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EXPERT VIEW

Merging genotypes: graft union formation and scion–rootstock interactions

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Abstract

Grafting has been utilised for at least the past 7000 years. Historically, grafting has been developed by growers without particular interest beyond the agronomical and ornamental effects, and thus knowledge about grafting has remained largely empirical. Much of the commercial production of fruit, and increasingly vegetables, relies upon grafting with rootstocks to provide resistance to soil-borne pathogens and abiotic stresses as well as to influence scion growth and performance. Although there is considerable agronomic knowledge about the use and selection of rootstocks for many species, we know little of the molecular mechanisms underlying rootstock adaptation to different soil environments and rootstock-conferred modifications of scion phenotypes. Furthermore, the processes involved in the formation of the graft union and graft compatibility are poorly understood despite over a hundred years of scientific study. In this paper, we provide an overview of what is known about grafting and the mechanisms underlying rootstock–scion interactions. We highlight recent studies that have advanced our understanding of graft union formation and outline subjects that require further development.

Keywords: Graft compatibility, grafting, phloem, plasmodesmata, rootstock, scion, xylem.

Introduction

Grafting is one of the most ancient horticultural techniques, originating prior to 7000 BC in China (Mudge *et al.*, 2009), and even today most commercial perennial fruit production is still dependent upon grafting with rootstocks. More recently, vegetable grafting has been increasing, especially in cucurbits and solanaceous crops (Bie *et al.*, 2017). The use of grafted plants provides flexibility, allowing growers to combine different scion and rootstock traits independently. During grafting, tissues cut from different genotypes are brought into contact so that the plants join together to form one composite organism. During this process two individuals are forced to interact, and their survival depends on the efficiency of this interaction. Integrating the tissues of two individuals implies that adult, differentiated tissues engage in a process during which they de-differentiate and form new conducting structures (as reviewed by Pina *et al.*, 2017). The underlying mechanisms must be significantly different for the xylem vessels (dead cells) and the sieve elements of the phloem (living cells). The mechanisms responsible for the integration remain poorly understood (as reviewed by Melnyk, 2017*a*, 2017*b*; Pina *et al.*, 2017) and the

ability to be grafted is not ubiquitous across taxa; dicotyledonous plants graft together easily, whereas grafting is not possible in monocots as they lack a vascular cambium.

Natural variation in the adaptation of different species or accessions to specific biotic and/or abiotic soil conditions has been exploited to generate many rootstocks (as reviewed by Warschefsky *et al.*, 2016; Colla *et al.* 2017). One of the most famous examples comes from the world of wine. Nearly all wine grapes are different varieties of the same European *Vitis* species, *V. vinifera*. At the end of the 19th century a soil-dwelling insect pest, *Phylloxera*, was accidentally introduced to Europe from North America, devastating European vineyards. Researchers at the time quickly realised that the roots of American *Vitis* species provided a natural tolerance and began grafting *V. vinifera* onto American *Vitis* species and hybrid derivates (Ollat *et al.*, 2016). Today, despite the continuing importance of *Phylloxera*-resistant rootstocks to viticulture, we know little of the molecular basis of this trait.

Phenotype modifications via scion–rootstock interactions

How do rootstocks modify scion phenotypes?

Rootstocks confer differences in salinity tolerance, drought tolerance, water-use efficiency, scion vigour, scion architecture, mineral element composition and use efficiency, phenology, and fruit quality and yield in a wide range of species (Warschefsky *et al.*, 2016; Colla *et al.*, 2017; Kumar *et al.*, 2017). Traditionally, rootstock-conferred differences in scion phenotypes have been determined empirically with little or no attention paid to the underlying mechanisms. However, it is clear that there is a genetic control of rootstock-conferred modifications of scion phenotypes, since the parentage of a given rootstock is frequently observed to indicate its behaviour in the field (Cordeau, 1998; Pico *et al.*, 2017) and quantitative trait loci (QTLs) for a variety of rootstock-conferred traits have been identified (Rusholme Pilcher *et al.*, 2008; Estañ *et al.*, 2009; Asins *et al.*, 2010, 2015, 2017; Marguerit *et al.*, 2012; Bert *et al.*, 2013; Fazio *et al.*, 2014; Raga *et al.*, 2014; Foster *et al.*, 2015; Knäbel *et al.*, 2015; Tandonnet *et al.*, 2018).

One important example of the use of rootstocks to confer traits to the scion is the use of dwarfing rootstocks of the Malling series in commercial apple orchards (Hatton, 1917). Dwarfing has been extensively studied with these rootstocks and three QTLs have been identified in populations containing the M9 dwarfing rootstock (Rusholme Pilcher *et al.*, 2008; Fazio *et al.*, 2014; Foster *et al.*, 2015; Harrison *et al.*, 2016*b*;). Recently, the apple WRKY transcription factor family has been targeted as candidate genes of dwarfing control in the M26 rootstock and MdWRKY9 has been identified as a potential candidate based on its differential expression between different dwarfing and non-dwarfing rootstocks (Zheng *et al.*, 2018). The over-expression of *MdWRKY9* represses *MdDWF4*, which controls the rate-limiting step in brassinosteroid synthesis, thereby reducing brassinosteroid production and triggering dwarfing (Zheng *et al.*, 2018). However, the ability of these transgenic rootstocks to confer dwarfing to a wild-type scion (given that brassinosteroids are not considered as long-distance signalling molecules; Symons *et al.*, 2008) and whether MdWRKY9 is the underlying cause of one of the QTLs of M9-conferred dwarfing in apple are still unknown.

Rootstocks could influence scion phenotypes via a variety of mechanisms, as follows.

(1) Rootstocks could differ in their functioning, i.e. their ability to capture soil resources (via differences in root system architecture, functioning, and interactions with the rhizosphere) and transport them to the scion. For example, both grapevine and citrus rootstocks have different root architectures (Sorgona *et al.*, 2007; Dumont *et al.*, 2016) and different capacities to take up phosphate and remobilise phosphorus reserves (Zambrosi *et al.*, 2012; Gautier *et al.*, 2018). Similarly, greater root length is associated with greater stomatal conductance and transpiration in grafted grapevine under low and moderate water deficit (Peccoux *et al.*, 2018). Furthermore, grapevine rootstocks differ in their pH exudation response to iron deficiency (Ollat *et al.*, 2003) and alter the microbiome of the soil (Marasco *et al.*, 2018).

(2) The graft interface itself could alter scion development directly; however, although frequently suggested in the literature, this seems unlikely in compatible grafts once the connections across the interface have been well established. In general, once established, the graft union offers little resistance to water movement (Clearwater *et al.*, 2004; Nardini *et al.*, 2006; Adams *et al.*, 2018) and there has been no clear evidence of the interface sequestering molecules despite there being numerous suggestions of this in the literature (Webster, 2004; Gregory *et al.*, 2013).

(3) Rootstocks could differ in their regulation of shoot–root signalling in terms of both the concentration and fluxes of signalling molecules. A number of reviews have been devoted to the potential roles of long-distance signalling molecules in regulating scion–rootstock interactions (Goldschmidt, 2014; Albacete *et al.*, 2015; Venema *et al.*, 2017). Grafting rootstocks that have been genetically modified to alter long-distance signal molecules (such as hormones) can affect scion phenotypes. For example, the overexpression of isopentenyltransferase (IPT), a key enzyme of cytokinins biosynthesis, in tomato rootstocks increases the cytokinin content of the scion and its resistance to salinity stress (Ghanem *et al.*, 2011). Similarly, a recent study has shown that methylation of the promoter of IPT5B is greater in roots of the dwarfing apple rootstock M9 compared to a high-vigour rootstock, and that this is correlated with reductions in IPT5B expression in the root and cytokinin content in the shoot (Feng *et al.*, 2017). There are also numerous examples of specific signals being associated with rootstock-conferred traits. For example, grapevine rootstocks can confer differences in shoot behaviour (e.g. shoot branching) that are consistent with differences in the biosynthesis of mobile signalling molecules (e.g. strigolactones) (Cochetel *et al.*, 2018). In tomato grafts, growth and plant responses to the abiotic environment have been associated with modifications of the concentration of certain hormones, for example under low potassium supply, shoot biomass is negatively correlated with the concentration of the ethylene precursor aminocyclopropane-1-carboxylic acid (Martínez-Andújar *et al.*, 2016). Similarly, in some cases, apple rootstocks have been shown to alter the concentration of gibberellins in the scion (Tworkoski and Fazio, 2016), and the over-expression of an artificially generated apple *Mhgai1*,

a GA-insensitive allele, in tomato rootstocks confers dwarfing to the wild-type tomato scion (Wang *et al.*, 2012); this suggests that gibberellin signalling could be a potential mechanism of dwarfing by apple rootstocks. As genetic resources are limited for most commercial crops, unequivocal experimental proof of the molecular mechanisms underlying genotypic variation in rootstock-conferred traits is difficult to obtain.

(4) In perennials crops, rootstocks and scions could differ in their perception of seasonal environmental signals related to dormancy; seasonal changes in climate have to be coordinated between two different species potentially adapted to different temperature regimes. There have been reports of rootstocks altering bud-break, leaf senescence, and the cessation of growth at the end of the growing season (Wang *et al.*, 1994; Dong *et al.*, 2008; Prassinos *et al.*, 2009; Loureiro *et al.*, 2016;), but the mechanisms remain unknown. In kiwifruit, rootstocks differ in the development of root pressure in the spring and this is associated with the vigour conferred to the scion, with high-vigour rootstocks more rapidly increasing root pressure (Clearwater *et al.*, 2007). Similarly, if grafted plants consist of two individuals with different biological clocks and rhythms, it is possible that rootstocks can influence the circadian rhythms of the scion, and vice versa.

How do scions modify rootstock phenotypes?

Rootstocks are known to alter a wide range of scion phenotypes, but little attention has been paid to scion effects on rootstock phenotypes despite the fact that such effects have long been recognised (Amos *et al.*, 1930). The characterisation of scion effects on rootstock development has been largely limited to effects on root biomass or total root length (Amos *et al.*, 1930; Tandonnet *et al.*, 2010; Harrison *et al.*, 2016*a*). There are numerous examples of shoot-borne signals regulating root development in model species (Ko and Helariutta, 2017), for example metabolites, hormones, peptides, HY5 (which regulates whole-plant carbon and nitrogen status; Chen *et al.*, 2016), microRNA 156 (which regulates tuber formation in potato; Bhogale *et al.*, 2014), and microRNA 399 (which regulates phosphate uptake and translocation under phosphorus starvation conditions; Lin *et al.*, 2014). Studying how scions alter rootstock phenotypes is of particular importance for root crops, and future work in this area is a priority.

Future research directions

Our knowledge of the signals associated with rootstock modifications of scion phenotypes is growing rapidly and many QTLs regulating conferred traits have been identified. However, experiments designed to understand the genetic architecture of rootstock-conferred traits have generally been restricted to the study of only one scion variety and have rarely included self-grafted controls. One exception is the study by Bert *et al.* (2013) on grapevine, in which tolerance to lime-induced iron deficiency in grafted rootstocks (with a unique scion) and un-grafted cuttings was compared. The authors found that the genetic architecture of rootstock versus whole-plant responses to iron deficiency were different; thus, future research directions should address the roles of both the shoot and the root in regulating traits of interest and plant responses to the environment.

The idea that the graft interface could sequester or physically alter the movement of signals between the scion and the rootstock originates from experiments on apple grafts without homo-grafted controls (Jones, 1974, 1976; Else *et al.*, 2018), but further experiments are required to confirm this hypothesis.

Numerous small RNAs are found in the phloem sap (Buhtz *et al.*, 2008) and are graft-transmissible (as reviewed by Tamiru *et al.*, 2018), suggesting that they could modify scion–rootstock signalling. As interspecific grafting can modify DNA methylation patterns in the grafted partner (Wu *et al.*, 2013), it is possible that epigenetic modifications underlie many rootstock-conferred traits in crop species and this topic will be of interest in the future.

Graft incompatibility and graft union formation

What are the causes of graft incompatibility?

The commercial use of grafting depends upon the degree of graft compatibility, i.e. the ability of the assembled scion–rootstock to form and sustain a successful graft union. It is generally considered that graft incompatibility increases with the taxonomic distance, but predicting compatibility is not always easy. Most intraspecific grafts and interspecific grafts (from within the same genus) are compatible; however interspecific graft incompatibility has been widely reported in fruit trees such as *Prunus* species (Pina *et al.*, 2017). Intrafamilial grafts are rarely compatible, except within the Solanaceae and Cucurbitaceae in which compatibility between different genera is exploited in commercial grafting. Similarly, in Rosaceae certain cultivars of pear (*Pyrus communis*) are compatible with rootstocks of quince (*Cydonia oblonga*). Interfamilial grafts are almost always incompatible; however, some may survive in the short-term (weeks) such as Arabidopsis–tomato grafts (Flaishman *et al.*, 2008). This short-term survival of interfamilial grafts can be used to graft-inoculate pathogens for scientific study (Vigne *et al.*, 2005; Aryan *et al.*, 2016). Graft incompatibility can express itself over various time-frames, from poor success soon after grafting to the dieback of grafted plants several years after planting in the field. This delayed dieback may simply be the appearance of incompatibility symptoms that have been progressing, unobserved, since shortly after the grafting was performed. Despite its importance in horticulture, little is known about the mechanisms that cause graft compatibility/incompatibility except for the special case of certain pear–quince grafts (Gur *et al.*, 1968). Certain quince rootstocks contain prunasin, a cyanogenic glycoside, which can move into the pear scion where hydrolysis by β-glicosidases releases toxic cyanide that causes tissue necrosis and graft incompatibility.

There have been a small number of studies of the transcripts or proteins accumulated at the graft interface during graft union formation that have been aimed at trying to understand the molecular basis of graft incompatibility and differences between hetero- and homo-grafting (Prassinos *et al.*, 2009; Cookson *et al.*, 2014; Wang *et al.*, 2016; Chen *et al.*, 2017; Ren *et al.*, 2018).

Generally, these studies have lacked appropriate controls (such as homo-grafts and cut, but un-grafted scions and rootstocks) and/or sufficient sampling to accurately identify the transcripts or proteins involved. This analysis is particularly complicated in perennial crops; grafting typically occurs when the wood is dormant during the winter months as such the graft union develops at the same time as the spring reactivation of the cambium (Cookson *et al.*, 2013). The activation of the cambium may be different between the different genotypes studied, thus requiring that changes in transcription expression are studied over time so that the profiles associated with differences in the reactivation of the cambium can be separated from those associated with the incompatibility responses. Furthermore, no attempts have been made so far to assign the transcripts harvested from the mixture of cells at the graft interface to either of the grafting partners, although this is theoretically possible if there is sufficient variation between the scion and rootstock genotype and long reads are used. A similar technique has been used to determine the parental origin of transcripts identified in RNA sequencing data from allopolyploid species (Peralta *et al.*, 2013).

How does the graft union form?

The process of graft union formation begins with formation of a necrotic layer, followed by adhesion of the two grafted partners, callus cell formation, and the establishment of a functional vascular system; this has been extensively studied in hypocotyl grafts of Arabidopsis (Box 1). The role of different hormones

Box 1. Key developments in understanding graft union formation

- **Auxin response genes are essential for phloem connection in Arabidopsis hypocotyl grafting, but less so for xylem connection**
  Melnyk *et al.* (2015) showed that in Arabidopsis hypocotyl grafts, auxin accumulated above the graft interface just after cutting and is key to forming vascular connections between the scion and rootstock. By grafting scions labelled with green fluorescent protein onto rootstocks defective in auxin signalling, they demonstrated that movement of the fluorescent protein from the scion to the rootstock was delayed up to 2-fold. However, transport assays from rootstock to scion showed that the xylem connection was not significantly impaired.

- **Genes are asymmetrically expressed between the scion and the rootstock around the graft interface of Arabidopsis hypocotyl grafts**
  Melnyk *et al.* (2018) characterised the genome-wide changes in gene expression induced during hypocotyl grafting in Arabidopsis (at 0–240 h after grafting). The authors observed that the gene expression response was very different between the scion and rootstock, and that many of the differences were driven by accumulation of carbon in the scion and limitation of carbon in the rootstock (until the phloem reconnected). Interestingly, many genes associated with vascular formation were up-regulated in grafted tissues in comparison to the cut and separated tissues before the formation of functional vascular connections, indicating that a recognition mechanism was activated.

- **Grafted partners can exchange nuclear and plastid genetic material in tobacco grafts**
  By grafting two transgenic tobacco lines, one carrying a kanamycin-resistance gene and the yellow fluorescent protein gene in its nuclear genome, and the other one carrying a spectinomycin-resistance gene and the green fluorescent protein gene in its chloroplast genome, Stegemann and Bock (2009) were able to select double-resistant cells from callus cells at the graft interface on selective media. Double-resistant lines could not be obtained from tissues far from the graft interface, suggesting that these genome transfer events were restricted to the graft interface itself. In a second paper (Fuentes *et al.* 2014), the same group showed in a similar fashion that not only chloroplast genomes were exchanged between the scion and rootstock at the graft interface, but nuclear genomes were also exchanged and that allopolyploid cells could be selected for in the callus tissues.

- **Mitochondria are able to move from cell to cell through the graft junction in tobacco grafts**
  To demonstrate the transfer of mitochondria at the graft interface, Gurdon *et al.* (2016) grafted two tobacco species, *Nicotiana tabacum*, which has male-sterile flowers due to cytoplasmic male sterility carried by mitochondria, and *N. sylvestris*, which has fertile flowers. As in the Stegemann and Bock (2009) and Fuentes *et al.* (2014) studies, thanks to resistant genes carried in the nucleus and the chloroplast, they selected and regenerated hybrid lines from the callus cells at the graft interface. The regenerated plants were chimeric, showing three types of flowers: sterile, fertile, and an intermediate phenotype, suggesting that a mitochondrial transfer also occurs at the graft interface.

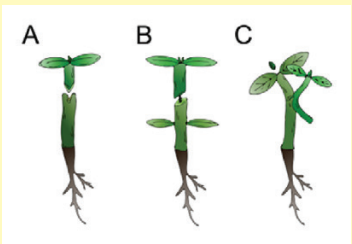
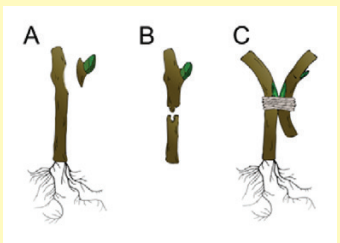
in graft union formation and wound healing has been recently reviewed by Nanda and Melnyk (2018). Although auxins have been used to improve grafting success in viticulture since 1934 (according to Fallot, 1970), their precise role in phloem reconnection during graft union formation has only recently been identified (Melnyk *et al.*, 2015). In perennial crops, grafting is traditionally performed on over-wintering woody tissues in the spring, whereas commercial vegetable grafting is generally done on hypocotyls or stems of actively growing plants soon after germination (Box 2), suggesting that the signalling processes involved may be different.

The development of the graft interface has been of scientific interest for nearly a hundred years, with the first classical microscopy studies being published in the 1920s (Bailey, 1923). More recently, 3D imaging techniques have improved our understanding of the graft union in perennial crops, but with limited resolution (Bahar *et al.*, 2010; Milien *et al.*, 2012). Significant progress has been made in understanding the early stages of vascular reconnection in hypocotyl grafts of Arabidopsis. Melnyk *et al.* (2015) showed that the scion and rootstock adhere 1–2 d after grafting, and the use of fluorescent dyes and proteins demonstrated that the phloem reconnects 3–4 d after grafting (which coincides the resumption of root growth). The xylem reconnects 6–7 d after grafting (Box 1). This study was restricted to the first week after grafting, so we still have no knowledge of the organisation of the limited secondary growth of grafted hypocotyls and how the newly formed xylem and phloem develop. In a subsequent paper, Melnyk *et al.* (2018) described in detail the genes differentially expressed during the time-course of graft union formation and highlighted the up-regulation of many genes associated with vascular regeneration (Box 1).

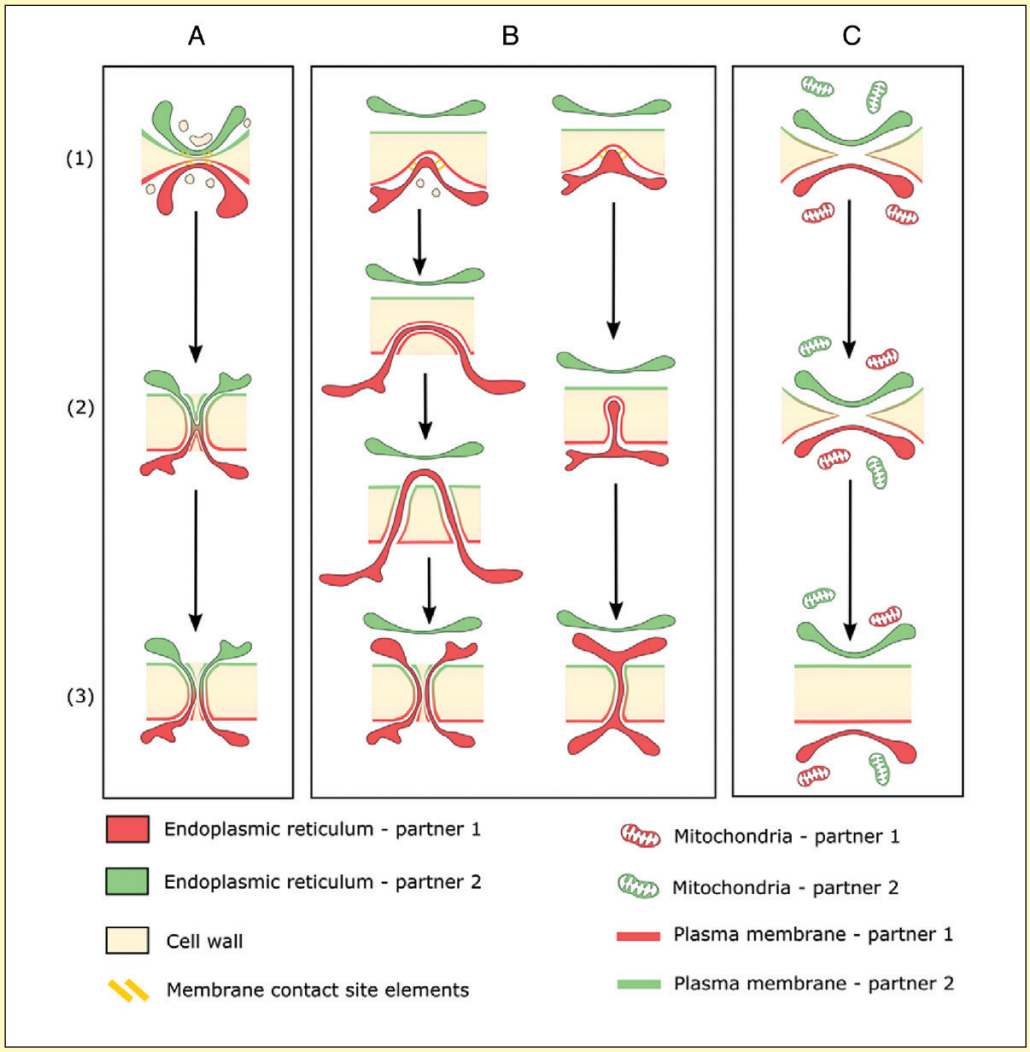
There are many difficulties associated with studying cellular development at the graft interface of woody perennial species: the tissues are large, very hard, and the identification of the exact location of the graft interface is impossible if the scion and rootstock have morphologically indistinct callus cells. Furthermore, using assays similar to those described by Melnyk *et al.* (2015) to quantify the function of xylem and phloem across the interface of woody grafts of perennial crops is technically challenging because plants are grafted before bud break, so there are no leaves to drive transpiration and movement of labelled molecules.

In addition to the connection of vascular tissues across the graft interface, cell-to-cell contacts via plasmodesmata are presumably also important to the function of grafted plants. Plasmodesmata are small membrane channels of about 30 nm in diameter that pass through the plant cell wall, and provide membrane and cytosolic continuity between most cells of the plant. They are composed of a central element originating from the endoplasmic reticulum, the desmotubule. The unequivocal presence of plasmodesmata at the scion–rootstock interface has only been demonstrated in one study, in which the scion and rootstock could be identified using electron microscopy thanks to histological differences between the two genotypes (Kollmann and Glockmann, 1985). Many questions remain concerning the formation of plasmodesmata at the graft interface: (1) are they essential for grafting success and/or long-term plant survival? (2) How do they form across the pre-existing cell walls of the scion and rootstock? (3) Where does the endoplasmic reticulum of the desmotubule originate, from the scion, from the rootstock, or from both grafted partners? Different models of plasmodesmata formation across the graft union are outlined in Box 3. In the first, (A), the

Box 2. Comparison of grafting in herbaceous versus woody plants

Herbaceous grafting	Woody grafting
	
Diagrams of (A) hypocotyl grafting, (B) stem grafting, and (C) approach grafting of herbaceous tissues	Diagrams of (A) chip budding, (B) omega table-top grafting, and (C) approach grafting of woody tissues
Tissues active and rapidly growing when grafted	Tissues dormant when grafted
Leaves and/or cotyledons photosynthesising and transpiring when grafted	Buds dormant when grafted
Carbon accumulates in the scion, whereas the rootstock is starved of carbon until the phloem reconnects	Large supplies of starch in dormant wood of scion and rootstock
Root growth depends upon phloem connections with scion	Bud-break and adventitious root formation largely independent of graft union development
Limited secondary growth	Considerable secondary growth

Box 3. Models of *de novo* plasmodesmata biogenesis and organelle transfer events at the graft interface



(A) Model of spatio-temporal coordination between both partners for plasmodesmata biogenesis. (1) Tethering of endoplasmic reticulum to the plasma membrane at both sides of the graft interface and thinning of the cell wall; (2) endoplasmic reticulum and plasma membrane fusions leading to the formation of branched plasmodesmata and thickening of the cell wall through callose deposition; (3) maturation from branched into twin plasmodesmata. (B) Biogenesis of plasmodesmata initiated by only one partner. (1) Tethering of endoplasmic reticulum to the plasma membrane at one side of the graft interface with thinning of the cell wall; (2) invagination of endoplasmic reticulum into the cell wall; (3) new twin or simple plasmodesmata are formed. (C) Organelle transfer events at the graft interface. (1) Cell wall opening at the graft interface; (2) organelles transfer; (3) pore closing.

scion and rootstock coordinate to form a new plasmodesmata by thinning the cell wall and tethering endoplasmic reticulum to it. This is followed by the fusion of the endoplasmic reticulum from both grafting partners, cell wall thickening, and the formation of a mature plasmodesmata. This would result in the formation of a desmotubule that originates from both grafted partners. In the second model, (B), the formation of plasmodesmata is initiated by only one of the grafted partners (the rootstock in the example shown). The endoplasmic reticulum of the rootstock invaginates into the cell wall and new plasmodesmata are formed with desmotubules originating from only one grafted partner.

The unprecedented work of Stegemann and Bock (2009) and Fuentes *et al.* (2014) has shown that entire chloroplast and nuclear genomes can be horizontally transferred across the graft union (Box 1). These authors suggest two potential mechanisms for this process: (1) fusion of neighbouring cells at the graft site, or (2) migration of nuclei from cell-to-cell through plasmodesmata in a cytomixis-like process. To date, we do not know the mechanisms of organelle exchange at the

graft interface (a model for this process is shown in Box 3C), or when, or in which cell type(s) it occurs. However, given that these exchanges appear to be fairly frequent, cell fusion or migration of nuclei may actually be an essential component of the graft union formation process.

Future research directions

We still have little understanding of how the physical connections across the graft interface are formed, how the vascular tissue is integrated, how plasmodesmata are formed, and how organelles are exchanged; future research should address these fundamental questions using fluorescent markers and correlative light-electron microscopy techniques.

In addition, to expand the range of rootstocks compatible with existing scion varieties, we need to advance the identification of the molecular elements underlying graft union formation and graft incompatibility. A clear understanding of the transcript or protein accumulation profiles at the graft interface associated with incompatibility is still lacking. Although it is logistically challenging to graft hundreds of different scion–rootstock combinations with a sufficient number of replicates to accurately quantify graft compatibility, QTL studies of graft compatibility should be done in the future.

Conclusions

Despite thousands of years of agronomic use, science is just beginning to reveal the mechanisms underlying graft union formation and scion–rootstock interactions. Understanding how rootstocks can modify scion phenotypes will be valuable in aiding plant adaptation to climate change via the creation of new rootstocks. The increasing commercial use of grafted vegetables has renewed scientific interest in grafting because it holds the promise of providing sustainable solutions to numerous agronomic challenges.

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