Title: The use of grafting to study systemic signaling in plants

Running head: Grafting for systemic signaling studies

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Abbreviations:

ABCG14, *ATP-binding cassette transporter subfamily G14*; *Acx1*, *acyl-coenzyme A oxidase 1*; AGO4, ARGONAUTE 4; CEP, C-TERMINALLY ENCODED PEPTIDE;

CEPD, CEP DOWNSTREAM; CEPR, CEP RECEPTOR; CK, cytokinin; CLE, CLAVATA3/ESR-related; CaMV, cauliflower mosaic virus; DCL, DICER-LIKE; DRM, DOMAINS REARRANGED METHYLTRANSFERASE; FT, FLOWERING LOCUS T; FTIP1, FT-INTERACTING PROTEIN 1; GA, gibberellin; *GA1*, *GIBBERELLIC ACID REQUIRING 1*; GA20OX, GIBBERELLIN 20-OXIDASE; IPT, isopentenyl transferase; JA, jasmonic acid; *Jai1*, *JASMONATE-INSENSITIVE1*; KAO, Ent-kaurenoic acid oxidase; LOX, lipoxygenase; miRNA, micro RNA; NaKR1, SODIUM POTASSIUM ROOT DEFECTIVE 1; nt, nucleotide; Pi, inorganic phosphate; *PI*, proteinase inhibitor; *PHT1*, *PHOSPHATE TRANSPORTER 1*; PUT3, polyamine uptake transporter 3; RdDM, RNA-directed DNA methylation; RS, root signal; SA, salicylic acid; siRNA, small interference RNA; TF, transcription factor; TGS, transcriptional gene silencing

Title: The use of grafting to study systemic signaling in plants

Abstract:

Grafting has long been an important technique in agriculture. Nowadays, grafting is a widely used technique to also study systemic long-distance signaling in plants. Plants respond to their surrounding environment, and at that time many of their physiologies are regulated systemically; these start from local input signals and are followed by the transmission of information to the rest of the plant. For example, soil nutrient conditions, light/photoperiod, and biotic and abiotic stresses affect plants heterogeneously, and plants perceive such information in specific plant tissues or organs. Such environmental cues are crucial determinants of plant growth and development, and plants drastically change their morphology and physiology to adapt to various events in their life. Hitherto, intensive studies have been conducted to understand systemic signaling in plants and grafting techniques have permitted advances in this field. The breakthrough technique of micrografting in *Arabidopsis thaliana* was established in 2002 and led to the use of molecular genetic tools in this field. Thereafter, various phenomena of systemic signaling have been identified at the molecular level, including

nutrient fixation, flowering, circadian clock, and defense against pathogens. The significance of grafting is that it can clarify the transmission of stimulus and molecules. At present, many micro- and macro-molecules have been identified as mobile signals, which are transported through plant vascular tissues to coordinate their physiology and development. In this review we introduce the various grafting techniques that have been developed, we report on the recent advances in the field of plant systemic signaling where grafting techniques have been applied, and provide insights for the future.

Introduction

Grafting is an advantageous technique in horticulture as well as agriculture. It has been used to propagate ornamental and fruit tree clones, promote tree growth, shorten juvenility, create dwarf trees, and so on. Grafting has a very long history evidence of its use has been found in ancient civilizations, e.g., in 1560 BC in China as discussed by Aristotle (384–322 BC), the Talmudic–Hellenistic times (ca 500 BC) in the Mediterranean region, and the Roman era (Lee and Oda 2003, Mudge et al. 2009). Even today, grafting is applied to propagate various fruit trees, such as apples, pears, citrus, persimmons, and grapes. The purpose of grafting is varied; the most common use is the clonal vegetative propagation of heterogeneic trees to harbor desirable traits. In addition, grafting is used to control size/growth, shorten juvenility, and gain biotic and abiotic stress tolerance. The methodology has been improved to enhance grafting success and achieve higher quality traits. While many methods are reported, there is a basic common principle, i.e., cutting and assembling the plant parts that have good tissue fitness and unifying them to grow on the grafted plants as a singular plant. Today, grafting is also applied to vegetable cultivation in Asia, Europe, the Middle East,

Northern Africa, and Central America. For example, *Cucubitaceae* spp. including cucumbers, watermelons, and pumpkins, and *Solanaceae* spp. including tomatoes (*Solanum lycopersicon*) have been grafted to enhance their resistance to soil-borne diseases/nematodes, increase yields, and enhance fruit quality (Kubota et al. 2008). Thus, grafting has long been an important and essential skill in agriculture. In addition, grafting has also recently been applied for the study of systemic signaling in plants. Many plant physiological responses are controlled through organ-to-organ long-distance communication, such as shoot-to-root, root-to-shoot, and shoot-to-shoot directed signaling. An overview of grafting processes and associated physiologies has been discussed in detail in other recent reviews (Wang 2011, Goldschmidt 2014, Melnyk 2016). In this review, we introduce the grafting techniques that have been developed for specific scientific fields and update the use of techniques to investigate phenomena that take place via systemic, long-distance signaling in plants.

Grafting techniques in model plants

Systemic signaling is largely known as a reaction and operation machinery to transmit information of abiotic (e.g., day length and nutrient conditions) and biotic (e.g., viruses, bacteria, fungi, and insects) factors from the external environment, and can be in theory categorized into four directional properties: shoot-to-root, root-to-shoot, shoot-to-shoot, and root-to-root. Grafting is a powerful and valuable tool that has been used to analyze various events related to systemic signaling. For example, grafting showed that day length information causes a floral stimulus in leaves and that a hypothetical signal substance called florigen is generated in the leaves and transmitted to the shoot apex to induce flowering (Lang 1965). Results from grafting experiments also suggested that the root-to-shoot-to-root directed signaling machinery controls nodulation in the roots, to signal the symbiotic interaction between legumes and soil bacteria (Nishimura et al. 2002, Okamoto et al. 2013, Sasaki et al. 2014). Some key molecules involved in systemic signaling have been identified in model plants such as *Arabidopsis thaliana* and *Lotus japonicus*, in which biological resources and transformation techniques are well established. Grafting of an *A. thaliana* inflorescence stem was first reported by Tsukaya et al. (1993). Later, a micrografting technique was

performed in both *Petunia* (Napoli 1996) and in *A. thaliana* (Turnbull et al. 2002), whereby young seedlings were cut to make a scion and a stock and assembled. This technique has been also demonstrated in *Nicotiana benthamiana* (Notaguchi et al. 2012) and in tomato (Marsch-Martínez et al. 2013). Improvements to the grafting method have also been reported. Notaguchi et al. (2009) proposed three technical tips: (1) Keeping the graft junction off the wet surface of the membrane that is placed on the growth medium or water-absorbed filter paper (seeds were sown on the membrane and germinated, and grafting manipulation was performed on the membrane), (2) performing micrografting procedures quickly, and (3) maintaining at 27ºC for a certain period after grafting to promote cell proliferation and repress adventitious root formation (5 days is preferable). Magori et al. (2009) reported on inverted-Y grafting, which has two root systems to test root-to-root signaling. Marsch-Martínez et al. (2013) reported efficient grafting without the use of a collar, which is generally used for holding the graft union. One step in their method was to cut the hypocotyl so that it has fine flat edges. To achieve a fine cut, a thin agarose plate with a narrow well was prepared and a graft union of the hypocotyl was positioned above the well. They also

suggested that sugar (0.5% sucrose) supported recovery after grafting. Yoo et al. (2013) reported the concept of "cotyledon grafting," where one cotyledon was grafted onto the cut petiole. Huang and Yu (2015) developed "pin-fasten grafting," where an insect pin was used to hold a scion on a stock. Since sterile conditions are not required for this grafting method, soil can be used as the growth medium. Thus, seedling graft techniques, so called micrografting, have been improved to fit the methods of scientific study.

We surveyed the number of papers and books that included terms related to this field on Google scholar. Using the terms "micrografting" and "plant" showed that the number of publications had significantly increased since the publication by Turnbull et al. (2002) (**Fig. 1A**). The number of publications that included these terms in the last 10 years was over 100 per year. The terms "systemic signaling" and "plant" or "long-distance signaling" and "plant" showed the same increasing tendency (the sum of these two criteria is shown in **Fig. 1B**). If we defined the term "*Arabidopsis*," which increased 1.6-fold from 2001 to 2016, as a mark of how plant biology has expanded, comparisons with publications for "systemic signaling" and "long-distance signaling"

showed a higher increase, 8.4- and 14-fold, respectively, over the same period. Thus, studies using micrografting techniques and studies on systemic signaling in plants are increasing each year (summarized in **Fig. 1C**). In the following sections, we discuss recent studies of systemic signaling and the mobile key molecules analyzed by micrografting.

Systemic signaling

Defense

Plants live in biologically diverse environments. The surrounding microbiota confer both positive and negative effects on plants. Hence, plants needed to evolve symbiotic relationships with microbes to receive benefits and defense systems to protect themselves. Microorganisms that effect plant growth include viruses, bacteria, fungi, and nematodes. Here we introduce recent findings of such plant-microbe interactions and signaling molecules involved in systemic defense responses.

Viral infection of plants induces RNA silencing to restrict virus propagation (Ratcliff et al. 1999, Ruiz et al. 1998), which results from virus-derived small

interference RNA (siRNA) generated by plant host factors, DICER-LIKE (DCL) proteins, and RNA-dependent RNA polymerases (Garcia-Ruiz et al. 2010). The RNA silencing effect can spread systemically (Voinnet and Baulcombe 1997) and is graft-transmissible (Brosnan et al. 2007, Fusaro et al. 2006, Palauqui et al. 1997). Production of siRNA against viruses in the transgenic rootstocks provided viral resistance to non-transgenic scions (Song et al. 2013, Zhao and Song 2014). Also, through the silencing of virus-supporting host factors, the plant can become resistant to viruses. *Nicotiana tabacum TOM1* and *NtTOM3* support *Tobamovirus* spp. multiplication, and the silencing of *NtTOM1/3* by siRNA provides resistance to *Tobamovirus* spp. (Asano et al. 2005). siRNA against *NtTOM1/3* can move from the rootstock expressing the hairpin RNA that generates siRNA to the scion, which results in RNA silencing of *NtTOM1/3* in the scion and virus-resistance (Ali et al. 2013).

Attack by bacteria also induces systemic defense signaling. Xia et al. (2004) reported that an apoplastic aspartic protease, encoded by *CONSTITUTIVE DISEASE RESISTANCE 1* (*CDR1*), was induced after bacterial inoculation and activated defense signaling systemically. In grafted plants consisting of a wild-type as a scion and a

CDR1 inducible transgenic plant as a stock, *CDR1* induction on the stocks promoted *pathogenesis-related protein 2* (*PR2*) expression in the scions (Xia et al. 2004). A low molecular weight (3–10 kDa) fraction of intercellular fluid collected from *CDR1*-induced plants had the ability to induce *PR2* expression, and the activity was reduced by heat and pronase treatments (Xia et al. 2004). These experiments indicate that CDR1 can generate a 3–10 kDa peptide elicitor(s) to induce a systemic defense response, including the expression of *PR2*.

Aphids (*Myzus persicae*) promote infestation by hijacking the lipoxygenase (LOX) pathway. In a normal situation, 9-LOX family genes in *A. thaliana* are involved in lateral root development (Vellosillo et al. 2007). However, once leaves were exposed to aphids, the expression level of one of the *9-LOX* family genes, *LOX5*, in the roots, was up-regulated within 3 hours and LOX5 synthesized oxylipin (Nalam et al. 2012). Oxylipin was detected in both petiole exudates and in aphids, suggesting that the root-to-shoot translocation of oxylipin resulted in the promotion of aphid fecundity (Nalam et al. 2012, Nalam et al. 2013). These studies suggest that aphids ingeniously use shoot-to-root-to-shoot systemic signaling for their infestation. An unknown factor(s)

can be released from aphids or aphid-damaged leaves to induce *LOX5* expression in the roots. Grafting of a wild-type scion onto a *lox5* mutant rootstock showed that the aphid population became smaller than that in a wild-type scion/wild-type stock or a *lox5* scion/wild-type stock (Nalam et al. 2012). These grafting experiments revealed that the *LOX5* genotype in the root influences aphid infestation, suggesting that root-derived oxylipin synthesized by LOX5 or its derivative can function as an aphid activator in the shoots.

Soil conditions

Primary resources for plant growth are photosynthetic carbon (C) fixation and absorption of nutrients in the shoot and root, respectively. These local events have to be precisely coordinated and integrated, and the output signals are then shared throughout the body for vigorous growth. The mechanisms of systemically coordinated macronutrient uptake and the signaling molecules involved have been unveiled by grafting experiments.

One macronutrient, phosphorus (P), which is a key component of ATP, nucleic acids, and phospholipids is absorbed in the form of inorganic phosphate (Pi)

(Schachtman et al. 1998). Pi is also the major form of P in the vascular system (Bieleski 1973). In response to Pi deficiency, its uptake is increased (Drew and Saker 1984) through the upregulation of *PHOSPHATE TRANSPORTER 1* (*PHT1*) expression via the microRNA399 (miR399)/PHO2 pathway (Bari et al. 2006, Fujii et al. 2005). Plants in Pi-starved conditions produced miR399 in the leaves and miR399 was detected in the phloem sap, which suggested that miR399 is a shoot-to-root mobile signal (Pant et al. 2008). miR399 targets *PHO2* transcripts encoding a ubiquitin-conjugating enzyme that mediates the degradation of *PHT1* (Huang et al. 2013). *PHO2* gene expression is decreased in Pi-starved conditions by miR399, resulting in the upregulation of *PHT1* in the roots and Pi accumulation in the leaves (Bari et al. 2006, Fujii et al. 2005). Thus in this miR399/PHO2 pathway, systemic signaling coordinates the Pi response and this Pi-responsive mechanism is likely conserved in plants (Bari et al. 2006, Branscheid et al. 2010). Similarly, in response to a sulfate deficiency, miR395 and some genes (including a sulfate transporter gene), are up-regulated (Kawashima et al. 2011, Takahashi et al. 1997).

A second example involves the uptake of nitrogen (N), a key component of nucleic acids and proteins. Nitrate a readily accessible form of N and details of a systemic signaling pathway in nitrate sensing have become increasingly clear from the use of micrografting. Root-to-shoot-to-root systemic signaling controls nitrate uptake from the roots, and a series of components are identified one after another in *A. thaliana*: root-derived peptide signals C-TERMINALLY ENCODED PEPTIDE (CEP), receptor CEP RECEPTOR (CEPR), and shoot-derived signals CEP DOWNSTREAM (CEPD) (Tabata et al. 2014, Ohkubo et al. 2017). Under nitrate-starved conditions, *CEP*s expression is induced in the roots and CEPs are transported to the shoots via the xylem. Subsequently, the expression levels of nitrate transporter genes are up-regulated in the roots to increase nitrate uptake (Tabata et al. 2014, Ohkubo et al. 2017). Grafting experiments demonstrated that the CEP signal was perceived by CEPRs in the shoots (Tabata et al. 2014). Grafted plants consisting of the *cepr1-1/2-1* mutant scion and the wild-type stock lost their responsiveness to nitrate-starvation. Thus, the perception of CEPs by CEPR triggers the production of a secondary signal translocated from the shoots to the roots, which promotes the expression of nitrate transporters in the roots.

Recently, CEPDs have been identified as the secondary signal (Ohkubo et al. 2017). The *cepd1 cepd2* double mutant did not show systemic upregulation of nitrate transporter genes under nitrate-deficient conditions. The expression of *CEPD*s was induced in response to CEP1 treatment in a wild-type, but not in the *cepr1* mutant, supporting the fact that CEPDs are secondary signals downstream of the CEP-CEPR pathway.

N fixation by rhizobium, another N uptake system based on systemic signaling, has been well studied in many plant species, including legumes. Legumes form nodules for the symbiosis with rhizobium through systemic regulation. Grafting experiments in *L. japonicas* have revealed the players in nodule formation or 'nodulation', as described below. Two CLAVATA3/ESR-related (CLE) family 13-amino acid length peptides with an arabinose chain, called LjCLE-Root Signal 1 (LjCLE-RS1) and LjCLE-RS2, were identified as root-to-shoot signals to suppress nodule formation that were up-regulated by rhizobial inoculation (Okamoto et al. 2009, Okamoto et al. 2013). LjCLE-RS2 is transported to the shoot via the xylem and binds to HAR1, a leucine-rich repeat receptor kinase, to regulate the number of nodules

(Okamoto et al. 2013). Grafting experiments showed that HAR1 activity in the shoots is required to regulate nodulation (Krusell et al. 2002, Nishimura et al. 2002, Okamoto and Kawaguchi 2015). The hypernodulation phenotype of the *har1* mutant is rescued by grafting of the wild-type scion onto the *har1* mutant rootstock, suggesting that there is a shoot-derived nodulation-inhibiting signal downstream of HAR1. Since the amount of cytokinin (CK) was decreased in the *har1* mutant, it has been proposed that CK is a signal molecule that suppresses nodulation (Sasaki et al. 2014). The following experiments by Sasaki et al. (2014) also support this hypothesis; the graft of a CK synthase, isopentenyl transferase 3 (IPT3), overexpressor suppressed nodulation in the wild-type stock. Conversely, a graft of the *ipt3* mutant promoted nodulation in the wild-type stock. CK applied to cotyledons was transported to roots (Sasaki et al. 2014). Altogether, nodulation was controlled by root-to-shoot-to-root signaling, whereas the transport of CK provided the molecular basis for shoot-to-root signaling.

Systemic signaling may also control the uptake of micronutrients. In the case of iron (Fe), grafting experiments were performed using two cultivars of field pea (*Pisum sativum*), one tolerant and the other intolerant to Fe-deficiency, and showed that

a shoot-derived signal in response to Fe-deficiency increased Fe-reductase activity and the amount of citrate in the roots to promote Fe uptake (Kabir et al. 2013). Self-grafted plants of the Fe-deficiency intolerant cultivar ‘Parafield’ displayed low ferric chelate reductase activity and a low amount of citrate in the roots and the xylem exudates under an Fe-deficient condition, whereas grafting of the Fe-deficiency intolerant cultivar ‘Santi’ scions onto ‘Parafield’ stocks increased the ferric chelate reductase activity and the amount of citrate in the ‘Parafield’ roots. This suggested that a tolerance to Fe-deficiency depends on signal(s) from the shoots and that the signal promotes the Fe-responsive metabolism pathway in the roots. Plants utilize various other micronutrients such as boron (B), copper (Cu), manganese (Mn), molybdenum (Mo), and nickel (Ni) (Hänsch and Mendel 2009). Some root-expressed transporters that are required for absorbing micronutrients have already been identified (Sancenón et al. 2004, Takano et al. 2002, Tomatsu et al. 2007). Since excessive amounts of micronutrients can be toxic, their uptake into plants should be strictly controlled though such regulatory uptake mechanisms still remain largely unknown. Proteome and transcriptome analyses revealed various miRNAs and peptides with unknown functions

that are expressed in roots (Kehr 2012, Petricka et al. 2012), which might play a key role in systemic regulation. As shown in the cases of P and N, systemic signaling through the transport of miRNAs or peptides might be a common regulatory mechanism for the coordinated uptake of nutrients below the ground.

Light/photoperiod

Another local environmental factor input in the plant body is light/ the photoperiod (day length). Several findings of light transmitting information to plants have been recently reported through the use of grafting. A bZIP transcription factor, ELONGATED HYPOCOTYL5 (HY5), regulates plant growth in response to light. Recently, grafting experiments using *hy5* loss-of-function mutants uncovered the systemic action of HY5 to regulate root growth and nitrate uptake depending on light illumination of shoots. Its transport was detected by grafting using the HY5-GFP fusion construct, and the authors concluded that C assimilation in shoots and N absorption in roots were coordinated through the transport of HY5 from shoot-to-root, which promoted root nitrate uptake by activating the expression of a nitrate transporter gene, *NRT2.1* (Chen et al. 2016).

The photoperiod is an important environmental cue that provides essential information for plants, allowing them to recognize seasons in broad areas on the planet. The information is mainly perceived in mature leaves and transmitted throughout the plant. Plant photoperiodic responses are varied and may include flowering in some species, tuberization in potatoes, and bud set in trees, and much progress has been made toward understanding the molecular mechanisms (Jackson 2009). Intensive studies on flowering involving grafting experiments critically revealed that an information signal called florigen is produced in the leaves and transmitted to the shoot apex, leading to a growth phase transition from vegetative to reproductive. It has been now well documented that the proteins encoded by *FLOWERING LOCUS T* (*FT*) in *A. thaliana* and its orthologs in other plants are the long-sought florigen, and the transport of the FT protein and its orthologs were also identified by grafting (Corbesier et al. 2007, Lin et al. 2007, Notaguchi et al. 2008). Following initial identification, the transport mechanism has been further dissected, and several components involved in FT transport have now been identified: FT-INTERACTING PROTEIN1 (FTIP1) (Liu et al. 2012), FE/ALTERED PHLOEM DEVELOPMENT - a transcriptional activator of FT and

FTIP (Abe et al. 2015), and SODIUM POTASSIUM ROOT DEFECTIVE 1 (NaKR1) (Zhu et al. 2016). Cotyledon grafting experiments were used to investigate the effect of NaKR1 on FT transport from the leaves to the shoot apex (Zhu et al. 2016). Like these, grafting experiments have provided strong evidence for the systemic action of flowering genes and movement of florigenic proteins.

Photoperiodic flowering is regulated through the function of the circadian clock. A recent study revealed that signals from the shoot apex were important for circadian oscillation in roots. The grafting of shoot apexes of arrhythmic mutants disrupted the rhythms of wild-type roots. The reverse experiments demonstrated that the graft of wild-type shoot apex partially restored the arrhythmic phenotype of mutant roots. These results indicate that the signals from the shoot apex can synchronize distal organs (Takahashi et al. 2015). On the tissue level scale, it has been suggested that in leaf tissues, the vasculature and mesophyll clocks asymmetrically regulate each other (Endo et al. 2014). It is therefore of interest to determine the importance of clock coupling between different tissues in local or separated organs, and what the underlying signals are.

Phytohormones and metabolites

Grafting has been a powerful tool to analyze the translocation of signal molecules. Here, we review some examples of molecules that were identified as mobile signals or their candidates other than the ones described in above sections. We introduce the cases of phytohormones and metabolites in this section and the cases of macromolecules in the next section.

In the original report regarding the micrografting method for *A. thaliana* (Turnbull et al. 2002), graft-transmissible regulation of shoot branching was illustrated. Two mutants, *max1* and *max3*, showed an increased branching phenotype, and the grafting between these mutant scions and wild-type stocks resulted in inhibition of shoot branching, even in the branching mutants. Together with previous grafting studies in the field pea *Pisum sativum* and petunia (Beveridge et al. 1994, Napoli 1996), a root-derived mobile signal(s) was inferred to regulate shoot branching. Subsequent studies revealed that *MAX1* and *MAX3* play roles in strigolactone biosynthesis and that the mobile signal is strigolactone (Gomez-Roldan et al. 2008, Umehara et al. 2008). After publication of the original method papers, studies using micrografting have

provided evidence that other phytohormones and metabolites are transported systemically in plants as discussed below.

(a) *Gibberellin (GA)*: GA is also a long-distance signaling molecule. Ragni et al. (2011) illustrated the importance of GA as a mobile signal in xylem expansion through micrografting experiments using a mutant of *GIBBERELLIC ACID REQUIRING 1* (*GA1*), *ga1-3*, which showed a deficiency in GA biosynthesis. *ga1-3* shows a shrinkage of the xylem area, but grafting of the wild-type scion restored this defect in *ga1-3* mutants, suggesting that shoot-derived GA promoted xylem expansion. The GA precursor, GA_{12}, which is a biologically inactive form, was proposed as a mobile signal that regulates plant growth (Regnault et al. 2015). Two different GA-deficient mutants were used in the test, a mutant of Ent-kaurenoic acid oxidase (KAO), *kao1 kao2*, which impairs GA_{12} synthesis, and a mutant of GIBBERELLIN 20-OXIDASE (GA20OX), *ga20ox1 ga20ox2 ga20ox3*, which impairs catalysis of GA_{12} resulting in the deficiency of bioactive GA synthesis (Hedden and Thomas 2012). Both mutants showed dwarf phenotypes, but grafting between the wild-type stock and the *kao1 kao2* scion could rescue the dwarf phenotype whereas grafting between the

wild-type stock and the *ga20ox1 ga20ox2 ga20ox3* scion could not (Regnault et al. 2015). Because only the GA_{12} synthesis mutant could be rescued, only GA_{12} is considered a mobile GA species.

(b) *Cytokinin (CK)*: CK species are thought to act as long-distance signals. Two types of CKs, iP-type and tZ-type, were detected in phloem sap and xylem sap, with the iP-type being the major form for the phloem translocation stream and the tZ-type for the xylem stream (Corbesier et al. 2003, Takei et al. 2001). tZ-type CK is synthesized by cytochrome P450 monooxygenases, CYP735A1 and CYP735A2, in the roots (Kiba et al. 2013). The double mutants are phenotypically retarded in their growth and development. Grafted plants of a *cyp735a1 cyp735a2* double mutant scion onto a wild-type rootstock restored the phenotype of the mutant shoot, which coincided with the restoration of tZ-type CK levels in the mutant shoot. This experiment indicates that root-derived tZ-type CK is sufficient for CK-controlled shoot growth and development. Ko et al. (2014) reported that *ATP-binding cassette transporter subfamily G14* (*ABCG14*), which is expressed in the stele, is required for the root-to-shoot translocation of CK in *A. thaliana*. Compared with the xylem sap of a wild-type, that of

the *abcg14* mutant contained a much lower amount of *tZ*-type CK. Grafting of an *abcg14* mutant scion onto a wild-type rootstock again restored the dwarf phenotype of mutants, but the reverse grafting did not indicating that xylem-mediated CK translocation from root to shoot promotes shoot growth.

(c) *Abscisic acid (ABA)*: Shoot-to-root translocation of ABA was reported to promote root growth in tomato and field pea under well-watered conditions (McAdam et al. 2016). In both species, root ABA levels and root biomass dramatically decreased in grafted plants of an ABA-deficient mutant scion and a wild-type root. These data suggest that shoot-derived ABA regulates root biomass. Since ABA is a major factor in the reaction pathways for abiotic and biotic stresses (Cutler et al. 2010), root growth and development might be comprehensively controlled via ABA, where various environmental signals are integrated.

(d) *Jasmonic acid (JA)*: JA is involved in wound response and is triggered by wounding such as insect damage and mechanical stimulus. The wound signal spreads systemically to induce the expression of defense genes in unwounded tissues (Stratmann 2003). This systemic signaling has been well studied in tomato. Wounding

induces the expression of defensive proteinase inhibitor (*PI*) genes (Graham et al. 1986, Lee and Howe 2003). Several JA response-related tomato mutants, such as systemin-insensitive mutants, *spr1* and *spr2*, *acyl-coenzyme A oxidase 1* (*acx1*), and *jasmonate-insensitive1* (*jai1*), were reported to be defective in the systemic induction of *PI*s (Lee and Howe 2003, Li et al. 2002, Li et al. 2005). Grafting experiments were performed using four-week-old wild-type and *spr1* mutant tomatoes where both the stock and the scion plants contained healthy leaves. A wild-type scion grafted onto an *spr1* stock showed a reduction of *PI* expression when the leaves of the stock plant were wounded, compared with wild-type self-grafts. Reverse grafting showed a normal *PI* expression at the scion (Lee and Howe 2003). The same held true for grafting using *spr2* and *acx1* mutants, and the results support the same scenario that *Spr1*, *Spr2*, and *Acx1* are essential for the production of the systemic wound signal, but not for its perception (Li et al. 2002, Li et al. 2005). By contrast, a grafting experiment using the *jai1* mutant indicated that JAI1 was required for the perception of the systemic wound signal, but was not required for its production (Li et al. 2002). JA accumulates in the vasculature in response to wounding (Stenzel et al. 2003). Taken together, this indicates

that JA is a systemic wound-induced signal that is transported through vascular tissues. In addition, other recent studies reported that JA-related systemic signaling pathways were also involved in the defense response (Nahar et al. 2011, Zhu et al. 2014), so in this respect it would be important to determine the relationship between the JA pathway and defense signaling cascades, as described above.

(e) *Salicylic acid (SA)*: SA is a central component of systemic acquired resistance (SAR), together with the methylated derivative of SA, MeSA. SA was initially thought to be a mobile signal because it can be detected in phloem sap, and SA accumulation in both local and distal tissues is induced by a pathogen (Métraux et al. 1990, Shulaev et al. 1995). However, data from grafting experiments using an SA-deficient mutant, where even mutant grafts can show SAR, goes against this hypothesis (Vernooij et al. 1994, Smirnov et al. 1997). Recent advances in the SAR field have identified the SA derivative MeSA, a phosphorylated sugar glycerol-3-phosphate, and other metabolites as mobile inducers of SAR (reviewed in Lucas et al. 2013).

(f) *Thiamine (vitamin B_1)*: Thiamine plays important cofactor roles in metabolic processes. Martinis et al. (2016) reported that shoot-derived thiamine was essential for root growth. The phloem-localized thiamine transporter, polyamine uptake transporter 3 (PUT3), mediates thiamine translocation from the shoots to the roots. Grafting of the *put3* mutant scion on the *put3* stock still showed impaired root growth, whereas grafting of a wild-type scion onto the *put3* stock restored proper root growth in the mutants, indicating the necessity of shoot-derived thiamine in the roots.

Macromolecules

The systemic movement of macromolecules, including RNAs, peptides, and proteins has been widely demonstrated in plants as follows. Such RNA includes si/miRNAs (reviewed in Kehr and Buhtz 2007, Mlotshwa et al. 2002), tRNAs, long non-coding RNA such as ribosomal RNAs and spliceosomal RNAs (Zhang et al. 2009), and mRNAs (reviewed in Notaguchi 2015, Ham and Lucas 2016). Grafting experiments have shown the long-distance mobility of such molecules. Here, we introduce the recent findings for (a) small RNAs, (b) mRNAs, and (c) peptide/proteins.

(a) *Small RNAs*: Recent exhaustive analyses by microarrays or next generation sequencing revealed that phloem sap contains various small RNAs, including siRNA and miRNA (Molnar et al. 2010, Buhtz et al. 2010). siRNA/miRNA was thought to move systemically via the phloem and act as an informative molecule to regulate gene silencing, responses to the environment, nutrient allocation, and development (Kehr and Buhtz 2007, Mlotshwa et al. 2002). The gene silencing effect by siRNA was initially shown to be graft-transmissible (Molnar et al. 2010). Transcriptional gene silencing (TGS) can be induced by systemic siRNAs, which leads to transcriptional repression by DNA methylation through RNA-directed DNA methylation (RdDM) or the inactive state of chromatin. Bai et al. (2011) performed grafting experiments using the scion-expressing hairpin RNA of a part of the cauliflower mosaic virus (CaMV) 35S promoter and the stock harboring the transgene (*CaMV 35Sp::GFP*). They demonstrated that this grafting resulted in GFP silencing in the roots. Here, the interesting notion was that the level of DNA methylation was increased at the 35S promoter in the roots, however the reverse grafting also showed GFP silencing at the shoots. When a DCL mutant, *dcl3*, was used as the siRNA donor tissue, the systemic

translocation of TGS was not detected (Melnyk et al. 2011). This result indicates that siRNAs derived from hairpin RNAs systemically spread through the graft union and induced TGS in the sites where siRNAs were transported. Further studies revealed the underlying mechanism of this RdDM pathway. DCL3 produces 24 nucleotide (nt) siRNAs (Qi et al. 2005) and the 24 nt siRNAs trigger RdDM (Law and Jacobsen 2010). Lewsey et al. (2016) performed a genome-wide analysis of DNA methylation in the roots by shoot-derived mobile siRNAs and revealed that RdDM by mobile siRNAs was dependent on DOMAINS REARRANGED METHYLTRANSFERASE 1 (DRM1) and DRM2. DRM2 interacts with ARGONAUTE 4 (AGO4) *in vivo* (Zhong et al. 2014). Taken together, the systemic TGS starts with 24 nt siRNA production by DCL3 and siRNAs are then translocated to the recipient tissues and loaded into AGO4. Subsequently, the complex including AGO4, 24 nt siRNA, and DRM2 induces RdDM and establishes TGS at the target locus.

DNA methylation changes have been reported after inter-species grafting within the *Cucurbitaceae* (Avramidou et al. 2015) and *Solanaceae* (Kasai et al. 2016, Wu et al. 2013). The same scenario was true for intra-species grafting using *A. thaliana*

accessions, where the grafting of C24 and Col-0 showed DNA methylation changes (Lewsey et al. 2016), demonstrating consequential epigenetic modifications taking place between cultivars by grafting. These studies suggest that a scion has the potential to give a useful trait to the stock by changing its epigenetic state, and vice versa. The partial use of transgenic plants will further enhance the opportunity to apply this TGS in practical non-transgenic cultivars in the field.

(b) *mRNAs*: In contrast to small RNAs, the biological role of mobile mRNAs is still largely unknown. The fusion transcript of a β subunit of pyrophosphate fructose-6-phosphate 1 phosphotransferase and *knotted1*-like homeobox genes in tomato was the first reported instance of a graft-transmissible signal that caused a morphological change (Kim et al. 2001). Recent studies of mobile mRNAs through genome wide next generation sequencing analyses revealed hundreds to thousands of mRNAs that were transmissible in the case of parasitism or grafting (Kim et al. 2014, Notaguchi et al. 2015, Thieme et al. 2015, Yang et al. 2015). In our previous investigation, no preferred transcript length and no previously known sequence motifs were found in the population of mobile mRNAs. Further, the transcript level seems not

to correspond to that in the source leaves (Notaguchi et al. 2015). Thieme et al. (2015) indicated that the mobile transcripts correspond to highly expressed genes, especially for the RNAs transported root-to-shoot. Calderwood et al. (2016) further examined the data generated by Thieme et al. (2015) and suggested that the majority of identified mobile transcripts could account for the transcript abundance and half-life. The authors proposed that most of the transcripts identified as mobile RNAs may be transported without selection. These findings evoke an insight that the phloem-based symplasmic translocation stream could have some level of leakage for the selection of cargoes. A similar representation for mobile proteins in grafting conditions was recently done by Paultre et al. (2016). The grafting experiments using plants expressing nucleus-localized GFP, actin-GFP, and GFP fused to transit peptides of chloroplasts and peroxisomes showed that such organelle retention signals were not enough to prevent their protein translocations. On the other hand, GFP fused to signals directing proteins to the endoplasmic reticulum and Golgi apparatus were not translocated (Paultre et al. 2016).

In addition, it is important to consider the artificial effect that grafting itself may have in such experiments. For instance, Yang et al. (2015) reported that the number

of grapevine mobile mRNAs of mature grafts 11 years after grafting were fewer than that of young *in vitro* grafts 1 month after grafting. This could be due to differences in growth stage and growth environments or may reflect the stability of the graft union. In the meantime, many studies using truncated or fusion constructs have provided concrete data to show the existence of a regulatory system for mobile mRNAs (Banerjee et al. 2009, Huang and Yu 2009, Thieme et al. 2015, Zhang et al. 2016). A recent advance was made by Zhang et al. (2016) who reported that tRNA-like structures enriched in the population of graft-mobile mRNAs were sufficient to mediate mRNA mobility. To further address the biological meaning of mobile mRNAs, secondary criteria to narrow down the candidate transcripts with true biological roles are required. One possible idea is to focus on ones only induced by specific environmental conditions, as tested by Thieme et al. (2015). In principle, systemic signals should transmit information from the site of reception to the responsive sites. Hence, local environmental cues are good targets to study. Many environmental parameters surrounding plants are in fact heterogeneous and input locally.

(c) *Peptides/proteins*: As described above, the essential roles of mobile peptides/proteins have been explored through grafting experiments over the last 10 years as information signals generated in response to external environments. More recently, a large-scale identification of mobile peptides/proteins was performed. A xylem sap analysis in soybean identified secreted oligopeptides that were transported from root-to-shoot via the xylem (Okamoto et al. 2015). Similarly, phloem sap analyses have long been performed in various plant species whereby hundreds of proteins have been identified (reviewed in Lucas et al. 2013, Notaguchi and Okamoto 2015). A study that focused more on the mobility of transcription factors (TFs) was done by Rim et al. (2011), where a series of fluorescent protein fused-TFs were expressed in cortical and endodermal root cells and their mobilities were tested. Of the 76 TFs tested, 22 TFs belonging to 17 TF families showed several different non-cell-autonomous patterns in root tips. Similar results were obtained by Lee et al. (2006) and these practices indicate the potential roles of TFs to coordinate events between tissues in a systemic manner. Thus, similar comprehensive studies will further unveil other mechanisms of mobile peptide/protein signals in plants.

Future perspectives

Grafting techniques have expanded the opportunities to study organ-to-organ communication in plants and their underlying mechanisms, including the transport of signaling molecules. In combination with other methods, such as using tissue-specific promoters or local substance treatment/manipulation, studies of systemic signaling can be enhanced. The advantage of grafting is that it provides strong evidence for the transmissibility of gene action and molecules. In particular, micrografting using young seedlings greatly contributes to the investigation of long-distance signaling during the early developmental stages. One difficulty of *A. thaliana* micrografting that we have found is that it takes time to acquire the technique. A publication search related to this field identified that the increasing rate of papers on micrografting was lower than that of papers on systemic long-distance signaling (**Fig. 1A, B**). If techniques are improved and become more readily accessible to researchers, then research in this field will also further expand. One possible solution is automation of the grafting procedure, as is the case for vegetable grafting (Kubota et al. 2008). To increase micrografting efficiency,

some supporters or devices that support the manipulation of tiny seedlings will reduce the fluctuations caused by hand work and further improve the accessibility and reproducibility of this technique.

From another viewpoint, understanding the mechanism of grafting itself is important when attempting to improve the technique. Recent molecular genetic studies on grafting processes indicated that deposition of phytohormones to the grafting region and some of the genes associated with phytohormone action, cell proliferation, and cell differentiation were important to establish a graft union (Wang et al. 2014, Melnyk et al. 2015, Matsuoka et al. 2016, Yin et al. 2012). The current state of these studies on grafting processes is discussed in other reviews (Melnyk 2016, Melnyk 2017). Grafting is established through a series of processes: wound response, hormone deposition, cell division, cell adhesion, tissue differentiation, and reconnection of apoplastic/symplasmic pathways, and these processes are spatiotemporally controlled. The complexity reflects gene expression, and we have observed many up- and down-regulated genes during grafting processes (authors' unpublished data). Another common important obstacle is graft compatibility, especially in practical agriculture.

Unfortunately, the determinant of compatibility or incompatibility is largely unknown and thus the recognition of self (or close species) or others (distant species), the transport/relocation/turnover of metabolites, and reconstruction machineries of injured sites are some of the many questions that remain unanswered. Overall, further studies are required to unveil the mechanical and molecular frameworks of grafting processes.

Finally, systemic signaling in plants is now widely researched in many fields of study and without doubt grafting techniques will continue to enhance our understanding of it. Moreover, an understanding of the physiology related to systemic signaling should lead to new horticultural techniques to control the growth and traits of actual crops, as is the case of florigen for controlling the timing of flowering or dwarfing to increase labor efficiency and increase yield/quality commonly seen in fruit tree cultivation. A visionary experiment performed by Kudo and Harada (2007) showed that mobile mRNA from a tomato rootstock can change the leaf morphology of potato scions. Not only above ground events, but also the responses to soil environments are quite important to be aware of because plant performance largely relies on nutrient availability and microbe interactions in the soil. Many studies have shown that microbe

biodiversity in soils greatly impact plant robustness (reviewed in Smith and Smith 2011). Like these, knowledge of systemic signaling, including mobile signals, the motifs conferring mobility, and the mechanisms of transport from the site of generation to the final destination will be fruitful in agriculture and compel us to further understand how plants have propagated around the world so vigorously.

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Figure legend

Fig. 1 Trend analysis of micrografting techniques in scientific fields. (A, B) The number of publications from 2001 onward for [“micrografting” and “plant”] (A) and [“systemic signaling” and “plant”] and [“long-distance signaling” and “plant”] (B). The approximate curves are drawn by polynomial approximation. (C) Micrografting

methods applied to scientific studies. Signaling directions and the phenomenon to which each method was applied are indicated.