Construction of an Arabidopsis/Nicotiana inter-order graft for studies of scion-rootstock interaction

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Zhuying Deng
Yangtze University

Mengting Jiang
Yangtze University

Mi Wang
Yangtze University

Dacheng Liang dachengliang@gmail.com
Yangtze University
Corresponding Author
ORCiD: 0000-0002-8898-3771

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Abstract

Background Scion-rootstock union formation is a critical step towards functional assemblage of heterogeneous plants. However, scion-rootstock interaction often results in graft incompatibility during the process of assemblage. So far, the lack of model heterografts involving both clear genetic backgrounds and taxonomically distant species greatly impedes insights into the mechanisms underlying scion-rootstock interaction.

Results In this report, we established an Arabidopsis (At)/Nicotiana benthamiana (Nb) heterografting system in which the model plant At and the model plant Nb for plant bioreactor was used as scion and rootstock respectively, to explore the interaction between the two model plants. Regarding to the At scion phenotypes, the At-Nb connection can be characterized into three groups: the mild-stressed, the albino and the dormant grafts. Examination of symplastic and apoplastic flow indicated that a functional inter-order grafting was established in the mild-stressed group, but not in the dormant group. What’s more, the free GFP movement in both At/At homograft and the At/Nb graft implicated that macromolecules moved across the heterograft union of the mild-stressed graft, but congealed at the union of dormant graft. These results accentuated the role of vascular connection in the establishment of compatible heterografts. Conclusions The present study established an inter-order model graft involving Arabidopsis and Nicotiana. The interactions from these two species resulted in three distinct grafting groups, which offer us a novel vista to explore many important issues such as grafting compatibility and biomolecule movement.

Background

Grafting is an ancient technique that allows an artificial combination of two different plants into a living biont and has been widely applied in horticultural and biological
research. Since grafting can separate one part of plant from one another, it is playing an increasingly important role in studying the inter-tissue/organ interaction and communication. For example, it was employed to study the physiological mechanisms of shoot branching [1], flowering [2-5], vegetable soil-borne disease [6-8], nutrient allocation [9-14] and other physical processes.

Construction of a successful graft in different positions of partners enables the studies of inter-organ or inter-tissue communication for different purposes. For example, the cotyledon grafting showed that the WT cotyledon, as the sources of florigen, can rescue the late flowering phenotype of ft-10 mutant by donating the mobile FT protein [15]. Inflorescence grafting was employed to study information flow between WT and acl1-1 mutant with premature arrest of the inflorescence meristem [16]. Recently, stem grafting was also used to demonstrate the antiflorigen NsCET1 mRNA movement from tobacco to tomato [17]. Another study involving the tobacco and tomato heterograft has adopted the same method to identify mobile transcripts from scion (Nicotiana) to rootstock (tomato) [18]. Grafting in the hypocotyl tissue is a good way to demonstrate root-to-shoot signaling, e.g. the long-distance mobile silencing [19, 20], root-to-shoot signals for branching [1] and root-derived bps signaling for regulating shoot development [21].

Insights engendered from above studies have sufficiently lightened the way the grafting is employed. However, the critical principle underlying grafting compatibility is poorly understood. Part of the reason is that graft partners have to be taxonomically (near-)equivalent. This rule dictates that most grafts are made within the same species (intraspecific) or the same genus (interspecific). With the increase of taxonomic distance, grafts are becoming less possible. As such, the intrafamilial grafts are found rarely compatible and interfamilial grafts are considered always incompatible [22]. However, an early study in the 1980s of last century have made attempts to construct interfamilial
grafts of *Helianthus tuberosus* and *Vicia faba* [23], and another study has made a graft combination of *Arabidopsis* and *Tomato* [24]. And most importantly, the contacted cells in the union of *Helianthus/Vicia* graft were connected by simple and branched plasmodesmata, heralding the two species communicated at supracellular level [23]. Presumably, the similar situation could also occur—probably at the parenchyma cells—in the graft union of *Arabidopsis/tomato* combination as plasmodesmata could complement the intercellular transport when lack of vascular connection between the two [24].

Noteworthily, these heterografts were all short-lived and displayed a certain degree of graft incompatibility. Therefore, constructing a life long-lived interfamilial graft would help us to further understand the grafting mechanisms.

Previously, Notaguchi et al. (2015) made *Nicotiana/Arabidopsis* grafts in the inflorescence stem to identify potential transcripts moving from source tissues of *Arabidopsis* into the *Nicotiana* sink tissues including inflorescence stems and floral buds. Nevertheless, the two species were combined in a short period (3 weeks) for sampling [25], and a long-term interaction between the two species haven’t been assessed, given the strong possibility of incompatibility may exist in these two distantly-related species. In this report, we constructed the life-long inter-order graft in which *Arabidopsis* was used as a scion and *Nicotiana* as a rootstock, and further dissected the compatible mechanisms between the two species. In addition, this grafting platform can be coopted for identifying long-distance mobile substances, such as mobile RNAs or proteins that serve as inter-tissue signaling.

**Results**

**Development of A. thaliana(At) / N. benthamiana (Nb) Heterografting System**

*Arabidopsis thaliana* (At) and *Nicotiana benthamiana* (Nb), belonging to rosids and
asterids taxa respectively, contain distinct genomic information, thus grafts between the
two most likely displayed incompatibility. In our initial attempt to construct an At-Nb graft
with the micrografting technique [1, 26], we were able to generate an At/Nb heterografting system in which At acted as scion and Nb as rootstock (Fig. 1a-c). However, a reciprocal graft with Nb as scion and At as a rootstock proved to be very challenging, with a scant grafting survival rate of less than 1% compared to At/At or Nb/Nb grafts (Fig. 1j, Log-rank test, p<0.0001). Although the At/Nb grafts showed a relative lower grafting survival rate than that of the self-grafts in At/At (Fig. 1d-f) and Nb/Nb (Fig. 1g-i), this combination still resulted in more than 60% grafting survival rate within a time course of 60 days (Fig. 1j). The relative low success rate in the At/Nb combination partly resulted from the adventitious root formation from the At scion. Adventitious roots at their first emergence from hypocotyl tissue were about 7 to 10 days after grafting (DAG). The emerging roots were immediately excised, this removal, however, did not stop their regeneration, and up to 29% of the scions with an average of 23% developed adventitious roots at 24 DAG (Table S1). Since adventitious roots can bypass the At/Nb union and fully support At own growth, grafts with any signs of adventitious roots were all discarded. Phenotypically, the remaining grafts can be classified into three distinct groups: the mild-stressed (Fig. 2a, Group A), the albino (Fig. 2b, Group B) and the retarded grafts (Fig. 2c, Group C). About 33% of the grafts varying from 22% to 45% in different experiments showed the mild-stressed phenotypes (Table 1). Under aseptic condition, the mild-stressed grafts can recover to normal growth and set seeds (Fig. 2d). Around 80% of the albino grafts were also gradually turning green, flowered and set seeds (Fig. 2e). However, the retarded grafts, accounting for 32% of the total (Table 1), remained in “dormancy” and eventually deceased (Fig. 2f).

The overall phenotypes of the mild-stressed scion were not essentially dissimilar from the
At/At self-grafts in respect to the leaf parameters (Fig. 2g, h). The At/Nb scion generated slightly more leaves than the At/At self-graft (Fig. 2g), which could be due to the late flowering of the At/Nb grafts (Fig. 2i). And most obviously, the time for scion in At/Nb graft (Group A) to reach seed-maturing stage was about 140±20 DAG, far more longer than that of At/At self-graft (60±10 DAG).

Vascular Reconnection In At/Nb Grafts

Once the grafting between At and Nb became feasible, we sought to test the vascular connection between At scion and Nb rootstock. We first used the apoplast dye, acid fuchsin, to investigate whether the xylem was connected [27, 28]. We found the red stain was readily seen in the veins of cotyledon of 7- and 10-DAG after 30-60 min application in the root tips (Fig. 3a), and the staining intensity was comparable to that of Nb self-grafts (Fig. 3b) but even more evident than that of At self-graft (Fig. 3c), implying a stronger rootstock in the At/Nb grafting combination. Although the stain was not obvious in the veins of 5 DAG, the longitudinal sectioning showed that the red dye had moved through the graft union in a weak flow (Fig. 3d), suggesting the effective xylem connection occurred at 7 DAG. Since the xylem connection for water and water-dissolved nutrients transport between scion and rootstock is fundamental to graft survival, the same method was used to examine those grafts that displayed a “dormant” state (Group C, Fig. 2c) in which the scion nearly ceased growth but rootstock is still alive at 30 DAG. As a result, the retarded grafts did not show any visible stains in the leaves, even incubated for prolonged time, e.g. 3hrs, indicating the xylem transport was interdicted. Further longitudinal section through the grafts showed that fuchsin was highly accumulated around the graft junction (Fig. 3e). These results indicated the grafts failure in the At/Nb combination could be attributed, at least partially, to the xylem opaqueness.

Phloem is an essential system for transporting not only photoassimilates, but also
proteins, RNAs and other signaling molecules. Thus, we sought to detect the phloem connectivity using phloem-mobile fluorescent tracer carboxyfluorescein (CF). By applying the non-cleaved form, or the 5(6)-carboxyfluorescein diacetate (CFDA) to the leaves of 5 DAG grafts, the CF signal is moving through the hypocotyle to the roots (Fig. 3f, g). These results suggested that phloem connection occurred around 5 DAG, agreeing with the Arabidopsis homografts [29]. However, for the majority of dormant grafts (Group C in Fig. 2), the down-streaming of CF is blocked at the graft union (Fig. 3h), and for a small part of these grafts (2 out of 11), the connection looks weak (Fig. S1). These results suggested that phloem connection is essential, but not sufficient to for a functional At/Nb graft.

**A GFP Signal Can Be Translocated Across the Graft Union**

To further explore the possibility of biomolecule movement in a functional At/Nb graft, we used GFP-expressing Arabidopsis as a scion to test the phloem connection. At 15 DAG, GFP fluorescent signal was detected in the root tip of Nb rootstock (Fig. 4a-c) when compared to Nb homograft control (Fig. 4d-f). Further longitudinal sectioning assay showed GFP fluorescence was detected in the graft union and the phloem strands of rootstock hypocotyl (Fig. 4g, h). However, the GFP signal was rarely if ever detected in the rootstock of dormant grafts (Fig. 4i, j).

What’s more, GFP signal was detected about 8 days earlier in the roots of At/At than in the roots of At(35S-GFP)/Nb (Fig. 4a-c, k-m), implying the phloem connection may be slower in the heterograft. We also observed that the intensity of GFP fluorescence in the rootstock of At(35S-GFP)/Nb is lower than that of At homograft (Fig. 4l-n). These results indicated that scion to rootstock protein movement indeed occurred, though in a relatively slow pace, in the functional At/Nb graft.

**Discussion**
From practical viewpoint, the grafting technique provides an alternative to securing food security [30], which requires a deeper understanding of the underlying mechanisms of grafting compatibility. Generally, grafting compatibility can be achieved within the same genus, but nearly non-applicable between interfamilial species [22]. Here, we have shown the inter-order graft was made, though at a relatively low rate as opposed to self-graft (Fig. 1j), from a far more distant taxonomic combination involving model plants Arabidopsis and Nicotiana (Fig. 1 and 2), implying that distant species could form a graft union but in a certain context as inter-order graft between Arabidopsis and tomato is short-living [24]. Furthermore, the At/Nb graft can be achieved only at the seedling stage by using At as scion and Nb as rootstock in this study; the reciprocal graft can be made at the bolting stage of the two species [25]. These results suggested other factors other than taxonomic discrepancy between the two species may also play a role in grafting compatibility.

Although the mechanisms to determine the grafting compatibility may vary between different species [22, 31, 32], but the vascular re-connection between scion and rootstock was considered as one of the critical elements in establishing a compatible union [33, 34]. Our results further corroborated the above proposed mechanisms as the compatible At/Nb plants showed comparable conductivity to the self-grafts whereas the symplastic and apoplastic dyes were completely blocked at the junction of the “dormant” grafts (Fig. 3). Similarly, the free GFP movement mainly via the phloem streaming was clearly detected in the phloem strands of Nb rootstock of Group A grafts, but blocked in the dormant grafts (Fig. 4g-j). Although xylem and phloem connection were both fundamental to successful grafts, noteworthily, the xylem connection seems to weigh more critical than that of phloem. This inference can be seen from the following three facts: (1) a small portion of dormant grafts also showed phloem connection (Fig. S1); (2) phloem connection occurred
slower in the At/Nb grafts than that of self-graft; (3) transmission efficiency in At/Nb is less than that of At homograft (Fig. 4k-n). Therefore, future investigation would be laid on issues such as what factors arising from xylem re-connection contributed to grafting compatibility.

Insights gained from above results did not fully address why the At scion and Nb stock combination, not the other way around, at the seedling stage did work. One possible clue came from the study by Melnyk et al. (2015) showing the vascular reconnection in Arabidopsis homo-grafting is associated with auxin perception and auxin signaling. Given the auxin transport is basipetal [35], the amount of auxin from the scion and response of the rootstock to the auxin input may work together to determine the vascular connection at the graft union. Since the perception of auxin in the Nicotiana and genomic organization of PIN genes in Nicotiana seems different to other plants [36, 37], it’s justifiable to speculate that the asymmetrical movement of auxin in the scion and the capability to respond auxin by the rootstock eventually leave the graft in a certain order. However, this order can be rearranged if grafting occurs in the stem for which there is different auxin responding ability and different vascular organization compared to the hypocotyl/root [38, 39].

Rootstock-scion interaction often results in the modification of both scion and rootstock phenotypes [22, 31]. In this study, the At/Nb combination indeed resulted in the modification of scion and rootstock phenotype (Fig. 2), particularly the flowering time and life cycle of At scion. These changes are most likely caused by the stress from At-Nb interaction as shown in Fig. 2. Nevertheless, this attribution does not fully explain the phenotypic changes occurring in the scion. For example, the stress often leads to early flowering in plants, the Arabidopsis scion, however, flowers very late, about 60 days after grafting, suggesting some other factors might play a role in this process.
One commonly-used practice in horticulture to promote flowering is through the imported florigen from a flowering rootstock that is connected to a nonflowering scion [40]. Functionally opposite to florigen, the floral inhibitor, or antiflorigen (CsAFT protein), was characterized to systemically inhibit scion flowering under a noninductive photoperiod in Chrysanthemum [41]. A recent report also showed that a tobacco antiflorigen NsCET1—in this case the RNA form—possessed the non-cell-autonomous inhibition function of flowering [17]. Future study can be performed to determine whether a Nb antiflorigen exists or in what form to inhibit At flowering.

Conclusion

In this study, we have used micro-grafting technique to present a heterografting system in which the grafted partners are genetically divergent species, belonging to two taxonomic orders respectively. A comparison between three types of grafts with regard to vascular reconnection suggested that xylem connection is prerequisite for a functional graft. Phloem connection between At and Nb always occur in a functional graft, although the transporting efficiency through phloem is less than in the homograft. Overall, the distinct At/Nb grafting groups provide us with high-potential working grip on the scion-rootstock interaction, particularly towards understanding the grafting compatibility and incompatibility between these two model plants.

Methods

Plant material and growth conditions

Seeds of the wild-type Arabidopsis thaliana (Col-0), the 35S-GFP transgenic line [20] and the wild-type Nicotiana benthamiana were sourced from Peter Waterhouse’s lab. These seeds were surface-sterilized in chlorine gas for 1h and then plated on sterile Murashige and Skoog (MS) medium supplemented with 3% (w/v) sucrose. Plates were grown vertically
in a growth room of long day conditions (16h light/ 8h dark) set at 22-23°C.

**Grating**

The grafting procedure was described in detail by Andersen et al (2014) [26]. Young seedlings that were grown on MS medium for 7-9 days, usually no more than 8 mm in total length for Nb plants and 2 cm for At plants, all with long straight hypocotyls were used for grafting. The cut was made halfway from the base of hypocotyl on the moisturized whatman paper. The scion and rootstock with smooth cut surface was pushed together with a certain tension. Grafts were grown on moisturized whatman paper for 2 days, then the grafts were gently lifted with forceps and placed vertically on the MS medium with 1% agar and 3% sucrose (w/v) in the growth room (16h light/ 8h dark) at 22-23 °C.

**Vascular re-connection analysis**

Xylem and phloem connectivity was measured with acid fuchsin and CFDA loading respectively. 1% (w/v) acid fuchsin solution (sigma) was introduced into the vascular system of heterografts and self-grafts by submerging the cut end of roots (the cut was made 2-3mm above the root tip) in the solution at room temperature [24, 27]. Make sure that the hypocotyl of rootstock was kept away from the solution. The cotyledon of the scion was examined under bright field microscope after 30-60min incubation. For CFDA staining, a fresh working solution of 5 μM CFDA in distill water was prepared from a 1 mM stock solution in dimethyl sulfoxide (DMSO). A syringe needle was used to gently puncture the newly grown leaf. 0.25 μl of 5 μM CFDA was pipetted onto the lightly damaged leaf and kept in the darkness at room temperature. Alternatively, the CFDA solution was applied to the lower part of roots that were put on the parafilm and incubated in the darkness at room temperature for 1-2 hour. The fluorescent signal was detected and imaged with Nikon ECLIPSE Ni fluorescence microscope and a Zeiss Axio Zoom V16 fluorescence microscope with a 1x/2x lens.
Abbreviations

At: Arabidopsis thaliana; Nb: Nicotiana benthamiana; CFDA: 5(6)-carboxyfluorescein diacetate; DAG: days after grafting.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

DL conceived the project and designed the experiments. ZD and MJ performed the experiments. ZD, MW and DL analyzed the data; ZD and DL wrote the manuscript.

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Table 1

Table 1. Three types of At/Nb grafts at 20 DAG: mild-stressed grafts, albino grafts and dormant grafts.

| No. of grafts | mild-stressed | percentage (%) | albino | percentage (%) | dormant | percentage (%) |
|---------------|---------------|----------------|--------|----------------|---------|----------------|
| 125           | 37            | 29.6           | 15     | 12             | 47      | 37.6           |
| 77            | 17            | 22.08          | 16     | 20.78          | 27      | 35.06          |
| 80            | 33            | 41.25          | 16     | 20             | 20      | 25             |
| 47            | 21            | 44.68          | 6      | 12.77          | 8       | 17.02          |
| 58            | 23            | 39.66          | 2      | 3.44           | 21      | 36.21          |
| 62            | 15            | 24.19          | 6      | 9.68           | 26      | 41.94          |

Figures
Figure 1

Phenotypic change of grafts in the different stage after grafting. The white arrow
indicates the graft union. Phenotypic comparison between Arabidopsis homograft (d,e,f), N. Benthamiana homograft (g,h,i), and Arabidopsis / N. benthamiana hetero-graft (a, b, c). a,d,g, 7 DAG. b,e,h, 15 DAG. c, f at flowering stage.
Three types of At/Nb heterografts grown under aseptic condition. (a) A representative plant of group A grafts that show mild stress at 20 DAG. (b) A representative plant of group B grafts with albino phenotype at 20 DAG. (c) A representative plant of group C grafts showing highly retarded growth or dormant at 20 DAG. (d) A 135 DAG graft from group A. (e) A 45 DAG graft from group B. (f) A 45 DAG graft from group C. (g) Leaves from At/Nb (bottom panel) and At/At graft (top panel). (h) Shoot, root and leaf measurement in grafts of At/At and At/Nb. (i) Flowering time comparison between At/At and At/Nb graft (P < 0.01).
Figure 3

Apoplastic and symplastic dye loading in At/Nb grafts. (a-e) 1% Acid fuchsin loading. (a) Fuchsin staining in the veins of 7-DAG At/Nb plant. (b) 7 DAG of Nb/Nb self-graft. (c) 7 DAG of At/At self-graft. (d) 5-DAG At/Nb plant. (e) 30 DAG of a dormant graft. Chevron indicates the accumulated fuchsin in the graft union of a retarded grafts. Arrow indicates the graft union. The black dotted line indicated where the fuchsin stops. (f-h) CFDA loading in the At/Nb grafts. (f) CFDA loading in a 5 DAG graft. Left: the longitudinal section under the bright field. Middle: the same section under the fluorescence. Right: the overlay image of Left and Middle image. (g) The CFDA signal in the root of 5 DAG At/Nb graft. Left: the root under the bright field. Middle: the same root under the fluorescence. Right: the overlay image of Left and the Middle. (h) CFDA loading in a dormant graft (30 DAG). Left: the longitudinal section under the bright field. Middle: the same section under the fluorescence. Right: the the overlay image of Left and the Middle. The red dotted line indicates where the CF stops.
Translocation of GFP from Arabidopsis (35S-GFP) scion to Nicotiana benthamiana rootstock. (a-c) The root tip of an At(35S-GFP)/ Nb graft at 15 DAG under bright field (a), fluorescence (b). (c), The overlay image of (a) and (b). (d-f) The root tip of Nb self-graft used as control. (g,h) The graft union of At(35S-GFP)/ Nb grafts from the mild-stressed group at 40 DAG. The arrow indicates the GFP signal at the graft union. The arrowhead indicates the GFP signal in the phloem strands of the rootstock hypocotyl. (i,j) The graft union of At(35S-GFP)/ Nb grafts from the dormant group at 40 DAG. (k) An At(35S-GFP)/At(WT) self-graft at 7 DAG. The arrowhead indicates the GFP signal. (l-n) The GFP fluorescence comparison between At(35S-GFP)/ Nb (l) and an At(35S-GFP)/At(WT) graft (m). The signal intensity in the root of At(35S-GFP)/ Nb is lower than the At(35S-GFP)/At(WT) graft (p<0.05) (n).
Supplementary Files

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