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THE COMMUNICATION OF ENDOGENOUS BIOMOLECULES (RNA, DNA, PROTEIN, HORMONE) VIA GRAFT UNION MIGHT PLAY KEY ROLES IN THE NEW TRAITS FORMATION OF GRAFT HYBRIDS

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Abstract

Plant graft hybridization is a fact ofasexual hybridization in which heritable changes trigger by grafting, it can produce hereditable variation types desired by breeders and can be used as a new method for germplasm innovation. This paper aimed to discuss new phenotypic variations such as phenotypic diversity and polyploidy formation discovered in recent years, new evidence supporting genetic material exchange between the rootstock and scion, and the timeliness, direction, and genetic stability of trait formation induced by graft hybridization. The unresolved mechanisms of trait variation were also discussed, and the relationship with exchange of chloroplast DNA (cpDNA) genetic information, horizontal gene transfer events of chloroplast and mitochondria, miRNA-mediated post-transcriptional regulation, and pressure-driven trait variations from graft hybridization were performed to clarify the relevant questions. Finally, the future research trend and key questions of graft hybridization were identified. Three clear conclusions for graft hybridization are presented: the new traits of graft hybrids have stable genetic characteristics and can be controlled via selection different genetic plant by grafting, and the products of graft hybrids were safer than those of transgenic plants.

Key words: Plant graft hybridization; Graft hybrids; Phenotypic variation; Hereditary character.

Introduction

Plant grafting is a method for not only seedling propagation and preservation of germplasm resources (Wang 2011a; Wang *et al.*, 2016a), but also cause changes in the traits of graft hybrids, which are stably inherited (Liu, 2006; Liu, 2013a; Zhou and Liu, 2015). These characteristics include phenotypic traits such as leaf color, leaf type, epidermal hair, and stem height (Li *et al.*, 2013; Tetsumura *et al.*, 2015; Khadivi-Khub & Anjam, 2016; Warschefsky *et al.*, 2016), flowering and juvenile periods (Chang *et al.*, 2010), and stress resistance(Abbas *et al.*, 2008; Temperini *et al.*, 2013; Calvo-Polanco *et al.*, 2016). Moreover, these characteristics can influence the yield and quality of produce (Wu *et al.*, 2014; Xu *et al.*, 2014; Soares *et al.*, 2015; Riga *et al.*, 2016) and improve physiological metabolism processes (Zhu *et al.*, 2010; Zhou, 2014; Huang *et al.*, 2015), thereby contributing to a new method for germplasm creation (Zhang *et al.*, 2002; Wang, 2011a; Wang *et al.*, 2011b; Zhu *et al.*, 2012; Liu *et al.*, 2013b). These phenomena might be attributed to genetic material exchange between scions and rootstocks (Stegemann& Bock, 2009; Liu *et al.*, 2010; Wang *et al.*, 2011b; Li *et al.*, 2013; Duan *et al.*, 2014; Sun *et al.*, 2014; Wang *et al.*, 2016a), leading to the formation of new traits from grafting, or adaptive variation caused by the selection pressure of graft hybridization (Wang, *et al.*, 2011b; Liu, 2013a; Huang *et al.*, 2015). Alternatively, graft hybridization might result in genomic (Zhang, 2002; Moreno *et al.*, 2014) and mitochondrial DNA methylation of both rootstocks and scions (Lewsey *et al.*, 2016), leading to the silencing of functional genes (Bazzini *et al.*, 2007). Numerous studies have shown that grafting leads to a two-way communication of chloroplast, mitochondrial horizontal gene transfer (HGT) (Stegemann *et al.*, 2012; Hao *et al.*, 2014; Gurdon *et al.*, 2015), miRNA/small RNA (Li *et al.*, 2013), proteins, and other signal molecules across the graft union (Liu, 2013a; Duan *et al.*, 2014; Melnyk & Meyerowitz, 2015; Wang *et al.*, 2016b).

With the advent of new research technologies, a series of groundbreaking findings was made for determining of the mechanism underlying the traditional technique, grafting. These results have promoted development of grafting for scientific direction. Thus, plant graft hybridization might become a new option for generating innovative germplasms (especially for plant breeding among internal or external species, genera and families), promoting species diversity, and ensuring food safety for the future (Albacete *et al.*, 2015).

Changes in phenotypes, physiologies, and genetic characters by graft hybridization is a common fact: Graft hybrids have significantly altered phenotypic traits. The *Compendium of Materia Medica*, *Darwin*'s "Pangenesis," and *Michurinism* have recorded the phenomenon of graft hybrids (Liu, 2006; Liu *et al.*, 2013b), which has rebuilt the interest of the scientist over the past 20 years. Fan (1999) found that graft *Prunus japonica* onto *P. armeniaca* rootstock can induce the rootstock to produce a *P. japonica*-like phenotypic variation. Grafted herbaceous *Solanumpimpinellifolium* onto the woody *Lycium chinense* showed that the vegetative hybrids from intergeneric graft hybridization (heterografting) were different with the same species (auto grafing). The fruit size, shape, weight and color of hybrids were all influenced by the rootstock of *L. chinense*, and the quality of the fruits was improved. After the scion of *Gossypium hirsutum* was grafted onto the *Gossypium barbadense* rootstock, the flowers and seeds showed mutations: the base of the flower changed from the original color of milky white without any spots to pale yellow with red spots (Hao *et al.*, 2014). The hairs on the seed coat disappeared, and they were replaced by numerous short fibers (Hao *et al.*, 2014). The vegetative hybrids exhibited intermediate characteristics between *G. hirsutum* and *G. barbadense*. The grafting between *Nicotiana glauca* and *N. tabacum* produced new polyploid species (belonging to graft chimera), and the plant height and leaf length of the new hybrid species were increased (Fuentes *et al.,* 2014). However, the phenotypic traits of the leaves and flowers as well as their size and color showed intermediate characteristics between the scion and rootstock. Thus, the phenotypic trait variation of this type tends to be of intermediate state between the scion and rootstock.

Graft hybridization can significantly alter the physiological processes of graft hybrids. For example, the net photosynthetic rate of periclinal chimeras from *Brassica juncea* and *B. oleracea* hybridization was 24.8% higher than that of the control cabbage group (Zhu *et al.,* 2010). In addition, the periclinal chimera produced a new leaf shape that was stably passed to its offspring. The rate of shoot apical meristem differentiation decreased from 74.52% to 3.01% after three successive generations of self-fertilization (Li *et al.,* 2013). A similar finding was noted in walnuts. Three years after the scion of walnut *Juglans regia* cv. Shangsong 14 ('*Shangsong*') was grafted onto '*Zhongningqi*' (bred from *Juglans hindsii* × *J. regia*) and *J. regia* cv. Ningyou ('*Ningyou*') rootstocks, the height of the '*Zhongningqi*' vegetative hybrid reached 5.33 m, whereas that of the '*Ningyou*' hybrid was only 4.18 m, which corresponded to an increase of 27.5% of the rootstock '*Zhongningqi*' (Zhou, 2014). Further, the two vegetative hybrids showed significant difference in photosynthesis. The net photosynthetic rate and chlorophyll obtained by graft hybridization between *S. pimpinellifolium* and *L. chinense* were significantly higher than auto grafting tomatoes (Huang *et al.,* 2015).

Graft hybridization can significantly alter the genetic characteristics of graft hybrids. In *Camellia*, when single-flowered *Camelliachangii* Ye was grafted onto the double-flowered *C.japonica* cv. Hongluzhen the expression of B-function gene was increased, leading to an increase in the number of petals in *C. changii* (Zhu *et al.,* 2011). Thus, grafting transformed single-flower to semi-double flower ones. This approach has expanded the origin of double-flower in *Camellia*. The grafting of plum *Prunus cerasifera* cv. Pissardii ('*Pissardi*') onto the green-leaved *Prunus salicina* cv. Yuhuang ('*Yuhuang'*) produced some graft hybrids with evident traits of red leaves. However, this phenomenon was not observed in the auto grafting of the offspring of '*Yuhuang'* (Zhou *et al.,* 2013), which suggested that the red leaf trait of '*Pissardi*' (gained from grafting) was inherited stably by the graft hybrids. The graft *Nicotiana glauca* with 24 chromosomes on *N. tabacum* with 48 chromosomes led to the formation of a new graft chimera with72 chromosomes (Fuentes *et al.,* 2014). When graft *N. tabacum* with mitochondrial-regulated male sterileon the fertile *N. sylvestris,* the reproductive capacity of *N. tabacum* was restored at thegraft union. Moreover, the horizontal transfer of mitochondrial DNA was observed, indicating that grafting accelerates the modification of mitochondrial DNA (Gurdon *et al.,* 2015). After the susceptible tomato scion was grafted onto the transgenic anti-cucumber mosaic virus (CMV) rootstock, the vegetative hybrids gained significant HR (highly resistant) capacity. The HR ratio increased from 66.7% of the T0 generation to over 80.0% of the T1 generation (Bai *et al.,* 2016). The resistance acquired from graft hybridization might be attributed to heterografting that upregulated the stress-related gene expression (Cookson *et al.,* 2014).

Thus, the rootstock and scion are known to influence each other, which can produce a series of changing of traits (phenotype). Plant graft hybrids were created from distant genetic relationship which resulted in the phenotypic, physiological, and genetic characteristics generated denoted more obvious variation. Conversely, the changes were more closely genetic relationship more smaller and less obvious. Plant graft hybridization breaks the reproductive limits of sexual hybridization and can lead to the inheritable variations at a high probability. In *Vigna radiate* and *Impatiens balsamina* L. graft hybrids, a 4% probability exists that phenotypic variation will occur, and variations occur in 6.25% of the inter-simple sequence repeat (ISSR) marker amplified bands (Zhao *et al.,* 2013). The mutation probability in the progenies of the red-leafed vegetative hybrids from '*Pissardii*' and '*Yuhuang*' was approximately 2.3–15.8% (Zhou *et al.,* 2013). Nonetheless, questions such as what is the mechanism of variation in graft hybrids; is stable inheritance of modified traits possible; and can the traits be artificially regulated have long puzzled the academia?

Exchanges in genetic material between rootstock and scion by graft hybridization through graft junction: Plant graft hybridization can result in the transfer of cpDNA genetic material and horizontal transfer of nuclear DNA between the rootstock and scion, but nuclear gene exchange does not occur. (Stegemann *et al.,* 2012) grafted transgenic tobacco with different resistance marker genes and reporter genes. In one group, the marker genes were mapped to the nuclear genome; however, in the other group, the marker genes were located in the chloroplast genome. After grafting, the fluorescent color reaction showed that the marker genes were frequently carried to the chloroplast genome at the graft union. The phenomenon of the exchange of these genes only occurred in this region, but not over a long-distance. (Fuentes *et al.,* 2014) simultaneously grafted two tobacco species with different chromosome numbers. In addition to the observed changes of phenotypic traits in graft hybrids, the graft union also produced a new heterologous polypoid (graft chimera), the *N. tabauca*, having a chromosome number that was the sum of the two species. However, they believed that it was HGT that caused this phenomenon, and nuclear genetic information exchange did not occur. (Tsaballa *et al.,* 2013) grafted *Capsicum annuum* cv Piperaki Long ('*Piperaki*') with elongated fruit shape on *C. annuum*cv Mytilini Round ('*Mytilini*'), which has a round fruit shape. The results showed that the fruit shape of the vegetative hybrids consecutively changed for two generations. However, the *CaOvate* gene sequence that controls the shape of pepper did not change. These findings indicated that, even though no exchange of genetic material occurs between graft hybridization, an unknown endogenous driving force can lead to phenotypic variation in graft hybrids. Thus, what are the factors that lead to this series of events? What are their respective roles?

Existing research findings showed that proteins, mRNA, small RNA (sRNA), micro–RNA (miRNA), and satellite RNAs were involved in the event of grafting (Li *et al.,* 2014; Yang *et al.,* 2015; Chang *et al.,* 2016; Wang *et al.,* 2016a; Warschefskv *et al.,* 2016). For example, in plant blossom, the FT protein (florigen) could induce plant flowering via the phloem of the graft union (Corbesier *et al.,* 2007; Putterill & Varkonyi-Gastic, 2016), this was likely related to the high homology rate (96.93%) of florigen protein in same family (Zhu *et al.,* 2014). However, which florigen contributed the most to the flowering of graft hybrids, from the rootstock or scion, is not yet known. If this trait is determined by one side, how is the gene of the other side silenced? What is the relationship between the two? In addition, large-scale movements of mRNA molecules were noted in graft hybrids. In the vegetative hybrids of *Vitis girdiana* and *V. palmata,* (Yang *et al.,* 2015) found that the mRNAs of more than 3000 genes were transported up and down via the graft union. Although the movement rates of these mRNAs were between 1.0% and 65.0%, (Yang *et al.,* 2015) believed that such large-scale movement of mRNAs would induce complex biological responses. (Liu *et al.,* 2016) also found differences in the expression of over 3485 genes in the vegetative hybrids of *Citrullus lanatus* and *C. moschata* cv. Shintozwa. Recent studies revealed that sRNAs/miRNA of 21–24 nt can directly induce genes silencing during grafting. Further, for example, in plant blossom, because flowering pathway have been relatively clear, (Wu *et al.,* 2013a) found that miR5200, a member of the miRNA family, regulates the expression of the FT protein. The induced expression of miR5200a by short daylight leads to the methylation of the *FT* gene and blocks florigen synthesis, thereby delaying flowering in *Brachypodium distachyon*. This indicates that miRNAs regulate the expression of plant proteins after transcription. Thus, the molecular regulation of flowering is moved to the post-transcriptional stage, which ultimately affects the expression of florigen. In the methylation map constructed using epiRIL (epigenetic recombinant inbred line) in *Arabidopsis thaliana*, three quantitative trait loci that might be associated with *FT* gene expression were found to be methylated (Cortijo *et al.,* 2014). This finding indicates that grafting between two plants can directly lead to silencing of the *FT* gene for one side, thereby altering its flowering. (Lewsey *et al.,* 2016) further showed that the interaction of miRNA in graft hybrids promoted the exchange of epialleles between the rootstock and scion, leading to the silencing of thousands of transposons. Thus, the expression of adjacent genes were affected. These suggested that the reliance of sRNAs on domains rearranged methylase DRM1 and DRM2 methylation pathways, as well as the independent chemically modified tetracycline CMT2 and CMT3 methylation pathways, resulting in a wide range of DNA methylations, causing inheritable trait variations (Lewsey *et al.,* 2016). The indirect effect of miRNAs on the abundant accumulation of florigen might also influence flowering. Further, miR156 is known to play an important role in the maintenance of the juvenile stage of plants (Bhogale *et al.,* 2013). In *Arabidopsis* (that leaves before flowering), the expression of squamosa promoter binding protein-like (*SPL*) is down-regulated by miR156, which indirectly affects the expression of *FT* gene and delays flowering (Kim *et al.,* 2012). Following the identification of miR399, miR395, miR172, and miR156 were also identified as mobile miRNAs that can move freely in the phloem of heterografting (Bhogale *et al.,* 2013). In *Rosaceae* (that typically flowers before leaf development), florigen synthesis and long-distance floral induction mechanism are different from those of monocotyledons, which synthesize florigen in the cotyledons and transport it to the apical shoot for floral induction (Yoo *et al.,* 2013). This is possibly because of the existence of the fifth regulatory pathway, the high-temperature induction pathway, which is different from the four established pathways involved in flowering—the photoperiodic pathway, gibberellin pathway, autonomous pathway, and vernalization pathway. The synthesis of florigen can be induced by temperature-sensitive miRNAs, which upregulate the expression of the FT protein. We found that flowering can be induced in *Armeniaca sibirica* by exposure to >15°C temperature over 5-7 consecutive days after a 600-800 h cold-induced dormancy period. Therefore, we believe that after *L. sibirica* completes the winter dormancy period, high temperature induction is one of the key pathways for the abundant accumulation of florigen. The mechanism underlying this phenomenon might be related to the fact that high temperature induction of miR399 can upregulate the expression of the *FT* gene, thereby promoting early flowering (Kim *et al.,* 2011). Conversely, the flowering period remains unchanged under low temperature conditions. In the comparative miRNA analysis of the tomato/ *Lycium barbarum* graft hybrid with the autografted tomatoes, 168 putative novel miRNAs were identified. In addition, 43 miRNAs were detected in the root and 163 were located in the fruit; these miRNAs showed significant expression differences. (Khaldun *et al.,* 2016) indicated that a long-distance exchange of miRNA signals between rootstock and scion regulates the complex biological processes of graft hybrids.

Plant grafting causes the previously independent rootstock and scion to form an integrated graft hybrids. In this hybrids, macromolecules such as proteins and hormones, as well as small signaling molecules such as sRNAs and miRNAs, can be exchanged. Further, cpDNA undergoes genetic material exchange, and horizontal transfer of nuclear genes occurs at the graft union. Under this complex selection pressure, a series of stress-related chain reactions is likely to occur in graft hybrids. However, still many questions regarding the timeliness, directionality, and genetic stability of this reaction remain unsolved.

On the timeliness, directionality, and genetic stability of new traits in graft hybrids: The timely occurrence of trait variations of graft hybrids can be unstable, i.e., they might occur in a short time, or require longer to occur; occasionally, it might not occur at all. These phenomena might be related to the competence of the scions, or the degree of lignification. It might also be related to the developmental stage of plants—juvenile or adult. (Flachowsky *et al.,* 2012) used micrografting techniques to graft the apple clone T355 clone with the CaMV35S::*gusA* reporter gene onto the *Malus domestica* Borkh with *hrp-gusA* reporter gene. The graft hybrids were cultivated under greenhouse condition, and *hrp-gusA* silencing was not observed. In the *G.*

barbadense and *G. hirsutum* graft hybridization experiments, three HGTs were detected. However, trait variation was only found after three generations (Hao *et al.,* 2014). In 1965, Fan *et al.* (1999) used the annual *P. armeniaca* as rootstock and mature *P.japonica* bud as a scion to produce a graft chimera. In 1986, a *P. japonica*-like phenotypic variation was first observed in the apricot rootstock. The phenotypic trait variations required 12 years to appear. Therefore, the occurrence of variations might require quantitative changes or the induction of appropriate conditions.

The directionality of trait variations by graft hybridization can also be difficult to determine, since it could be unidirectional or bidirectional. Most studies consider the mutual influence of rootstock and scion as a unidirectional effect. In *Arabidopsis,* the FT mRNA related to flowering was transferred from the rootstock to scion in order to influence flowering (Li *et al.,* 2011). In *J. regia* experiments performed by Zhou (2014), the rootstock was found to affect the photosynthesis and growth of the scions. In *Arabidopsis,* the auxin-related *Aux/IAA* mRNA became active after it was transferred from the scion to rootstock (Li *et al.,* 2011). Fan (1999) also showed that the scion had a unidirectional effect on the rootstock, leading to trait variations resembling the scion. However, some studies support the bidirectional effect. For example, (Banerjee *et al.,* 2009) found that the *StBEL5* mRNA was the key factor that was involved in the bidirectional regulation of the formation of chimeric potato tubers. (Fuentes *et al.,* 2014) created polyploid tobacco by superimposing the rootstock and scion genomes onto each other.

The heterosis obtained by graft hybridization might be stably inherited. After *Jatropha curcas* was grafted onto the rootstock with extraneous gene *pAnos*, the random amplified polymorphic DNA technique was used to confirm that the genetic stability of the graft hybrid could reach 100% (Jaganath *et al.,* 2014). (Zhang *et al.,* 2002) grafted self-pollinated *Vigna radiate* onto cross-pollinated *Impatiens batatas*. The acquired seeds of vegetative hybrids showed significant genetic variation after successive generations. Although the authors did not describe the number of seeding required, their results indicated that these mutations were stably inherited for at least 3 generations in the offspring of self-pollinated mung beans. Wang (2011c) showed that the leaf shape variation of interspecific chimeras formed by purple cabbage and mustard tuber was stably inherited for 3 generations. When '*Mytilini*' was grafted onto '*Piperaki*', a new, oval-shaped fruit was produced, which could be stably inherited for at least 2 generations in this self-pollinated crop (Tsaballa *et al.,* 2013). *Arabidopsis thaliana,* a typical self-pollinating plant, is known to have an outcrossing rate of 0.74% within the group and that of 0.1%–8.0% between groups (Cortijo *et al.,* 2014). In the study on epiRIL, environmentally induced epiallele state stability, phenotypic variations caused by the epiallele were found to exist stably in *A. thaliana* for at least 8 generations (Cortijo *et al.,* 2014). Similarly, when the CMV-infected tomato was grafted onto the rootstock of resistant transgenic tomato, the acquired viral resistance material was retained for at least 2 generations (Bai *et al.,* 2016). However, the level of resistance increases with each generation. PIn our study, after the *Vitis amurensis* scion was grafted onto *Schisandra sphenanthera* rootstock, variations were found in the leaf size, vein shape, leaf hair, leaf color, fruit traits, flowering, and fruit development period of the vegetative hybrid (Fig. 1). These traits remain unchanged and can be stably inherited after cutting propagation.

Fig. 1. A new graft hybrids union between *Vitis amurensis* (scion) and *Schisandra sphenanthera* (rootstock).
(A) Vegetative hybrids from *Vitisamurensis* and *Schisandra sphenanthera* (*VSc*). (B) Graft union. (C) The fruits of vegetative hybrids from *VSc*. (D) *V. amurensis* has a longer internode. (E) The internode length of *VSc* is between those of *V. amurensis* and *S. sphenanthera*. (F) The internodes of *S. sphenanthera* are shorter. (G) The leaf phenotype of *S. sphenanthera*. (H) The leaf phenotype of *V. amurensis*. (I) The leaf phenotype of *VSc* shows that the leaf vein is change from reticulate to radial shape. (J) The fruits of *S. sphenanthera*. (K) The fruits of *V. amurensis*. (L) The characteristics such as size and pigmentation of the fruits of *VSc* differ according to the scion and stock. The arrow shows the difference in places between scion and stock, the scale plate of background for figs. 1D-1F, 1J-1L indicate 1 cm.

Discussions

One of the main reasons for changes in leaf traits could be cpDNA genetic material exchange at graft union: Plant cpDNA is known to be highly conserved because the impact of external selection pressure on cpDNA is considerably small. In rice, the mutation rate of cpDNA is only one-tenth of that of the nuclear genome (Wang, 2011a). However, cpDNAs regulate some very important plant traits such as the leaf spot color and their inheritance (Hagemann, 2000), and photosynthetic efficiency (Peng *et al.*, 2017). Thus, the creation of new cpDNA varieties can be a challenging task. (Stegemann *et al.*, 2009) showed that graft hybridization could accelerate the probability of cpDNA varieties. (Zhou *et al.*, 2013) found that, after grafting '*Pissardii*' to'*Yuhuang*,' the seedlings of the offspring of '*Yuhuang*' exhibited an evident trait of violet leaves. We think that this phenomenon could be attributed to the imbalance in the regulation of gene expression controlling tetrapyrrole and carotenoid metabolism in the chloroplast (Wang, 2015). Alternatively, the exchange and formation of new genes or proteins could result in abnormal color expression in the graft hybrids. This implies that graft hybridization might lead to the exchange of cpDNA genetic material at the graft union, thereby affecting the variation and inheritance of leaf color. This finding might become a new method to determine the mechanism of leaf color formation and plant breeding. (Zhu *et al.*, 2010) and Zhou, 2014) found that graft hybridization caused difference in the photosynthetic mechanism and biomass of the graft hybrid. This could be because of the regulation of the ATP synthase gene expression of the chloroplast at the graft union because ATP synthase exhibits a relatively high mutation rate (Wang, 2011a), thereby promoting adaptive mutation to also occur in its chloroplast genome in the graft chimeras.

The signal substances at the synthesis part (rootstocks or scions) determine the new traits of receptor part (scions or stocks) in graft hybrids: The scion and rootstock contribute differently to the graft hybrids (Kümpers *et al.*, 2015; Nelson, 2004), leading to the formation of a primary and secondary relationship in the determination of graft hybrid characteristics. In plants, the synthesis of some signal substances is known to be determined by specific locations. Thus, we think that the synthetic location of these signal substances determines the phenotypic trait variation of the receptor.

We hypothesized that the scions were located in the upper part of the plant, where they could receive more light/photoperiod signals, temperature signals, and exogenous pollen than the rootstock. Thus, photosynthetic products and signal substances were continuously transported down to the rootstock. Because signal transmission occurs in an upper position, the scion became the "transmitter" of the signaling materials. Conversely, the rootstock only provided nutrients, water, and related substances to the scion. The signal transmission occurred in a lower position, and hence the rootstock cells were in a state of receiving. Thus, they became a "receptor" of signals. Moreover, in the study by (Fan *et al.* (1999), as well as in actual production, ensuring that scions receive sufficient nutrients, requires that all the lateral branches of rootstocks are removed. This action further weakened the abundance of signal substance production in the rootstocks. Therefore, we believe that the rootstock was more affected by the scion. This resulted in the ability of the scion to determine the trait of the rootstocks. This perspective is different from that of (Taller *et al.*, 1998) who proposed that rootstock tends to decide the variation of scion's phenotypic traits.

The miRNAs negatively regulate the expression of target genes after transcription at the synthesis part (Liu, 2013a) that notably affects the trait of the "receptor". miR319 can regulate the formation of *Arabidopsis* leaves by influencing the expression of genes *Lanceolate* (Ori *et al.*, 2007) and *Knotted1-like homeobox* (Li *et al.*, 2012). In addition, miR16/166 negatively regulates the HD-ZIP transcription factor by forming a complex with the argonaute protein, which affects the differentiation and development of *A. thaliana* apical meristem cells, resulting in the changes in the morphological development of leaves (Sun *et al.*, 2012; Zhang *et al.*, 2013). External heat stress induced the expression of ABA in roottcocks, leading to inhibition of the expression of csa-miR159b in cucumbers. Moreover, the expression of target genes such as *CsGAMYB1,CsMYB29*, and *CsHSP70* was up-regulated, which improved heat resistance of the graft hybrids (Li *et al.*, 2016). In addition, the preference of different rootstocks to nutrient elements, water content and miRNA can affect the nutrient and water requirement of the scions, which can significantly restrict the formation of scion-like phenotypic traits. Thus, the graft hybrids often show rootstock-like phenotypic variations. For example, graft '*Mytilini*' onto '*Piperaki*' led the fruit shape to become an intermediate between the two varieties, i.e., the round fruit of '*Mytilini*' was significantly elongated in the longitudinal axis and reduced in the horizontal axis, thereby forming an oval shaped fruit (Tsaballa *et al.*, 2013). Intergenic grafting of *Solanoideae* and *Lycium* in the study of (Huang *et al.*, 2015) showed that trait variation of graft hybrid existed between tomato and wolfberry, e.g., the fruit color pigmentation changed from that of tomato to that of wolfberry. Hence, the formation of new characteristics was at an intermediate state between the two. Although the authors did not conduct studies at the molecular level, we deduced that the molecular mechanism underlying this phenomenon might be related to the miRNA produced by wolfberry (rootstock) that caused the silencing of genes that regulated the traits (scion) of tomato via DNA methylation. Further, (Khaldun *et al.*, 2016) found that the *Solyc07g06284.2*, *Solyc11g027650.1*, and *Solyc03g121000.2* genes in the wolfberry/tomato hybrid and three equivalent miRNAs with unknown functions tono50, tono95, and tono97 could be involved in the long-distance transport from the rootstocks to scions thus the traits of rootstock determined the scion in a graft hybrid, This result is the same as that of (Taller *et al.*, 1998), but t different from the wide-spread DNA methylation events caused by the transfer of sRNA from the scion to rootstock as observed in the auto grafting of *Arabidopsis* by (Lewsey *et al.*, 2016). It is also very unlikely to be related to HGT, as revealed by Stegemann & Bock (2009) and (Fuentes *et al.*, 2014). This could be because, during the intergenic grafting with large phenotypic trait differences, the resultant DNA methylation event has a stronger deterministic effect on trait variations than auto grafing or HGT (limited to the graft union).

The long-distance transport of hormones through the graft union plays an important role in the graft hybridization signal transduction (Kümpers *et al.*, 2015; Wang *et al.*, 2016b). Therefore, the hormone-dependent trait variation depends on whether the hormone is synthesized in the scion or rootstock. Cytokinin is synthesized in the plant roots and transported to the apical meristem through the phloem. Conversely, auxin is synthesized in the plant leaves and transported to the roots via the phloem for its physiological action. The ethylene precursors (ACC) are synthesized in the xylem of the rootstock and transferred to the scion via the graft union, which simultaneously inhibit the biomass accumulation of the *Solanum pimpinellifolium* leaves and increase the water use efficiency of chimeras (Cantero-Navarro *et al.*, 2016), reduce the dependency for potassium, and improve the growth in soil lacking potassium (Martínez-Andújar *et al.*, 2016). Therefore, we speculate that, for the determination of graft hybrid phenotypes, the traits maintained by cytokinin and ethylene are determined by the rootstock for the scion, and the directionality of the new trait is closer to the rootstock. Conversely, the traits maintained by auxin are influenced by the scion, and the new trait variations in the rootstock are closer to the scion in terms of orientation. This phenomenon might be related to the expression of some genes related to plant hormones, which affect the formation of new traits or trait variations in graft hybrids. Fundamentally, because of the basic needs of plants for developmental stage progression or trait maintenance, the endogenous driving factors induced by the synthetic sites need to determine the directionality of the traits of graft hybrids.

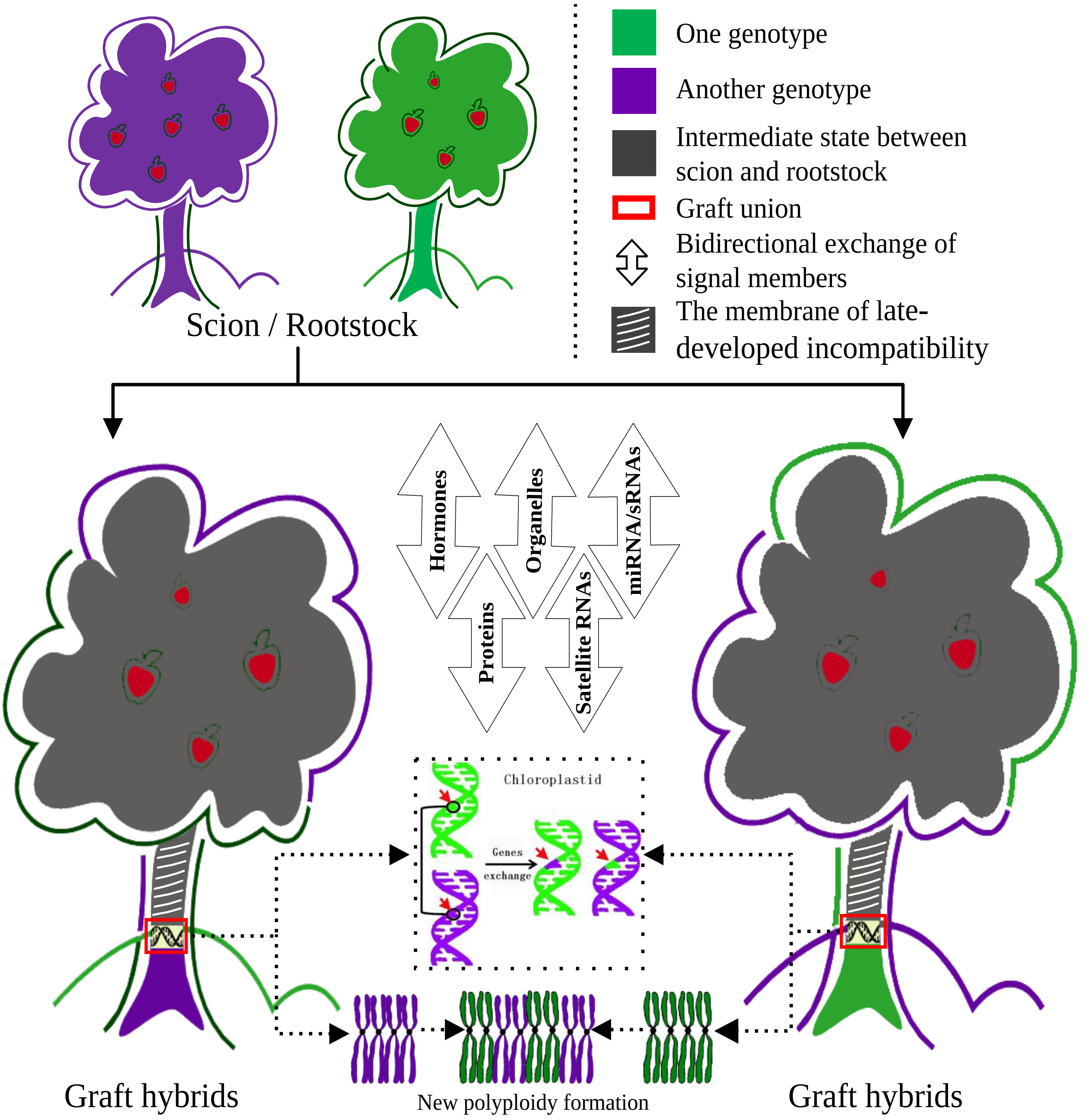

Fig. 2. Schematic viewpoint of the molecular mechanism of graft hybridization.

The mechanism of phenotypic changes caused by graft hybridization might involve the following two factors (Fig. 2). First, the selective pressure of grafting, with the interaction of proteins, hormones, miRNAs, as well as organelles across the graft union (Hyssen *et al.*, 2012), causes the DNA to become methylated in graft hybrids (Wu *et al.*, 2013b). This could lead to the extensive methylation of scion genes determined by the rootstock, which directly leads to the phenotypic variation. The other factor is the HGT caused by grafting. It breaks the traditional barrier of vertical gene transfer, where parents pass genetic information to offspring. Thus, genetic material exchanges in graft hybrids are genetic material transfer events that differ significantly from the insertion and recombination events of the transgenic technology. Therefore, elucidating this mechanism has become very complex. Nonetheless, graft hybridization has provided a new approach for breeding of polyploids. Furthermore, interestingly, the farther is the phylogenic relationship of the species, the higher is the likelihood of HGT. This also explains why HGT caused by grafting of distant species can lead to the change in phenotypic traits. In addition, the probability of generating new trait variations is higher than after conventional breeding (Fraser *et al.*, 2007). HGT could significantly contribute to the evolution of receptor phylogeny (Huang & Yue, 2013; Wu *et al.*, 2015). Therefore, creating new germplasms from grafting requires that varieties with large phenotypic differences and distant genetic relationships are selected to obtain the desired variation types.

Conclusion

Plant graft hybridization is a process that more facts are unknown with two difference genotype adhesion at the graft union (Zhou & Liu, 2015). The use of grafting is becoming increasingly common since it easily changes phenotype, improves disease resistance, stress resistance and fruit quality without resorting to genetic modifications or lengthy plant breeding processes (Melnyk *et al.*, 2017). Although the mechanisms of graft hybridization are becoming clear, many questions have not yet been well addressed, e.g., what are the consequences of cpDNA gene exchange at the graft union? Are new chloroplast proteins produced? What roles do these proteins play in graft hybrids? Whether the rootstock or scion plays a primary induction role? (Wu *et al.*, 2013b) showed that, in heterografting, the rootstock caused the methylation of specific sites in the scion DNA, which became inheritable trait variations. This indicated that the rootstock determined the traits of the scions. However, in a more than 10-year study, Fan (1999) found the obvious influence of scion on the phenotypic traits of the rootstock. Thus, in addition to endogenous driving factors, interactions with the environment might also exist (Albacete *et al.*, 2015). In addition, what are the tendency patterns for the formation of phenotypic traits of graft hybridization? Whether the formation of a trait can be achieved by selecting rootstocks with different properties to ensure directional control of the desired traits? How long does it require to form and fix these types of traits? Whether this is related to the formation of grafted hybrid traits, and whether it is a dynamic or a static process? The studies by (Taller *et al.*, 1998) suggested that the phenotypic variation of scions becomes closer to rootstock with an increase in the number of graft times, indicating that this is likely a dynamic process. Can these types of phenotypic trait variations be inherited stably after auto grafing or homografting? If so, for how many grafting times can they be inherited? What are the characteristics of the new tissues formed from cell fusions at the graft union? Monocotyledons have been shown to have lower graft hybridization compatibility than dicotyledonous plants. This could be because the vascular bundles of monocotyledons are scattered and have no cambium (Melnyk&Meyerowitz, 2015). This raised a new question, i.e., for monocotyledons and dicotyledonous plants, especially the short-lived herbaceous plants and long-lived woody plants, what are the key differences in their graft hybridization mechanisms? These are very interesting and not yet completely explored scientific problems, which mandate in-depth studies. In addition, although most of the current studies focus on improving the resistance of the graft hybrids, the next major question is, what are the factors or genetic background that distantly related rootstocks and scions need to have in order to be successfully connection for producing healthy graft hybrids? In closely related varieties, hormones (Huang *et al.*, 2002; Goldschmidt, 2014; Corso *et al.*, 2016), enzymes (Huang *et al.*, 2014; Irisarri *et al.*, 2016) or reactive oxygen (Irisarri *et al.*, 2015), and temperature/light (Wang *et al.*, 2016b; Yang *et al.*, 2016) are known to play an important role in the vascular bundle connected and wound healing in grafting compatibility. In particular, auxin is the most commonly studied plant hormone. For example, exogenous auxin was found to significantly promote the survival rate of pecan grafting. In *Arabidopsis*, endogenous auxin and its related genes *ABERRANT LATERAL ROOT FORMATION 4* and *AUXIN-RESISTANT 1* were necessary for the connection of the graft union (Goldschmidt *et al.*, 2014). In grape rootstock, the types of auxin-related *AFR* and *AUX* genes were directly associated with fruit development (Corso *et al.*, 2016). However, the key factors that promoted compatibility in plants at the exterspecies level have not yet been identified. Therefore, we believe that future studies should focus on the mechanism of compatibility between distantly related species during graft hybridization, in particular, compatibility forecast and diagnosis before grafting might become a popular study in the future. The results of present studies suggest that plants with similar phenotypic traits (e.g., *Vitis. amurensis* and *Schisandra sphenanthera*, *S. pimpinellifolium*, and *L. chinense* have similar berries, fruit shape, fruit color, and growth model) might have better grafting compatibility. Although these plants are known to belong to different lineages in the long evolutionary paths, they appear to have maintained a certain type of “kinship.” This relationship breaks the limitations of reproductive isolation and allows them to connect via grafting. We believe that this relationship might be related to the high degree of homology of the structural genes of phenotypic traits that determine trait formation and development. Therefore, we believe that the application of distant grafting to determine the origin and evolution of plants is a good research direction. Nonetheless, the finding of current studies, reveal these three conclusions:

The traits of graft hybrid were transmitted dynamically and showed stable genetic characteristics: If the characteristics of germplasms need to be maintained, autografting and homografting should be applied to control the genetic relationship of the rootstocks at the level interspecies. The extent of trait maintenance is proportional to the phylogenetic relationship between the rootstock and scion, i.e., the closer the relationship, the more stable is the trait maintenance. In contrast, in germplasm innovation, heterografting should be applied to rootstocks with the exterspecies. The degree of trait variation is inversely proportional to the genetic relationship between the rootstock and scion. However, this might create a new problem, i.e., cutting propagation, tissue culture, and self-pollination can be applied to maintain the genetic stability of the new variant trait from distant grafting. However, for the plants that do not meet these three conditions, determining whether the lower (stock) or upper part (scion) of the signal material of veretative hybrid should be used as a rootstock is difficult. This involves the genetic stability and grafting compatibilityof the new phenotype. Theoretically, we believe that maintaining the stability of scion traits in plants (excluding asexual and self-pollinating plants) requires the use of seeds of the graft hybrid as rootstock to maintain the stability of new traits. Since their genetic origins are similar, the likelihood of developing variations from graft hybridization is lower.

The traits of graft hybrids can be designed by human control: In general, the variation in phenotypic traits in graft hybrids tends to be at an intermediate state between the rootstock and scion, with a tendency toward rootstock-like traits. However, the overall trends of new traits tend to compromise the phenotypic traits of the rootstock and scion, resulting in the intermediate state. This indicated that the general direction of variations of graft hybridization traits could be achieved by artificial control.

The agricultural products from graft hybrids are safer than transgenic plants: While scions can be grafted onto transgenic rootstocks to obtain the desired traits of graft hybrid could no doubt follow the rule of natural selection (cytomembrane filtration) and compatibility. Moreover, this approach reduced the public concern regarding transgenic food safety, considering that, comparison of the desired traits obtained from graft hybridization with those obtained from transgenic techniques shows that the former clearly involves an additional step of natural selection in the later development in graft hybrids.

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