago, IL) for 2 weeks. After this time, the Parafilm® was removed. Four weeks after the budding event, the WT scion was pruned just above the uppermost bud graft. If two buds flushed on the same tree, they were both allowed to develop on the stem. Ungrafted (UG) trees were kept as additional controls for the detection of *gafp-1* mRNA and protein. Tissues from 4J and 4I lines were analyzed for the presence or absence of *gafp-1* mRNA and protein.

Materials and Methods

Generation of chimeric-grafted and autografted trees. Transgenic plum (*Prunus domestica* var. ‘Stanley’) lines 4J and 4I (Nagel et al., 2008) and nontransformed WT plum lines were used in this study. Both transgenic 4J and 4I lines express the *gafp-1* gene under the control of the CaMV-35S promoter sequence (Plant Genetic Systems N.V., Gent, Belgium). Trees from 4J and 4I lines were cloned propagated from their respective mother (T₀) lines. WT trees, however, originated from different ‘Stanley’ seeds and thus represented some, albeit limited, inherent genetic variation within the WT population. WT scion tissue was chip-budded (hereafter referred to as “budded”) onto three transgenic 4J and 4I trees and three nontransformed WT trees (Fig. 1). Briefly, dormant bud tissue was excised from the scions of donor (WT) and recipient (4J, 4I, or WT) rootstocks. The donor bud was then placed into the chipped area of the recipient rootstock stem. Buds from 1-year-old WT scions were budded onto the stems of 1-year-old 4J and 4I lines to create chimeric-grafted (CG) trees. Buds from 1-year-old WT plum were budded onto stems of 1-year-old WT plum (originating from different seeds) to create autografted (AG) trees. AG trees serve as negative controls for the detection of *gafp-1* mRNA and protein. Three tree replicates received two buds each for a total of six budding attempts per line. Buds were wrapped in Parafilm® (Pechiney Plastic Packaging Company, Chicago, IL) for 2 weeks. After this time, the Parafilm® was removed. Four weeks after the budding event, the WT scion was pruned just above the uppermost bud graft. If two buds flushed on the same tree, they were both allowed to develop on the stem. Ungrafted (UG) trees were kept as additional controls for the detection of *gafp-1* mRNA and protein. Tissues from 4J and 4I lines were analyzed for the presence or absence of *gafp-1* mRNA and protein.

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CG and AG trees. Root tissues were sampled from corresponding trees. When possible, leaf tissue was sampled from grafted scions on separate trees. Leaf and root tissues were also sampled from three UG 4J, three UG 4I, and three UG WT trees. Soft shoots were sampled from two grafted scions on separate CG, AG, and UG trees from each line.

Detection of gafp-1 transcripts. Reverse transcription–polymerase chain reaction (RT-PCR) was used to determine if gafp-1 transcripts were present in leaf and root tissues of CG, AG, and UG trees. Total protein was extracted from leaf, shoot, and root tissues. Plant tissues (300 mg) were homogenized in liquid nitrogen and total cellular protein was extracted with TRIZol Reagent (Invitrogen Corporation) according to the manufacturer’s instructions. Bovine serum albumin was used as a standard. Sample absorbance was quantified at 650 nm using an Emax® precision microplate reader (MDS Analytical technologies, Sunnyvale, CA).

Total protein (20 μg) was loaded onto a 15% Tris–HCl gel and separated by SDS-PAGE. Protein molecular weight markers were included in all analyses (Precision Plus Protein Standards; Bio-Rad Laboratories). Proteins were tank-transferred to a PVDF membrane for 18 h at 30 V. Membranes were incubated with purified, polyclonal GAFP-1 antibodies (1:1000 dilution) then incubated with AP-conjugated secondary antibody (Bio-Rad Laboratories). Bound antigen was visualized using a fast 3′-diaminobenzidine (DAB) substrate kit (Bio-Rad Laboratories). Membranes were developed with BCIP/NBT solution (Sigma–Aldrich, St. Louis, MO). The entire procedure (protein isolation and immunoblot analysis) was repeated for all tissues. The AP-conjugated secondary antibody is documented to detect as little as 10 pg of protein (Bio-Rad Laboratories).

Results

With one exception, at least one of the two budding attempts became successfully established on each replicate tree. One tree from line 4J failed to yield successful bud grafts. Successfully grafted buds began to flush ≈3 to 4 weeks after budding.

The expected 367-bp gafp-1 and 498-bp α-tub fragments were successfully amplified from root tissue cDNAs of CG 4J and CG 4I trees (Fig. 2A). However, even after 35 cycles of amplification, we were not able to detect gafp-1 transcripts by RT-PCR in leaf tissues taken from WT scions of CG 4J or CG 4I trees (Fig. 2B). Successful amplification of the expected 572-bp cat2 fragment confirmed the quality of the RNA isolation from these leaf tissues (Fig. 2B). Transcripts of gafp-1 were not detected in the leaf or root tissues of UG WT or AG trees, but gafp-1 fragments were consistently amplified from leaf and root tissue cDNAs of transgenic UG 4J and UG 4I trees (Table 2). Cat2 and α-tub transcripts were detected in leaf and root tissues, respectively, from all CG, AG, and UG trees. Duplicates of each sample were created at the RNA extraction stage and subjected to the entire procedure without the inclusion of the RT enzyme. This confirmed that cDNA-derived amplicons were not a result of genomic DNA contamination (Fig. 2A–B).

GAFP-1 (expected size 12 kDa) was detected in roots of CG 4J and CG 4I trees but not in the leaf or soft shoot tissues of grafted, WT scions (Fig. 3A–B). In contrast, the lectin was detected consistently in the leaf and root tissues of UG 4J and UG 4I trees (Fig. 3C). A GAFP-1 signal was not extracted in leaf, soft shoot, or root tissues from UG WT or AG trees (Table 2; Fig. 3A–C). Lignified tissues from grafted, WT scions of CG trees were not analyzed for gafp-1 products in this study.

GAFP-1 antibodies showed cross-reactivity with other proteins on the immunoblots; however, cross-reactivity was not observed at the 12-kDa position. Antibody crossreaction occurred with an unknown 14 kDa protein in protein extracts taken from leaf, shoot, and root tissues and with a 15 kDa protein in protein extracts from shoot and root tissues. Although the binding specificity of the GAFP-1 polyclonal antibody may have been optimized by loading a smaller amount of protein on the gels, we chose to load higher amounts of total protein (20 μg) in an effort to resolve small amounts of GAFP-1 that may have been moving from the rootstock into the leaf tissues of the grafted, WT scions. On the immunoblots, the intensity of GAFP-1 bands

Table 1. Primers used for amplification of cDNAs from leaf and root tissues.

| Primer | Target gene | Size (bp) | Orientation | Sequence |
|--------|-------------|----------|-------------|----------|
| 1      | gafp-1      | 367      | forward     | 5′-CGCTGGCTTTCGCTGGACTGCAAGAT-3′ |
| 2      | gafp-1      | 572      | reverse     | 5′-GTGGTGTTCTGGCCAAATGCTGATT-3′ |
| 3      | cat2        | 498      | forward     | 5′-ACCTCTTCTACCTCGTTGACTGAAA-3′ |
| 4      | α-tubulin   | 498      | reverse     | 5′-TGTCGATTGGAGGACCCACCTACA-3′ |
| 5      | α-tubulin   | 572      | reverse     | 5′-TGTCGATTGGAGGACCCACCTACA-3′ |


**Table 2. Detection of gafp-1 mRNA and protein in tissues of autografted (AG), ungrafted (UG), and chimeric-grafted (CG) trees.**

| Rootstock | Graft type | Observations | Leaf mRNA | Protein | Root mRNA | Protein | Shoot mRNA | Protein | Observations | Protein |
|-----------|------------|--------------|-----------|---------|-----------|---------|------------|---------|--------------|---------|
| Wildtype  | UG         | n = 3        | –         | –       | –         | –       | –          | –       | n = 2        | –       |
| Wildtype  | AG         | n = 2        | –         | –       | –         | –       | –          | –       | n = 2        | –       |
| 4J        | CG         | n = 2        | –         | –       | –         | –       | –          | –       | n = 2        | –       |
| 4I        | UG         | n = 3        | +         | +       | +         | +       | –          | –       | +            | +       |
| 4I        | CG         | n = 3        | –         | –       | –         | –       | +          | +       | –            | –       |

*Leaf, shoot, and root tissues were analyzed twice for the presence (+) or absence (−) of gafp-1 transcripts or protein.

*Number of trees from which leaf and root tissues were sampled.

*Protein bands in kilodaltons.

Discussion

Many phloem-mobile macromolecules have been shown to traverse a graft union formed between compatible plant tissues. In situ RT-PCR studies demonstrated that pumpkin-derived CmNACP mRNA was present within the sieve elements (SEs) of grafted cucumber scions (Ruiz-Medrano et al., 1999). Gomez et al. (2005) showed that an RNA-binding phloem lectin from melon, CmLec17, could be detected within the phloem exudate of heterografted pumpkin tissues. Grafting experiments between transgenic and WT tissues have demonstrated that transcripts may move through graft junctions and elicit responses in plant cells that do not contain the causal gene. Transcripts of the tomato PFP-LeT6 gene, a sequence fusion found exclusively in the dominant mutant *Mouse ears*, were able to move across a graft union and induce changes in leaf pigmentation in WT tissues (Kim et al., 2001). St BELL5 transcripts were translocated across grafts made between potato overexpression lines and WT rootstocks, and localization of St BELL5 in stolons tips resulted in a twofold increase in tuber yields (Banerjee et al., 2006). Macromolecules have even been observed to move across graft unions established in host–parasite relationships. After colonization of transgenic tobacco by the adventitious plant species *Cuscuta reflexa*, green fluorescent protein expressed within tobacco companion cells was detectable within the SEs of the associated parasite (Haupt et al., 2001).

Conversely, this study provides strong evidence that gafp-1 transcripts and protein may not be moving into the grafted, WT scions of a CG tree species. These results contrast previous research that supports the phloem mobility of GAFP-1 within its host, the aclorophyllic orchid *Gastrodia elata*. Immunofluorescence studies demonstrated that the GAFP lectin is present within the vascular tissue of terminal combs, and it was proposed that the lectin is transported from the primary (nutritive) to the secondary (terminal) comb of the orchid as well as into the developing flower stem through the SEs (Hu and Huang, 1994). There are no data, however, to indicate that gafp-1 transcripts are phloem-mobile in *G. elata*.

As emerging leaves develop on the scion, they make the transition from “sink” to “source,” and at this point, they begin to contribute phloem assimilates to the scion translocation stream (Haywood et al., 2005). By routinely pruning the trees, we strive to keep the grafted scions in a “sink” state. There was not a single instance in which our detection procedures gave any indication of gafp-1 mRNA or protein in the grafted, WT leaf tissues of CG trees, even when tissues were sampled shortly (2 weeks) after maintenance pruning. Similarly, soft shoots, which were sampled from scions of CG trees between 2 and 6 weeks postpruning, never showed a protein signal at the 12-kDa position. Immunobots performed on protein extracts from WT leaves of CG 4J and CG 4I trees 8 weeks after budding (4 weeks postbud-flush) did not show GAFP-1 protein signals (data not shown).

We began to sample leaves and soft shoots for the detection of gafp-1 mRNA and protein 2 weeks after maintenance pruning. This should have been an adequate amount of time for the hypothetically phloem-mobile gafp-1 molecular products to move into grafted tissues. Several studies have demonstrated that macromolecules using phloem channels...
will spread relatively quickly within the plant. It has been reported that phloem-mobile gene-silencing signals are distributed systemically within a few days in tomato (Voinnet et al., 1998), and in herbaceous heterografts, 3 weeks has been sufficient for the detection of various, imported phloem-mobile transcripts and proteins (Gomez et al., 2005; Haywood et al., 2005; Ruiz-Medrano et al., 1999). Certain phloem-mobile plant viruses, which are thought to travel through the translocation stream as ribonucleoprotein complexes (Santa Cruz, 1999), are capable of spreading systemically in a matter of hours (Gal-On et al., 1994; Ismail et al., 1987) or days (Bennett, 1940; Capoor, 1949; Helms and Wardlaw, 1976; Mäst and Pallas, 1996) in herbaceous species. If virus movement is being assessed by symptom emergence, fruit tree seedling double-budding experiments have shown that virus particles can move from infected to noninfected tissues in 4 weeks (Fridlund, 1980). Most grafting studies conducted with woody plant material do not determine on a molecular level the amount of time it takes for phloem-mobile virus particles to move into budded tissues (S. Scott, personal communication); however, it has been shown in herbaceous systems that manifestation of disease symptoms in grafted tissues is preceded by the delivery of virus RNA (Mäst and Pallas, 1996).

It is possible that the GAFP-1 lectin may target transport of the phloem transportation machinery of P. domestica. Being in a different genetic background, the GAFP-1 lectin may move into the translocation stream only to be quickly degraded, or the protein may not be entering the translocation stream at all. In higher plants, the noncell-autonomous activity of signaling proteins and transcripts has a significant impact on the coordination of complex developmental and physiological events (Nakajima et al., 2001; Palaquvi et al., 1997; Ruiz-Medrano et al., 1999; Xocconostle-Cázarres et al., 1999; Yoo et al., 2004) and is likely subject to a certain degree of regulation. Indeed, targeted expression of knottin-like homeodomains as well as nonspecific diffusion has been observed within symplasmically connected cells and SEs (Crawford and Zambrany, 2000; Itaya et al., 2002; Lucas et al., 1995; Stadler et al., 2005). The concept of a surveillance network of the phloem mobile RNA (Ma, 1996). Microinjection of sonchus yellow net virus within infected Nicotiana tabacum, p. 283–293. In: Wardlaw, I.F. and J.B. Passioura (eds.). Transport and transfer processes in plants. Academic Press, New York, NY.

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