**Title:** Cell-to-Cell Connection in Plant Grafting – Molecular Insights into Symplasmic Reconstruction

**Running Title:** Cell-to-Cell Connection in Plant Grafting

**Corresponding Author:** M. Notaguchi

Bioscience and Biotechnology Center, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, Aichi, 464-8601 Japan

Tel: +81-52-789-5714

Fax: +81-52-789-5714

Email: notaguchi.michitaka@b.mbox.nagoya-u.ac.jp

**Subject Areas**: (6) structure and function of cells, (7) membrane and transport

**Number of black and white figures, color figures, tables and type and number of supplementary materials**:

Black and white figures: 0

Color figures: 1

Tables: 1

Type and number of supplementary materials: 0

**Title:** Cell-to-Cell Connection in Plant Grafting – Molecular Insights into Symplasmic Reconstruction

**Running Title:** Cell-to-Cell Connection in Plant Grafting

**Authors:** Ken-ichi Kurotani[1], Michitaka Notaguchi[1,2,3,*]

[1]Bioscience and Biotechnology Center, Nagoya University, Nagoya, Japan

[2]Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya, Japan

[3]Institute of Transformative Bio-Molecules (ITbM), Nagoya University, Nagoya, Japan

*__Corresponding Author:__ Michitaka Notaguchi

Email: notaguchi.michitaka@b.mbox.nagoya-u.ac.jp

Fax: +81-52-789-5714

**Abstract**

Grafting is a means to connect tissues from two individual plants and grow a single chimeric plant through establishment of both apoplasmic and symplasmic connections. Recent molecular studies using RNA-sequencing data have provided genetic information on the processes involved in tissue reunion, including wound response, cell division, cell-cell adhesion, cell differentiation, and vascular formation. Thus, studies on grafting increase our understanding of various aspects of plant biology. Grafting has also been used to study systemic signaling and transport of micro- and macromolecules in the plant body. Given that graft viability and molecular transport across graft junctions largely depend on vascular formation, a major focus in grafting biology has been the mechanism of vascular development. In addition, it has been thought that symplasmic connections via plasmodesmata are fundamentally important to share cellular information among newly proliferated cells at the graft interface and to accomplish tissue differentiation correctly. Therefore, this review focuses on plasmodesmata formation during grafting. We take advantage of interfamily grafts for unambiguous identification of the graft interface and summarize morphological aspects of *de novo* formation of plasmodesmata. Important molecular events are addressed by re-examining the time-course transcriptome of interfamily grafts, from which we recently identified the cell-cell adhesion mechanism. Plasmodesmata-associated genes upregulated during graft healing that may provide a link to symplasm establishment are described. We also discuss future research directions.

**Keywords: cell-to-cell connection, grafting, plasmodesmata, symplasm, symplasmic transport, transcriptome**

## Introduction

Grafting has been an important technique in agriculture for millennia to propagate tree species that have valuable traits and are mostly genetically heterogeneous. In recent years, certain vegetables have been propagated by grafting to increase disease resistance, regulate fruit quality, and improve yields (Lee and Oda, 2003). In plant biological research, grafting has been frequently used to generate genetic chimeras, and to study systemic signaling and interorgan transport of diverse endogenous molecules, including phytohormones, metabolites, small RNA species, mRNAs, secreted peptides, and proteins (Goldschmidt, 2014, Tsutsui and Notaguchi, 2017, Thomas and Frank, 2019). Given its agricultural and scientific importance, molecular analysis of the graft healing process has been initiated to determine the mechanism underlying tissue reunion at the graft wound surfaces.

In practice, grafting is mostly performed by joining a stem from one plant (the scion) onto the root system of another plant (the stock). Vascular formation is required to establish graft reunion and viability of the grafts. During graft healing, the apoplasmic and symplasmic domains are gradually rebuilt within several days to weeks. For completion of vascular formation, a sequence of events, such as cell division, cell-cell adhesion, and cell differentiation, in appropriate positions occurs beforehand in the graft region to reconnect each tissue (Goldschmidt, 2014, Melnyk, 2017). Molecular analyses have been conducted whereby *Arabidopsis thaliana* micrografting has been mainly used as a model system. Auxin action is a primary step in grafting. Polar auxin transport is abolished when the stem is cut, resulting in accumulation of a high concentration of auxin at the base of the scion (Yin et al. 2012, Melnyk et al. 2015, Matsuoka et al. 2016, Melnyk et al. 2018). The graft efficiency of numerous mutants defective in auxin signaling has been examined and some mutants show reduced phloem transport (Melnyk et al. 2015). Similarly, sugar also accumulates in the scion part after grafting, thus the involvement of sugar in graft healing has been proposed (Marsch-Martínez et al. 2013, Melnyk et al. 2018, Tsutsui et al. 2020). Transcriptome analysis of Arabidopsis micrografting has revealed up- or downregulation of sugar-responsive genes, such as ADP-glucose pyrophosphorylase-encoding gene

(*ApL3*), *DARK INDUCIBLE 6* (*DIN6*), *GLUTAMATE DEHYDROGENASE 1* (*GDH1*) and *SUGAR TRANSPORTER 1* (*STP1*) (Melnyk et al. 2018). In the early stages of grafting, elongated and expanded cells in the epidermis, cortex, and endodermis tissues fill the space at the graft boundary, followed by proliferation of cells from cambial and vascular tissues (Matsuoka et al. 2016). Auxin-induced NAC transcription factors, ANAC071 and ANAC096, promote the proliferation of vascular tissues in the graft region (Matsuoka et al. 2016). Cell-cell adhesion at the graft boundary occurs, especially in the cell division area. Genes that encode cell wall modification enzymes are highly upregulated after grafting (Cookson et al. 2013, Ren et al. 2018, Xie et al. 2019, Notaguchi et al. 2020). Elevated expression of β-1,4-glucanase, β-1,3-glucanase, xyloglucan hydrolase, and expansin is detectable by 1 day after grafting (DAG) and thereafter the expression level increases gradually. Laser microdissection (LMD) analysis detected a high expression level of these genes in proliferated cells in the vascular region at the graft boundary compared with that of cells of other tissues (Notaguchi et al. 2020). Crucial roles of these enzymes in cell-cell adhesion have been demonstrated by analyses of the glycosyl hydrolase 9B3 clade of β-1,4-glucanases (Notaguchi et al. 2020, Kurotani et al. 2020). After sequential tissue-dependent cellular events, vascular tissues differentiate and connect the severed vasculature to restore the systemic transport system. Transcriptome analyses have identified cambial activity genes, such as *WUSCHEL RELATED HOMEOBOX 4* (*WOX4*), *PHLOEM INTERCALATED WITH XYLEM* (*PXY*) and *TARGET OF MONOPTEROS 6* (*TMO6*), master genes for xylem formation, such as *VASCULAR RELATED NAC-DOMAIN PROTEIN 7* (*VND7*) and *BIFUNCTIONAL NUCLEASE I* (*BFN1*), and marker genes associated with phloem formation, such as *NAC020*, *NAC086*, and *OCTOPUS* (*OPS*) (Melnyk et al. 2018, Notaguchi et al. 2020, Kurotani et al. 2020). Thus, the process of vascular formation during grafting has been intensively studied and molecular knowledge of the process has increased dramatically in recent years.

From a cellular perspective, the symplasmic domains are considered important for sharing cellular information among proliferated cells in the grafted region as found in normal plant growth and

development and probably for tissue differentiation in the correct positions. Plant cells construct cell-to-cell symplasmic connections, termed plasmodesmata, between adjacent cells to facilitate the transport of low-molecular-weight nutrients and macromolecules, such as proteins, mRNAs, and small RNAs. Plasmodesmata, the membrane-lined channels that perforate the plant cell wall and exchange molecules between neighboring cells, are important in the formation of symplasmic domains to coordinate plant growth at all stages of development. Plasmodesmata are formed primarily on the cell plates during cell division, and increase in number secondarily and/or change their structure. To understand the molecular mechanism of nano-scale microchannel formation and factors that regulate plasmodesmata permeability, proteome analysis and genetic screening have indicated that many molecules are fundamentally associated with plasmodesmata function (Oparka, 2004, Brunkard et al. 2015, Nicolas et al. 2017a, Sun et al. 2019, Pankratenko et al. 2020). In addition, ultrastructural observation of plasmodesmata using transmission electron microscopy (TEM) has provided insights into their detailed structure, molecular components, and functional aspects in time and space (Stonebloom et al. 2009, Xu et al. 2012, Nicolas et al. 2017b, Ross-Elliott et al. 2017). The dynamics of structural changes and the complex molecular regulation of plasmodesmata function remain largely unknown. For an improved understanding of the roles of plant symplasmic domains, it is preferable to monitor plasmodesmata behavior in plant cells using tracer molecules, and to identify genes associated with the development and function of plasmodesmata. In grafting, plasmodesmata are formed *de novo* at the graft interface and molecules are transported between grafted tissues through the plasmodesmata. Therefore, understanding the molecular mechanism of grafting will provide insights into the development and function of plasmodesmata.

Practical observation of secondary plasmodesmata formation at the graft boundary has been conducted using interfamily graft combinations. In interfamily grafts, the boundary between two grafted plant species can be identified by observation of organelles characteristic of the scion and stock cells, whereas distinguishing the graft boundary has been a challenge in conventional grafts between

close relatives. Hence, we hereafter summarize previous observations of symplasmic reconstruction and describe plasmodesmata morphology in the interfamily graft interface. Upregulated genes potentially associated with symplasmic domain formation during interfamily grafting are listed to gain insights into the formation and function of cell-to-cell connections in plants.

**Functional Aspect – Symplasm in Interfamily Grafting**

In general, it has been thought that symplasmic connections are constructed in the graft interface, and traffic micro- and macromolecules between cells, tissues and, ultimately, distant organs. Typically, grafting is accomplished between closely related species within a family, but occasional interfamily graft combinations have been reported and the morphological and physiological features characterized. Funk (1929) and Simon (1930) performed grafting between *Solanum melongena* (Solanaceae) and *Iresine lindenii* (Amaranthaceae) and observed that one or two cells attached tightly between the two species at the graft boundary. Cell-cell attachment was observed only in a small partial region of the graft boundary, whereas the remaining region was lined with a necrotic layer and no tissue adhesion was noted. Nickell (1948) further demonstrated the potential for interfamily grafting. Grafting of *Melilotus alba* (Fabaceae) onto *Helianthus annuus* (Asteraceae) was performed, and the development of normal healthy plants and vascular differentiation in the pith parenchyma at 3 weeks after grafting was reported. An additional interfamily combination of *Vicia faba* (Fabaceae) and *H. annuus* was intensively analyzed by Kollmann and colleagues in the 1980s and 1990s. In this interfamily graft, cells that proliferated at the graft junction were well attached. The cell wall between adjacent cells of the two species was often structurally indistinguishable from division walls (Kollmann and Glockmann, 1985). Sieve element formation was initiated approximately 4–6 DAG and increased over 1 month in the interfamily graft, but the number of sieve elements was fewer than that of intrafamily grafts (Rachow-Brandt and Kollmann, 1992). Symplasmic transport was tested using radioactive carbon isotopes. In the interfamily graft, translocation into the stock across the graft junction comprised only

2% of the photosynthates in the leaves of the isotope-fed scion. Translocation in the corresponding homografts attained approximately 30%–40% of the photosynthates (Rachow-Brandt and Kollmann, 1992). Thus, the limited graft formation in interfamily grafts leads to reduced detection of symplasmic connections.

We recently demonstrated that certain plant groups, such as the Solanaceae genus *Nicotiana* and parasitic plants in the Orobanchaceae and Convolvulaceae families, show an exceptional capability for tissue connection by interfamily grafting (Notaguchi et al. 2020, Kurotani et al. 2020, Okayasu et al. 2021, Fig. 1A). As demonstrated previously, one characteristic of interfamily grafting involving *Nicotiana* and parasitic species is that scion viability is preserved by parenchymatous tissue in the graft interface. Therefore, the healing processes in interfamily grafting tend to be prolonged compared with those of conventional intrafamily grafting, thereby allowing description of the concomitant, sequential biological events that occur during tissue healing. Another characteristic is that the range of potential graft partners for these species is broadly expanded to angiosperms; interfamily grafting of *Nicotiana* has been successful with 73 species from 38 families. Thus, we further investigated the morphology of interfamily grafts and tested whether symplasmic connection is established in interfamily grafting using several methods. Our observations of interfamily grafts revealed that tissue union occurred less uniformly in the graft interface and was likely to be patchy compared with that of intrafamily graft combinations. Generally, when members of different families are grafted, a necrotic layer is formed in the graft interface, and the scion and stock tissues do not connect. However, in interfamily grafting with *Nicotiana* and parasitic species, partial tissue adhesion occurs at the graft boundary. Dye tracer experiments using carboxyfluorescein, a symplasmic tracer, provided evidence for establishment of symplasmic transport of micromolecules in interfamily grafting using *Nicotiana* (Notaguchi et al. 2020) and an Orobanchaceae member, *Phtheirospermum japonicum* (Kurotani et al. 2020). Transport of mRNAs and green fluorescent proteins (GFP) across the graft junction was also detected in *Nicotiana* interfamily grafting, thus indicating that the established symplasmic connection can traffic

macromolecules (Notaguchi et al. 2015, Notaguchi et al. 2020). The timing of symplasmic reconstruction was estimated to be 3 DAG or later in time-course experiments using a tracer dye (Notaguchi et al. 2020). Although the transport of molecules across the graft interface was reduced compared with that of conventional intrafamily grafting, these observations indicate that reconstruction of symplasmic domains is accomplished in interfamily grafting.

In nature, plants that parasitize species belonging to a different plant family have evolved a haustorium, a specialized organ that invades host plant tissues and absorbs nutrients following tissue adhesion (Westwood et al. 2010). Symplasmic domains are also constructed between parasitic plants and their host plants. Therefore, such plants potentially have the ability to establish symplasmic connections among members of different families.

**Morphological Aspect – *De novo* Formation of Plasmodesmata in the Graft Interface**

On the basis of the observation of symplasmic molecular transport, symplasmic domains are predicted to be constructed at the stock–scion interface in intrafamily grafting. However, morphological analysis has revealed that vascular connection is limited in previously identified and *Nicotiana* interfamily grafts (Kollmann and Glockmann, 1985, Notaguchi et al. 2020). In *Nicotiana* interfamily grafts, cell proliferation and thin xylem bridge formation is observed in the graft interface region, but phloem continuity has not been clearly observed. Similar phenomena are detected at the contact site of parasitic plant and host tissues. No clear phloem connection has been also observed in some cases of host–parasite interaction, such as hemiparasites *Striga* and *Triphysaria* spp (Yoshida et al. 2016). Symplasmic transport of endogenous mRNAs, GFP proteins and carboxyfluorescein tracer between host and parasite plants have demonstrated that symplasmic domains are formed (e.g. David-Schwartz et al, 2008, Aly et al. 2011, Kim et al. 2014).

The question then is whether plasmodesmata formation is achieved at the interface of interfamily grafts. At the tissue-connecting sites, newly formed plasmodesmata bridging cell walls in

interspecific graft unions have been detected with TEM. In the interfamily graft between *V. faba* and *H. annuus*, single and branched plasmodesmata were observed in areas where the cell wall was thinner than elsewhere (Kollmann and Glockmann, 1985). In addition to continuous plasmodesmata, incomplete half plasmodesmata were observed in the interfamily graft (Kollmann et al, 1985). In our recent studies, electron microscopic observation of the graft interface between *N. benthamiana* and *A. thaliana* 4 DAG showed that the cell walls were clearly digested resulting in cell-cell attachment (Notaguchi et al. 2020, describing the detailed method) and that, if we observed such adhesive region, structures considered to be plasmodesmata were newly formed between cells of *N. benthamiana* and *A. thaliana* (Fig. 1B, C), similar to those formed between cells of *A. thaliana* (Fig. 1D) and between cells of *N. benthamiana* (Fig. 1E). At 14 DAG, the cell walls of the two species were more closely adjacent at the interface (Fig. 1F, G). The graft boundary is identifiable by the chloroplast characteristics of each plant cell (Fig. 1H, I). *De novo* formation of plasmodesmata between cells of the two species was observed especially in a region where the cell walls were highly degraded. The *de novo* plasmodesmata may serve as symplasmic domains in interfamily grafting. The plasmodesmata observed in Fig. 1G appeared to extend from a *N. benthamiana* cell to the *A. thaliana* cell. The detection of entire plasmodesmata structure by TEM is sometimes difficult because the plane on which the section is made must be exactly aligned with the axis of the plasmodesmata to be observed in its entirety. Recently, Nicolas et al (2017b) used electron tomography to obtain 3-dimensional structure of plasmodesmata and showed that the plasmodesmata structure, including the contact sites of endoplasmic reticulum and plasma membrane, undergoes substantial remodeling during cell differentiation. This indicates that plasmodesmata ultrastructure dynamically changes to regulate their function as symplasmic pathways for molecular exchange between neighboring cells. In our observations of interfamily grafting, sequential sections were prepared from areas with signs of plasmodesmata penetration and observed by TEM (Fig. 1J, K). We observed slightly wavy structures between *N. benthamiana* and *A. thaliana* cells, which were not completely continuous in each section,

and were considered to be plasmodesmata formed between cells of the two species (Fig. 1L–N). Half plasmodesmata reaching to cell wall middle lamella were also observed to be formed from *N. benthamiana* and *A. thaliana* cells (Fig. 1O, P). Thus, plasmodesmata are newly formed at the graft boundary and structural variation is observed.

**Molecular Aspect – Grafting-induced Genes Associated with Plasmodesmata Biology**

Given that reconstruction of tissue connections seems to be prolonged in interfamily grafting, the transcriptional reaction is also considered to be dynamic and significant. Indeed, transcriptome analyses of interfamily grafting have enabled identification of a large suite of genes associated with cell-cell adhesion as well as tissue reconstruction (Notaguchi et al. 2020). Hence, such gene profiles in interfamily grafting may also provide meaningful information to understand symplasmic domain construction. Since cells are newly proliferated through cell division at graft boundary, genetic information related to both primary and secondary plasmodesmata formation should be included in transcriptome data. Given that we observed that the symplasm of interfamily-grafted *Nicotiana* began to be established gradually around 3 DAG in a previous study, we reanalyzed our transcriptome data sets from the stems of *N. benthamiana* grafted onto the inflorescence stem of *A. thaliana* with a focus on the 189 genes upregulated by 1 DAG and that continued to be upregulated at 3 and 7 DAG.

Among the 189 upregulated genes, the gene ontology (GO) terms enriched in the cellular component category included “Extracellular region”, “Cell wall”, “Apoplast”, and “Plasmodesmata” (Notaguchi et al. 2020). Fourteen genes were annotated with the GO term “Plasmodesmata” (Table 1). These genes, further described below, encode proteins involved in binding to the plasma membrane, cell wall modification, transport of signals and substances, and response to abiotic stresses such as wounding. Their expression was confirmed in the connection boundary region of the graft by LMD. Seven of the 14 genes were expressed in the vasculature (Vas) and pith (Pith) areas near the graft surface, rather than inside tissue (In), which is farther from the graft surface (Table 1). Eight of the 14

genes encode proteins included in the 1341 proteins detected in the proteome analysis of plasmodesmata purified from the cell wall fraction of Arabidopsis suspension cells (Fernandez-Calvino et al. 2011, Table 1). In addition to the previously known plasmodesmata proteins such as plasmodesmata-located proteins1 (PDLP1), β-1-3 glucanase (AtBG_PPAP), calreticulin and remorin, this proteome data contains plasmodesmata-associated regulators, such as glycosylphosphatidylinositol (GPI)-anchored proteins, receptor-like kinases (RLKs) and transmembrane proteins. Another plasmodesmata proteome analysis reported 41 proteins, including plasmodesmata callose binding proteins (PDCBs) and callose synthases (CalSs). One of these genes, *tetraspanin1* (*TET1*), was included in the 14 grafting-induced genes (Grison et al. 2015). It was noted that almost no direct association of these genes with plasmodesmata has been found, and hence no annotation has been provided in previous reports on the proteome, with the exception of tetraspanin (Fernandez-Calvino et al. 2011, Grison et al. 2015). Therefore, these genes are expected to provide insights to understanding the hidden aspects of plasmodesmata biology.

Niben101Scf15391g05004 is an ortholog of Arabidopsis *TET1*. *TETRASPANINs* encode conserved transmembrane proteins that function in intracellular communication in animals. In plants, *tornado*, a mutant of *TET1*, participates in root morphogenesis (Cnops et al. 2000, Wang et al. 2015). It was suggested that tetraspanin recruits specific proteins to plasmodesmata and forms tetraspanin-enriched microdomains in plasmodesmata in plants (Fernandez-Calvino et al. 2011), similar to lipid rafts in animal cells, which play roles in the membrane compartmentalization of biological processes such as cell-cell adhesion, signal transduction, and intracellular trafficking (Stipp et al. 2003, Yunta and Lazo, 2003).

Niben101Scf04102g03015 and Niben101Scf11091g00001 are orthologs of AT4G28250 and encode expansin B3, an enzyme that binds to the plasma membrane and loosens the cell wall (Cosgrove, 2000, Lee et al. 2001). The expression of these genes was particularly enriched in Vas rather than In (Table 1). The plasmodesmata observed on the interfamily graft surface are located at sites where the

cell wall is highly degraded and the distance between adjacent cells is reduced (Fig. 1F, G). Hence, loosening of the cell wall may precede *de novo* formation of plasmodesmata. In addition, we showed in our previous report that a clade of the large glycosyl hydrolase family, designated GH9B3, which degrades cellulose in the cell wall, is important for graft establishment (Notaguchi et al. 2020).

Niben101Scf03226g01002 and Niben101Scf06743g00014 are orthologs of AT4G00430 and are considered to encode PLASMA MEMBRANE INTRINSIC PROTEIN 1;4 (PIP1;4). This protein, also termed aquaporin, is involved in the formation of water channels (Jang et al. 2007). Activation of this gene is logical because ensuring water communication between cells is vital where the xylem is severed. A related gene, Niben101Scf21986g00008, is also activated. This gene encodes a MIZU-KUSSEI-like protein, which is involved in root hydrotropism (Kobayashi et al. 2007, Yamazaki et al. 2012), and may be induced by insufficient water supply during grafting to inquire water location. The genes Niben101Scf02914g02043, Niben101Scf02145g09006, Niben101Scf03483g00002, and Niben101Scf06017g00002 encode GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE 2 (GAPC-2), ASPARTIC PROTEASE A1 (APA1), L-ascorbate oxidase, and PHOSPHATE TRANSPORTER 1;4 (PHT1;4), respectively. These proteins may also be involved in the response to disruption of water and inorganic nutrient supply immediately after grafting (Cruz de Carvalho et al. 2001, Yamamoto et al. 2005, Arnaud et al. 2014, Han et al. 2015, Kim et al. 2020, Sebastián et al. 2020).

Another gene for which the molecular function in grafting has not been examined is Niben101Scf00207g02007, which encodes MULTIPLE C2 DOMAIN AND TRANSMEMBRANE REGION PROTEIN 16 (MCTP16). Arabidopsis *MCTP16* is phylogenetically most closely related to *QUIRKY* (*MCTP15*), which is a well-characterized member of the *MCTP* gene family. QUIRKY, together with the receptor-like kinase STRUBBELIG, co-localizes in plasmodesmata and is considered to regulate root morphogenesis (Vaddepalli et al. 2014). The aforementioned genes are considered to

cooperatively regulate morphogenesis by sharing information between cells, but more detailed analyses of their functions are required.

*Glycine max* is a plant species that cannot be successfully grafted in interfamily combinations. Among the 189 genes upregulated in *Nicotiana* interfamily grafts, 79 genes were not clearly upregulated in the interfamily grafts of *G. max* (Notaguchi et al. 2020). Thus, these 79 genes are characteristically upregulated in *N. benthamiana* and are likely to be essential for establishment of interfamily graft unions. Two of the 14 plasmodesmata-associated genes were among the genes that show *N. benthamiana*-characteristic expression patterns. These genes, Niben101Scf06483g00007 and Niben101Scf06628g01013, are orthologs of *A. thaliana* AT2G34790 and AT4G15800, which encode a FAD-binding Berberine family protein and a RAPID ALKALINIZATION FACTOR (RALF) protein, respectively. In *A. thaliana*, AT2G34790 has been identified as ATBBE-LIKE 15, and the protein X-ray crystallography and activity as a monolignol oxidoreductase have been reported (Daniel et al. 2015). The locus AT4G15800 encodes RALF-LIKE 33, a secreted small-peptide signal that is widely conserved in plants and has the ability to alkalinize extracellular media. The physiological roles of RALF-LIKE 33 are activation of a MAP kinase, mobilization of calcium, inhibition of root growth, and cell expansion (Campbell and Turner, 2017). In addition, the ANJEA-FERONIA receptor kinase complex functions as a stamen gatekeeper by binding to RALF23 and 33 to induce production of reactive oxygen species in the papillae of stamens (Liu et al. 2021). These two genes may play roles in the establishment of interfamily grafts through the reconstruction of cell walls, but the association with plasmodesmata is unclear. Further studies on their molecular functions are required to understand the mechanism of wound healing in interfamily grafting.

Of the 189 upregulated genes, in addition to the 14 genes annotated with the “Plasmodesmata” GO term, we identified other genes that may be involved in the formation and functional regulation of plasmodesmata. For example, Niben101Scf14799g01012 encodes a member of the glycosyl hydrolase superfamily, a relatively closely related to *AtBG_PPAP* in phylogenetic tree. *AtBG_PPAP* is a crucial

regulator of callose turnover in plasmodesmata and may be involved in the formation of plasmodesmata and/or regulation of plasmodesmata permeability (Benitez-Alfonso et al. 2013, Storme and Geelen, 2014). Niben101Scf02069g00018 encodes expansin A24. Expansin has been described above for EXPB3, but there have been reports of *Nicotiana benthamiana* α-expansin (NbEXPA1) in relation to plasmodesmata (Park et al. 2017). NbEXPA1, a protein that loosens the cell wall, was shown to localize to plasmodesmata in response to viral infection and to promote virus migration. This suggests that the regulation of plasmodesmata permeability correlates with the defense response. However, the EXPA genes form a very large superfamily, and Niben101Scf02069g00018 and Niben101Scf20887g00008 (NbEXPA1) are not very close in the phylogenetic tree.

Parasitic plants, which survive by forming a vascular union with the host plant to absorb water and nutrients, also show capabilities for interfamily grafting (Kurotani et al. 2020, Okayasu et al. 2021). Symplasmic connection in parasitism has been observed through detection of the transport of macromolecules, such as GFP and RNAs (Aly et al. 2011, Tomilov et al. 2008, Roney et al. 2007). In interfamily grafting with *P. japonicum*, symplasmic connection was observed using a tracer dye (Kurotani et al. 2020). In *P. japonicum*, 1699 genes showed overlapping temporal expression patterns between parasitism and grafting (Kurotani et al. 2020). The GO analysis of these 1699 genes revealed that the most enriched GO terms in the cellular component category were “Nucleolus”, “Ribosome”, and “Cytosol”, but “Plasmodesmata” also showed significant enrichment ($p < 0.01$). Ninety plasmodesmata-associated genes were detected in *P. japonicum*. Although no direct overlap in annotation with the 14 plasmodesmata-associated genes detected in *N. benthamiana* interfamily grafting was observed, some functional similarities, such as water stress response, signal peptides, modification of cell wall components, and cell elongation, were apparent. Therefore, elucidation of the potential relationships of these identified biological processes will facilitate an improved understanding of plasmodesmata development and regulation. These functionally similar 13 genes found in *P.*

*japonicum* interfamily grafting are also listed in Table 1. It should be noted that seven of the 13 genes listed here also encode proteins included in the plasmodesmata proteome (Fernandez-Calvino et al. 2011, Table 1).

Thus, although many of the genes extracted by transcriptome analysis of grafted plants have not been shown to be directly related to plasmodeamata, their predicted functions, phenotypes of the mutants, and sequence homology suggest that they may be involved in the structure formation and functionality of plasmodesmata. In the future, localization analysis of these genes and evaluation of the effects of deletion and overexpression of these genes on the permeability of plasmodesmata will be required.

**Future Perspectives**

Grafting is achieved through a series of biological processes occurring sequentially, such as cell proliferation, cell-cell attachment at the interface, and tissue reunion accompanied with apoplasmic and symplasmic transports. In addition to moving water, phytohormones, sugars and nutrients, the vascular tissues transport many macromolecules. In grafts, it has been shown that proteins with organelle targeting sequences, such as peroxisomes, actin and nucleus, are transported over graft junction (Paultre et al. 2016). Molecular transports over grafted junction can sometime change physiology and morphology as represented by flowering time control (Goldschmidt, 2014, Tsutsui and Notaguchi, 2017, Thomas and Frank, 2019). Horizontal transfer of entire plastid, mitochondrial, or nuclear genomes have been also found at the graft boundary region which could be a future application to generate new hybrids, especially providing advantage for plants which cannot be crossed through pollination (Stegemann and Bock, 2009, Thyssen et al. 2012, Fuentes et al. 2014, Gurdon et al. 2016). , The mechanism of genome transfer has been studied and recently reported that plastid movement into neighboring cells at the graft boundary has been captured using organelle marker lines. Intercellular connections newly emerge by partial cell wall disintegration, forming connective pores through which

plastids move between cells (Hertle et al. 2021). Thus, in addition to new plasmodesmata formation, it seems that grafting can induce symplasmic domains in other typical manner. It is possible that high activity of cell wall modifications enzymes found in grafting may localize in the cell wall and trigger the disintegration of cell wall in part (Cookson et al, 2013, Ren et al, 2018, Xie et al, 2019, Notaguchi et al. 2020). In addition, it is interesting to ask the relation between the emergence of such intercellular connections and factors involved in plasmodesmata formation. How much graft formation relies on each symplasmic domain/pathway is remained to be clarified.

Observations of tissue/cellular events occurring in inner tissues at graft junction help us to understand the complex reunion process. Micro-Computed Tomography (Micro-CT) imaging and magnetic resonance imaging (MRI) are powerful tools to scan tissues in time course without invasion, although further improvement in their spatial resolution is demanded specially to aim at observing specific tissue/cellular domains such as symplasmic pathways. Combining genetical approach would be a powerful way to investigate these questions. This article summarizes information accumulated from interfamily grafting together with the gene information. Genes associated with plasmodesmata formation and regulation have been screened from the transcriptome of graft unions, tissue healing after wounding, and tissue parasitized by parasitic plants. The other data sets for grafting (Chen et al. 2017, Melnyk et al. 2018, Assunção et al. 2019, Xie et al. 2019) will be helpful to detect candidate genes crucial for plasmodesmata biology. To determine the roles of each gene, investigation of the effects of gene mutation or overexpression on plasmodesmata formation and function is required. Electron microscopic analysis is an ideal method with which to observe plasmodesmata ultrastructure. Moreover, super-resolution imaging using fluorescent markers has been developed and applied for plasmodesmata observation (Fitzgibbon et al. 2010, Bell and Oparka, 2015, Bell et al. 2016). Cell-to-cell molecular transport also must be tested to assess plasmodesmata permeability. We recently established a system to trap plant cultured cells in a microfluidic device for stable observation without moving the sample and examined plasmodesmata permeability (Shimizu et al. 2021). Use of such a

sample fixing system in combination with genetic tools and markers is well suited to quantify plasmodesmata permeability. Insights from transcriptome analyses of grafting will further enhance knowledge of the molecular mechanism of primary and secondary plasmodesmata formation, molecular components that regulate plasmodesmata structure, and modification of the transport capacities of plasmodesmata.

## Data Availability

The RNA-seq data underlying this article are available in the DNA Data Bank of Japan (www.ddbj.nig.ac.jp) , and can be accessed with DRA009936 and DRA010010.


## Funding

This work was supported by grants from the Japan Society for the Promotion of Science Grants-in-Aid for Scientific Research (18H03950 and 21H00368 to MN and 20H03273 to KK and MN) and the Project of the NARO Bio-oriented Technology Research Advancement Institution (Research Program on Development of Innovative Technology 28001AB to MN).


## Author Contributions

KK and MN conceptualized, wrote, and approved the manuscript.

## References


Abuqamar, S., Ajeb, S., Sham, A., Enan, M.R. and Iratni, R. (2013). A mutation in the *expansin-like A2* gene enhances resistance to necrotrophic fungi and hypersensitivity to abiotic stress in *Arabidopsis thaliana*. *Mol. Plant Pathol.* 14, 813–27.

Aly, R., Hamamouch, N., Abu-Nassar, J., Wolf, S., Joel, D.M., Eizenberg, H. et al. (2011). Movement of protein and macromolecules between host plants and the parasitic weed *Phelipanche aegyptiaca* Pers, *Plant Cell Rep.* 30: 2233–41.

Arnaud, C., Clément, M., Thibaud, M-C., Javot, H., Chiarenza,S., Delannoy, E. et al. (2014). Identification of Phosphatin, a Drug Alleviating Phosphate Starvation Responses in Arabidopsis. *Plant Physiol.* 166: 1479–1491.

Assunção, M., Santos, C., Brazão, J., Eiras-Dias, J.E. and Fevereiro, P. (2019). Understanding the molecular mechanisms underlying graft success in grapevine. *BMC Plant Biol.* 19: 396.

Bar, M., Leibman, M., Schuster, S., Pitzhadza, H. and Avni, A. (2013). EHD1 Functions in Endosomal Recycling and Confers Salt Tolerance. *PLOS ONE* 8: e54533.

Bell, K. and Oparka, K. (2015). "Preparative Methods for Imaging Plasmodesmata at Super-resolution", in Plasmodesmata: Methods and Protocols, ed. M. Heinlein (New York, NY: Springer New York), 67–79.

Bell, K., Oparka K. and Knox, K. (2016). Super-resolution Imaging of Live BY2 Cells Using 3D-structured Illumination Microscopy. *Bio-protocol* 6, e1697.

Benitez-Alfonso, Y., Faulkner, C., Pendle, A., Miyashima, S., Helariutta, Y. and Maule, A. (2013). Symplastic intercellular connectivity regulates lateral root patterning. *Dev. Cell* 26: 136–147.

Brunkard, J.O., Runkel, A.M. and Zambryski, P.C. (2015). The cytosol must flow: intercellular transport through plasmodesmata. *Curr. Opin. Cell Biol.* 35: 13–20.

Campbell, L. and Turner, S.R.A. (2017). Comprehensive Analysis of RALF Proteins in Green Plants Suggests There Are Two Distinct Functional Groups. *Front. Plant Sci.* 24.

Chen, Z., Zhao, J., Hu, F., Qin, Y., Wang, X. and Hu, G. (2017). Transcriptome changes between compatible and incompatible graft combination of Litchi chinensis by digital gene expression profile. *Sci. Rep.* 7: 3954.

Cnops, G., Wang, X., Linstead, P., Van Montagu, M., Van Lijsebettens, M. and Dolan, L. (2000). Tornado1 and tornado2 are required for the specification of radial and circumferential pattern in the Arabidopsis root. *Development* 127: 3385–3394.

Cookson, S.J., Moreno, M.J.C., Hevin, C., Mendome, L.Z.N., Delrot, S., Trossat-Magnin, C. et al. (2013). Graft union formation in grapevine induces transcriptional changes related to cell wall modification, wounding, hormone signalling, and secondary metabolism. J. Exp. Botany 64: 2997–3008.

Cosgrove, D.J. (2000). Loosening of plant cell walls by expansins. *Nature*, 407: 321-326.

Cruz de Carvalho, M.H., d'Arcy-Lameta, A., Roy-Macauley, H., Gareil, M., El Maarouf, H., Pham-Thi, A.T. et al. (2001). Aspartic protease in leaves of common bean (Phaseolus vulgaris L.) and cowpea (Vigna unguiculata L. Walp): enzymatic activity, gene expression and relation to drought susceptibility. *FEBS Lett*. 492: 242–246.

Goldschmidt E.E. (2014). Plant grafting: new mechanisms, evolutionary implications. *Front Plant Sci* 5: 727.

Daniel, B., Pavkov-Keller, T., Steiner, B., Dordic, A., Gutmann, A., Nidetzky, B. et al. (2015). Oxidation of Monolignols by Members of the Berberine Bridge Enzyme Family Suggests a Role in Plant Cell Wall Metabolism. *J. Biol. Chem.* 290: 18770–18781.

David-Schwartz, R., Runo, S., Townsley, B., MacHuka, J. and Sinha, N. (2008). Long-distance transport of mRNA via parenchyma cells and phloem across the host-parasite junction in *Cuscuta*. *New Phytol.* 179:1133–41.

Fernandez-Calvino, L., Faulkner, C., Walshaw, J., Saalbach, G., Bayer, E., Benitez-Alfonso, Y. et al. (2011). Arabidopsis plasmodesmal proteome. *PLoS ONE* 6: e18880.

Fitzgibbon, J., Bell, K., King, E., and Oparka, K. (2010). Super-resolution imaging of plasmodesmata using three-dimensional structured illumination microscopy. *Plant Physiol.* 153: 1453–1463.

476 Fuentes, I., Stegemann, S., Golczyk, H., Karcher, D. and Bock, R. (2014) Horizontal genome transfer
477 as an asexual path to the formation of new species. *Nature* 511: 232–235.
478 Funck, R. (1929). Untersuchengen über heteroplastische transplantationen bei Solanaceen und
479 Cactaceen. *Beitr Biol Pflanzen* 17: 404–468.
480 Grison, M.S., Brocard, L., Fouillen, L., Nicolas, W., Wewer, V., Dörmann, P. et al. (2015). Specific
481 membrane lipid composition is important for plasmodesmata function in Arabidopsis. *Plant Cell*
482 27: 1228–1250.
483 Gurdon, C., Svab, Z., Feng, Y., Kumar, D. and Maliga, P. (2016). Cell-to-cell movement of
484 mitochondria in plants. *Proc. Natl. Acad. Sci. U.S.A.* 113: 3395–3400.
485 Han, S., Wang, Y., Zheng, X., Jia, Q., Zhao, J., Bai, F. et al. (2015). Cytoplastic glyceraldehyde-3-
486 phosphate dehydrogenases interact with ATG3 to negatively regulate autophagy and immunity in
487 *Nicotiana benthamiana*. *Plant Cell* 27: 1316–1331.
488 Hertle, A.P., Haberl, B., and Bock, R. (2021). Horizontal genome transfer by cell-to-cell travel of whole
489 organelles. *Science Advances*, 7: eabd8215.
490 Jang, J.Y., Lee, S.H., Rhee, J.Y., Chung, G.C., Ahn, S.J. and Kang, H. (2007). Transgenic *Arabidopsis*
491 and tobacco plants overexpressing an aquaporin respond differently to various abiotic stresses.
492 *Plant Mol. Biol.* 64: 621–632.
493 Kim, D., Yang, J., Gu, F., Park, S., Combs, J., Adams, A. et al. (2021). A temperature-sensitive
494 *FERONIA* mutant allele that alters root hair growth. *Plant Physiol.* 185: 405–423.
495 Kim, G., LeBlanc, M., Wafula, E.K., Depamphilis, C.W. and Westwood, J.H. (2014). Genomic-scale
496 exchange of mRNA between a parasitic plant and its hosts. *Science* 345:808–11.
497 Kim, S.C., Guo, L. and Wang, X. (2020). Nuclear moonlighting of cytosolic glyceraldehyde-3-
498 phosphate dehydrogenase regulates Arabidopsis response to heat stress. *Nat Commun.* 11: 3439.
499 Kobayashi, A., Takahashi, A., Kakimoto, Y., Miyazawa, Y., Fujii, N., Higashitani, A. et al. (2007). A
500 gene essential for hydrotropism in roots. *Proc. Natl. Acad. Sci.* 104: 4724–4729.

Kollmann, R. and Glockmann, C. (1985). Studies on graft unions. I. Plasmodesmata between cells of plants belonging to different unrelated taxa. *Protoplasma* 124: 224–235.

Kollmann, R., Yang, S. and Glockmann, C. (1985). Studies on graft unions II. Continuous and half plasmodesmata in different regions of the graft interface. *Protoplasma* 126: 19-29.

Kurotani K., Wakatake T., Ichihashi Y., Okayasu, K., Sawai, Y., Ogawa, S. et al. (2020). Host-parasite tissue adhesion by a secreted type of β-1,4-glucanase in the parasitic plant *Phtheirospermum japonicum*. *Commun. Biol.* 3: 407.

Lee, J. and Oda, M. (2003). Grafting of herbaceous vegetable and ornamental crop. *Hort. Rev.* 28: 61–124.

Lee, Y., Choi, D. and Kende, H. (2001). Expansins: ever-expanding numbers and functions. *Curr. Opin. Plant Biol*., 4: 527–532.

Linster, E., Stephan, I., Bienvenut, W. V., Maple-Grødem, J., Myklebust, L. M., Huber, M. et al. (2015). Downregulation of N-terminal acetylation triggers ABA-mediated drought responses in *Arabidopsis*. *Nat. Commun.* 6: 7640.

Liu, C., Shen, L., Xiao, Y., Vyshedsky, D., Peng, C., Sun, X. et al. (2021). Pollen PCP-B peptides unlock a stigma peptide-receptor kinase gating mechanism for pollination. *Science* 372: 171–175.

Maaroufi-Dguimi, H., Debouba, M., Gaufichon, L., Clément, G., Gouia, H., Hajjaji, A. et al. (2011). An Arabidopsis mutant disrupted in *ASN2* encoding asparagine synthetase 2 exhibits low salt stress tolerance. *Plant Physiol. Biochem.* 49: 623–628.

Marsch-Martinez, N., Franken, J., Gonzalez-Aguilera, K. L., de Folter, S., Angenent, G., & Alvarez-Buylla, E. R. (2013). An efficient flat-surface collar-free grafting method for *Arabidopsis thaliana* seedlings. Plant Methods, 9: 14.

523 Matsuoka, K., Sugawara, E., Aoki, R., Takuma, K., Terao-Morita, M., Satoh, S. et al. (2016).
524 Differential Cellular Control by Cotyledon-Derived Phytohormones Involved in Graft Reunion of
525 Arabidopsis Hypocotyls. *Plant Cell Physiol.* 57: 2620–2631.
526 Melnyk C.W., Schuster C., Leyser O. and Meyerowitz E.M. (2015). A developmental framework for
527 graft formation and vascular reconnection in Arabidopsis thaliana. *Current Biol.* 25:1306–1318.
528 Melnyk, C.W. (2017). Plant grafting: insights into tissue regeneration. *Regeneration* 4: 3–14.
529 Melnyk C.W., Gabel, A., Hardcastle, T.J., Robinson, S., Miyashima, S., Grosse, I. et al. (2018).
530 Transcriptome dynamics at *Arabidopsis* graft junctions reveal an intertissue recognition
531 mechanism that activates vascular regeneration. *Proc. Natl. Acad. Sci. U.S.A.* 115: E2447–E2456.
532 Minami, A., Tominaga, Y., Furuto, A., Kondo, M., Kawamura, Y. and Uemura, M. (2015). Arabidopsis
533 dynamin-related protein 1E in sphingolipid-enriched plasma membrane domains is associated with
534 the development of freezing tolerance. *Plant J.* 83: 501–514
535 Moneo-Sánchez, M., Alonso-Chico, A., Knox, J.P., Dopico, B., Labrador, E. and Martín, I. (2018). β-
536 (1,4)-Galactan remodelling in Arabidopsis cell walls affects the xyloglucan structure during
537 elongation. *PLANTA* 249: 351–362.
538 Narendra, S., Venkataramani, S., Shen, G., Wang, J., Pasapula, V., Lin, Y.et al. (2006). The
539 *Arabidopsis* ascorbate peroxidase 3 is a peroxisomal membrane-bound antioxidant enzyme and is
540 dispensable for *Arabidopsis* growth and development. *J. Exp. Botany* 57: 3033–3042.
541 Nickell, L.G. (1948) Heteroplastic Grafts. *Science* 108: 389.
542 Nicolas, W.J., Grison, M.S. and Bayer, E.M. (2017a). Shaping intercellular channels of
543 plasmodesmata: the structure-to-function missing link. *J. Exp. Bot.* 69: 91–103.
544 Nicolas, W.J., Grison, M.S., Trépout, S., Gaston, A., Fouché, M., Cordelières, F.P. et al. (2017b).
545 Architecture and permeability of post-cytokinesis plasmodesmata lacking cytoplasmic sleeves.
546 *Nat. Plants* 3: 17082.

547 Notaguchi, M., Higashiyama, T. and Suzuki, T. (2015). Identification of mRNAs that move over long
548 distances using an RNA-Seq analysis of Arabidopsis/Nicotiana benthamiana heterografts. *Plant*
549 *Cell Physiol.* 56: 311–321.
550 Notaguchi M, Kurotani K, Sato Y, Tabata, R., Kawakatsu, Y., Okayasu, K. et al. (2020). Cell–cell
551 adhesion in plant grafting is facilitated by β-1,4-glucanases. *Science,* 369: 698–702.
552 Okayasu, K., Aoki, K., Kurotani, K. and Notaguchi, M. (2021). Tissue adhesion between distant plant
553 species in parasitism and grafting. *Commun. Integ. Biol.* 14: 21–23.
554 Oparka, K.J. (2004). Getting the message across: how do plant cells exchange macromolecular
555 complexes? *Trends Plant Sci.* 9: 33–41.
556 Pankratenko, A.V., Atabekova, A.K., Morozov, S.Y., and Solovyev, A.G. (2020). Membrane Contacts
557 in Plasmodesmata: Structural Components and Their Functions. *Biochemistry (Mosc)* 85: 531–
558 544.
559 Paultre, D.S.G., Gustin, M.P., Molnar, A., Oparka, K.J. (2016). Lost in transit: long-distance trafficking
560 and phloem unloading of protein signals in Arabidopsis homografts. *Plant Cell* 28: 2016–2025.
561 Rachow-Brandt, G. and Kollmann, R. (1992). Studies on Graft Unions IV. Assimilate Transport and
562 Sieve Element Restitution in Homo- and Heterografts. *J. Plant Physiol.* 139: 579-583.
563 Ren, Y., Xu, Q., Wang, L., Guo, S., Shu, S., Lu, N. et al. (2018). Involvement of metabolic,
564 physiological and hormonal responses in the graft-compatible process of cucumber/pumpkin
565 combinations was revealed through the integrative analysis of mRNA and miRNA expression,
566 *Plant Physiol. Biochem.* 129, 368–380.
567 Roney, J.K., Khatibi, P.A. and Westwood, J.H. (2007). Cross-species translocation of mRNA from
568 host plants into the parasitic plant dodder, *Plant Physiol.* 143: 1037–43.
569 Ross-Elliott, T.J., Jensen, K.H., Haaning, K.S., Wager, B.M., Knoblauch, J., Howell, A.H. et al. (2017).
570 Phloem unloading in Arabidopsis roots is convective and regulated by the phloem-pole pericycle.
571 *Elife* 6: 02 2017.

572 Sampedro Jimenez, J., Valdivia, E.R., Fraga, P., Iglesias, N., Revilla, G. and Zarra, I. (2016). Soluble
573 and membrane-bound beta-glucosidases are involved in trimming the xyloglucan backbone. *Plant*
574 *physiol.* 173: 1017–1030.
575 Sebastián, D., Fernando, F.D., Raúl, D.G. and Gabriela, G.M. (2020). Overexpression of Arabidopsis
576 aspartic protease APA1 gene confers drought tolerance, *Plant Science* 292: 110406.
577 Shimizu, K., Kikkawa, M., Tabata, R., Kurihara, D., Kurotani, K., Honda, H. et al. (2021). Quantitative
578 Analysis of Plasmodesmata Permeability using Entrapped Cultured Tobacco BY-2 cells in
579 Microfluidic Chips. *bioRxiv.* 2021.02.19.431975
580 Simon, S.V. (1930). Transplantationsversuche zwischen Solanum melongena und Iresine Lindeni. *Jb.*
581 *Wiss Bot.* 72: 137–160.
582 Stegemann, S. and Bock, R. (2009). Exchange of genetic material between cells in plant tissue grafts.
583 *Science* 324, 649–651.
584 Stipp, C.S., Kolesnikova, T.V. and Hemler, M.E. (2003). Functional domains in tetraspanin proteins.
585 *Trends Biochem. Sci.* 28: 106–112.
586 Stonebloom, S., Burch-Smith, T., Kim, I., Meinke, D., Mindrinos, M. and Zambryski, P. (2009). Loss
587 of the plant DEAD-box protein ISE1 leads to defective mitochondria and increased cell-to-cell
588 transport via plasmodesmata. *Proc. Natl. Acad. Sci. U. S. A.* 106: 17229–34.
589 Storme, N. and Geelen, D. (2014). Callose homeostasis at plasmodesmata: molecular regulators and
590 developmental relevance. *Front. Plant Sci.* 21.
591 Sun, Y., Huang, D. and Chen, X. (2019). Dynamic regulation of plasmodesmatal permeability and its
592 application to horticultural research. *Hortic. Res.* 6: 47.
593 Thomas, H.R. and Frank, M.H. (2019). Connecting the pieces: uncovering the molecular basis for
594 long‐distance communication through plant grafting. *New Phytol*. 223: 582-589.

595 Thyssen, G., Svab, Z. and Maliga, P. (2012). Cell-to-cell movement of plastids in plants. *Proc. Natl.*
596 *Acad. Sci. U.S.A.* 109: 2439–2443.
597 Tomilov, A.A., Tomilova, N.B., Wroblewski, T., Michelmore, R. and Yoder, J. I. (2008). Trans-
598 specific gene silencing between host and parasitic plants, *Plant J.* 56: 389–97.
599 Tsutsui, H. and Notaguchi, M. (2017). The use of grafting to study systemic signaling in plants. *Plant*
600 *Cell Physiol*. 58: 1291-1301.
601 Tsutsui, H., Yanagisawa, N., Kawakatsu, Y., Ikematsu, S., Sawai, Y., Tabata, R. et al. (2020).
602 Micrografting device for testing environmental conditions for grafting and systemic signaling in
603 Arabidopsis. *Plant J.* 103: 918–929.
604 Vaddepalli, P., Fulton, L., Wieland, J., Wassmer, K., Schaeffer, M., Ranf, S. et al. (2017). The cell
605 wall-localized atypical β-1,3 glucanase ZERZAUST controls tissue morphogenesis in *Arabidopsis*
606 *thaliana*. *Development* 144:2259–2269.
607 Vaddepalli, P., Herrmann, A., Fulton, L., Oelschner, M., Hillmer, S., Stratil, T.F. et al. (2014). The C2-
608 domain protein QUIRKY and the receptor-like kinase STRUBBELIG localize to plasmodesmata
609 and mediate tissue morphogenesis in Arabidopsis thaliana. *Development* 141: 4139–4148.
610 Wang, F., Muto, A., Van de Velde, J., Neyt, P., Himanen, K., Vandepoele, K. et al. (2015). Functional
611 analysis of the Arabidopsis TETRASPANIN gene family in plant growth and development. *Plant*
612 *Physiol.* 169: 2200–2214.
613 Westwood, J.H., Yoder, J.I., Timko, M.P. and dePamphilis, C.W. (2010). The evolution of parasitism
614 in plants. *Trends Plant Sci.* 15: 227–235.
615 Xie, L., Dong, C. and Shang, Q. (2019). Gene co-expression network analysis reveals pathways
616 associated with graft healing by asymmetric profiling in tomato. *BMC Plant Biol.* 19: 373.
617 Xu, M., Cho, E., Burch-Smith, T.M. and Zambryski, P.C. (2012). Plasmodesmata formation and cell-
618 to-cell transport are reduced in decreased size exclusion limit 1 during embryogenesis in
619 Arabidopsis. *Proc. Natl. Acad. Sci. U. S. A.* 109: 5098–103.

620 Yamamoto, A., Bhuiyan, Md. N.H., Waditee, R., Tanaka, Y., Esaka, M., Oba, K. et al. (2005).
621 Suppressed expression of the apoplastic ascorbate oxidase gene increases salt tolerance in tobacco
622 and Arabidopsis plants, *J. Exp. Botany* 56: 1785–1796.
623 Yamazaki, T., Miyazawa, Y., Kobayashi, A., Moriwaki, T., Fujii N. and Takahashi, H. (2012). MIZ1,
624 an essential protein for root hydrotropism, is associated with the cytoplasmic face of the
625 endoplasmic reticulum membrane in Arabidopsis root cells. *FEBS Lett.* 586: 398–402.
626 Yin, H., Yan, B., Sun, J., Jia, P., Zhang, Z., Yan, X. et al. (2012). Graft-union development: a delicate
627 process that involves cell-cell communication between scion and stock for local auxin
628 accumulation. *J. Exp. Botany* 63: 4219–4232.
629 Yunta, M. and Lazo, P.A. (2003). Tetraspanin proteins as organisers of membrane microdomains and
630 signalling complexes. *Cell. Signal.* 15: 559–564.
631 Zhang, Q., Lin, F., Mao, T., Nie, J., Yan, M., Yuan, M. et al. (2012). Phosphatidic Acid Regulates
632 Microtubule Organization by Interacting with MAP65-1 in Response to Salt Stress in *Arabidopsis*.
633 *Plant Cell* 24: 4555–4576.
634
635 **Figure legends**
636 **Figure 1.** Plasmodesmata are formed between interfamily grafted tissues. (A) Interfamily graft
637 between *Nicotiana benthamiana* and *Arabidopsis thaliana*. Arrow indicates the sampling area for the
638 transcriptome analysis. (B) to (P) Transmission electron micrographs (TEMs) of near the graft interface
639 between *N. benthamiana* (*Nb*) and *A. thaliana* (*At*). (B) Near the graft interface at 4 days after grafting
640 (DAG). (C) and (D) High-magnification TEMs of plasmodesmata formed in the cell wall between *N.*
641 *benthamiana* and *A. thaliana* (C), and between two *A. thaliana* cells (D) (regions in B enclosed by
642 solid and dashed rectangles, respectively). (E) Plasmodesmata between *N. benthamiana* cells. (F) Near
643 the graft interface at 14 DAG. The area indicated by red arrows is a region where the cell wall is
644 digested and the distance between cells of the two plant species is reduced. CW, cell wall. Arrowheads

indicate plastids. (G) High-magnification TEM of the plasmodesmata in the dashed rectangle in (F). Arrowheads indicate plasmodesmata formed between *N. benthamiana* and *A. thaliana* cells. (H) and (I) TEMs of plastids of *N. benthamiana* (H) and *A. thaliana* (I). (J) to (N) TEMs of plasmodesmata formed between *N. benthamiana* and *A. thaliana* cells at 4 DAG. Light blue arrowheads indicate plasmodesmata in *A. thaliana* cells, and magenta arrowheads indicate those in *N. benthamiana* cells. (K) Schematic diagram of TEMs overlaid as sequential sections. (L) to (N) Sequential TEMs of the area enclosed in the dashed rectangle in (J). (O) and (P) Half plasmodesmata formed between *N. benthamiana* and *A. thaliana* cells. Arrowheads indicate plasmodesmata formed from a *N. benthamiana* cell (O) and an *A. thaliana* cell (P). All samples were prepared according to the methods described by Notaguchi et al (2020). Scale bars, (A) 1 cm, (B) and (F) 10 μm, (C) to (E), (G) to (P) 1 μm.

**Table 1** Upregulated genes annotated with the GO term "Plasmodesmata" detected in the transcriptome of *Nicotiana benthamiana* and *Phtheirospermum japonicum* under interfamily grafting.

| ***Nb* or *Pj* gene ID (*At* gene ID)** | **Gene description** | **Keywords** | **Expression patterns** | **References** |
|---|---|---|---|---|
| Niben101Scf04102g03015, Niben101Scf11091g00001 (AT4G28250 *) | EXPB3, expansin B3 | cell wall | Vas<br>Vas | Cosgrove, 2000,<br>Lee et al. 2001 |
| Niben101Scf02145g09006 (AT1G11910 *) | APA1, aspartic proteinase A1 | drought tolerance | In | Cruz de Carvalho et al. 2001, Sebastián et al. 2020 |
| Niben101Scf21986g00008 (AT3G25640) | MIZU-KUSSEI-like protein | hydrotropism | Pith,Vas | Kobayashi et al. 2007, Yamazaki et al. 2012 |
| Niben101Scf06483g00007 (AT2G34790) | ATBBE-LIKE 15, FAD-binding Berberine family protein | lignin | In | Daniel et al. 2015 |
| Niben101Scf00207g02007 (AT5G17980 *) | MCTP16, C2 calcium/lipid-binding plant phosphoribosyltransferase family protein | non-cell-autonomous tissue morphogenesis | Vas | Vaddepalli et al. 2014 |
| Niben101Scf06628g01013 (AT4G15800 *) | RALF-LIKE 33 | peptide signal | In | Campbell and Turner, 2017, Liu et al. 2021 |
| Niben101Scf06017g00002 (AT2G38940 *) | PHT1,4, phosphate transporter 1;4 | Pi starvation | In,Vas | Arnaud et al. 2014 |
| Niben101Scf15391g05004 (AT5G46700 *†) | TET1, Tetraspanin family protein | root development | Vas | Cnops et al. 2000,<br>Wang et al. 2015 |
| Niben101Scf03483g00002 (AT5G21105) | Plant L-ascorbate oxidase | salt tolerance | Vas | Yamamoto et al. 2005 |

| | | | | |
|---|---|---|---|---|
| Niben101Scf03226g01002, Niben101Scf06743g00014 (AT4G00430) | PIP1;4, plasma membrane intrinsic protein 1;4 | water cannel | In<br>In | Jang et al. 2007 |
| Niben101Scf02914g02043 (AT1G13440 *) | GAPC-2, glyceraldehyde-3-phosphate dehydrogenase C2 | water loss | In | Han et al. 2015,<br>kim et al. 2020 |
| Niben101Scf01241g02002 (AT2G37870) | Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein | | In | |
| Pjv1_00006974-RA (AT3G13750 *) | BGAL1, Beta galactosidase 1 | cell wall | | Moneo-Sánchez et al. 2018 |
| Pjv1_00020551-RA (AT4G38400) | EXLA2, Expansin-like A2 | cell wall | | Abuqamar et al. 2013 |
| Pjv1_00010594-RA (AT5G20950 *) | BGLC1, Glycosyl hydrolase family protein | cell wall | | Sampedro et al. 2016 |
| Pjv1_00001160-RA (AT1G64760 *) | ZERZAUST, O-Glycosyl hydrolases family 17 protein | cell wall | | Vaddepalli et al. 2017 |
| Pjv1_00006904-RA (AT3G16570) | RALF23, Rapid alkalinization factor 23 | peptide signal | | Liu et al. 2021,<br>Kim et al. 2021 |
| Pjv1_00010337-RA (AT4G35000) | APX3, Ascorbate peroxidase 3 | antioxidation | | Narendra |
| Pjv1_00008376-RA (AT5G65010 *) | ASN2, Asparagine synthetase 2 | salt tolerance | | Maaroufi-Dguimi et al. 2011 |
| Pjv1_00010056-RA (AT3G57530 *) | CPK32, Calcium-dependent protein kinase 32 | nutrient signalling | | Liu et al. 2017 |
| Pjv1_00012706-RA (AT3G60190 *) | DL1E, DYNAMIN-like 1E | freezing tolerance | | Minami et al. 2015 |
| Pjv1_00012393-RA (AT3G20290) | EHD1, EPS15 homology domain 1 | salt tolerance | | Bar et al. 2013 |
| Pjv1_00022953-RA (AT5G03870 *) | Glutaredoxin family protein | stress response | | |
| Pjv1_00012821-RA (AT2G38720) | MAP65-5, Microtubule-associated protein 65-5 | salt response | | Zhang et al. 2012 |
| Pjv1_00018162-RA (AT1G80410) | NAA15, Tetratricopeptide repeat (TPR)-containing protein | drought response | | Linster et al. 2015 |

Asterisks and dagger indicate whose proteins have been detected in plasmodesmata proteome previously reported, respectively (Fernandez-Calvino et al. 2011, Grison et al. 2015). Expression patterns: In, inside tissue; Vas, vasculature; Pith, pith.

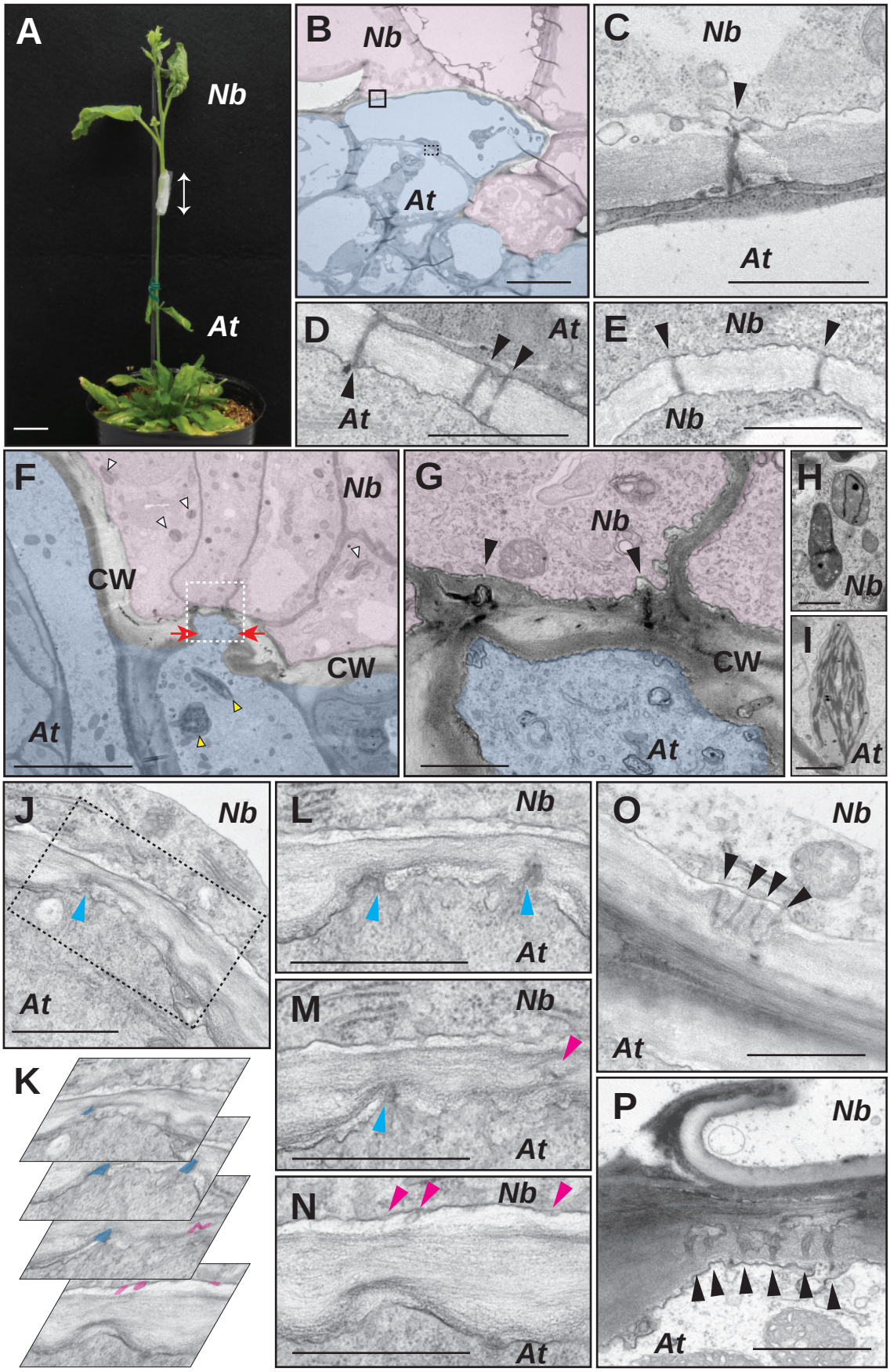
A
Nb
At
B
Nb
At
C
Nb
At
D
At
At
E
Nb
Nb
F
Nb
CW
CW
At
G
Nb
CW
At
H
Nb
I
At
J
Nb
At
K
L
Nb
At
M
Nb
At
N
Nb
At
O
Nb
At
P
Nb
At

**Figure 1.**