Graft-union development: a delicate process that involves cell–cell communication between scion and stock for local auxin accumulation

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Abstract

Grafting is an ancient cloning method that has been used widely for thousands of years in agricultural practices. Graft-union development is also an intricate process that involves substantial changes such as organ regeneration and genetic material exchange. However, the molecular mechanisms for graft-union development are still largely unknown. Here, a micrografting method that has been used widely in Arabidopsis was improved to adapt it a smooth procedure to facilitate sample analysis and to allow it to easily be applied to various dioctyledonous plants. The developmental stage of the graft union was characterized based on this method. Histological analysis suggested that the transport activities of vasculature were recovered at 3 days after grafting (dag) and that auxin modulated the vascular reconnection at 2 dag. Microarray data revealed a signal-exchange process between cells of the scion and stock at 1 dag, which re-established the communication network in the graft union. This process was concomitant with the clearing of cell debris, and both processes were initiated by a wound-induced programme. The results demonstrate the feasibility and potential power of investigating various plant developmental processes by this method, and represent a primary and significant step in interpretation of the molecular mechanisms underlying graft-union development.

Key words: Arabidopsis thaliana, cell–cell communication, endomembrane system, graft-union development, local auxin accumulation, micrografting, vascular reconnection.

Introduction

Grafting is an asexual plant propagation technique in agriculture that has been widely used for thousands of years. Woody plants such as apple trees are grafted for the purpose of dwarfing, ease of propagation, and sturdiness, while herbaceous grafting can increase productivity and control damage caused by soil-borne disease and abiotic stress, such as in tomato, cucumber, and melon (Estañ et al., 2005; Sigüëenza et al., 2005; Zhou et al., 2009). It has been used widely for decades as a convenient technique in long-distance signalling research and has generated persuasive evidence in various plant processes. For example, the most important discovery using grafting was that FLOWERING LOCUS T (FT) protein was confirmed as the florigen (Corbesier et al., 2007). The epigenetic state can also be converted, as mobile small RNAs from the scion could direct epigenetic modifications in the genome of stock in Arabidopsis (Molnar et al., 2010). The findings that morphological and physiological changes could occur with a specific stock–scion combination have also been reported, as grafting altered leaf morphology (Kim et al., 2001) and improved salinity tolerance (Estañ et al., 2005) in tomato. In cherry trees, dwarfing rootstock can trigger an early

Abbreviations: CIPK, CBL interacting protein kinase; cyclB, cyclin B; dag, days after grafting; DIC, differential interference contrast; GFP, green fluorescence protein; GO, Gene Ontology; GUS, β-glucuronidase; HSP, heat-shock protein; KEGG, Kyoto Encyclopedia of Genes and Genomes; PDLP, plasmodesmata-located protein; PS-PI, modified pseudo-Schiff propidium iodide; WT, wild type.

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cessation of terminal meristem growth of the scion, conferring up to a 50% reduction in scion size compared with standard rootstocks (Prassinos et al., 2009). Aside from these applications, graft-union development is an intricate process during which histological and physiological changes, such as organ regeneration, are initiated and progress dramatically, and even the genetic interaction between different cells of the scion and stock can be exchanged (Stegemann and Bock, 2009). Thus, grafting is not only a useful technique but also an attractive research subject. Although a number of reports have depicted the process with regard to the histological and physiological aspects (Kollmann and Glockmann 1985; Fernandez-Garcia et al., 2003; Flashman et al., 2008), the molecular mechanisms related to the process remain elusive.

In the model organism *Arabidopsis thaliana*, grafting techniques were developed early in 1993 (Tsukaya et al., 1993) but were restricted to the grafting of inflorescence stems late in development when most key processes had already been determined. In 2002, Colin Turnbull and colleagues developed a method using 3–4-d-old seedlings, allowing experiments to be conducted on many aspects of long-distance signalling for the first time (Turnbull et al., 2002; Bainbridge et al., 2006). This seedling grafting technique has been applied to the study of many plant processes including flowering (Corbier et al., 2007), shoot branching (Turnbull et al., 2002), disease resistance (Xia et al., 2004; Thatcher et al., 2009), nutrient allocation (Rus et al., 2005), post-transcriptional gene silencing (Brosnan et al., 2007; Molnar et al., 2010), cytokinin activity (Foo et al., 2007; Matsumoto-Kitano et al., 2008), and root-to-shoot hydraulic signalling (Christmann et al., 2007).

Here, a simple and effective seedling micrografting method that improved on the previous protocol (Turnbull et al., 2002; Bainbridge et al., 2006) was developed. This approach reduced the operational work and facilitated sample analysis, yet yielded higher success rates than the previous method. It could also easily be applied to the seedling micrografting methods of other dicotyledonous plants such as tomato, alfalfa, and tobacco. This method was further used to model graft-union development in *Arabidopsis*. Anatomic and transcriptomic analysis uncovered elaborate stages for graft-union healing processes and highlighted a cell–cell communication process during its early stage.

**Materials and methods**

**Plant materials and grafting procedure**

*A. thaliana* ecotype Col-0 was used as the wild-type (WT) plant. The transgenic lines proDR5:GUS and procyclB:GUS expressing GUS under the control of the DR5 (a synthetic auxin response element) and cyclin B (*cyclB*) promoters, respectively, were purchased from NASC (European *Arabidopsis* Stock Centre, http://arabidopsis.info/). The transgenic line proSUC2::GFP expressing green fluorescent protein (GFP) under the control of the sucrose-proton symporter 2 (*SUC2*) promoter was a kind gift from Dr Norbert Sauer.

Grafting in *Arabidopsis* was conducted as follows. Plates were tilted using a glass rod of 0.7–1 cm in diameter. Seeds were sown on 1% agar medium containing the macro-nutrients 5 mM KNO₃, 2 mM Ca(NO₃)₂·4H₂O, 2 mM MgSO₄·7H₂O, 2.4 mM KH₂PO₄, 0.1 mM K₂HPO₄, Fe·EDTA, 50 μM FeSO₄·7H₂O, 50 μM EDTA-Na₂·2H₂O, and the micro-nutrients 0.5 μM KI, 10 μM H₂BO₃, 10 μM MnSO₄·4H₂O, 3 μM ZnSO₄·7H₂O, 0.1 μM Na₃MoO₄·2H₂O, 0.01 μM CuSO₄·5H₂O and 0.01 μM CoCl₂·6H₂O at pH 5.7–5.8. The plates were incubated in the dark at 4 °C for 2–3 d and then placed vertically in a growth room (temperature 22–25 °C; light intensity 6000 lux; photoperiod 16 h light/8 h dark) with the thin side pointing downward and the thick side upward. Grafting was performed using a dissecting microscope at 4 d post-germination. Seedlings were chosen with long straight hypocotyls and the hypocotyl was cut transversely while on the agar. The stock donor was cut near the shoot apical meristem and the scion donor was cut halfway from the base of the hypocotyl (the hypocotyl consequently appeared longer than usual). Forceps were used to keep the seedling stable while cutting the hypocotyl with the razor blade under a dissecting microscope. It was important to make the cut quickly and to push the blade rather than pull it, so that the cut surface was clean and smooth. The scion was lifted up to bring it to the stock, and the stock was carefully picked up to approach the scion and connect them together. Note that it is important to be aware of raising the graft union up away from the agar surface (the oblique surface will provide enough space to make it possible). The scion or stock was carefully and slightly pushed to adjust the relative position of the two parts, and the graft was inspected from all sides of the graft junction to make sure that the two parts connected and supported each other thoroughly. Initially, this process may take some practice to connect the scion and stock together successfully while lifting the union up, but in our experience the operation went smoothly after about 1–2 weeks of practice. The plate was returned to the growth room to the same vertical position with the thin side downward and the thick side upward.

Grafting details for other plant species are illustrated in Supplementary Methods at *JXB* online.

**Pseudo-Schiff propidium iodide (PS-PI) staining**

PS-PI staining followed the method of Truernit et al. (2008) with some modifications. Samples were fixed in 50% ethanol and 10% acetic acid at 4 °C overnight and then transferred to 80% ethanol and treated in a water bath at 80 °C for 1 min. The tissues were then transferred back to fixative and incubated for 1 h at room temperature. Next, the tissues were rehydrated in an ethanol series (50, 30, and 10% for 20 min each), and rinsed with distilled water three times. Samples were then incubated in 1% (w/v) periodic acid at room temperature for 40 min and rinsed with water again. The tissues were incubated in PI-Schiff solution (100 mM sodium metabisulphite, 150 mM HCl, 30 μg/ml propidium iodide) for 1 h. The samples were rinsed with water and incubated in chloral hydrate solution (chloral hydrate:glycerol:water, 8:1:2) for 2–3 d. Sample observation was performed with a Zeiss confocal laser scanning microscope to record the images.

**β-Glucuronidase (GUS) staining**

Collected tissues were fixed in cold 90% acetone at 4 °C for 10 min and rinsed five to six times with staining buffer (50 mM sodium phosphate buffer, 0.1% Triton X-100, 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, 10 mM EDTA-Na₂). The staining buffer was removed from the samples and staining solution containing 2 mM 5-bromo-4-chloro-3-indolyl-β-d-glucuronide (X-Glu) was added. The samples were incubated at 37 °C. The exact time course for incubation depended on GUS activity in the chosen line (for example, 1 h for DR5/WT and 4 h for WT/DR5). Samples were then dehydrated in a progressive series of ethanol dilutions (10, 30, 50 and 70%) and incubated in some modifications. Samples were fixed in 50% ethanol and 10% acetic acid at 4 °C overnight and then transferred to 80% ethanol and treated in a water bath at 80 °C for 1 min. The tissues were then transferred back to fixative and incubated for 1 h at room temperature. Next, the tissues were rehydrated in an ethanol series (50, 30, and 10% for 20 min each), and rinsed with distilled water three times. Samples were then incubated in 1% (w/v) periodic acid at room temperature for 40 min and rinsed with water again. The tissues were incubated in PI-Schiff solution (100 mM sodium metabisulphite, 150 mM HCl, 30 μg/ml propidium iodide) for 1 h. The samples were rinsed with water and incubated in chloral hydrate solution (chloral hydrate:glycerol:water, 8:1:2) for 2–3 d. Sample observation was performed with a Zeiss confocal laser scanning microscope to record the images.
70% ethanol overnight. Next day, the ethanol was removed, chloral hydrate solution was added and the samples were incubated overnight at room temperature. Samples were then placed onto a slide, a coverslip was added and images were obtained as described above.

**Acid fuchsin staining**

An aqueous solution of 0.1% (w/v) acid fuchsin (Sigma) was introduced into the vascular system of the grafts (SUC2/WT) by submerging the roots in the solution at room temperature (Flashman et al., 2008), taking care that the hypocotyl of the stock was above the solution. The cotyledon of the scion should become red in the vascular veins over 1–2 h, indicating that the xylems of the scion and stock have been connected.

**Microscopy**

Fluorescence was viewed with a confocal laser scanning microscope (LSM 510, Zeiss). PI and GFP fluorescence were excited with a 543 and 488 nm argon laser, and emission was detected with 565–615 and 500–550 nm band-pass filter combinations, respectively. GUS-stained images were recorded using a Nikon Eclipse 80i microscope.

**Plasmid construction and plant transformation**

A region of approximate 2000 bp upstream of the ATG of heat-shock *HSP21* (At4G27670) gene was amplified with the high-fidelity polymerase PrimeSTAR™ (TaKaRa) using the gene-specific primers 5′-CTGCCAGCTGACTCTTTGGCAATAG-3′ (forward) and 5′-GGATCTTTGTTITCGAGTAGCCGAC-3′ (reverse) and cloned into the vector pCM1300-GUS. *Arabidopsis* transformation was carried out using the floral dip method. Transgenic plants were obtained by screening successive generations in terms of hygromycin resistance.

**Microarray analysis and quantitative RT-PCR**

WT/WT grafts at 22–26 h after grafting were collected for microarray, with intact WT seedlings and ungrafted scions and stocks used as controls. For sampling, all three types of sample—grafts (group A: whole grafts), ungrafted scions and stocks (group B: mixture of ungrafted scions and stocks), and intact seedlings (group C: whole intact seedlings)—were collected in liquid (group B: mixture of ungrafted scions and stocks), and intact grafts (group A: whole grafts), ungrafted scions and stocks. WT/WT grafts at 22–26 h after grafting were collected for Microarray analysis and quantitative RT-PCR.

Normalized expression data was subjected to log 2 transformation. Data were normalized between arrays using the quantile method. Normalized expression data was subjected to log2 transformation. For differential expression analysis, Student’s t-test assumed that the variance of the two classes not being the same was applied. First, data were analysed between groups A and C with a *P* value ≤0.01, *q*-value ≤0.05, and fold change ≥2. Differential probes were subsequently compared between A and B with *P* value ≤0.01 and *q*-value ≤0.05 (see Supplementary Fig. S4). Fold change was not applied in the comparison of groups A and B due to the high similarity of expression profiles between the two groups (see Supplementary Fig. S5). Probe annotation was according to Agilent’s web instructions (https://earray.chem.agilent.com/). Gene-enrichment tests for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) categories were performed in R program with the fisher.test function. Quantitative RT-PCR was carried out as described by Mu et al. (2011).

**Results**

**Improvement of the micrografting method in Arabidopsis**

For a single successful graft, a good union between the scion and stock is the most important key point. In the previous protocol (Turnbull et al., 2002; Bainbridge et al., 2006), 0.3 mm diameter silicon tubing was used as a collar to support the graft and hold the stock and scion together during graft healing. The collar greatly increased the success rate but also interfered with the observation of the graft union. There are some reported methods that do not use such collars, but they do not have satisfactory success rates (Turnbull et al., 2002; Bainbridge et al., 2006).

Following the ‘good union’ principle, the protocol was improved in terms of enhancing the connection between the scion and stock. The cut surface of the scion and stock cannot be placed closely together due to their different morphology—expanded cotyledons will raise the hypocotyl of the scion and result in a poor connection between the scion and stock. Moreover, the water on the medium surface will infiltrate the graft union and consequently interfere with the union healing. An oblique medium surface was found to overcome these problems through trial and error. In the first step, a glass rod was used to prop up one side of the plate before pouring the liquid medium into it (Fig. 1A). The agar solidified as an inclined plane at approximately 15° relative to the plate and formed an oblique surface that was thick at one side and thin at the other. Seeds were sown on this oblique surface, and after stratification at 4°C for 2–3 d, the plate was placed vertically in the growth room with the thin side downward and the thick side upward. On the oblique surface (Fig. 1B, reversed triangle), enough space (Fig. 1B, dotted line) was provided for the cut surfaces to connect the scion and stock thoroughly and completely (Fig. 1B, arrowhead). Therefore, there were strong mutual-effect forces on the joint when the plate was placed vertically in the growth room (Fig. 1B, white arrows), supporting a tight connection between the scion and stock. As it was easy to cut adventitious roots at their first emergence and to get rid of grafts that fail to form a good union immediately after grafting, because the graft union could easily be inspected from all sides (Fig. 2A–L),
The series of histological changes that took place during graft-union healing was examined by modified PS-PI staining (Truernit et al., 2008) and pro\textit{cyCB}:GUS expression in \textit{Arabidopsis}. For the purpose of homogenization, all grafts were examined under a dissecting microscope at 1 dag before sampling. The grafts that were connected tightly and with no obvious gaps were fixed and used for subsequent treatments. At 1 dag, there were no apparent changes were recorded (Fig. 3A, D, J). In the cyCB/cyCB (scion/stock) union, there was no GUS staining observed in the graft union at 1 dag, suggesting the absence of cell division at this time point (Fig. 3G). The first evidence of cell division became apparent at 2 dag in both WT/WT and cyCB/cyCB grafts (Fig. 3B, E, H, K). By 3 dag, substantial cell division occurred on both the scion and stock at the graft interface (Fig. 3I, L). A united vessel was also detected in the graft union (Fig. 3C, F, I, arrow), implicating functional recovery of the vasculature.

To determine experimentally whether the transport activity of vascular tissue was restored, grafting with the combination of SUC2/WT was conducted. The pro\textit{SUC2}:GFP transgenic line has been used as a marker for detection of source-to-sink transition (Imlau et al., 1999; Oparka et al., 1999). Under the control of the \textit{AtSUC2} promoter, soluble GFP was expressed specifically in phloem companion cells, in which GFP was diffused into the phloem and passively transported with the photoassimilates in the direction of source to sink. Thus, detection of GFP fluorescence in the WT stock root of SUC2/WT grafts could be indicative of the recovery of phloem transport activity. Consistent with the histological results, GFP fluorescence was observed in WT stock root, and its distribution pattern was the same as in SUC2 intact seedling roots (Fig. 4A–C). SUC2/WT grafts in which GFP was detected in the stock root were subsequently stained with acid fuchsin to explore the recovery of xylem activity. The dye transport was inspected in cotyledons, shown as a red leaf vein (Fig. 4D–F). The results showed that, as in WT intact seedlings (Fig. 4F), the SUC2 scion was stained red in the leaf vein (Fig. 4D, E), suggesting the functional transport activity of the xylem in the graft. Taken together, these results demonstrated functional recovery of the graft vasculature at 3 dag.

**Functional recovery of vasculature at 3 d after grafting (dag) in Arabidopsis**

From the histological experiments, a vascular differentiation between 2 and 3 dag could be inferred. Auxin significantly contributes to the pattern formation of vascular tissue (Sauer et al., 2006; Scarpella et al., 2009; Caño-Delgado et al., 2010), so the distribution of GUS staining of pro\textit{DR5}:GUS was investigated during graft-union development.

In ungrafted DR5 scions, there was no detection of GUS staining close to the graft union at 1–3 dag. GUS activity was observed mainly in the vasculature of the hypocotyls, which is the site for adventitious root initiation (Supplementary Fig. S3 at \textit{JXB} online). For DR5/WT scions, GUS staining
was absent from the graft union and was seen mainly in the middle of hypocotyls at 1 dag (Fig. 5A). A deep staining was observed in vascular tissue adjacent to the graft union at 2 dag (Fig. 5B, arrow). PI staining demonstrated that cell division was initiated at 2 dag (Fig. 3B), and no GUS staining of the cycB/cycB union was recorded before 2 dag in the graft union (Fig. 3G). In DR5/WT grafts, GUS staining could also be detected at 2 dag (Fig. 5B). These results demonstrated that auxin initiated cell division and differentiation at 2 dag, as well as its function in organ formation (Benkova et al., 2003). At 3 dag, GUS staining could be observed across the graft union (Fig. 5C). The significance of auxin was further emphasized by WT/DR5 grafts. In contrast to the extensive distribution in the scion, accumulation of auxin appeared as a concentrated pattern that was distributed exclusively in a group of cells within the graft union (Fig. 5D–F). This specific pattern was detected as early as 2 dag, approximate 40 h after grafting (Fig. 5D), and was maintained throughout the healing process until the reconnection of vasculature (Fig. 5D–F). Furthermore, the tracheary elements were

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**Fig. 2.** Grafts in Arabidopsis and tomato. (A–L) Time-lapse images demonstrating the phenotypic development of a graft union of a WT/WT combination in Arabidopsis. Arrowheads indicate the graft union. Images were ordered as dag (days after grafting). The graft union was examined on all sides: (A), (C), (E), (G), (I), and (K) show the front view, which was observed perpendicular to the medium surface, while (B), (D), (F), (H), (J), and (L) show the side view, which was observed parallel to the medium surface. (M–T) Graft union development in tomato. White arrowheads indicate the graft union. Tomato grafts recover faster than other species studied. At 3 dag, the scion and stock were connected completely. Bars, 200 μm.
detected to differentiate towards the auxin accumulation site (Fig. 5E, arrows) and eventually reconnected the vessels at the site (Fig. 5F, arrow). Taking these into account, a central organizer role of auxin acting as a director for reconnection of the vascular tissue in the graft union was concluded.

Transcriptional analysis demonstrates that cell–cell communication accompanies clearing of cell debris under the control of a wound-induced programme at 1 dag.

Comparing the GUS distribution of ungrafted DR5 scion (Supplementary Fig. S3, 2 dag) with DR5/WT grafts (Fig. 5B, arrow), it was apparent that some unknown signals were being transported from the stock to the scion to induce the auxin distribution changes. These results suggested that a communication network had been established between the scion and stock at 2 dag. A cell–cell communication process that represents signal exchange between cells of scion and stock is obviously compulsory for subsequent processes in graft-union development. In addition, no obvious changes could be detected by anatomic analysis (Fig. 3A, D, G, J) at 1 dag, implicating a potential signal-exchange process. To confirm this hypothesis and to

Fig. 3. Anatomical analysis of graft union development in Arabidopsis. (A–C) PI stained WT/WT grafts. Panels are projections from confocal z-stacks. The approximate time points of sampling of the grafts are indicated. The box in (A) indicates the graft union. A complete union resulted in a poor staining at 3 dag. (D–E) Differential interference contrast (DIC) images of the same graft shown in (A–C). The dotted line in the DIC images shows the graft union. Arrows indicate cell divisions and the united vessels at 2 and 3 dag, respectively. Note that the good union of the 3 dag graft caused poor staining for PI permeation. (G–I) GUS staining of cycB/cycB grafts. GUS activity in the graft union is indicated by arrows at 2 dag. At 3 dag, the arrow indicates the united vessels, while the arrowhead indicates deep staining showing an adventitious root. (J–L) Sections of grafts in Arabidopsis. Samples were embedded in Technovit 7100 (Kulzer) and 5μm sections were cut. Arrows indicate the graft unions. SC, Scion; ST, stock. Bars, 50 μm.
characterize the molecular identity of the graft-healing processes on a genome-wide scale, microarray analysis was performed to investigate the transcriptome changes at 1 dag in WT/WT grafts. Samples were grouped as follow: group A comprised grafts, group B was ungrafted scions and stocks, and group C was intact seedlings. Microarray data were first compared between groups A and C, and differentially expressed probe sets were then tested based on groups A and B to obtain graft-specific probes (Supplementary Fig. S4 at JXB online). Differential probes were annotated for a total of 306 genes, including 120 upregulated and 186 downregulated genes according to the fold change in group A relative to group C (Supplementary Fig. S5 and Table S2, and Supplementary File 2 at JXB online). Several genes showing expression-level changes were confirmed by real-time PCR (Fig. 6A).

Fig. 4. Functional analysis of vascular transport activity in SUC2/WT grafts. (A–C) GFP fluorescence detected in WT stock roots. Images are the merges of GFP and DIC. WT and SUC2 represent the intact seedlings, respectively. Bar, 100 µm. (D–F) Acid fuchsin transport experiment. (D) and (E) represent the graft, and (F) represents a WT intact seedling. The roots were submerged in fuchsin solution, as shown in (D), with a higher magnification shown in (D). Arrows indicate the red leaf veins stained by fuchsin.

Fig. 5. DR5 grafts show a pivotal role for auxin in vascular reconnection. (A–C) DR5/WT grafts. At 1 dag (A), no obvious GUS activity was detected closed to the graft union, but by 2 dag, there was a deep staining adjacent to the union (white arrow; B). Vessel union was apparent by 3 dag (white arrow; C). Arrowheads indicate adventitious root initiation. Bars, 100 µm. (D–F) WT/DR5 grafts. The earliest detection of GUS staining was at 40 h after grafting (D). The arrows in (E) indicate the vessel elements that differentiated towards the accumulation site at 62 h. The arrow in (F) represents the united vessels at the auxin accumulation site. Dotted lines show the graft union and the scions and stocks were as indicated. Bars, 50 µm.
Furthermore, the promoter region of *HSP21* (At4G27670) was fused upstream of the GUS coding sequence to control enzyme activity, and GUS staining also suggested a graft-specific pattern (Fig. 6B–E).

Significant genes were classified according to GO and KEGG (Tables 1–Tables 4) to explore their functional significance. Among the GO categories, 15 and eight groups were significantly over-represented for up- and downregulated genes, respectively, based on a gene-enrichment test (Tables 1 and 2). In downregulated genes, the cell growth (GO:0016049) category was present, indicating a logical consequence for regeneration after wounding (Table 2). The upregulated genes (Table 1) were more noteworthy than the downregulated genes. Lyase activity (GO:0016829), hydrolase activity (GO:0016787) and oxidoreductase activity (GO:0016491) demonstrated that cells were sweeping away the cell debris caused by wounding. A set of stimulus-response categories was significantly over-represented, suggesting that a wound-induced programme took part in the graft-union developmental processes. This was further confirmed by over-representation of the biosynthesis of ethylene and jasmonic acid in the KEGG plant hormone biosynthesis pathway (Table 3, Fig. 7), as these phytohormones are typical responses involved in wounding (Schilmiller and Howe, 2005). More importantly, the endomembrane system (GO:0012505) was calculated as having the lowest P value and q-value for both up- and downregulated genes (Tables 1 and 2), suggesting a crucial contribution to graft-union development. The endomembrane system consists of serial membranous organelles essential for various aspects of plant development and signal transduction, and is an important passageway for cells to communicate with the environment (Surpin and Raikhel, 2004). *In planta*, the endomembrane system offers many regulated activities for reproductive processes, which involve substantial signal exchanges between pollen and the pistil (Cheung and Wu, 2008; Kumar and McClure, 2010).

For grafts, there is no existing route for communication between the scion and stock cells, which consequently rely on signal transduction across the plasma membrane. In accordance with this, most of the five protein kinases out of the 120 upregulated genes participated in acceptance of extracellular signals. Two of them, At4G04490 and At1G70250, had receptor-like protein kinase activity (Table 5). CIPK5 (At5G10930) and CIPK14 (At5G01820) belong to the calcium-neurin B-like (CBL)-interacting protein kinase (CIPK) family (Table 5), which represents a critical signalling system in response to a wide range of environmental stimuli (Weinl and Kudla, 2009). Taken together, the microarray data revealed that a cell-cell communication process accompanied the clearing of cell debris during the early stage of the graft-healing process, and that both were initiated by a wound-induced programme.

### Model refinement for graft-union developmental stages

Summarizing the results, a concise model was proposed to illustrate graft-union development in general (Fig. 8). At the beginning, wounding elicited the programmes that stimulated the undamaged cells to eliminate cell debris at the cut surface. Accompanying this clearing process was signal exchange between intimate contacting cells of the scion and stock at the graft interface. This reconstructed the communication network in the graft union to give rise to an integrated background for subsequent events. Once the communication was reconstructed, auxin accumulated, contributing in particular to vascular reconnection. Finally, the cells differentiated into vascular tissues with the auxin in control to reconnect the vasculature between scion and stock. At this point, the key event in the graft-healing process was accomplished, indicating a successful grafting.

### Discussion

**Applications of seedling micrografting towards a research platform for diverse aspects of plant biology**

In recent decades, grafting has been used to study the long-distance signalling between the shoot apical meristem and root apical meristem, which is essential for plant development and adaptation to environmental change (Turnbull et al., 2002; Xia et al., 2004; Estañ et al., 2005; Rus et al., 2005; Siguenza et al., 2005; Brosnan et al., 2007; Christmann et al., 2007; Foo et al., 2007; Matsumoto-Kitano et al., 2008; Thatcher et al., 2009; Zhou et al., 2009; Molnar et al., 2010). In this report, an improved seedling micrografting process was characterized and validated in plants. In our long-term experience with this practice, this method is satisfactory in sample analysis and has a high success rate, and also saves labour, time, and reagents. At the same time, there is a high degree of application similarity in other dicotyledonous plants. There are reasons to believe that this method would function better in long-distance signalling research than current methods.

In graft-union development, substantial changes occur, such as cell differentiation and organ regeneration, and even genetic material can be exchanged between the scion and stock at the graft site (Stegemann and Bock 2009). Compared with traditional grafting methods used previously (Kollmann and Glockmann 1985; Napoli, 1996; Fernandez-Garcia et al., 2003; Estañ et al., 2005; Siguenza et al., 2005; Flashman et al., 2008; Stegemann and Bock 2009; Zhou et al., 2009), this seedling micrografting method is better suited for sample analysis. The simple procedure and high success rate will ensure that researchers can generate abundant and homogeneous grafts within a short period of time to fulfil the requirements of an experiment. A similar micrografting method was reported recently in *Nicotiana attenuata* plants (Fragoso et al., 2011), but its use of agar blocks would obviously interfere with observation of the union. Using our improved protocol, the graft union could be inspected easily and flexibly, which will facilitate investigations based on numerous scion/stock combinations with various experimental techniques to interpret the mechanisms underlying the healing process.
Recently, Molnar et al. (2010) reported that a substantial proportion of endogenous small RNAs had moved across the graft union, and these mobile small RNAs directed DNA methylation in the genome of the recipient cells. DNA methylation differences can be stably inherited across generations in the absence of extensive DNA sequence
polymorphisms, and these epigenetic variations influence the plant phenotype impressively and even affect some quantitative traits, such as plant height and yield (Hauben et al., 2009; Johannes et al., 2009; Reinders et al., 2009). These findings open up a new avenue for testing the epigenetic impacts on plant development and heredity by creating desirable combinations of shoot and root phenotypes or genotypes with grafting. Bringing the full power of the seedling micrografting method into play will be critical for rapid progress in understanding plant epigenetics, and for developing new and better ways for plant improvement.

**Hormone functions in the union process**

The microarray data showed that ethylene and jasmonic acid synthesis were elevated. This early activation of ethylene and jasmonic acid is consistent with findings made in the tissue-reunion process in partially incised inflorescence stems of *Arabidopsis* (Asahina et al., 2011). In contrast, giberellin is required for cell division during tissue reunion of cucumber and tomato hypocotyls (Asahina et al., 2002). Although we used hypocotyls of *Arabidopsis* for grafting, the hormones involved in the healing process are similar to those of inflorescence stems rather than the hypocotyls in cucumber and tomato.

The histological data showed that cell division commenced after auxin accumulation (Figs 3 and 5). The precedence of ethylene and jasmonic acid elevation to auxin accumulation remind us of the importance of ethylene and jasmonic acid during the signal-exchange process between scions and stocks. Unlike partially incised inflorescence stems, signal exchange is indispensable for subsequent events during graft-union healing. It is conceivable that similar mechanisms activated by ethylene and jasmonic acid after wounding may play a part in the early responses of both grafted hypocotyls and injured stems in *Arabidopsis*.

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**Table 1.** Significant categories for upregulated genes in GO

| GO ID     | Name                          | Hits | Total  | P value<sup>a</sup> | q-value<sup>b</sup> |
|-----------|-------------------------------|------|--------|---------------------|---------------------|
| GO:0003674 | Molecular function            | 166  | 29448  | 0.9753              | 0.6903              |
| GO:0016787 | Hydrolase activity           | 32   | 2933   | 5.0E–4              | 0.0039              |
| GO:0005199 | Structural constituent of cell wall | 4   | 39     | 1.0E–4              | 0.0011              |
| GO:0046900 | Tetrapyrrole binding         | 8    | 320    | 7.0E–4              | 0.0048              |
| GO:0004601 | Peroxidase activity         | 5    | 109    | 6.0E–4              | 0.0040              |
| GO:0005575 | Cellular component           | 165  | 28657  | 0.8255              | 0.6903              |
| GO:0012505 | Endomembrane system         | 53   | 4046   | <1.0E–4             | <1.0E–4             |
| GO:0008150 | Biological process           | 44   | 7802   | 0.0029              | 0.0080              |
| GO:0006955 | Immune response              | 7    | 312    | 2.0E–4              | 0.0013              |
| GO:0044238 | Primary metabolic process    | 6    | 332    | 0.0019              | 0.0060              |

<sup>a</sup> P ≤0.01.  
<sup>b</sup> q ≤0.01.

**Table 2.** Significant categories for down-regulated genes in GO

| GO ID     | Name                          | Hits | Total  | P value<sup>a</sup> | q-value<sup>b</sup> |
|-----------|-------------------------------|------|--------|---------------------|---------------------|
| GO:0003674 | Molecular function            | 166  | 29448  | 0.9753              | 0.6903              |
| GO:0016787 | Hydrolase activity           | 32   | 2933   | 5.0E–4              | 0.0039              |
| GO:0005199 | Structural constituent of cell wall | 4   | 39     | 1.0E–4              | 0.0011              |
| GO:0046900 | Tetrapyrrole binding         | 8    | 320    | 7.0E–4              | 0.0048              |
| GO:0004601 | Peroxidase activity         | 5    | 109    | 6.0E–4              | 0.0040              |
| GO:0005575 | Cellular component           | 165  | 28657  | 0.8255              | 0.6903              |
| GO:0012505 | Endomembrane system         | 53   | 4046   | <1.0E–4             | <1.0E–4             |
| GO:0008150 | Biological process           | 44   | 7802   | 0.0029              | 0.0080              |
| GO:0006955 | Immune response              | 7    | 312    | 2.0E–4              | 0.0013              |
| GO:0044238 | Primary metabolic process    | 6    | 332    | 0.0019              | 0.0060              |

<sup>a</sup> P ≤0.01.  
<sup>b</sup> q ≤0.01.

**Table 3.** Significant pathways for up-regulated genes in KEGG

| Pathway name                      | Hits | Total  | P value<sup>a</sup> | q-value<sup>b</sup> |
|-----------------------------------|------|--------|---------------------|---------------------|
| α-Linolenic acid metabolism       | 3    | 28     | 2.0E–4              | 3.0E–4              |
| Biosynthesis of plant hormones    | 4    | 304    | 0.0267              | 0.0055              |
| Flavone and flavonol biosynthesis | 1    | 4      | 0.0181              | 0.0043              |
| Metabolic pathways                | 12   | 1257   | 0.0024              | 0.0017              |
| Pentose and glucurionate interconversions | 2 | 32 | 0.0069 | 0.0027 |
| Starch and sucrose metabolism    | 3    | 106    | 0.0075              | 0.0027              |
| Taurine and hypotaurine metabolism | 1   | 4      | 0.0181              | 0.0043              |

<sup>a</sup> P ≤0.05.  
<sup>b</sup> q ≤0.05.

**Table 4.** Significant pathways for down-regulated genes in KEGG

| Pathway name                      | Hits | Total  | P value<sup>a</sup> | q-value<sup>b</sup> |
|-----------------------------------|------|--------|---------------------|---------------------|
| Biosynthesis of phenylpropanoids   | 5    | 247    | 0.0139              | 0.0139              |
| Methane metabolism                | 5    | 83     | 1.0E–4              | 2.0E–4              |
| Phenylalanine metabolism          | 5    | 81     | 1.0E–4              | 2.0E–4              |
| Phenylpropanoid biosynthesis      | 6    | 105    | <1.0E–4             | 2.0E–4              |
| Starch and sucrose metabolism     | 3    | 106    | 0.0234              | 0.0195              |
| Zeatin biosynthesis               | 2    | 22     | 0.0079              | 0.0099              |

<sup>a</sup> P ≤0.05.  
<sup>b</sup> q ≤0.05.
but the subsequent signal transduction is different between these two processes.

**Similarity of expression profiling between groups A and B**

The microarray data showed a high similarity of expression profiling between sample groups A and B (Supplementary Fig. S5). This was a reflection of the high similarity between these two groups. The only difference between groups A and B was grafting, which may be less influential at such an early developmental stage (1 dag). This high similarity also demonstrated that plants utilized the wound-induced programme to accomplish communication between the scion and stock. Activation of the ethylene and jasmonic acid biosynthesis pathway (Fig. 7) and the significant stimulus-response categories (Table 1) confirmed this assumption, and were also a reflection of the high similarity of expression profiling between groups A and B.

**What is the signal exchanged between scion and stock?**

In the model developed in this report, the development of seedling grafts involved several stages in Arabidopsis. However, the necrotic layer and callus reported previously in inflorescence stem grafts (Flaishman et al., 2008) were not observed. This was probably because of the high potential for division and differentiation of the young...
seedling cells. The reconnection of vasculature between the scion and stock is essential for a successful graft, and the results indicated a director role for auxin in vascular differentiation and reconnection. A well-established cell–cell communication network is obviously essential in exercising the appropriate functions of auxin, and this was corroborated by the results of the transcriptome analysis. The subsequent question of the molecular nature of the signals exchanged between scion and stock is becoming clearer.

In the differentially expressed genes, SWEET15 (AT5G13170, also named senescence-associated protein SAG29) and SWEET9 (AT2G39060) were recently identified as a new class of sugar transporters (Chen et al., 2010), and SWEET15 is located at the plasma membrane (Seo et al., 2011). CIPK14 (AT5G01820) is regulated by sucrose (Chikano et al., 2001). Sugars, as the energy source for plant growth and development, are also fundamental in coordinating metabolic fluxes in response to the changing environment (Ramon et al., 2008). It is conceivable that SWEET15 and SWEET9 perceive extracellular sucrose from the scion and transduce them into cells of the stock at the graft interface, in which CIPK14 incorporates the signals and transmits them to appropriate downstream effectors, thereby initiating cell–cell communication between cells of the scion and stock.

Oligosaccharides released from plant cell walls are powerful signalling molecules capable of conveying information to elicit multiple changes in plant defence responses (John et al., 1997). In the clearing of cell debris, oligosaccharides would be generated from the hydrolysis of cell walls and perceived by specific receptors, which will activate the signal cascade and consequently achieve cell–cell communication.

Plasmodesmata are important channels for signal transmission between plant cells in various plant processes, such as pollen mother-cell development (Wang et al., 1998). Secondary plasmodesmata will be substantially generated during graft-union development (Kollmann and Glockmann 1985). The expression levels of plasmodesmata-located protein PDLPIA (At5g43980) and At2g41870 were elevated in the microarray data. PDLPIA targets to the plasmodesmata and participates in plasmodesmal trafficking (Thomas et al., 2008). At2g41870 belongs to the remorin family, which clusters at plasmodesmata and is involved in potato virus X cell–cell movement, suggesting functionality in macromolecular trafficking through plasmodesmata (Raffaele et al., 2009). These results implicate that plasmodesmata may contribute to cell–cell communication in graft-union development.

These hypotheses need to be pursued further in future experiments.

Supplementary data

Supplementary data are available at JXB online.

Fig. S1. Grafts in tobacco.
Fig. S2. Grafts in alfalfa.
Fig. S3. GUS staining in DR5 scions.
Fig. S4. Procedure of microarray data analysis.

Fig. S5. Heatmap for differential expressed genes.
Table S1. Primers used in quantitative RT-PCR.
Table S2. The 306 graft-specific genes.

Supplementary methods. Operational procedures for grafting in tobacco, tomato, and alfalfa.

Supplementary file 2. This Excel document lists the 306 significantly altered genes.

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