Final Scientific Report

Cover Page

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Project Title:

Rootstock Delivery of PGIP for Pathogen Protection in Grafted Tomatoes

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Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.

Fruit, scion, *Botrytis cinerea*, yield

Abbreviations commonly used in the report, in alphabetical order:

ARO, Agricultural Research Organization

BARD, US-Israel Binational Agricultural Research and Development Fund

B. cinerea, *Botrytis cinerea*

CA, California

Fig., figure

hr, hour

kg, kilogram

Obj., objective

PCR, polymerase chain reaction

PG, polygalacturonase

PGIP, PG-inhibiting protein

pPGIP, 'Bartlett' pear fruit PGIP

PTC, Plant Transformation Facility at UC Davis

RNA, ribonucleic acid

T5pPGIP, T5 tomato cultivar expressing pPGIP with the CaMV 35S promoter

UC, University of California

US, United States

USDA, United States Department of Agriculture

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Abstract

Grafting horticultural plants is an opportunity to incorporate important characteristics that allow for the production of a high value crops. Disease symptoms caused by pests and pathogens can be reduced by the delivery of anti-pathogen/pest factors from rootstocks. Many pests and pathogens that cause rotting in fruit and other harvested portions of crop plants produce polygalacturonases (PGs), an enzyme that degrades the plant cell wall. Polygalacturonase-inhibiting proteins (PGIPs) produced by plants are part of the defenses of fruit and leaves against damage caused by pest and pathogen PGs; production of these extracellular defense proteins can be enhanced by genetic modification and reductions in pathogen and pest caused disease result. Our aim is to expand the utility of this form of resistance by grafting a food crop-producing, wild-type scion onto a rootstock that has been modified to produce PGIPs that move across the graft union and into the scion tissues. The feasibility project was based on the hypothesis that (1) plant proteins that inhibit damage-causing polygalacturonases, PGIPs, can be transported from rootstocks via the xylem to grafted scions and (2) sufficient PGIP protein is, therefore, present in the scion to enhance defenses against pests and pathogens. Our one-year feasibility study demonstrated that tomato fruit and leaf defenses against *Botrytis cinerea*, *Alternaria alternaria* and *Cladosporium fulvum* are enhanced by the production of pear fruit (p)PGIP in rootstocks and movement of the pPGIP to grafted scions. In our recently submitted full BARD project, we propose to determine whether production of PGIPs in tomato rootstocks results in improved resistance to root-knot nematodes (*Meloidogyne*) in the roots and reduced damage caused by leaf miners (*Tuta absoluta*), and vascular wilts (*Fusarium oxysporum*) in grafted scions. The three objectives of our feasibility study were to:

Objective 1: Use existing tomato lines expressing pPGIP as rootstocks to determine whether the pPGIP is mobilized from the rootstock via the xylem and distributed into scion tissues.

Objective 2: Evaluate whether pPGIP protects the scion against pathogens, including *B. cinerea*

Objective 3: Transform tomato rootstock germplasm to express the pPGIP gene.

Objective 1 was expanded to evaluate several rootstock/scion graft combinations in Israel and in the US. The Israeli team focused on commercial scion varieties grafted onto pPGIP expressing rootstocks and the US team focused on heirloom scion varieties grafted onto new and traditional rootstock varieties.

The major conclusions are that pPGIP expression in rootstocks effectively improves the defenses of the scion, including fruit, but specific rootstock/scion combinations influence the yield of marketable fruit. This technology is likely useful with other horticultural crops, but consumer and grower acceptance should be evaluated, as we have proposed. If the approach is acceptable, impacts on pest and pathogen control in diverse horticultural crops eventually could be substantial.

Achievements:

Obj. 1. Demonstration of pPGIP protein movement from rootstocks to scion portion of grafted plants and evaluation of rootstock-scion compatibility.

Tomato grafting trials in Israel and Davis, CA were done. For two years in Israel, work with three rootstock varieties ('Resistor', 'Maxifort' and 'Spirit') grafted with scions of commercial cultivars '870', '1402', '4103' confirmed that rootstocks control soil-borne diseases (*Fusarium* and *Verticillium* wilts and *Fusarium* crown and root rot) and increase fruit yield to 170-188 ton/ha compared to 113 ton/ha for non-grafted plants. Fruit quality (texture, blemishes and lack of external defects) was better than that of fruit from non-grafted plants. Plants with 'Maxifort' rootstocks had the highest yield and the fruit had fruit total soluble solids and were firmer. In the feasibility study, the Israel group compared fruit yield and plant vigor (Table 1) of plants grafted to a 'T5' cultivar rootstock producing pPGIP (T5:pPGIP). They found that there was good compatibility (e.g. improved yield, plant dry weight, and chlorophyll contents) between the T5:pPGIP rootstock and scion cultivars '870', '1335', '1402' and 'IKRAM'.

Table 1 Treatment	Fruit yield (gr/plant)	Plant dry weight (gr)	Chlorophyll Content
870	3228 ab	182 ab	18.1 f
870/Maxifort	3339 ab	233 a	16.9 f
870/T5PGIP	3329 ab	175 ab	22.7 f
870/WT	2952 b	196 ab	26.9 c-f
870/T5	3882 ab	205 ab	44.8 a-c
1335	1909 b	190 ab	19.7 f
1335/Maxifort	2504 ab	212 ab	22.5 ef
1335/T5PGIP	1671 b	181 ab	27.3 c-f
1335/WT	2581 ab	175 ab	21.6 d-f
1335/T5	2322 ab	227 ab	28.0 b-f
1402	2404 ab	182 ab	29.2 b-f
1402/Maxifort	3907 ab	269 a	41.3 a-e
1402/T5PGIP	3592 ab	227 ab	42.6 a-c
1402/WT	3562 ab	230 ab	45.2 a-c
1402/T5	3715 ab	199 ab	53.1 a
IKRAM	2823 ab	206 ab	51.3 a
IKRAM/Maxifort	3712 ab	219 ab	29.0 c-f
IKRAM/T5PGIP	3178 ab	274 a	56.1 a
IKRAM/WT	3110 ab	198 ab	42.8 a-d
IKRAM/T5	3118 b	234 a	48.1 ab
T5	2713 ab	137 ab	51.5 a
T5/Maxifort	3049 ab	165 ab	44.3 a-d
T5/T5PGIP	2766 b	162 ab	50.1 a
T5/WT	3366 a	152 ab	55.5 a
T5/T5	2798 ab	152 ab	54.7 a
T5PGIP	1224 b	105 b	49.5 a

Table 1. Fruit yield, plant dry weight and leaf chlorophyll index of non-grafted '870', '1335', '1402', 'IKRAM' and 'T5' and scion/rootstocks combinations after 4.5 mos. Letters indicate significance.

Figure 1. Fruit (A and B) yield mass and number and dry biomass yield (C) of 'La Roma' (LR), 'Costuloto Genovese' (CLG), 'Brandywine Red' (BW) grafted onto 'Cheong Gang' (CG), 'Maxifort' (MF) or 'Beaufort 35' (BF) rootstocks.

The US group monitored plant vigor, yield and total soluble solids in field trials of ‘Costuloto Genovese’, ‘Brandywine Red’, ‘La Roma’, ‘Ace’, ‘T5’ and ‘Ailsa Craig’ scions (fresh market, processing, commercial or heirloom cultivars) grafted on to ‘Cheong Gang’, ‘Beaufort 35’, ‘Maxifort’, ‘T5’, ‘Shady Lady’ and ‘Ailsa Craig’ rootstocks. Yield and vigor results of a field trial (n>12 of each scion, rootstock combination) in the 2011 season in Davis are shown in Fig. 1.

Figure 2. Western blot detecting pPGIP protein in the scion (top) and rootstock (bottom) of grafted tomatoes.

With grafted plants expressing pPGIP in the rootstock, pPGIP protein was detected in scion leaves (Fig. 2) and susceptibility was monitored (see

below). Thus, defense factors in roots (e.g. pPGIP) can be made available to scion via grafting to improve the vigor, quality and pathogen and pest resistance of the food crop. Prior to evaluations, genotypes of the rootstocks and scions were confirmed by PCR of genomic DNA and amplification of the pPGIP sequence.

Conclusions: Fruit yield and plant weights in all combinations using commercial rootstocks were similar and the grafted plants looked healthier and were greener (Table 1). In grafts involving heirloom varieties as scions, yield varied considerably depending on the rootstock. The benefit of grafting depended on the rootstock onto which a particular heirloom variety was grafted. It can be concluded that (1) there is **good** compatibility between four commercial tomato scions and the T5:pPGIP rootstock (2) there is **good** compatibility between heirloom varieties and some new rootstock varieties, particularly ‘Cheong Gang’, and (3) the pPGIP protein is moved into the scion from a grafted rootstock (Fig. 2).

Objective 2. Evaluating the protection provided by ‘T5’ rootstock expressing pPGIP against plant pathogens

Tomato foliar and ripe fruit resistance to *B. cinerea* is improved about 40% by the constitutive over-expression of pPGIP in tomatoes (Powell et al., 2000). We observed that grafting with ‘T5’ rootstocks expressing pPGIP (T5:pPGIP) reduces pathogen damage (Figs. 3-5, Table 2).

Figure 3. Symptoms (48 h post inoculation) of *B. cinerea* on detached leaves of tomato cv. ‘870’ (left) as compared to cv. ‘870’ grafted onto T5:pPGIP rootstock (right). Damage is only observed on the ‘870’ that was not grafted.

Figure 4. Disease severity 3 days after inoculation with *B. cinerea* spores into small puncture wound sites of ripe fruit. ‘T5’ control (ungrafted) showed signs of tissue maceration and fungal growth but fruit from scions grafted to the T5:pPGIP rootstock showed reduced symptoms.

A. *Botrytis cinerea*

Conclusions: Leaves and ripe fruit of cvs. 'T5', '870' and 'IKRAM' (Fig. 3-5, Table 2) grafted on T5:pPGIP rootstocks were significantly more resistant to *B. cinerea* than the non-grafted lines.

<u>Treatment</u>	<u>Lesion diameter</u>	
	48 hrs	72 hrs
Scion/Rootstock		
IKRAM	9.22 a	14.22 a
IKRAM/T5	8.67 ab	14.22 a
IKRAM/T5pPGIP	5.38 c	10.25 b
IKRAM/WT	6.89 bc	13.44 ab
IKRAM/Maxifort	7.89 ab	16.44 a

Table 2. Lesion diameter (mm) of detached leaves from non-grafted and grafted 'IKRAM', on rootstocks 48 and 72 hrs after *B. cinerea* inoculation. Letters indicate significant differences.

B. *Alternaria alternaria* and *Cladosporium fulvum*

Conclusions: The tomato rootstock T5 expressing pPGIP can protect tomato scions against infections caused by the introduction of *B. cinerea* spores and also by natural infections by pathogens, such as *A. alternata* and *C. fulvum* (Fig. 5).

Figure 5. Leaf spots and necrosis caused by natural inoculations with *Alternaria alternaria* and *Cladosporium fulvum** on un-grafted 1402 (left), and grafted (right) on T5pPGIP tomato rootstocks.

*Identified by the Plant Protection and Inspection Services, Ministry of Agriculture and Rural Development, State of Israel.

Objective 3. Transform tomato rootstock germplasm to express the pPGIP gene.

In our feasibility proposal we intended to transform the 'Maxifort' tomato genotype, a popular choice as a tomato rootstock, so that it would express the pear fruit PGIP gene. After our proposal won support from BARD we learned that 'Maxifort' was a hybrid tomato line. Thus, a plan to transform 'Maxifort' germplasm and subsequently self-cross the T₀ transgenic lines would not maintain the "rootstock qualities" of the 'Maxifort' germplasm. Therefore we chose an alternative variety for generating the pear PGIP-expressing rootstock that will be used in the full BARD proposal that we submitted in September, 2011. We selected 'Cuatomate', a *Solanum lycopersicon* v. *cerasiforme* tomato line that is a common rootstock for tomatoes grown in Mexico. Drs. Edelstein and Fallick approve of this change. We prepared the transformation construct used to introduce the pPGIP-encoding gene and delivered the gene construct and 'Cuatomate' seeds to the UC Davis Plant Transformation Facility (PTC). In mid-December 2011, we received from the PTC twelve transformed, regenerated 'Cuatomate' tomato plants that should be expressing pPGIP. We hope to be able to analyze and utilize these plants with full BARD proposal funding in 2012.

Details of Cooperation:

In the first year of the project, Dr. Menahem Edelstein came to UC Davis for 1 month to instruct the US group in grafting techniques and to learn about pPGIP detection and function. The US group provided the Israeli group with seeds from the T5 line expressing pPGIP. A visiting fellow, Dr. Javier Lopez from Oaxaca, Mexico, continued the grafting work in Davis for about six months and supervised a UC Davis undergraduate student, Mar Jo Odias, in the grafting of tomato plants, detecting the pPGIP protein and assaying fruit susceptibility. Jo Odias presented the work as part of his senior project for graduation from UC Davis. In mid-2011, Dr. Hakan Aktas from Suleyman Demirel University, Isparta, Turkey, continued the project in UC Davis as Dr. Lopez was on medical leave and then had to return to Mexico. Dr. Aktas had collaborated with Dr. Edelstein on other aspects of vegetable plant grafting prior to coming to UC Davis, and thus, his participation enhanced our cooperation. Drs. Lopez and Aktas were supported by funds from their university institutions. The Israeli and US group have corresponded regularly by email and jointly submitted a request for funding for a full BARD proposal in September, 2011.

Publications:

A review manuscript, "Delivery of Nucleic Acids and Proteins from Grafted Rootstocks for Pathogen and Pest Control" (Authors: Victor Haroldsen, Mark W. Szczerba, Hakan Aktas, Javier Lopez, Mar Joseph Odias, Cecilia L. Chi-Ham, John Labavitch, Alan B. Bennett, Ann L.T. Powell) was submitted 22 December, 2011, by the US group has been submitted to a special issue on nucleic acids and plant pathogen resistance by the journal, *Frontiers in Plant Sciences*. The manuscript is currently under review. A copy of the submitted manuscript is included in the appendix.

Delivery of Nucleic Acids and Proteins from Grafted Rootstocks for Pathogen and Pest Control

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Running title: Trans-grafting for scion improvement

Abstract

Grafting has been used in agriculture for over two thousand years. With modern advances in biotechnology, new options for utilizing this proven horticultural technique are emerging. Specifically, trans-grafting, the combination of a genetically engineered (GE) rootstock with a wild-type (WT) scion, has the potential to provide biotic and abiotic stress tolerance or to increase plant vigor and productivity. Yet, it is unclear whether nucleic acids and proteins will be transmitted across a graft and, if so, whether this movement may affect the efficacy of the trans-grafting approach and/or regulatory oversight of the WT scion and its products. There are reports of the movement of organellar DNA and cellular mRNAs and proteins across graft unions, but to what extent these molecules affect performance of the scion is not clear. Strategies that can be used to limit or enhance nucleic acid or protein movement in order to maximize trans-grafting benefits have not been defined. This paper reviews, using specific examples, the transport of nucleic acids and proteins between rootstock and scion with the objectives of increasing the benefits grafting, particularly for pathogen resistance and defining how use of GE rootstocks effectively extends the horticultural utility of grafting.

Key words: Genetically engineered; protein; mRNA; siRNA; rootstock; scion; trans-grafting

Introduction

In agriculture today, the ancient and well-established technique of plant grafting is an indispensable tool that offers an opportunity for combining beneficial root and shoot characteristics for the production of high value horticultural crops. Plants growing in close proximity can undergo “natural grafting” or inosculation through physical contacts. For example, California black oaks are known to share root systems through natural root grafts and fortuitous contacts between strawberry or potato plants can result in stem grafts. Given that “natural grafting” occurs without human intervention, it is likely the art of grafting arose through discovery rather than by innovation, although it is unclear when or where grafting originated (Mudge et al., 2009). Chinese writings mention grafting of peach varieties as early as 1560 BC, and grafting was knowledgeably discussed by Aristotle, Theophrastus, Cato, and Varro from the 1st-4th centuries BC (Roberts, 1949).

Grafting provides a number of critical horticultural benefits. Grafting two woody species, each with desirable traits was instrumental in the domestication of a variety of tree species (e.g., apples, pears, and plum trees) that, otherwise, were recalcitrant to asexual propagation techniques (Mudge et al., 2009). In perennial species, grafting is used for clonal propagation and production of specialized ornamental trees. Grafting provides the means to repair or bypass damaged trunks, hasten development of fruiting varieties or improve vigor or dwarfing characteristics determined by the rootstock. Rootstocks also provide resistance to pests and pathogens, including insects and soil-borne diseases, and tolerance of abiotic stress conditions, such as thermal shock, low root temperature, boron toxicity, and salinity (Bulder et al., 1991; Bulder et al., 1991; Ahn et al., 1999; Rivero et al., 2003; Edelstein et al., 2005; Dolgov and Hanke, 2006; Edelstein et al., 2007; Venema et al., 2008). Currently, almost every commercial fruit or nut tree production system uses grafting to increase yields or avoid disease (Kubota et al., 2008).

In many parts of the world, grafting is used in vegetable production (Edelstein et al., 1999; Romano and Paratore, 2001; Fernandez-Garcia et al., 2004; Khah et al., 2006; Balliu et al., 2008; Davis et al., 2008; King et al., 2008; Kubota et al., 2008; Misovic et al., 2009; Di Gioia et al., 2010). Grafting is used widely with Solanaceae and Cucurbitaceae crops to reduce infections by soil-borne pathogens and to enhance the tolerance against abiotic stresses (Colla et al., 2010; Justus and Kubota, 2010; Lee et al., 2010; Rouphael et al., 2010; Savvas et al., 2010; Schwarz et al., 2010). In the Middle East, grafted vegetable rootstocks are used in order to utilize poorer soils, and in Japan, almost 95% of the watermelons, oriental melons, eggplants, cucumbers and tomatoes are grafted before transplantation to fields or greenhouses (Lee, 1994). Grafting vegetable and fruit plants became increasingly important for disease control in Europe and Israel after the soil fumigant methyl bromide was banned under the Montreal Protocol in 2005 (Cohen et al., 2007; Davis et al., 2008). In Israel, approximately 20% of the cultivated tomato plants are grafted. Grafting scions to vigorous, disease-resistant rootstocks is considered as an alternative to chemical control methods, a particularly appealing feature for organic cultivation of crops. An economic advantage of grafting is that a few rootstock lines may be utilized with multiple scion varieties, although the success of particular rootstock:scion combinations may be variable.

For farmers who grow heirloom tomato varieties organically, grafting is an emerging option, since, while tasteful and attractive, these varieties lack many of the disease resistance and vigor traits of conventionally produced varieties. The use of grafted tomatoes in the US has grown (Kubota, 2008; Kubota et al., 2008) as the demand for elite and heirloom varieties from small organic farms has increased. In North America, >95% of the grafted tomatoes are for greenhouse and high tunnel production, reflecting the markets for high-value fresh tomatoes. While the cost per grafted tomato seedling in the US is 1.5-2 times greater than for conventional seedlings, the possibility of introducing advantageous horticultural traits without compromising fruit characteristics offers significant value. Currently, most propagators supplying grafted seedlings to the North American market are in Canada and Mexico, although there are some specialty market suppliers in Ohio and North Carolina. At least 40 million tomato plants now are grafted in North America, with the US being the largest user (Kubota et al., 2008).

While conventional breeding has developed disease resistant rootstock genotypes for grafting, the additional approach of trans-grafting resistant GE rootstocks with WT scions provides a potentially valuable merger of ancient and modern technologies. In this paper, we discuss how trans-grafting can increase the options to provide agricultural solutions. We review our understanding of the mobilization of transgenes and the products they encode across graft junctions.

Trans-grafting

Trans-grafting (the use of a GE-rootstock with a WT-scion) has the potential to expand the traits provided by rootstocks by utilizing benefits derived from transgenes. These improvements could be accomplished while maintaining a scion that is free of transgenic DNA. A trans-grafted plant would allow agricultural industries to benefit from a transgenic trait expressed in a rootstock while addressing consumer concerns about food derived from GE-crops, because the scion would not have been genetically engineered. Trans-grafting also offers advantages for the environment, since under proper orchard maintenance, pollen flow concerns would be minimized because the non-engineered scion would be the only source of pollen. In addition, deregulation of a single or a small number of rootstocks that could be used with multiple scion cultivars or varieties would be preferable over engineering each scion genotype, especially given that the cost to develop each GE line is \$6 to 15 million in the US (Kalaitzandonakes et al., 2007). A deregulated rootstock could be utilized by different scion cultivars and, in some cases, by multiple genera. This could facilitate the entry of GE specialty crops into commercial production since deregulation of each scion cultivar would likely not be necessary, lowering the burden placed on specialty crop producers.

One consideration for the use of trans-grafted plants is the identification of transgene product(s) that have the potential to move from the rootstock to the scion. With GE rootstocks, the potential movement of DNA and RNA genetic or epigenetic factors and translocation of proteins can be evaluated because of their identifiable and unique characteristics. While grafted scions and rootstocks are generally assumed to conserve their own genetic identity, it is becoming evident that certain transcription factors, mRNAs, regulatory microRNAs (miRNAs), small interfering RNAs (siRNAs), peptides and proteins are mobile in the plant vascular system and thus, may cross the graft union. Examples of this will be discussed in the

following sections. Potentially, however, delivery of any of these products from a GE rootstock can be advantageous for the scion, particularly for the enhancement of pathogen and pest resistance.

Mobility of genetic components

Historically, nucleic acids were believed to be cell-autonomous (i.e., contained in the cell of origin), unable to move beyond the point of synthesis. However, this paradigm has evolved as sensitive methods have become available and been used to demonstrate that nucleic acids are present, and even have a role, outside of the cells where they are synthesized. Proteins are also known to cross cellular barriers and exert developmental control beyond their site of synthesis. The possibility that these molecules move and the regulation of their movement is shaping the way we think about transgenic rootstocks and their potential applications.

DNA

While there is no current evidence that would support the movement of genomic DNA through the vascular system of a grafted plant (apart from DNA-based plant viruses), movement of plastid DNA across cellular barriers *immediately adjacent to the graft junction* has been demonstrated (Stegemann and Bock, 2009). In this study, two cultivars of tobacco were each transformed with antibiotic-resistance selectable and visual markers. One cultivar was transformed with a kanamycin resistance gene and the nuclear-encoded yellow fluorescent protein (YFP) and another cultivar was transformed with a spectinomycin resistance gene and a plastid-encoded green fluorescent protein (GFP) marker. Explants taken from tissue immediately adjacent to the graft junction were able to grow on selective media for both constructs and fluorescence from both nuclei and plastids was detected. This outcome was not due to cellular fusion but rather due to the exchange of large sections of plastid (but not nuclear) DNA. However, the molecular studies did not exclude the possibility that entire organelles were transferred. While this effect was restricted to a few cell layers near the graft junction, it, nevertheless, challenges the idea that rootstock and scion strictly maintain their individual genetic identities. It has been suggested that exchange of genetic material might occur during graft healing as the cell wall and vascular system are being remodeled. The formation of new plasmodesmata could allow the symplasts of rootstock and scion cells to merge and, perhaps, exchange organelles (i.e., chloroplasts in this example); this would thus accomplish transfer of organellar genes. It is important to emphasize that the resulting chimera was not due to cellular fusion, because through single nucleotide polymorphism (SNP) genotyping and partial sequencing, scion cells were shown to have incorporated only a large piece of the rootstock plastid DNA.

While it is extremely unlikely that genomic or organellar DNA would be mobile over long-distances, as suggested by some researchers (Ohta, 1991), it is possible that heritable changes induced by epigenetic modifications of genomic DNA may occur as a result of movement. Heritable changes can result from RNA-mediated silencing mechanisms; siRNA can induce epigenetic effects such as sequence-specific DNA methylation (Jones et al., 2001). Present knowledge of heritable epigenetic influences might explain earlier claims of graft hybridization that alleged phenotypic changes in grafted pepper progeny due to mobility of DNA through the graft junction and into the seeds (Taller et al., 1998; Liu et al., 2010). Although grafting applications that take advantage of epigenetic modifications have not been developed, epigenetic changes present an opportunity to endow progeny with characteristics that result from transcriptional down-regulation or gene silencing without introduction of *heritable* transgenic DNA. Furthermore, based on

previous epigenesis experiments (Jones et al., 2001), subsequent generations should revert back to non-silenced phenotypes, thereby limiting the duration of the original modification.

mRNA

Evidence of a highly regulated and selective process involving long-distance trafficking of mRNA has been demonstrated. Observations have been made of differential localization and accumulation of transcripts in sink tissues, presence of mRNA-binding proteins in phloem sap and sequence-specific motifs of mobile mRNAs that interact with transcript-binding proteins. Messenger RNAs encoding transcriptional regulators and cell fate/cycle-related, hormone response, and metabolic genes have been identified in pumpkin and tomato sieve tube elements (Ruiz-Medrano et al., 1999; Kim et al., 2001; Haywood et al., 2005).

For example, the transcripts of pumpkin *CmNACP*, a member of the family of NAC transcription factors that are involved in apical meristem development and leaf senescence, have been identified in scion tissues from pumpkin rootstock–cucumber scion (i.e., heterografted) plants. This observation supports the idea of long-distance transport and accumulation of *CmNACP* RNA in vegetative, floral and root meristematic tissues. Data for this experiment were gathered using *in situ* RT-PCR and confirmed by *in situ* hybridization studies. Further experiments with seven other phloem sap-localized transcripts gave similar results, demonstrating the existence of delivery systems of specific transcripts to shoot and root apices (Ruiz-Medrano et al., 1999).

In another pumpkin rootstock/cucumber scion heterograft experiment, a phloem-mobile pumpkin RNA, *CmPP16*, was found in stem, leaves, and floral tissues of the scion. It was determined that the translated protein product CmPP16 bound sense and antisense *CmPP16* transcripts and, thus, mediated the transport of its own mRNA into the phloem translocation stream (Xoconostle-Cazares et al., 1999). Due to this self-mobility characteristic, the protein was termed a “plant paralog to viral movement protein”.

In grafted tomato example, a line carrying the dominant mutation, *Mouse ears (Me)*, which causes rounded and unlobed leaflets, was used as the rootstock and grafted to a semi-dominant *Xanthophylllic (Xa)* mutant scion with yellow, lobed leaves. Eleven of 13 graft plants demonstrated the *Me* phenotype in the scion. Interestingly, the *Me* gene is a fusion of two separate genes, *PFP* and *Let6*, that produces two transcript splice variants, but only the longer transcript is in-frame with the *Let-6* homeodomain and only this transcript was detectable in the scion. Fluorescent *in situ* RT-PCR confirmed accumulation of the longer *Me* transcript that had been detected in scion phloem sieve tubes and associated companion cells by Northern blots and confocal imaging. It was concluded that the *Me* phenotype of the scion was caused by movement of the *Me* transcript from the rootstock. The authors suggested that patterns of transcript accumulation observed by *in situ* experiments may not be entirely due to promoters expressing locally, but also may be attributed to transport of transcripts (Kim et al., 2001). In a follow up experiment, the *Me* tomato genotype was used as a heterografted rootstock with potato as the scion. Again, leaf morphological changes in the scion were observed and DNA gel blot analysis of the RT-PCR products revealed translocation of the *Me* transcript across the graft junction (Kudo and Harada, 2007).

Two mutant transcripts from the GRAS gene family, *CmGAIP* and *GAI*, were used to examine processes underlying mRNA mobility in pumpkin and *Arabidopsis*. In pumpkin these genes influence responses to gibberellin hormones. The pumpkin *CmGAIP* transcript, with a deleted DELLA domain, and the equivalent *Arabidopsis* mutant *gai*, with a non-functional DELLA domain, were analyzed because the DELLA domain mutations offer an easily trackable semi-dominant, dark-leaved, dwarf phenotype. The *CmGAIP* transcripts were found in stem, leaf, and floral tissues of heterografted plants with pumpkin rootstocks, particularly in the stem companion cells and sieve tube elements. Long-distance trafficking of these transcripts influenced development and leaf morphology in the scion. While the *CmGAIP* transcripts could be found in floral tissues, they were never detected in maturing fruit tissue; thus, it was concluded that tissue sink strength did not necessarily affect localization and delivery. To confirm specificity and selectivity and to rule out promoter effects, enhanced GFP (EGFP) was transformed into rootstocks under the companion cell-specific *SUC2* promoter. Although fluorescent signal from the EGFP protein could be detected in grafted scions, the *EGFP* transcript was not detected, suggesting that inherent properties of particular transcripts were likely responsible for their mobility or lack of mobility. That is, companion cells were able to retain *EGFP* transcript, but allowed the EGFP protein product to enter the phloem and the companion cells did not retain the *CmGAIP* transcripts. The observations suggested a complex, regulated, and tissue-specific process underlying mRNA phloem mobility (Haywood et al., 2005). Furthermore, the 3'UTR of the *GAI* transcript was shown to be necessary and sufficient to target *GFP* RNA for long-distance movement (Haywood et al., 2005). A mutated, movement-defective *GAI* transcript could be partially rescued by restoring nucleotides involved in the formation of predicted stem-loop structures. Thus, in addition to the nucleic acid sequence, the macromolecular structure of the mRNA may also contribute to its ability to be mobilized (Huang and Yu, 2009).

Aside from studies of individual transcripts, large scale experiments have identified families of mobile RNAs. Transcripts within the extracellular apoplastic compartments would be candidate mobile RNAs, particularly in the vascular fluids. Out of 1830 expressed sequence tags (ESTs) isolated and sequenced from melon sap, 986 were shown to be unique and many transcripts associated with biotic responses, stress and defense responses, metal-ion binding, and signal transduction were detected. Only three of the 1830 ESTs were identified as encoding Rubisco or chlorophyll-related proteins. Thus, the authors of this experiment concluded that the results were not due to contamination from surrounding cells. Heterografting with cucumber rootstocks revealed that 43 of the 986 unique transcripts were mobile and translocated through the vascular system into the pumpkin scion, perhaps suggesting conservation among RNA trafficking motifs, at least between the Cucurbits (Omid et al., 2007).

In addition to the heterografting studies, there is evidence for cross species mRNA mobility in the parasite-host interaction between *Cuscuta* and tomato (Roney et al., 2007). RT-PCR and microarray analyses showed the presence of over 400 tomato transcripts in *Cuscuta* tissue. Earlier studies had shown that one of the transcripts, *LeGAI*, was mobile in tomato phloem (Haywood et al., 2005).

It is clear that RNA sequences specify their mobility. Both the 3' and 5' untranslated regions (UTRs) appear to contain *cis*-acting sequences termed "zip codes" that provide competence for mRNA vascular transport, transcript stability, and translational regulation (Bassell et al., 1999; Jansen, 2001; Lucas et al., 2001;

Banerjee et al., 2009). It is known that mobile mRNAs can influence phenotypes (Kim et al., 2001; Kudo and Harada, 2007) and, at least in one case, this was demonstrated to be the direct result of translation of the mobile mRNA (Schmelzer et al., 2005). The experimental evidence makes it clear that mRNAs are present in the vascular stream and can be transported with a high degree of specificity. There have been no studies yet that demonstrate the non-regulated mobility or diffusion of mRNA into the vasculature. Given the relative instability of nascent mRNAs (Shyu et al., 2008) and the identification of protein binding regions in mobile mRNAs (Gomez et al., 2005), it is generally believed that mRNA transport is mediated via a ribonuclear protein complex (RNP) and movement of isolated single-stranded RNA transcripts has not been reported (Lucas et al., 2001; Gomez et al., 2005; Lough and Lucas, 2006). Aside from providing protection against endogenous ribonucleases, RNP proteins may provide additional information for targeting functions. Utilization of the mRNA transit mechanisms with specific anti-pathogen transcripts may be a viable strategy for improving pathogen resistance of scions, although no specific examples for this approach have been described at present.

Future applications will likely involve the addition of "zip codes" to target rootstock-generated transcripts to specific scion tissues or organs. Under the control of temporal, developmental, or inducible promoters in a rootstock, the effects of the transgene in the scion would be evident while maintaining the shoot, as well as its seed and pollen, free from transgenic DNA. In *Arabidopsis*, the mean and median half-lives of mRNA are 5.9 and 3.8 hours, respectively, but this varies with mRNA function and sub-cellular localization (Narsai et al., 2007). Given the relatively short half-lives of RNA transcripts, it is possible that once fruit and other products are harvested by removal from the plant, any transgenic RNAs in the scion tissues would degrade because the attachments to the sites of RNA synthesis, the source rootstocks, are severed. Much remains to be discovered in the field of nucleic acid movement and associations before applications that can utilize mobile, scion-targeted mRNAs are sufficiently defined to permit their exploitation.

Small non-coding RNAs

Small double stranded RNAs (sRNAs, less than 200 nucleotides (nt)) that participate in gene-silencing can be divided into two major groups: short/small interfering RNA (siRNA) and micro RNA (miRNA). siRNAs are generated from perfect double stranded RNAs produced by RNA-dependent RNA polymerase and can be induced by viruses, genetic constructs, or experimentally introduced. miRNAs are derived from non-coding, imperfect stem-loop RNAs and transcribed from their own promoters by RNA polymerase II. Both are processed by the RNA-induced silencing complex, but while siRNAs have a strictly silencing or quenching effect on gene expression, miRNAs are able to regulate gene expression in a much more tunable manner (Vazquez et al., 2010).

The silencing effect can be cell autonomous or non-autonomous, the latter indicating that silencing effects can be exerted over long-distances from the site of synthesis. With endogenous miRNAs, evidence indicates that most appear to be cell autonomous (Parizotto et al., 2004; Alvarez et al., 2006). There are exceptions; for instance, the gradual spreading and accumulation of miRNA166 in phloem tissue has been observed during leaf development (Juarez et al., 2004). In addition, regulation of transcription factors in roots and xylem patterning due to crosstalk between miRNA166 and miRNA165 and transcription factors have been observed (Carlsbecker et al., 2010). Additionally, long-distance movement of miRNA399 is

essential for inorganic phosphate uptake in the roots of phosphate-stressed *Arabidopsis*, rapeseed and pumpkin (Lin et al., 2008; Pant et al., 2008).

Non-cell autonomous gene silencing was first shown in tobacco with the nitrate reductase gene (Palauqui and Vaucheret, 1995). Subsequent grafting experiments confirmed that, in addition to its non-cell autonomous nature, the effect could spread unidirectionally from the tobacco rootstock to tobacco scions across a 30 cm WT-grafted “bridge” (Palauqui et al., 1997). Similar results were later confirmed in grafted sunflower using a GUS marker gene (Hewezi et al., 2005). In both tobacco and sunflower, the silencing effect was unidirectional, from rootstock to scion. Three-week-old embryos (seeds) derived from self-fertilized graft-silenced scions in sunflower showed restored GUS activity, demonstrating that, at least in this case, the silencing signal was not transmitted to the progeny through the graft. Both of these examples used sense transgenes, this type of silencing effect commonly referred to as co-suppression. Several other groups working with similar systems have reported analogous results (Voinnet et al., 1998; Sonoda and Nishiguchi, 2000; Crete et al., 2001; Mallory et al., 2003; Tournier et al., 2006).

In contrast, antisense silencing was shown in tobacco to be not graft-transmissible regardless of whether the signal originated in the scion or rootstock (Crete et al., 2001). This was initially the case also in tomatoes grafting experiments with the ACC oxidase gene. However, antisense silencing of the ACC oxidase was eventually shown to be graft transmissible in tomato, although it took several weeks to be evident (Shaharuddin et al., 2006). This time lag may account for why the earlier experiments concluded that there was no silencing in grafted antisense lines. A high level of expression of the target gene in the scion was necessary for the silencing due to expression of the antisense construct in the rootstock to be detectable by Northern hybridization, a requirement also noted in the nitrate reductase silencing experiments discussed above (Palauqui et al., 1997). Thus, experimental time lines, the levels of target gene(s), and the model organisms used may be important determinants of the efficacy of antisense silencing in grafted systems.

Using a hairpin construct, Molnar et al. (Molnar et al., 2010) used mutants deficient in RNAi biogenesis to show that graft-recipients accumulated sRNAs from donors through the vascular system. *Arabidopsis* containing a GFP inverted-repeat silencing construct as the donor were grafted with WT or GFP-expressing scions as recipients. The sRNAs identified in scion tissues included the GFP construct and a substantial population of endogenous sRNAs from the rootstock donor as well. Size classes ranging from 21-25 nt were most abundant, and the 24 nt class directed epigenetic modification of the GFP signal in the scion. The massively parallel deep sequencing methods used by this group showed that if a silencing target was not present in the recipient (i.e., completely WT-scion without GFP), then siRNAs generated from hairpin-GFP in the rootstock were still present in the scion, albeit at several orders of magnitude lower levels. This could be why previous experiments using less sensitive detection techniques, such as Northern blots, have not detected mobility of the signal. A recent report has shown that beyond the 24 nt siRNAs mentioned above, all size classes of siRNAs can trigger homologous sequence-specific methylation of targets at long-distances, at least in *Arabidopsis* (Dunoyer et al., 2010).

What facilitates the movement of sRNAs? sRNAs (~15 kDa) and associated RNPs are small enough to be translocated based on their size, since experiments have shown that a 27 kDa GFP is able to diffuse into the vascular system (Imlau et al., 1999; Kim et al., 2005). Results of experiments where movement proteins are included indicate that spreading of the silencing signal is at least partially dependent on the size of the plasmodesmata apertures (Kobayashi and Zambryski, 2007). Alternatively, movement of the silencing signal might be selective, perhaps requiring protein-protein, or protein-nucleic acid interactions in order to obviate the apparent plasmodesmata aperture size exclusion limit. This view is supported by experiments involving mutants defective or deficient in the ability to move signals (Dunoyer et al., 2005; Dunoyer et al., 2007; Yelina et al., 2010). Regardless of the mechanics behind movement of the sRNAs, the evidence demonstrates that movement does indeed occur through the phloem component of the vascular system and is mediated by plasmodesmata, at least to some degree.

Many experiments have been performed regarding the mobility of RNAs, both large and small, but whether the same pathways that are used for the movement of mRNA are used for miRNA or siRNA movement has not been determined. The emerging idea that sRNAs are involved in physiology, defense, and development, both cell autonomously and for long-distance signaling, is becoming more widely accepted (Buhtz et al., 2010). Given the variability in mobilities detected across several studies, it seems that plasmodesmata-based transport of sRNAs is a regulated process. However, the molecular mechanisms that mediate sRNA mobility and whether they are *cis* or *trans*-acting, are unknown.

Researchers have begun to use transgenic rootstocks that express siRNAs to protect the root zone from pathogens (Escobar et al., 2002), however use of grafting to deliver siRNAs to the scion for scion pathogen protection has only been reported in tobacco (Smirnov et al., 1997). Aside from pathogen resistance, down regulation and/or epigenetic modification of transcripts and genetic networks in the scion also appear to be possible through the use of siRNAs and could influence, for example, flowering time, fruit production or fruit quality characteristics.

Protein

In addition to RNAs, proteins may be transported over long distances in a regulated fashion. Certain motifs, reminiscent of nuclear localization signals, allow protein entry into companion cells and subsequently into the phloem for long-distance movement. Despite the evidence for selective and regulated processes for protein long-distance translocation, there is also evidence that shows non-specific “leakage” of supposedly cell-autonomous proteins into sieve tubes and subsequently into sink tissues. Xylem vessels, which mainly transport water and low molecular weight inorganic and organic solutes, have been shown to contain proteins, although at lower concentrations than in phloem sap (Aguero et al., 2008; Buhtz et al., 2010). Proteins targeted to the apoplast may inadvertently enter xylem or phloem vasculature and subsequently be transported to and unloaded in sink tissues.

Examples of movement of proteins include exogenous viral movement proteins, endogenous transcription factors and xylem/phloem proteins. Some of the first studies of xylem protein transport involved viral

movement proteins (Wolf et al., 1989), but as knowledge has progressed, more researchers have been able to demonstrate mobility of endogenous plant proteins. For many years, proteins had been observed in the phloem, but the idea of a coordinated, selective and regulated process of trafficking, influencing not only development, but plant responses to environmental cues is a more recent idea that has gained support (Kehr, 2009). Mobile proteins or non-cell autonomous proteins (NCAPs) may be encoded by as many as 20% of the genes in *Arabidopsis* (Lee et al., 2006). A comprehensive analysis of phloem sap proteins in pumpkin and cucumber using high resolution 2-D gel electrophoresis and partial sequencing by mass spectrometry identified several hundred proteins in the phloem, and the majority of these proteins may have roles in stress and defense reactions (Walz et al., 2004).

Models of the mechanics underlying protein mobility in the vasculature include the structures associated with the vascular tissue. Within the phloem, sieve tube elements (SE), which lack a nucleus, ribosomes and a vacuole, depend on neighboring companion cells (CC) for maintenance of their metabolic tasks (Fisher et al., 1992). Because mature SE cells cannot synthesize proteins, the likely origins of proteins in the phloem are immature SE or CC cells. Structurally different from the plasmodesmata that connect mesophyll cells, specialized plasmodesmata between CC and SE are branched with all of the branches on the CC side funneling to a single opening on the SE membrane side. The requirements for specificity of transport between CC and SE are not completely known but accumulating evidence points to the importance of these branched plasmodesmata. Reviews from two research groups establish plasmodesmata as the “gatekeepers” of macromolecular transport into the SE (Zambryski and Crawford, 2000; Lough and Lucas, 2006). The specific mechanics behind the regulation of plasmodesmatal apertures are still a mystery, but fluorescently labeled dextrans or expression of GFPs have been used to study plasmodesmatal size exclusion limits and their function under differing conditions. Through grafting, the vascular networks (phloem and xylem) of both rootstock and scion become connected and what is mobile in the rootstock vascular networks is likely to become mobile in the vascular networks of the scion.

In a thorough heterografting experiment involving 11 interspecific and intergeneric Cucurbit graft combinations, several structural phloem proteins (P-proteins) appeared in the recipient phloem exudate, as shown by SDS-PAGE and Coomassie staining. The results effectively demonstrated the direction of transmission was dependent on the combination of heterograft used, with some graft partners taking the role of donor, some acceptor, and some both (Golecki et al., 1998). This has clear implications for choosing of graft partners for GE-modified rootstocks. Fluorescent microscopy of graft junctions has shown sieve-tube bridges connecting scion external bundle phloem to internal bundle rootstock phloem when mobility was demonstrated. This observation identified physical continuity within the phloem as a prerequisite for mobility of proteins, but did not resolve the selective directionality observed. Golecki *et al.* (Golecki et al., 1999) examined in closer detail two Cucurbit structural P-proteins, PP1 and PP2, in intergeneric grafts. RT-PCR and Northern blots demonstrated that protein products rather than mRNA transcripts were translocated across the graft junctions.

In addition to structural proteins, RNA-binding proteins appear to be abundant in the phloem translocation stream. Phloem sap collected and analyzed from four different sources (cucumber, lupine, castor bean, and yucca) all contained sRNAs of 18-25 nt sizes with various abundance profiles for each species. Fractionation

of the phloem sap from pumpkin, cucumber, and lupine also identified a small ~27 kDa protein (PSPR1) that bound strongly to 18-24 nt single-stranded RNAs (ssRNA). After cloning the pumpkin *PSPR1* gene, microinjection studies demonstrated that PSPR1 specifically shuttled a high percentage of the ssRNAs across cell boundaries. In these studies, co-injection and subsequent movement of a 20 kDa fluorescent dextran showed that plasmodesmatal aperture was at least 20 kDa. Apparently, dilated plasmodesmata alone were not sufficient to allow the movement of ssRNAs between cells, since use of another protein shown to increase plasmodesmatal apertures (KN1) was not sufficient to allow the movement of the ssRNAs (Yoo et al., 2004). Given that the ssRNAs were approximately 8 kDa, their lack of movement when KN1 was provided suggests some sort of sequestration mechanism or perhaps a more complex interaction between ssRNAs and their binding proteins than is currently presumed.

In an informative experiment, rice thioredoxin (RPP13-1) a major phloem sieve tube protein with basic antioxidant functions, was expressed in *E. coli* and fluorescently labeled with FITC (Ishiwatari et al., 1995). In tobacco, the purified, labeled, heterologously expressed RPP13-1 protein was observed to migrate beyond the site of injection. However, the similarly purified and labeled *E. coli* homologue of RPP13-1 was not phloem-mobile under duplicate conditions, suggesting significant sequence or structure requirements for movement. Co-injection of rice RPP13-1 and FITC-labeled dextrans established that RPP13-1 increased the plasmodesmatal size exclusion limit to 9-20 kDa, from ~1 kDa. Furthermore, two mutants of RPP13-1 that were deficient for mobility were identified and crystal structure prediction studies suggested that charged clusters of residues on the outer surface were responsible for binding and/or transport of RPP13-1 through the companion cell-plasmodesmata complex (Ishiwatari et al., 1998).

Aoki and colleagues (Aoki et al., 2002) demonstrated the importance of protein structure for mobility using two heat shock proteins (HSPs), CmHsc70-1 and CmHsc70-2, that had been isolated from pumpkin phloem sap. In microinjection experiments, CmHsc70-1 and CmHsc70-2, interacted with plasmodesmata, increasing the size exclusion limit and thereby, enhanced their own cell-to-cell transport. The C-terminal region of these HSPs potentiated their non-cell-autonomous mobility through the plasmodesmata. A gain-of-function experiment in which the C-terminal cucumber HSP motif was fused to a human Hsp70 protein established that the fusion protein, but not WT human Hsp70, could move from cell to cell following microinjection of pumpkin cotyledons, much like the movement of injected intact CmHsc70-1 and CmHsc70-2. Interestingly, fusing the HSP C-terminal motif to GFP did not result in cell-to-cell migration, suggesting that at least in this case, the targeting motif was only active in the context of highly conserved HSPs (Aoki et al., 2002). Unlike nuclear localization signals or ER-targeting peptides, vascular targeting peptides may have several different motifs, probably suggesting specialized interactions with different families of proteins.

While targeting motifs appear to be important in regulating mobility, a non-regulated diffusion-based mechanism in the symplast from one cell to another is supported by the observation that protein size influences non-targeted movement of GFP but differences appear to be species- and developmentally dependent. Earlier studies indicated that non-regulated diffusion is limited to ~50 kDa proteins in mature leaves and 60 kDa proteins in developing leaves (Oparka et al., 1999; Crawford and Zambryski, 2000).

Unregulated diffusion-based movement across the sieve tube element-companion cell complex has been observed when CC-specific promoters (e.g., AtSUC2) regulate 27 kDa GFP expression. GFP was detected in the sieve tube elements and carried to sink tissue in the translocation stream (Imlau et al., 1999). While it was perhaps not surprising to detect the GFP in the vascular system due to the porous end plates of the sieve tube elements, unloading of the GFP into the mesophyll sink cells was unexpected. Using the same promoter, GFP-fusions as large as 67 kDa subsequently were shown to traffic from companion cells to sieve tube elements in root tips, although larger variants were restricted to a zone of cells adjacent to the mature protophloem. Only the smaller GFP variants (27-36 kDa) moved beyond this zone (Stadler et al., 2005).

To add further complexity to protein trafficking and regulation, phosphorylation and glycosylation are required for pumpkin CmPP16 to interact and form a stable complex with the mobility-endowing protein, Nt-NCAPP1, prior to phloem trafficking (Taoka et al., 2007). Discrepancies in observed mobility from one study to another could be attributed to phosphorylation and glycosylation since earlier studies did not take these post-translational, covalent modifications into consideration.

Two groups have demonstrated that non-endogenous proteins are retained in the rootstock. The *Gastrodia* antifungal protein (GAFP-1, a lectin) expressed by transgenic plum rootstocks under the control of the constitutive CaMV35S promoter was identified in roots by immunoblot, but not in the soft shoot or leaf tissues of grafted, WT-scions. This suggested that GAFP-1 was not moving into the WT-scion tissues of trans-grafted plum trees (Nagel et al., 2010). In the other example, transgenic watermelon rootstocks over-expressing a cucumber mottle mosaic virus coat protein (CGMMV-CP) gene were trans-grafted with WT watermelon as the scion. Protein expression and mRNA levels were detected in the transgenic rootstock but not in the non-transgenic scion (Youk et al., 2009). Detection limits of the techniques utilized were not reported in either of these studies. A pokeweed antiviral protein from *Phytolacca americana* was expressed in transgenic *Nicotiana tabacum* rootstocks and provided resistance to potato virus X in *NN* and *nn* grafted non-transgenic scions. However, the antiviral protein was detected only in the rootstocks and not in the grafted scion tissues (Smirnov et al., 1997). The basis for resistance expression in this situation is not clear.

Protein translocation from a transgenic rootstock to a WT-scion will likely depend on the species and/or type of protein in the transgene construct. Should transgenic proteins manage to migrate to the scion, their longevity is a consideration. NPTII and GUS proteins have estimated half-lives of 6-7 minutes and 36 hours, respectively, *in planta* (Lo et al., 2005). If NPTII were translocated to scions it would be lost rapidly, but the GUS protein would not be reduced to 1% of the initial level accumulated in scions for 12 days.

Research on the production of transgenic proteins in rootstocks for delivery to scions arguably is more advanced than analogous work with the use of nucleic acids. For example, researchers at University of Florida have engineered grape rootstocks that deliver hybrid lytic peptides to control bacterial and fungal

diseases (Dutt et al., 2007; Gray et al., 2007). Work in our lab has shown that delivery of a protein that inhibits microbial maceration of plant cell walls is possible (below). While advances to date have focused on delivery of single gene products with specific function to scions, future advances may target transport of transcription factors that influence expression of multiple genes, which could coordinate concerted scion responses to complex challenges such as pathogen, pest or abiotic stresses.

Delivery of anti-pathogen proteins from rootstocks to scion: the pPGIP example

Proteins that are delivered to and function in the apoplast can provide protection against pathogens, particularly those that target the extracellular cell wall. The plant cell wall is the site where the molecular conversations that determine the host plant's fate are begun (Cantu et al., 2008; Cantu et al., 2008). In many plant–microbe/pathogen/pest interactions, the plant cell walls are a major obstacle to colonization or expansion within plant tissues. To overcome this barrier, most fungal pathogens produce a variety of enzymes, which degrade the host cell wall. Polygalacturonases (PGs) (EC 3.2.1.15) are often the first enzymes secreted during the infections (Collmer and Keen, 1986). PGs cleave α -(1→4) linkages between D-galacturosyl residues in pectic homogalacturonan, causing cell separation and tissue maceration. *Botrytis cinerea* expresses six PGs during infection and growth on plant hosts (Wubben et al., 1999) and the PG-inhibiting protein (PGIP) produced in pear fruit (pPGIP), inhibits some but not all of these PGs (Sharrock and Labavitch, 1994).

Given the importance of PGs in pest and pathogen interactions with plants, it is not surprising that PGIPs are components of the defenses against invasion by pathogens and pests (Powell et al., 2000; Ferrari et al., 2003; Aguero et al., 2005). Tomato foliar and ripe fruit resistance to *B. cinerea* is improved about 40% by the constitutive over-expression of pPGIP in tomatoes (Powell et al., 2000). PGIPs expressed in rootstocks, therefore, are potential anti-pathogen proteins that could be delivered from the rootstock to the scion in trans-grafted plants.

Our work has shown that pPGIP expression reduces the effects of Pierce's disease in grapevines, caused by the bacterium, *Xylella fastidiosa* (Aguero et al., 2005) because it inhibits the *X. fastidiosa* virulence factor, PG (Roper et al., 2007; Perez-Donoso et al., 2010). As with other vascular pathogens, the *X. fastidiosa* PG contributes to disease development by digesting the polysaccharides in the pit membranes of the xylem network. When intact, these membranes help to prevent the pathogen's vessel-to-vessel spread from the initial sites of infection of grapevines (Sun et al., 2011). Because pPGIP inhibits the *X. fastidiosa* PG and because pPGIP can enter the xylem, PGIPs in the xylem of both the rootstock and the scion could provide protection against pathogens in the water transport system is logical.

We have observed that when pPGIP expressing transgenic plants are used as rootstocks onto which non-expressing scions are grafted, the pPGIP protein, but not the pPGIP-encoding nucleic acids, are exported to the scion, crossing the graft union via the xylem system (Aguero et al., 2005). In grafted tomato plants expressing pPGIP in the rootstock, pPGIP protein has been detected in scion leaves (Fig. 1). Similarly, in grafted grapevines, we have observed the pPGIP protein in the wild-type scion tissue grafted onto pPGIP-expressing rootstocks (Fig. 2).

Furthermore, we have observed that expression of pPGIP in rootstocks reduces pathogen damage in scion tissues (Figs. 3). Thus, defense factors in roots (e.g., pPGIP) can be made available to scions via grafting, improving the vigor, quality and pathogen/pest resistance of the food-producing scion and its crop.

Concluding Remarks

Grafting has been used extensively to improve productivity, mainly in woody perennial horticultural crops like fruits and nuts, but is increasingly used to enhance the productivity and disease resistance of high value vegetable crops. Trans-grafting should extend the utility and value of the grafting strategy, enabling the utilization, in rootstocks, of transgenes whose products serve novel, potentially powerful functions. By deploying the transgene products to scion tissues, the performance of scion-borne crops should be improved. Obvious examples are the introduction of genes with demonstrated efficacy in disease resistance (e.g., PGIPs), but also may include traits that target developmental events, metabolic pathways or fruit quality. In designing these strategies it will be important to consider the mechanisms that regulate long distance translocation of DNA, RNA, sRNAs and proteins to assess the durability and efficacy of alternative strategies.

There are no precedents for how a GE-rootstock and non-GE scion may be regulated, but the practice would raise interesting questions. Based on the current regulatory system for GE crops, one might expect that the rootstock would require full “deregulation” but that the WT-scion would not since it is not GE. Regulatory agencies (USDA, EPA and FDA) may want to consider the status of the scion depending on what transgene-derived products are detectable in it. For example, movement of nucleic acids into the scion may not trigger a response as nucleic acid molecules are generally recognized as safe and thus, exempt from regulation. However, trafficking of proteins into a WT-scion may have to be examined analogously to examinations of typical GE-plants, addressing whether the transgene-encoded proteins present allergenicity threats or affect the composition of consumed fruits. Since the GE-rootstock is not intended to flower or fruit and is often sterile, regulatory considerations related to pollen-flow should be minimized. Consumable fruit or vegetable organs harvested from the WT-scion are not genetically modified, but questions related to whether the food products would require labeling may remain since they may contain transgene products. Plant biologists are increasing our understanding of the mobilization of transgene-encoded molecules and expanding our ability to deliver agronomic improvements to food products. The development of this technology will likely require rethinking of specific regulatory issues as well as the design of efficacious ways to achieve a higher level of food quality and consumer acceptance and trust.

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LEGENDS

Figure 1. (A) pPGIP PCR products from genomic DNA and (B) Western blots detecting pPGIP protein from grafted tomato plants. Lane 1, pPGIP expressing rootstock and scion; Lane 2, WT rootstock and pPGIP expressing scion; Lane 3, WT rootstock and scion; Lanes 4-6, pPGIP expressing rootstocks with wild-type scion. The upper row of images is results from scion tissue and the lower row is from rootstock material. The detection limit with the pPGIP polyclonal antibody is 40 ng protein. Stacked images in (A) visually represents the fruit, leaf and root material from grafted plants, with dark boxes representing transgenic portions of the plant and light boxes representing WT.

Figure 2. Western blots of extracts taken from rootstock tissue and scion leaves of grafted ‘Thompson Seedless’ grapevines. pPGIP is visualized with a polyclonal antibody whose limit of detection is 40 ng pPGIP protein. The pPGIP protein detected in the scion leaves has moved from transgenic rootstocks into wild-type (WT) scion tissue (lanes 4-6). This movement is not seen in the reciprocal graft (lane 2).

Figure 3. Disease severity of *B. cinerea* infections of T5 control (ungrafted) and transgrafted (T5:pPGIP rootstock) light red fruit. Disease severity is measured as the diameter of the macerated tissue around the

sites of inoculation. Each fruit was inoculated at 5 puncture sites with 1000 *B. cinerea* spores in 10 µl water. Fruit were maintained in moist crispers at 20°C and lesion diameter was measured daily (Cantu et al., 2008; Cantu et al., 2009). Standard error of the mean (SEM) are indicated.

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A **B**

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