Grafting of Genetically Engineered Plants

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ABSTRACT. Grafting is a well-established agricultural practice, and it now has implications for the commercialization of transgenic plants. In transgrafted plants, only one part (scion or rootstock) is transgenic with the other part untransformed. However, transgenes may affect both mobile and immobile endogenous metabolites (e.g., RNAs, proteins, and phytohormones) and mobility has implications for transgrafting. In the phloem, long-distance transport of mobile metabolites can play important roles in plant development and signaling. In a transgrafted plant, an immobile transgene product (ITP) is not likely to be translocated across the graft union. In contrast, mobile transgene products (MTP) may be translocated across the graft. Regardless of the mobility of transgene products (TP), interaction of transgenic and nontransgenic parts in transgrafted plants through either the MTP or ITP has been demonstrated to be effective in facilitating changes in nontransgenic portions of the plant. Consequently, and particularly in fruit crops, transgrafting provides the potential for improving products from their nontransgenic parts with the possibility of minimizing the controversy over transgenic crops. This review focuses mainly on the mobility of TP and effects on the whole transgrafted plant.

Currently, all commercialized genetically engineered (GE) crops contain exogenous genes (transgenes) not naturally present in the host crops. Since first commercialized in 1996, GE crops have been adopted worldwide with an average annual increase in acreage of about 10%. By 2012 about 11.3% of the earth's arable land was growing GE crops [e.g., soybean (*Glycine max*), corn (*Zea mays*), canola (*Brassica napus*), cotton (*Gossypium hirsutum*), and sugar beet (*Beta vulgaris*)] that were engineered mainly with herbicide- or insect-resistance traits (Camacho et al., 2014). However, rapid adoption of the technology has been accompanied by controversy (Gillam, 2014).

Questions about food safety and environmental health have arisen, and these concerns include misunderstandings, hostilities to, and uncertainties about the safety of transgenes, TP, and transgene flow to the environment (Fernandez-Cornejo et al., 2014; Gillam, 2014). Compared with agronomic crops (e.g., soybean, corn, cotton, etc.), genetic engineering of specialty food crops (i.e., fruits and vegetables) and ornamentals has been particularly impacted by these concerns. However, other factors, including technical barriers, investments required and profit potentials, have also been important. To date, of the 27 crop species registered in the GE Approval Database of the International Service for the Acquisition of Agri-biotech Applications (ISAAA, 2015), only eight vegetable and fruit crops [i.e., eggplant (*Solanum melongena*), melon (*Cucumis melo*), papaya (*Carica papaya*), plum (*Prunus domestica*), potato (*Solanum tuberosum*), squash (*Cucurbita pepo*), sweet pepper (*Capsicum annuum*), and tomato (*Solanum lycopersicum*)] are listed. Transgenic papaya with resistance to *Papaya ringspot virus* is the only commercialized GE fruit crop. Transgenic plum with resistance to *Plum pox virus* (PPV) was finally registered in 2011, but has not been commercialized

in the United States because PPV is not spreading and the need for resistance is limited (Polák et al., 2012). Recently, 'Arctic^R' (nonbrowning) apple cultivars have been developed using RNAi-mediated intragenic technology (Okanagan Specialty Fruits, 2015), which subsequently provoked a new wave of the debate over GE fruits, and thus, commercialization has stalled. For vegetables, only squash with transgenic virus resistance has been commercialized. The U.S. Department of Agriculture (USDA) and U.S. Food and Drug Administration (FDA) have also deregulated Simplot's InnateTM potato that contains only potato genes (Simplot Company, 2015).

Genetically engineered organisms can be transgenic as well as nontransgenic. New biotechnology tools (i.e., intragenesis, cisgenesis, and genome editing) promise the production of nontransgenic GE crops. For example, intragenesis and cisgenesis enable genetic modification using target genes from the same or closely related species (Holme et al., 2013; Rommens, 2004; Schouten et al., 2006). A cisgene is defined as an identical copy of gene that exists in the sexually compatible pool: no further in vitro manipulation of the cisgene is allowed except that the addition of the borders of transfer DNA (T-DNA) required for agrobacterium-mediated transformation (Schouten et al., 2006). An intragene is an engineered gene within the T-DNA borders in which the gene elements (i.e., promoter, coding regions, and noncoding regions) are from the sexually compatible pool and have been recombined into novel arrangements (Rommens, 2004). Accordingly, intragenic and cisgenic products are little different from the products of traditional breeding and are arguably more likely to be acceptable to the public than transgenic products. The USDA is currently not regulating several intragenic crops (Ledford, 2013); but no cisgenic plants have been reported (Holme et al., 2013). Indeed, it has been suggested that cisgenic or intragenic plants should not be regulated as transgenic but perhaps by a separate, but as yet undefined, regulatory framework (Camacho et al., 2014). Alternatively, transgrafted plants, which are defined as grafted plants where transgenic parts are joined with nontransgenic parts, have potential to expand the use of transgenesis for

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horticulture crops (Haroldsen et al., 2012a, 2012b; Lev-Yadun and Sederoff, 2001). Although current regulations may still apply to transgated crops, collectively, the next generation of transgenic transgated GE crops may avoid some of the controversy over conventional transgenic GE crops, and by boosting the value (yield, quality, sustainability, and uniqueness) of vegetables, fruits, and ornamentals, will offer benefits to both growers and consumers. This article considers the issues involving transgated plants.

Transgating: Joining a GE Plant with a Non-GE Plant

For over 3000 years, grafting has been used to produce plants by artificially conjoining different vascular systems (i.e., rootstock and scion) for multiple purposes, such as asexual propagation, disease/pest-resistance, altered plant vigor and architecture, increased tolerance to abiotic stresses, precocity, and higher yields (Aloni et al., 2010; Gonçalves et al., 2006; Koepke and Dhirga, 2013; Kubota et al., 2008; Lee, 1994; Martinez-Ballesta et al., 2010). The rootstock interacts directly with soil through which water and nutrients are absorbed and transferred to the scion; however, endogenous products of roots (e.g., hormones and stored carbohydrates) also have impact on the scion. The scion leaves photosynthesize and produce carbohydrates and other metabolites that can be translocated to the roots. Apparently, rootstock-scion interactions play an essential role in regulating growth, development, and productivity of a grafted plant. Numerous factors (e.g., water, hormones, nutrients, DNA, RNAs, and proteins) contribute to the rootstock-scion interactions. Accordingly, there have been many hypotheses on the mechanisms involved in long-distance signaling and rootstock-scion interactions (for details see the following sections).

In this review, transgating refers to grafting a GE part with a non-GE part. The GE parts can be transgenic, intragenic, or cisgenic. GE-root plants refer to transgated plants where non-GE scions are grafted on GE rootstocks. Conversely, GE-scion plants are transgated plants where GE scions are grafted on non-GE rootstocks. Transgated plant/food products refer to the edible products from non-GE parts of the transgated plants. For example, GE-root plants provide the potential of using transgenic rootstocks (TR) to improve performance of commercially approved scion varieties and produce non-GE products (e.g., perennial fruit crops). Similarly, there is a potential to use GE-scions to enhance the production of conventional cultivars of root or tuber crops [e.g., cassava (*Manihot esculenta*) and potato]. Transgated plants also have the potential to address concerns about transgene-flow and exogenous TP in most transgenic organisms.

Movement of GE Products in Plants

Endogenous molecules in higher plants are either mobile or immobile through the vascular system. Phloem sap assays have demonstrated the exchanges of many substances (i.e., water, mineral nutrients, hormones, sugars, proteins, RNAs, lipids, and fatty acids) between the scion and rootstock of grafted plants (Aloni et al., 2010; Dinant and Suárez-López, 2012; Guelette et al., 2012; Ham and Lucas, 2014; Pyott and Molnar, 2015). Overwhelming evidence has shown that mobile macromolecules (e.g., proteins and RNAs) in the phloem function as signaling molecules that play important roles in plant

development (Chitwood and Timmermans, 2010; Citovsky and Zambryski, 2000; De Schepper et al., 2013; Ham and Lucas, 2014; Hannapel et al., 2013; Kehr and Buhtz, 2008). Similarly, transgenes produce either MTP or ITPs. The ITP may improve transgenic parts (e.g., abiotic and biotic stress tolerance) and could indirectly affect non-GE parts in transgated plants. When the MTP are produced in either part (scion or rootstock) of the grafted plant, translocation of signal MTP (e.g., proteins/peptides, RNAs, and hormones) across the graft union will occur and could thus enable the strategy of using the transgene product from the transgenic part to positively affect the nontransgenic part of a grafted plant.

Mobility of GE Proteins/Peptides

Some proteins/peptides serve as signal molecules in plants. Mobility of many endogenous proteins, including intracellular movement through plasmodesmata or long-distance trafficking in phloem, is well documented (Atkins et al., 2011; Golecki et al., 1999; Han et al., 2014; Ham and Lucas, 2014; Lucas et al., 2009; Wu and Gallagher, 2011; Zeevaart, 2006). Of the long-distance transported proteins, the role of transcription factor families is essential for plant development (Ayre and Turgeon, 2004; Ham and Lucas, 2014; Han et al., 2014; Rim et al., 2011; Wu and Gallagher, 2011). For example, the well-studied flowering locus T (FT) protein family is a phloem-mobile signal from leaves that regulates plant flowering, bulbing, and storage tuber development (Lee et al., 2013; Lifschitz et al., 2006; McGarry and Kragler, 2013; Navarro et al., 2011; Pin and Nilsson, 2012; Taoka et al., 2013; Yoo et al., 2013). Grafting experiments in tomato demonstrated that the tomato FT (SFT: single-flower truss) signals produced in the GE scion are able to cross the graft union and rescue flowering and morphogenetic defects of the receptor *sft* rootstock-derived shoots (Lifschitz et al., 2006). In grafted potato plants, FT protein (but not the RNA) produced in the scion by ectopically expressing the rice (*Oryza sativa*) *FT* (*Hd3a*) gene was transported to wild type rootstocks promoting tuberization (Navarro et al., 2011). In addition, there is also evidence showing that *FT* RNA is involved in long-distance transport and floral induction (Li et al., 2011; Lu et al., 2012). In our recent study, we found the highbush blueberry (*Vaccinium corymbosum*)-derived *FT*-like gene, *VcFT*, hastens flowering when ectopically expressed in tobacco (Song et al., 2013b). However, when the GE tobacco plants were used as rootstocks, they did not promote flowering in non-GE scions, probably due to the low amount of *VcFT* transported to the shoot apical meristem (Walworth et al., 2014). Studies in many herbaceous plants have demonstrated similar scion-to-root or source-to-sink transfer of phloem-mobile, signal proteins (like FT) and their potential application for plant breeding (Jiang et al., 2013; McGarry and Kragler, 2013). In woody plants, overexpressing *FT* or *FT*-likes clearly promotes flowering in several systems [e.g., apple, highbush blueberry, sweet orange (*Citrus sinensis*), plum, poplar (*Populus* sp.), peach (*Prunus persica*), and trifoliate orange (*Poncirus trifoliata*), etc.] (Böhlenius et al., 2006; Endo et al., 2005; Hsu et al., 2011; Matsuda et al., 2009; Song et al., 2013b; Srinivasan et al., 2012; Tränkner et al., 2010). More recently, Ye et al. (2014) reported that overexpressing a barbados nut (*Jatropha curcas*)-derived *FT* (*JcFT*) promotes flowering of not only the transgenic plant but also the grafted, nontransgenic scion. However, it is still unclear if the

transported JcFT directly caused early flowering in the non-transgenic scions.

In general, large proteins are not involved in transport either from cell-to-cell through plasmodesmata or over long distance across graft unions (Crawford and Zambryski, 2001). Therefore, large transgenic proteins in a transgrafting plant are not likely in the products of the non-GE part. Examples include the Cry1Ab protein (about 100 kDa) for insect resistance and the bar protein (about 22 kDa) for herbicide resistance) where there is no documentation of long-distance transport of these proteins. Consequently, little has been achieved in using transported transgene proteins to improve grafted woody plants, although the potential has been reported in grapevine (*Vitis vinifera*), where transgenic peptides/proteins produced in rootstocks for disease resistance were present in nontransgenic scions (Agüero et al., 2005; Dutt et al., 2007). However, rootstock-to-scion movement of transgenic proteins was not detected in transgenic plum containing the *Gastrodia* antifungal protein (GAFP-1) (Nagel et al., 2010) or from the 'M26' apple rootstock containing the root-promoting *rolB* gene (Smolka et al., 2010), indicating that engineering rootstocks with low mobility transgenic proteins may result in less concern about transgenic products in nontransgenic scions.

Mobility of RNA Signals

Phloem flow clearly facilitates transport of RNAs (i.e., viral RNAs, mRNAs, rRNAs, tRNAs, siRNAs, and microRNAs) over long-distances. These transported RNA molecules mediate systemic signaling and play essential roles in plant development, growth and responses to biotic and abiotic stressors. Several groups have reviewed the studies on the mobility and function of these RNA signals (Bai et al., 2011; Baulcombe, 2004; Citovsky and Zambryski, 2000; Deeken et al., 2008; Eckardt, 2002; Hannapel et al., 2013; Harada, 2010; Huang and Yu, 2009; Kehr and Buhtz, 2008; Kragler, 2010; Marín-González and Suárez-López, 2012; Molnar et al., 2011; Sarkies and Miska, 2014; Uddin and Kim, 2013; Varkonyi-Gasic et al., 2010). So far, major evidence of long-distance transport of RNA molecules includes: 1) the presence of a range of RNA molecules in phloem sap of numerous plants, suggesting likely transport of these RNA molecules through source-sink interactions (Banerjee et al., 2006; Buhtz et al., 2008; LeBlanc et al., 2012; Omid et al., 2007; Zhang et al., 2009); 2) functional analyses of phloem-mediated long-distance transport of RNAs in grafted plants (Table 1), indicating that some RNA molecules are systemic signals capable of regulating target genes/traits; and 3) plant virus vector systems that have been widely used for RNA production and delivery in woody plants (Yamagishi et al., 2014).

Scion-to-rootstock translocation of RNA molecules has been reported in grafted arabidopsis (*Arabidopsis thaliana*), tobacco, wild tobacco (*Nicotiana benthamiana*), and potato, in which mobile RNA molecules [including exogenous green fluorescent protein (GFP) siRNAs and endogenous microRNAs, siRNAs, and transcription factor RNAs] have been documented (Table 1). Using high-throughput sequencing, translocated small RNAs of both exogenous and endogenous sRNAs in arabidopsis have been profiled (Dunoyer et al., 2010; Molnar et al., 2010). In grafted potato plants, the transcription factor *St BEL5* RNA and miR172 have been found to be long-distance signals transported from scions to regulate tuber/stem formation of the rootstock (Martin et al., 2009).

Rootstock-to-scion transport of RNA molecules has been reported in grafted arabidopsis, tobacco, wild tobacco, tomato, cucumber (*Cucumis sativus*), potato, pumpkin (*Cucurbita maxima*), apple, pear (*Pyrus betulaefolia*), and sweet cherry (*Prunus avium*) (Table 1). Transported RNA molecules that have been documented include exogenous siRNAs and endogenous microRNAs, siRNAs, and other functionally undocumented RNAs (Table 1). In phloem sap assays, endogenous RNAs of rootstocks have been found in cucumber grafted on pumpkin, pumpkin grafted on cucumber, potato grafted on tomato, apple grafted on chinese crabapple (*Malus prunifolia*), and pear grafted on birch-leaved pear (*Pyrus betulaefolia*) (Table 1). Using transgrafting approaches, rootstock-to-scion transport of exogenous gene-derived RNAs (e.g., marker genes *GFP* and *uidA*) has also been demonstrated in model plants. In cucumber plants, grafted on a transgenic squash expressing a viral coat protein (CP) gene, presence of the CP siRNAs in the cucumber sap has provided direct proof that virus siRNAs can move in the phloem stream (Yoo et al., 2004). In grafted tobacco plants, hpRNA-derived siRNAs of viruses and viroids in rootstocks were transported to nontransgenic scions and suppressed virus accumulation following virus inoculation (Ali et al., 2013; Kasai et al., 2011, 2013). For grafted woody plants, we reported profiles of transported siRNAs detected in the nontransgenic scion of sweet cherry grafted on a transgenic rootstock expressing short hairpin RNAs of genomic RNA3 of *Prunus necrotic ringspot virus* (PNRSV-hpRNA). Importantly, the transported siRNAs could enhance PNRSV resistance in the scions (Zhao and Song, 2014). Such observations pose the question as to whether such scions would be "regulated" by the USDA Animal and Plant Health Inspection Service (APHIS) due to the presence of a plant pest control agent. Interestingly, Fuentes et al. (2014) reported that the transfer of entire nuclear genomes between scion and rootstock cells occurs at the graft union; however, instead of conventional rootstock-scion interactions, horizontal gene transfer or gene movement throughout the scion or stock, this event appears to have resulted from nuclear fusion with the investigators subsequently able to regenerate plants from callus tissues formed at the graft union with one result an amphipolyploid.

Movement of Phytohormones

Phytohormones play essential roles in plant growth and development. Many phytohormones (e.g., auxin, cytokinin, gibberellin, abscisic acid, jasmonic acid, and salicylic acid) are phloem-mobile signals, able to move from cell-to-cell and over long distances (Bishopp et al., 2011; Erb et al., 2012; Ghanem et al., 2011; Hoad, 1995; Ko et al., 2014; Leng et al., 2014; Li et al., 2014; Moubayidin et al., 2009; Perilli et al., 2010; Petrášek and Friml, 2009; Proebsting et al., 1992; Rocher et al., 2009; Sun, 2000; Thaler et al., 2012; van Berkel et al., 2013; Yin et al., 2012; Zhang et al., 2014a). Accordingly, phytohormone-related genes are important targets of genetic engineering for various purposes. Examples include modifying plant architecture, size, rooting ability, flowering development, leaf abscission, and plant responses to abiotic and biotic stresses.

To date, few studies have reported on direct application of plant hormone genes to modify woody plants. However, utilization of hormone related genes can modify woody rootstocks (Table 2). For example, several root locus (*rol*)

Table 1. Summary of the evidence of phloem-mediated long-distance transport of RNAs in grafted plants.

Material	RNA	Movement direction	Experimental evidence	Reference
Scion: St BEL5 lines of papa nativa (<i>Solanum tuberosum</i>) Rootstock: papa nativa line 7540	The full-length transcripts of St BEL5 (≈ 2000 nucleotides) across a graft union	Scion-to-rootstock	Reverse transcription polymerase chain reaction (RT-PCR) and situ hybridization	Banerjee et al., 2006
Scion: silenced green fluorescent protein (GFP) line of wild tobacco Rootstock: GFP-expressing line of wild tobacco	The hairpin RNA (hpRNA)-derived GFP small interfering RNAs (siRNAs) <i>MiR399d</i>	Scion-to-rootstock	Using radioactive phosphate as a phloem tracer	Tournier et al., 2006
Scion: arabidopsis 'Columbia' expressing the phosphate (Pi) starvation-induced microRNA399d (<i>miR399d</i>) Rootstock: wild type arabidopsis 'Columbia' Scion: transgenic papa nativa line 7540 expressing a putative potato microRNA172 (<i>miR172</i>) precursor Rootstock: wild papa nativa line 7540 Scion: wild type arabidopsis Rootstock: arabidopsis miRNA processing <i>hen1-1</i> mutant	<i>MiR172</i> <i>MiRNAs</i> 393 and 399 are phloem translocatable but not <i>miR171</i> Endogenous <i>IR71</i> -derived siRNAs	Scion-to-rootstock Scion-to-rootstock Scion-to-rootstock	Quantitative RT-PCR (qRT-PCR) and Northern blot Tuberization times Microarray hybridization	Pant et al., 2008 Martin et al., 2009 Buhtz et al., 2010
Scion: wild type arabidopsis 'Columbia' (silencing donor) Rootstock: arabidopsis 'Columbia' mutant with the inverted repeat 71 (<i>IR71</i>)-transfer DNA (T-DNA) (silencing recipient) Scion: silenced GFP line of arabidopsis 'Columbia' Rootstock: wild type arabidopsis 'Columbia' and mutant Col- <i>dc12</i> , 3, 4	The hpRNA-derived GFP siRNAs and a substantial proportion of the endogenous siRNA Post-transcriptional silencing signals of <i>Nia2</i> , <i>Ni11</i> , and <i>uid4</i>	Scion-to- rootstock Scion-to-rootstock	Small RNA sequencing Small RNA sequencing	Dunoyer et al., 2010 Mohar et al., 2010
Scion: nontransgenic tobacco 'Paraguay PBD6' Rootstock: three types of transgenic tobacco plants carrying silencing signals of nitrate reductase (<i>Nia2</i>), nitrite reductase (<i>Ni11</i>), and <i>uid4</i> Scion: cucumber 'Straight Eight' Rootstock: squash lines expressing a viral coat protein (CP) siRNAs	Viral CP siRNAs	Rootstock-to-scion Rootstock-to-scion	Northern blot Northern blot and RT-PCR	Palauqui et al., 1997 Yoo et al., 2004
Scion: cucumber 'Straight Eight' Rootstock: pumpkin 'Big Max' Scion: wild type tomato 'Money-maker' Rootstock: transgenic tomato 'Money-maker' expressing <i>CmGAIP</i> or <i>ΔDELLA-gai</i> Scion: wild type arabidopsis Rootstock: transgenic arabidopsis expressing <i>CmGAIP</i> or <i>ΔDELLA-gai</i> Scion: arabidopsis 'Columbia' expressing GFP (silencing recipient) Rootstock: arabidopsis 'Columbia' expressing silencer of the GFP (silencing donor)	Cucumber <i>CmGAIP</i> and <i>CmGAIP-B</i> transcripts Cucumber <i>CmGAIP</i> and <i>CmGAIP-B</i> transcripts Cucumber <i>CmGAIP</i> and <i>CmGAIP-B</i> transcripts Cucumber <i>CmGAIP</i> and <i>CmGAIP-B</i> transcripts The hpRNA-derived GFP siRNAs	Rootstock-to-scion Rootstock-to-scion Rootstock-to-scion Rootstock-to-scion Rootstock-to-scion	RT-PCR RT-PCR and phenotype changes RT-PCR and phenotype changes Northern blot	Haywood et al., 2005 Haywood et al., 2005 Haywood et al., 2005 Haywood et al., 2005 Brosnan et al., 2007

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Table 1. Continued.

Material	RNA	Movement direction	Experimental evidence	Reference
Scion: potato Rootstock: mouse ear tomato (<i>Solanum lycopersicum</i>)	Transcripts of pyrophosphate-dependent phosphofructokinase (PFK)-LeT6 Six out of 43 examined transcripts transported from melon stocks to pumpkin scions	Rootstock-to-scion	RT-PCR; altered morphology of the scion	Kudo and Harada, 2007
Scion: pumpkin 'INT 7B RS' Rootstock: melon 'Noy Israel'	Endogenous <i>IR71</i> -derived siRNAs	Rootstock-to-scion	EST sequencing	Omid et al., 2007
Scion: arabidopsis 'Columbia' mutant with the inverted repeat 71 (<i>IR71</i>)-T-DNA (silencing recipient) Rootstock: wild type arabidopsis 'Columbia' (silencing donor) Scion: apple 'Fuji' Rootstock: Wild apple rootstock (<i>Malus prunifolia</i>) 'Ringo Asami Mo 84-A'		Rootstock-to-scion	Small RNA sequencing	Dunoyer et al., 2010
Scion: Chinese white pear (<i>Pyrus bretschneideri</i>) 'Yali' Rootstock: birch-leaved pear 'Bunge' Scion: nontransgenic plants of tobacco and wild tobacco Rootstock: transgenic tobacco	<i>MpSLR1A114</i> Gibberellic acid-insensitive (<i>gai</i>) mRNA The hpRNA-derived siRNAs of <i>NtTOM1</i> and <i>NtTOM3</i> The hpRNA-derived siRNAs of the PSTVd	Rootstock-to-scion	Complementary DNA (cDNA) sequencing, RT-PCR, and situ hybridization analysis RT-PCR	Kanehira et al., 2010
Scion: nontransgenic wild tobacco Rootstock: transgenic wild tobacco expressing a hairpin RNA (hpRNA) of potato spindle tuber viroid (PSTVd) in companion cells		Rootstock-to-scion	RT-PCR and Northern hybridization	Zhang et al., 2012
Scion: nontransgenic wild tobacco Rootstock: transgenic wild tobacco expressing arabidopsis <i>gai</i> Scion: sweet cherry 'Emperor Francis' Rootstock: transgenic cherry rootstock 'Gisela 6' expressing short hairpin RNAs of genomic RNA 3 of <i>Prunus necrotic ringspot virus</i> (PNRSV-hpRNA)	The <i>gai</i> mRNA The <i>PNRSV-hpRNA</i> -derived siRNAs	Rootstock-to-scion	qRT-PCR, dot blot and Northern hybridization	Ali et al., 2013
Scion: nontransgenic or transgenic (35S:YFP-ΔN DMCI) tobacco expressing the yellow fluorescent protein (YFP) fused with the meiosis factor, disrupted meiotic cDNA 1 (DMCI) protein Rootstock: transgenic tobacco expressing the <i>DMCI-1-hpRNA</i> Scion: pear 'Yali' Rootstock: birch-leaved pear 'Duli' Scion: wild tobacco Rootstock: arabidopsis	The <i>DMCI-1-hpRNA</i> -derived siRNAs The <i>KNOTTED1</i> -like mRNAs 138 arabidopsis transcripts	Rootstock-to-scion & scion-to-rootstock Rootstock-to-scion	Scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) PCR RNA sequencing	Xu et al., 2013 Zhao and Song, 2014 Zhang et al., 2014b Duan et al., 2015 Notaguchi et al., 2015

*Bold text indicates transgenic parts.

Table 2. Application of transgenic rootstocks in grafting woody plants.

Nontransgenic scion	Transgenic rootstock	Trait	Movement direction	Experimental evidence	Reference
Five apple cultivars	Transgenic apple rootstock 'M26'-rolB and 'M9'-rolB1	The rolB transgenic rootstocks showed increased rooting ability and reduced plant size. The rolB transgenic rootstocks altered growth and development of nontransgenic scion cultivars.	<i>RollB</i> mRNA was not detected in the tissues of nontransgenic scion cultivars.	Reverse transcription polymerase chain reaction (RT-PCR)	Welander et al., 1998; Zhu et al., 2000; Zhu et al., 2001; Smolka et al., 2010
Not applicable (NA)	Transgenic common pear 'BP10030' containing the <i>rolB</i>	The rolB transgenic rootstocks showed the increased rooting ability.	NA	NA	Zhu et al., 2003
NA	Transgenic apple rootstock 'Alharp 2' overexpressing the arabidopsis gibberellic acid insensitive gene (<i>gai</i>)	Transgenic 'Alharp 2' showed reduced plant size and rooting ability.	NA	NA	Zhu et al., 2008
Nontransgenic scion grape 'Chardonnay'	Transgenic lines of the grapevine rootstocks '41B' (<i>Vitis vinifera</i> × <i>V. berlandieri</i>) expressing the coat protein gene of <i>Grapevine fanleaf virus</i> (GFLV)	Nontransgenic scions on three out of 16 independent transgenic rootstock lines showed GFLV resistance.	Rootstock-to-scion transfer of the overexpressing coat protein of the GFLV	NA	Vigne et al., 2004
Walnut (<i>Juglans regia</i>) 'Chandler'	Walnut hybrid (<i>Juglans hindsii</i> × <i>J. regia</i>) rootstock expressing <i>rolABC</i>	Transgenic rootstock showed phenotype changes but did not affect the phenotype of the scion.	NA	NA	Vahdati et al., 2002
NA	Transgenic citrange (<i>Citrus sinensis</i> × <i>Poncirus trifoliata</i>) rootstock 'Carrizo' overproducing proline	Transgenic plants had proline accumulation but did not show morphological alterations.	NA	NA	Molinari et al., 2004
Grapevine 'Cabernet Sauvignon'	Transgenic european grapevine 'Thompson Seedless' expressing the Shiva-1 lytic peptide gene	Presence of the Shiva-1 peptide was detected in xylem sap of the nontransgenic scion.	Rootstock-to-scion transfer of the Shiva-1 peptide	Enzyme-linked immunoabsorbent assay (ELISA)	Dutt et al., 2007
Sweet orange 'Tarocco Nucellare'	Transgenic citrange 'Troyer' overexpressing <i>rolABC</i> genes of <i>agrobacterium</i> (<i>Agrobacterium rhizogenes</i>)	Transgenic rootstock showed increased rooting ability and reduced plant size. Nontransgenic scion grafted on transgenic rootstock had reduced plant size and altered hormone levels.	NA	NA	La Malfa et al., 2011
Sweet cherry 'Emperor Francis'	Transgenic cherry rootstocks 'Gisela 6' and 'Gisela 7' expressing short hairpin RNAs of genomic RNA3 of <i>Prunus necrotic ringspot virus</i> (PNRSV-hpRNA)	PNRSV resistance in transgenic rootstock as well as nontransgenic scion.	Rootstock-to-scion transfer of hpRNA-derived PNRSV-specific siRNAs	Small RNA sequencing and PNRSV tolerance	Song et al., 2013a; Zhao and Song, 2014

genes of *Agrobacterium rhizogenes* have been demonstrated to be effective in modifying phenotypes (i.e., plant architecture, size, and rooting ability) of transgenic plants through altering the sensitivity of plants to the phytohormone (Chilton et al., 1982; Vahdati et al., 2002). In grafted plants, overexpressing *rolB* and *rolABC* in rootstocks altered development of nontransgenic apple scion cultivars and sweet orange (La Malfa et al., 2011; Zhu et al., 2001), although no convincing evidence shows transfer/change of any hormone in either the scions or rootstocks. On the other hand, the overall impact of the transgene-derived hormones depends on the TPs (e.g., types and quantity) and the plant species. For example, overexpressing *rolABC* in walnut hybrid (*Juglans hindsii* × *J. regia*) rootstocks did not affect the scions (Vahdati et al., 2002) (Table 2).

Biosafety Considerations of Transgated Plants

It has been argued that science-based regulations for GE organisms should target the product rather than the process of genetic engineering by which the GE organisms are created (Fedoroff and Brown, 2004). From this perspective, rationales for the regulation of intragenic and cisgenic products do not seem to exist, although the current regulation of APHIS to these products is on a case-by-case basis (Camacho et al., 2014; Ledford, 2013).

Also, as indicated above, depending on the transgenes, not all macromolecules (proteins and RNAs) of TP are mobile over a long distance (Youk et al., 2009). Thus, transgrafting could enable the harvest of non-GE food/fruit products from transgrafted plants (herein transgrafted food products, e.g., non-GE fruits from non-GE scions grafted on GE rootstocks). To date, none of the GE rootstocks have been commercialized. It remains uncertain as to whether GE rootstocks should be regulated as GE crops or not, because GE rootstocks themselves are generally not used to produce food products and thus may arguably pose little concern for food safety. Given that rootstocks are not usually allowed to flower, environmental concerns on transgene flow would also be minimal. For transgrafted food products, if the GE parts produce immobile GE products, no GE components should be present in the transgrafted food products, which therefore should pose no food safety concerns. In addition, as indicated above when intragenic or cisgenic materials are the GE parts of transgrafted plants, the transgrafted food products may be interpreted as cause for less concern regardless of the impact of GE products across graft union. To date, it is not clear whether these transgrafted food products are inside or outside the regulation scopes of the USDA-APHIS and the FDA (Camacho et al., 2014).

An argument may still be made in favor of regulating transgrafted materials generated by *agrobacterium* or gene gun methods, because the random site of transgene insertion may impact nontarget genes. Although this is comparable to other currently accepted practices in conventional breeding where a desirable phenotype is derived through a similar manner of nonspecific DNA interruption, such as X-ray or ethyl methanesulfonate (EMS) mutations.

Transgrafting has the potential to expand the use of transgenesis for production of food crops (Harada, 2010; Haroldsen et al., 2012a, 2012b; Kalantidis, 2004; Koepke and Dhingra, 2013; Lemgo et al., 2013; Lev-Yadun and Sederoff, 2001; Martinez-Ballesta et al., 2010). The mobile product of transgenic plants can be endogenous plant

metabolites (e.g., phytohormones, RNAs, and proteins/peptides). Transgrafted food products containing these metabolites could be considered as posing no new risks to environmental and food safety. For example, the MTP of the *iaaM* gene of *agrobacterium*, indoleacetic acid (IAA), is an endogenous, mobile plant auxin (Klee et al., 1987); the MTP of the *Escherichia coli* mannitol-1-phosphate dehydrogenase gene (*mtlD*), mannitol, is a six-carbon sugar alcohol found in many plants (Tarczynski et al., 1992); and the MTP of hairpin plant RNAs or plant microRNAs driven by the CaMV 35S promoter, or small interference RNAs (siRNAs) or microRNAs, are endogenous plant RNAs (Dunoyer et al., 2010; Pant et al., 2008).

The MTP can also be exogenous plant products (e.g., RNAs and proteins/peptides) (Table 1). As such, when the newly TP are introduced, the consideration reverts back to the general concerns about whether these TP are safe or not when used as food. To date, the amount of MTP in transgrafted food products has not been well documented due mainly to the lack of a reliable method to quantify extremely low amounts of the target products. Even if detectable, i.e., when using high-throughput sequencing, our recent study in sweet cherry suggests that the transported (rootstock-to-scion) small interfering RNAs (siRNAs) derived from TR that enable PNRSV resistance in nontransgenic scions arguably pose no new risks to food safety (Zhao and Song, 2014). First, the sweet cherries currently on the market are not necessarily PNRSV-free, and they are not considered as a food safety concern because no evidence to date has shown that shows the PNRSV are toxic to mammals. According to documentation from the National Clean Plant Network-Fruit Trees, the PNRSV is a major virus that can reduce yield and is common in all sweet cherry production areas of the world. Indeed, presence of PNRSV or *Prune dwarf virus* (PDV) is common in most 12- to 14-year-old sweet cherry orchards, despite nursery certification programs that enable early detection and elimination of virus-infected plants (Smith and Eastwell, 2015). Consequently, PNRSV containing fruit products can only with difficulty be prevented from entering our food chain due to often-unseen PNRSV symptoms associated with a higher PNRSV tolerance of certain plants [e.g., ‘Gisela 6’ rootstocks (*P. cerasus* × *P. canescens*) are more tolerant to PNRSV than ‘Gisela 7’] (Song et al., 2013a). Second, the PNRSV-specific small RNAs are present in all PNRSV-infected plants. The types of these naturally occurring small RNAs at various sizes from the entire PNRSV RNAs are much more common than those of hairpin-RNA-derived siRNAs, the majority of which are 24-nucleotide (nt) and 21-nt from a specific region (RNA3) of the PNRSV genome. For example, the authors found 761 reads/million read (MR) of naturally occurring PNRSV sRNAs (20–24 nt) in the whole RNA3 region (1959 nt), of which 188 reads/MR were mapped to the PNRSV-hpRNA region (414 nt) (Zhao and Song, 2014). Third, the amount of PNRSV siRNAs transferred to the nontransgenic scion (15.7 reads/MR) is much lower than those of PNRSV small RNAs found in the PNRSV infected plants (761 reads/MR: the figure of only RNA3 region) (Zhao and Song, 2014). The amount of transported PNRSV siRNAs in the nontransgenic scion is highly correlated ($y = 0.005x$, $R^2 = 0.92$) with the total amount of PNRSV siRNAs produced in the transgenic rootstock (Fig. 1). Apparently, compared with naturally occurring PNRSV products (761 reads/MR plus a large amount of PNRSV RNAs and proteins), the transferred PNRSV siRNAs suggest low food safety concerns if using

Fig. 1. The correlation of the number of *Prunus necrotic ringspot virus* hair-pin-RNA derived 20- to 24-nucleotide (nt) siRNAs per million reads (MR) in a transgenic rootstock (x-axis) plant and a nontransgenic scion grafted on a transgenic rootstock (y-axis).

nontransgenic scions grafted on TR to produce nontransgenic fruits. However, as already mentioned, this might still be interpreted as a plant pest control product.

Concluding Remarks

Both mobile and immobile GE products have potential to improve transgated plants. In terms of the food products from the nontransgenic parts of the transgated plants, immobile GE products are not present in the nontransgenic part, and mobile intragenic/cisgenic products are equivalent to endogenous plant products and thus might be considered as posing no new risks. Although presence of mobile transgenic products in transgated plants may be a food-safety issue, new evidence suggests that the mobile transgenic products could have little impact on environmental and food safety (Zhao and Song, 2014). Hence, transgating is a way to expand the use of genetic engineering for horticultural crops, although regulation of transgated foods by the USDA-APHIS and FDA could be on a case-by-case basis (Camacho et al., 2014).

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