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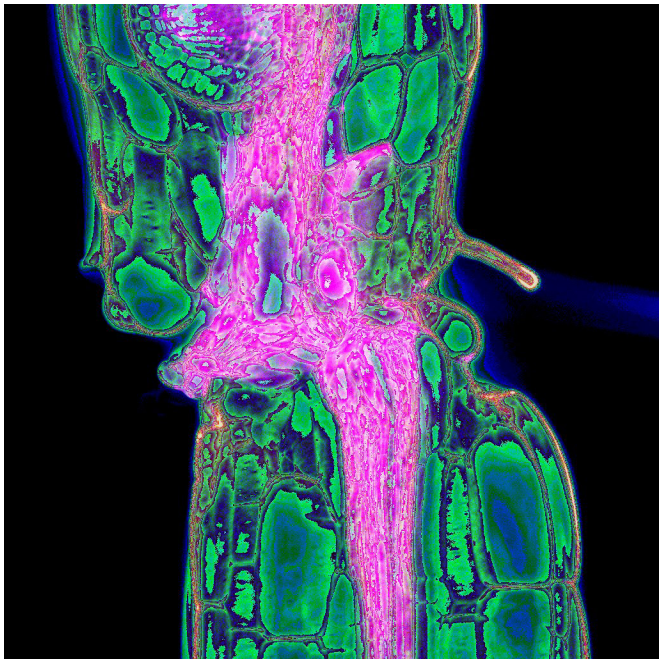
Cellular mechanisms of plant tissue regeneration

Head over heals

Phanu Theodore Serivichyaswat



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Phanu Theodore Serivichyaswat
Faculty of Natural Resources and Agricultural Sciences
Department of Plant biology
Uppsala

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Cover: Regenerating cells in Arabidopsis graft junction as seen by the author during one of his meditation trips.
(photo: P.T. Serivichyaswat)

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Abstract

Plants are remarkable at healing. Humankind has long known about this and has exploited it by grafting different plants together for crop propagation and crop improvement. During graft formation, the surfaces of the cut tissues attach, the cells proliferate, and the vasculatures reconnect to form a chimeric plant. Although grafting is extensively used in fruit and vegetable crop production, our fundamental understanding of how a graft forms is limited. In this current thesis, we described the cellular mechanisms of graft regeneration. We found that auxin signalling is crucial in the procambial cells during tissue adhesion, callus formation, phloem reconnection, to form a successful graft connection (Paper I). Investigating the effects of environmental factors on graft regeneration demonstrated that high temperatures promoted graft formation via leaf-derived auxin signaling (Paper II). Additionally, we found that plant parasitism, a grafting-like process, also shared a common feature by enhancing inter-plant vascular connections upon elevated temperatures. Lastly, we showed that parasitic plants regulated their infection organ development in the presence of nitrogen via abscisic acid (Paper III). Altogether, the work in this thesis expands our fundamental knowledge of tissue regeneration by highlighting plant's developmental plasticity.

Keywords: regeneration, grafting, auxin signaling, elevated temperature, Arabidopsis, parasitic plant, developmental biology, plant adaptation.

Cellulära mekanismer för växtvävnadsregenerering

Abstrakt

Växter har en anmärkningsvärd förmåga att läka. Detta har vi känt till länge och utnyttjat genom att ympa samman delar från olika individer för förökning och för ökad kvalitet. Vid ympning fäster snittytorna vid varandra, cellerna förökar sig och kärlen återansluts för att bilda en chimär växt. Även om ympning används flitigt i produktion av frukt- och grönsaker, är vår grundläggande förståelse för hur en ymp bildas begränsad. I den här avhandlingen beskrivs de cellulära mekanismerna för regeneration vid ympning. Vi fann att auxinsignalering är avgörande i de prokambiala cellerna under vävnadsvidhäftning, kallusbildning, floemåterkoppling, vilket bidrar till en framgångsrik ympanslutning (Paper I). Undersökning av miljöfaktorernas effekt på regeneration vid ympning visade att höga temperaturer främjar ympanslutning via bladmedierad auxinsignalering (Paper II). Dessutom fann vi att förhöjda temperaturer också förbättrar kärlkopplingar mellan växter vid växtparasitism, som är en ympliknande process. Slutligen visade vi att parasitiska växter reglerar utvecklingen av sina infektionsorgan i närvaro av kväve via abscisinsyra (Paper III). Sammantaget utökar arbetet i denna avhandling vår grundläggande kunskap om vävnadsregeneration genom att påvisa växternas utvecklingsmässiga plasticitet.

Nyckelord: ympning, regenerering, auxinsignalering, förhöjd temperatur, Arabidopsis, parasitväxt, utvecklingsbiologi, växtanpassning.

Dedication

To my British Shorthair Björnando Serivichyaswat de Tailandia y Suecia, you are my joy and delight.

“*บางครั้งเราชนะ บางคลาวเราได้เรียนรู้*”

- ซาร่า

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This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. **Serivichyaswat P.T.** and **Melnyk C.W**. (2022). Cellular mechanisms of graft development in *Arabidopsis thaliana*. (Manuscript)

II. **Serivichyaswat P.T, Bartusch K, Leso M, Musseau C, Iwase A, Chen Y, Sugimoto K, Quint M, Melnyk C.W.** (2022). High temperature perception in leaves promotes vascular regeneration and graft formation in distant tissues. Development, 149 (5).

III. **Kokla, A., Leso, M., Zhang, X., Simura, J., Phanu T. Serivichyaswat, Cui, S., Ljung, K., Yoshida, S., and Melnyk, C.W.** (2022). Nitrogen represses haustoria formation through abscisic acid in the parasitic plant *Phtheirospermum japonicum*. Nature communications, 13 (2976).

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The contribution of Phanu Theodore Serivichyaswat to the papers included in this thesis was as follows:

I. Planned, performed, analyzed the experiments, and wrote the manuscript.

II. Planned, performed, analyzed the experiments, and wrote the manuscript.

III. Performed gene cloning and plasmid constructions for generation of transgenic *Phtheirospermum japonicum* roots.

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1. Introduction

Domestication of wild plants into food crops marks the origin of agriculture, an invention that transformed humankind from a hunter-gather to a sedentary species. Since then, we have been collectively progressing with innovations in plant production. Early domestication of trees plants involved clonal propagation, as some plant species root from cuttings easily. Ancient gardeners took notes that other woody species did not root as easily, and invented grafting to allow asexual propagation of desirable tree varieties that could not otherwise be propagated (Mudge *et al.* 2009). The grafting technique is defined by the cutting and joining of two plants resulting in a single chimeric plant with different genetic composites. Grafting involves attaching the cut shoot, referred to as the scion, that will continue to grow branches and the cut root or stem, referred to as the rootstock, that will function as the root system. The scion-rootstock interphase is where the tissues and vascular connection occurs, referred as the graft junction. Grafting is still being practiced today for crop production, from trees to vegetables (Warschefsky *et al.* 2016). Grafting is therefore a significant tool in the history of plant domestication and modern-day agriculture. Even though grafting has been practiced for millennia and largely contributed to humankind, our fundamental understanding of how plants graft is still not well described. The work presented in this thesis aims to understand the biological basis of graft development.

1.1 Grafting in agriculture

Arguably one of the most ancient plant production technologies, the origin of grafting is still uncertain due to the lack of substantial written records. To date, the earliest evidence of deliberate grafting has been suggested to be from ancient Chinese literature dating back to as early as 2205-2197 BCE, a mention of grafting citrus plants likely for dwarfing (Cooper & Chapot 1978). Thereafter, the transfer of technology westward may have been done though the silk industry with mulberry grafting in 300 BCE (Juniper & Mabberley 2006), then became wide spread throughout the Roman era (Mudge *et al.* 2009) (Fig 1A). Grafting was first practiced with tree species such as citrus, apple, and plum (Fig 1B-C, E), and was later also adopted for vegetable production, including pepper, eggplants, tomato, and melons (Lee *et al.* 2010b). Today, vegetable grafting on the commercial scale is done through nurseries rather than individual farmers. Grafting robots have been developed for robust graft production, with as little as 4.5 sec per graft with 95% survival rates (Lee & Oda 2002; Kubota *et al.* 2008) (Fig 1D).

Traditionally, grafting has been used for vegetative propagation where clonal propagation is difficult to achieve (Mudge *et al.* 2009), but grafters later discovered other desirable effects of grafting. These include size manipulation such as apple scion dwarfing when a size-controlled rootstock is used (Juniper & Mabberley 2006). Grafting can also promote systemic vigor, for instance, the amount of fertilizers needed for grafted watermelon is reduced by half (Lee & Oda 2002). Rootstocks are often used to confer resistance against several soil-borne pathogens including *Fusarium*, *Verticillium*, *Phytophthora*, *Pseudomonas*, *Didymella bryoniae*, *Monosporascus cannonballus*, and nematodes (Edelstein *et al.* 1999; Ioannou 2001; Cohen *et al.* 2005). Rootstocks can also improve abiotic stress tolerance such as extreme temperature, heavy metal contaminant, salinity, and flooding (Lee *et al.* 2010b). In many vegetable crops, significant yield increase, regardless of diseases and abiotic stresses, can be achieved by grafting vegetables crops with selected rootstocks, as reported in tomato (Chung & Lee 2007), watermelon, and cucumber (Lee & Oda 2002). Besides

food production, ornamental plants are also grafted to create unusual growth form, called arboriculture, for aesthetic purposes (Mudge *et al.* 2009) (Fig 1F).

Figure 1 Evolution of grafting methods. (A) A third-century Roman mosaic showing tree grafting from St. Roman-en-gal, France (Source: last-of-the-romans.tumblr.com). (B) The 17th-century grafting technique technique (Sharrock 1672). (C) Modern day apple grafting (University of Massachusetts Amherst). (D) Automated grafting machine developed in the Netherlands for tomato and eggplant grafting (Bayer AG). (E) Grafted vines in a vineyard in Queenstown, New Zealand (Photo taken by P.T. Serivichyaswat). (F) Tree of Forty Fruit #84 at The RockWell Museum created by an artist Sam Van Aken producing over 40 varieties of fruit including peach, plum, nectarine, apricot, cherry, and almond (Source: rockwellmuseum.org).

Grafting may be an invaluable tool to agriculture, but it also comes with dark sides. Although grafting has been used extensively to introduce resistance against several diseases, ironically, it is also responsible for the spread of many important plant diseases that have serious impacts on crop production. Several plant viruses and bacteria can be transmitted through graft junctions. Even mechanical contact from using contaminated grafting tools can easily spread pathogens (Barbosa *et al.* 2005; Bausher 2013). Apple phytoplasma

diseases are transmitted from the infected rootstock to the scion (Aldaghi *et al.* 2007). Grafting was reported to introduce a citrus viroid disease to avocado from an interspecific graft (Hadas *et al.* 1992; Thomas *et al.* 2022). Another practical challenge is graft incompatibility. Intra-species grafting is generally compatible and successful, but inter-species grafting is often problematic and incompatible. The scion of incompatible grafts may survive for years but without forming vasculature with the rootstock, resulting in stunted growth and reduced fruit size (Proebsting 1928). For example, peppers and tomato graft combinations result in a complete graft incompatibility (Kawaguchi *et al.* 2008; Thomas *et al.* 2022). Eggplant rootstock is compatible with tomato scion but not *vice versa*, as eggplant scion on tomato rootstock produces reduced eggplant fruit size (Suzuki & Komochi 1974). Furthermore, plant parasitism, a process which is biologically similar to grafting (Fig 2A-B), is tremendously devastating to agriculture. Like grafting, parasitic plants, such as dodder and mistletoes, fuse their tissues and form vascular connections with their host (Kokla & Melnyk 2018). Through the connected vasculatures, the plant parasites draw water and nutrients from their hosts (Birschwilks *et al.* 2006). They are also a pathogen vector that can readily transmit pathogens between their infecting host plants, even among different host species (Mikona & Jelkmann 2010; Leblanc *et al.* 2012)

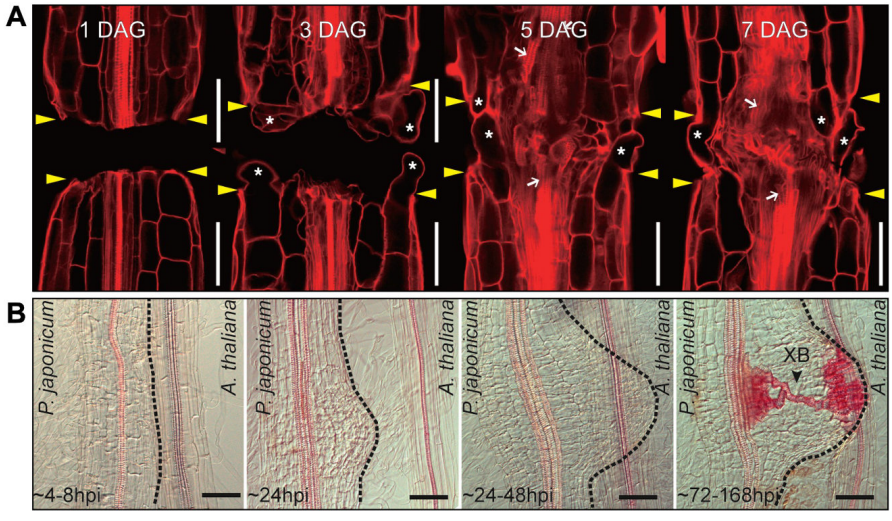

Figure 2. Comparing and contrasting graft formation and plant parasitism. (A) Morphology of Arabidopsis graft development at 1, 3, 5, 7 days after grafting. Yellow arrowheads: cut surface positions. White asterisks: cortical cell expansion. White arrows: differentiated xylem (Matsuoka et al 2016). (B) Development of parasitic plant *Phtheirospermum japonicum* (left) infecting its host Arabidopsis (right). XB: xylem bridge. Dashed line indicates the epidermis of the parasite. Scale bars = 100 µm (Kokla & Melnyk 2018)

1.2 Grafting in science

Grafting is an invaluable tool in agriculture as well as in scientific research. Because the grafted plant is comprised of two or more plant parts, botanists are keen to use the technique to study the movement of biological molecules and communication between the plant organs. However, the use of grafting for scientific purposes was not popular because most grafting techniques were mostly reported in crop plants that have limited genetic information and manipulation tools. Attempts have been made to develop grafting techniques in model organisms for this purpose. The first report of Arabidopsis grafting was done with inflorescence stems (Tsukaya *et al.* 1993) and later a grafting protocol in petunia was established (Napoli 1996). However, it was not until the report of micrografting technique, ie grafting of seedlings, in Arabidopsis (Turnbull *et al.* 2002), that the plant science community started widely

adopting this grafting technique into their studies, demonstrated by more than 100 publications including 'plant micrografting' per year (Tsutsui & Notaguchi 2017). Following this, we saw a rapid improvement of the Arabidopsis grafting protocol (Notaguchi *et al.* 2008; Bausher 2013; Marsch-Martinez *et al.* 2013; Yoo *et al.* 2013; Melnyk 2017) as well as development of micro-grafting in other model species such as tobacco and tomato (Marsch-Martinez *et al.* 2013; Notaguchi *et al.* 2020).

The advancements in our understanding of systemic and long-distance signaling were acclerated since the early 2000s largely due to grafting. Grafting of model species has enabled botanists to study systemic and long-distance signaling in plants including hormones, systemic pathogen resistance, flowering, stress responses, as well as macromolecule transport. In the original report of Arabidopsis micrografting, Turnbull et al (2002) showed that the shoot branching phenotypes of *max1* (*more axillary growth*) and *max3* mutants could be restored by a wild-type rootstock. Later studies demonstrated that *MAX1* and *MAX3* are strigolactone biosynthesis genes, and stringolactone is the mobile hormone (Gomez-Roldan *et al.* 2008; Umehara *et al.* 2008). Later works have used micrografting to provide supports for the mobile nature of other classes of phytohormones including auxin, gibberellin, cytokinin, and jasmonic acid (Lee & Howe 2003; Ragni *et al.* 2011; Ko *et al.* 2014; Li *et al.* 2017; Serivichyaswat *et al.* 2022).

The plant defense system and biotic interactions are thought to rely on systemic signaling. Upon infection, infected tissues signal to surrounding and distant organs for the activation of defense responses to prevent further infection. Plants infected with viruses produce small interfering RNAs (siRNAs) to contain viral spread (Ruiz *et al.* 1998), and this effect was observed to spread systemically (Voinnet & Baulcombe 1997). The movement of siRNAs against viruses was demonstrated by grafting transgenic rootstock producing siRNA to confer virus resistance in the wild-type scion (Song *et al.* 2013). Pathogenic bacteria also induce plant systemic defense. Plants express *CONSTITUTIVE DISEASE RESISTANCE 1* (*CDR1*) upon *Pseudomonas syringae* infection to activate defense (Xia *et al.* 2004). Transgenic rootstocks expressing *CDR1* could activate defense responses and confer resistance to the wild-type scions, but whether or not CDR1 is graft-transmissible is unclear. Grafting experiments were used to better

describe the symbiotic interaction between legumes and nitrogen-fixing bacteria through cytokinin signaling (Sasaki *et al.* 2014). Another ground-breaking discovery in plant biology is the identification of a mobile flowering inducing agent, ie florigen. Grafting was used to initially demonstrate that a hypothetical florigen was produced in leaves and then tranmitted to the shoot apical meristem (SAM) to induce flower development (Chailakhyan 1937). Later works then discovered the FLOWERING LOCUS T (FT) protein as the long-sought florogen, and its transport from leaves to SAM was also identified in part by using grafting (Corbesier *et al.* 2007; Jaeger & Wigge 2007; Lin *et al.* 2007; Notaguchi *et al.* 2008). Using cotyledon grafting, researchers found that a cotyledon of wild-type plant rescues the late-flowering phenotype of *ft* mutants, showing that the floral signaling from cotyledons is sufficient for early flowering in Arabidopsis seedlings under the long-day conditions (Yoo *et al.* 2013).

Movement of other macromolecues that systemically regulate long-distance gene expression such as peptides, including small-sized proteins and transcription factors (Rim *et al.* 2011; Okamoto *et al.* 2015), and RNAs, including small RNAs, miRNAs, mRNAs (Kim *et al.* 2001; Molnar *et al.* 2010), was all demonstrated using grafting. Exchange of genetic material between the scion and rootstock was also reported, but the movement is restricted to the tissues surrounding the graft juntion (Stegemann & Bock 2009). A work later showed that cells at the graft junction of two different tobacco varieties can fuse their genomes and generate a new stable polyploid species (Fuentes *et al.* 2014). Recent evidence has shown the long-distance movement of entire plastid organnels across the graft junction (Hertle *et al.* 2021). All of these observations suggest that grafting may contribute to horizontal gene transfer and speciation events during the evolution of plants.

1.3 Biology of graft formation

Although plant grafting has been adopted and extensively used to facilitate agriculture and scientific discoveries, to date, our understanding of graft development as a biological process is still lacking. Graft formation is a unique developmental process that requires tissue damage to activate developmental programming. Understanding graft junction formation could provide a better understanding of plant tissue regeneration, but studies are

limited by the lack of non-destructive and precise experimental methods. Nonetheless, several attempts have been made with available tools to elucidate the cellular and genetic mechanisms of grafting. The cellular structure of grafted plant tissues were first observed and reported in tomato and potato using a light microscope, describing the formation of a callus mass between the cut tissues aiding the reconnection (Fig 3A) (Wright 1893). The parenchyma cells just beneath the wounded sites were proposed to be giving rise to undifferentiated cells which then formed the callus. With the invention of scanning electron microscopy in the following century, we could observe the formation of pectinaceous beads on the cell surface during early graft formation, and also plasmodesmata connections between the opposing cells, followed by vascular formation (Fig 3B) (Jeffree & Yeoman 1983). After the establishment of Arabidopsis as a model organism, anatomical and histological analyses were used to examine graft junctions of Arabidopsis inflorescence grafts (Flaishman *et al.* 2008). The observations led to the proposal of four sequential developmental stages of graft healing: initial attachment of cut surfaces, callus proliferation at the graft junction, differentiation of new vasculatures within the scion, and full vascular connection.

The plant vascular system is critical for plant function as it is responsible for the relocation of resources throughout the plant as well as providing structural support. Upon being damaged, plants rapidly initiate regeneration processes to resume vascular transport. After cutting and re-joining, damaged cell walls at the cut site sites secrete oligosaccharide substances to activate defence and regeneration responses (Nuhse 2012). These oligosaccharides are thought to be the first step in graft healing by gluing the scion and rootstock together. The damage tissues also induce expression of auxin-induced cell-wall modifying enzymes XTH19 (xyloglucan endotransglucosylase/hydrolases19) and XTH20 to catalyse cell wall reconstruction and facilitate tissue adhesion (Pitaksaringkarn *et al.* 2014). A recent study done in a tobacco relative *Nicotiana benthamiana* (*N. benthamiana*) has shown that β-1,4-glucanases, a different group of cell-wall modifying enzymes, are also extracellularly released to the graft interface to facilitate the attachment. Transgenic Arabidopsis overexpressing this enzyme shows improved grafting. Moreover, using *N. benthamiana* as a middle section, called interstock, can facilitate grafting between a wide range

of distantly related species, concluding that initial tissue attachment is the key factor in graft success (Notaguchi *et al.* 2020). However, tissues of incompatible grafts also adhere similarly to their compatible counterparts (Moore 1984; Thomas & Frank 2019), arguing that tissue attachment is unlikely the sole factor for successful graft healing. Damage and modification of cellulose and pectin in the cell wall matrix also activates DNA-binding with one finger (*DOF*) transcription factors, which in turn up-regulate downstream regeneration genes to facilitate the subsequent graft healing processes (Zhang *et al.* 2022). In addition to grafting, wounding in Arabidopsis roots activates the transcriptional responses in the wound-adjacent cells to activate healing through auxin signaling (Hoermayer *et al.* 2020). These reports consistently suggest that perception of damaged tissues leads to activation of regeneration mechanism.

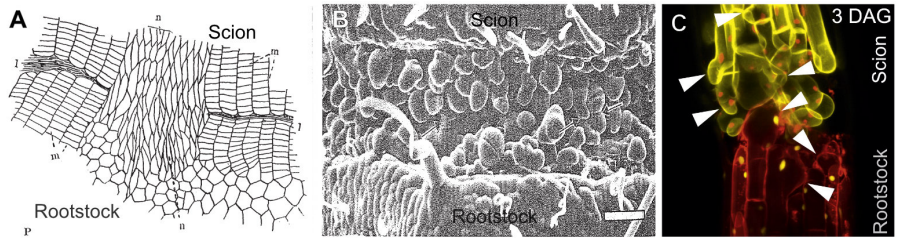

Figure 3. History of graft junction imaging. (A) A geranium graft junction drawn from an observation made under a light microscope (Wright 1893). (B) An image of tomato graft junction at 48 hours after grafting taken by SEM. Scale bar: 200μm (Jeffree & Yeoman 1983). (C) Fluorescent image of Arabidopsis graft junction at 3 days after grafting taken on a laser scanning confocal microscope (Melnyk et al 2015).

After successful tissue attachment, cells at the cut tissues expand and a mass of undifferentiated cells, referred to as callus, forms to fill the intercellular space, and promotes adhesion between the scion and rootstock (Jeffree & Yeoman 1983). While plants can generally develop callus from either wounded tissues or from exogenous hormone treatments, each of which has a different developmental mechanism (Ikeuchi *et al.* 2013), the developmental identity of the graft junction callus is still unclear. Because both wound-induced and graft junction formation has a common activating factor, ie mechanical damaging, it is speculated that graft-junction callus is similar to the wound-induced callus, as large amounts of callus are observed at the cut but ungrafted shoot (Melnyk *et al.* 2015). However, suppressing would-induced callus genes resulting in reduced callus size does not affect

graft formation (Melnyk *et al.* 2015), which may suggest that the size of callus formed is not necessarily corelated with grafting efficiency. On the other hand, *ABERRANT LATERAL ROOT FORMATION 4* (*ALF4*), a gene that is important for hormone-induced callus, is also required in the rootstock for graft formation (Sugimoto *et al.* 2010; Melnyk & Meyerowitz 2015), showing their similarity to a certain degree. Altogether, the literature suggests that graft-junction callus may follow a unique developmental program.

For over a century, researchers have been trying to investigate and find an answer to what cell types or tissues give rise to the graft junction callus. The first documented investigation reported an elongation of parenchyma cells toward the graft interphase and thus proposed that the parenchymal cells in the cortex may be responsible for callus formation at the graft junction. Later works using confocal microscopy of Arabidopsis graft junction revealed that, in addition to cortex, the epidermal cells are also expanding during graft healing (Melnyk *et al.* 2015; Matsuoka *et al.* 2016; Melnyk *et al.* 2018). The vascular cells of the scion near the graft junction start to proliferate prior to those in the rootstock during graft connection (Melnyk *et al.* 2015), consistent with the shoot-to-root directional auxin transport (Lomax *et al.* 1995). Meristem is a plant tissue consists of undifferentiated cells, also known as plant stem cells, that are capable of cell division and differentiation, ultimately gives rise to all plant tissue. Shoot apical meristem (SAM) generate the above-ground organs while root apical meristem is responsible for root development during the primary growth (Traas & Bohn-Courseau 2005). The meristem in the vasculature, called vascular cambium, accounts for secondary growth. In the vascular bundle, cambium is the layer of meristematic tissue that separates xylem and phloem. Through coordinated and oriented cell division programs, cambium divides and differentiate into xylem and phloem cells. *PHLOEM INTERCALATED WITH XYLEM* (*PXY*) is an important cambial regulator gene that maintains the cell polarity and division orientation during vascular development. Vascular bundles of *pxy* mutant lose the cell polarity, and thus phloem and xylem are interspersed and not separated by a distinct cambial layer (Fisher & Turner 2007). Because of its totipotency, cambium is widely assumed to give rise to the graft junction callus. However, up to date, the evidence supporting this assumption is still insufficient. This is largely due to the

technical difficulty of physically removing the cambial tissue and its function without killing the plant.

One key hormone in graft formation is auxin (Melnyk *et al.* 2015). Auxin is known for its role in promoting cell expansion, division, and wound healing (Asahina *et al.* 2011). Auxin functions through the TRANSPORT INHIBITOR RESPONSE1/ AUXIN SIGNALING F-BOX (TIR1/AFB) auxin response pathway. In low auxin conditions, Auxin/INDOLE-3-ACETIC ACID (Aux/IAA) and TOPLESS (TPL) proteins repress the activity of auxin response factors (ARFs) (Chapman & Estelle 2009; Leydon *et al.* 2021). When cellular levels of auxin elevate, TIR1/AFB binds to the conserved degradation domain II on Aux/IAA, and consequently triggers Aux/IAA polyubiquitylation and degradation by the proteasome. Once released from the repressors TPL and Aux/IAA, ARFs are free to recruit transcriptional machinery and initiate or repress gene expression (Kepinski & Leyser 2005; Chapman & Estelle 2009; Figueiredo & Strader 2022). In Arabidopsis, mutations in several Aux/IAA genes have been identified. One example is a gain-of-function mutation in *INDOLE-3-ACETIC ACID INDUCIBLE 12* (*IAA12*) called *bodenlos* (*bdl*). *bdl* blocks the auxin signaling pathway with a point mutation in its degradation domain, which prevents the binding of TIR1/AFB, and therefore prevents the subsequent protein degradation and ARFs remain sequestered (Hamann *et al.* 2002; Figueiredo & Strader 2022). Several works have suggested the involvement of auxin in graft formation. The activity of the auxin-responsive promoter *DR5* was detected at the graft junction, and mutants that perturb auxin related genes also showed graft failure (Melnyk *et al.* 2015). Consistently, inhibiting auxin transport from the shoot suppresses expression of an auxin responsive gene, *NAC DOMAIN CONTAINING PROTEIN 71* (*ANAC071*), which is involved in graft formation, and reduces cell proliferation and vascular reconnection at the graft junction (Matsuoka *et al.* 2016). Auxin is also crucial for vascular formation and phloem and xylem cell differentiation (Scarpella *et al.* 2006; Heo *et al.* 2014; Mäkilä *et al.* 2022), however increasing auxin levels by grafting with transgenic plants overexpressing auxin biosynthesis genes or exogeneous application of auxin did not improve vascular reconnection rates (Melnyk *et al.* 2015). In contrast, grafting with auxin-signaling mutants such as *aberrant lateral root formation 4* (*alf4*), *auxin resistany 1* (*axr1*), and *bodenlos* (*bdl*) mutants significantly inhibit

graft formation, suggesting that auxin signaling, rather than the excess cellular auxin levels, is critical for vascular reconnection (Melnyk *et al.* 2015). Auxin signaling is also required for coordination of wound healing in the outer layer of roots by activating a regeneration transcription factor ETHYLENE RESPONSE FACTOR 115 (ERF115) in the wound-adjacent cells to replenish the wound (Hoermayer *et al.* 2020). Moreover, parasitic plant infection, a process highly similar to graft connection, also employs auxin to facilitate the development of the infection organ (Ishida *et al.* 2016), known as the haustorium, highlighting the fundamental and conserved role of auxin signaling in plant regeneration.

Although our current knowledge of vascular reconnection during graft formation is still limited, understanding a closely related developmental process such as vein development can shed some light on how graft vascular reconnection occurs. During plant growth and development, veins form and connect new organs to the existing vasculatures. Vein patterning in leaves is controlled by auxin, as the hormone transported from the existing veins activates *ARABIDOPSIS THALIANA HOMEOBOX8* (*ATHB-8*) gene, a marker for procambial specification, in the adjacent undifferentiated ground meristematic cells, which consequently differentiate into procambium and veins, respectively (Scarpella *et al.* 2004; Donner *et al.* 2009). Root and leaf vascular developments share similar genetic mechanisms, and identification of several leaf vascular development genes were aided by root gene expression profiles (Gardiner *et al.* 2011). Based on the conserved developmental regulation between leaf and root vascular development, it has therefore been proposed that vascular formation during graft formation may also rely on the same genetic mechanisms (Melnyk 2016).

Graft regeneration is a coordination of multiple sequential regenerative processes, including initial tissue adhesion, callus proliferation, and vasculature formation, each of which employs a different genetic programming. Since auxin signaling is crucial in graft development, one creative approach to untangle the grafting role of different tissues without devastating the plant is by blocking auxin signaling in each cell type during each step of graft formation.

1.4 Environment and tissue regeneration

Plants are immobile and are hence constantly exposed to fluctuating environments. To survive and thrive, plants have an exceptional intrinsic ability to detect changes from the surroundings, such as gravity, light, temperature, humidity, and nutrients, then compute them to accurately optimize their growth and developmental programs. Tissue regeneration is a developmental response to physical damage, induced by stresses including wind, snow, herbivory, cutting, requiring to maintain tissue integrity and functionality (Ikeuchi *et al.* 2019) for completion of their life cycle. Although environment is a source of physical damage, certain conditions can encourage tissue regeneration.

Plants live in environments with variations in temperatures both daily and annually. Temperature is a key signal that governs plant growth and development. According to the thermodynamic principle, the rate of a biochemical reaction increases proportionally to the rise of temperature within the biological relevant range. Temperatures affect properties of biomolecules such as lipid membrane fluidity and protein conformations (Somero 1995; Mansilla *et al.* 2004). However, to maintain their physiological functions at multiple temperatures despites the nature of thermodynamic entropy, plants also actively regulate their biochemical reactions by integrating changes in physical properties of biomolecules to the downstream signal transduction to create a signal regulating process (Franklin *et al.* 2014). Well described examples of temperature signaling in plant physiology and development include seed germination rates (Hageseth & Joyner 1975), respiration rate (Hansen *et al.* 1994), hypocotyl elongation (Gray *et al.* 1998), and flowering time (Blazquez *et al.* 2003; Amasino 2010). Through cascades of signal networks, plants use the active signal transduction to fine tune the magnitude of temperature effects to adjust their physiology to adapt to different temperature environment. Arabidopsis hypocotyl and petiole elongate upon sensing warm temperatures and move upwards (Quint *et al.* 2016) to promote cooling by allowing better air circulation (Crawford *et al.* 2012). Under long day conditions, warm temperatures can also substantially induce early flowering (Balasubramanian *et al.* 2006). Both thermo-responsive hypocotyl elongation and flowering are controlled by a common transcription factor regulator, PHYTHOCHROME

INTERACTING FACTOR 4 (PIF4) (Proveniers & van Zanten 2013), which serves as the central hub for warm temperature responses (Wigge 2013). Upon sensing warm temperatures, plants increase *PIF4* expression which in turn coordinates the morphological responses through converging transcriptional and post translational regulations of auxin biosynthesis and auxin signalling genes, essentially resulting in SMALL AUXIN UP RNA (SAUR)- and YUCCA (YUC)-mediated elongation growth and induction of EXPASIN genes (Quint *et al.* 2016). PIF4 is regulated by EARLY FLOWERING 3 (ELF3) at the transcriptional and post translational levels (Nieto *et al.* 2015), and a recent work shows that temperatures directly change ELF3 protein biophysical property and its functions correspondingly to temperature responsiveness of the plant, suggesting that ELF3 serves as a thermosensor (Jung *et al.* 2020). Moreover, chromatin modification also plays a role in temperature regulation as nucleosomes containing H2A.Z histone protein sequester the promoter regions from RNA polymerase II and suppress gene expression at low temperatures (Kumar & Wigge 2010). The expression and structural modification of several microRNAs are also influenced by temperature (Kim *et al.* 2016; Serivichyaswat *et al.* 2017), suggesting that plants also employ post-transcriptional mechanisms to regulate temperature responses.

While the genetic networks and molecular mechanisms of temperature sensing and response in plant growth and development have been well described, the current understanding of how environmental conditions and temperature impact regeneration has not yet been thoroughly investigated. A growing number of recent reports show that temperatures also regulate several aspects of tissue regeneration. During *in vitro* tissue culture for micropropagation, incubation of explants in high temperature increases callus formation efficiency in several plant species (Skoog 1944; Wang *et al.* 2014; Sharma *et al.* 2018). Investigations in Arabidopsis showed that exposure to warm temperatures upregulates expression of *BRASSINOSTEROID-INSENSITIVE 2* (*BIN2*), an activator of ARF transcription factors, which subsequently activates callus formation genes *LATERAL ORGAN BOUNDARIES-DOMAIN 16* (*LBD16*) and *LBD29*, resulting in a higher callus formation rate. Conversely, *bin2* and *arf* mutants show temperature insensitivity of callus formation, suggesting that thermo-induced callus formation relies on the BIN2-ARF-LBD pathway (Lee & Seo

2017). A recent study has reported that *de novo* shoot organogenesis is temperature dependent. High temperatures during shoot regeneration enhance expression of several regeneration genes, including a transcription factor involved in shoot meristem formation encoded by *CUP-SHAPED COTYLEDON 1* (*CUC1*) and auxin biosynthesis genes *YUC*s, as well as reducing transcriptional repressor H2A.Z occupancy on several loci (Lambolez *et al.* 2022).

In commercial grafting, elevating temperatures during graft recovery promotes graft success rates in plants such as watermelon, eggplants, walnut, and tomato (Avanzato & Tamponi 1988; Shibuya *et al.* 2007; Shibuya *et al.* 2008; Yang *et al.* 2016). Graft success in Arabidopsis, both in the hypocotyl and cotyledon, is also promoted by warm temperatures (Turnbull *et al.* 2002; Bartusch *et al.* 2020; Serivichyaswat *et al.* 2022). As mentioned in the previous chapter, several regeneration processes occur during graft formation, and it is therefore highly likely that temperature regulates multiple levels of graft formation including callus formation and vascular regeneration. The central role of PIF4 and auxin signaling in warm temperature responses further suggests that graft development may employ the same genetic pathway for temperature enhancement. The molecular mechanism by which plants use to enhance graft reconnection in response to warm environments has yet to be thoroughly investigated.

Parasitic plants are plants that invade other plants to uptake their resources such as nutrients, water, and photosynthate. Parasitic plants that are completely dependent on their hosts to complete their life cycles are referred to as obligate parasites, while those that can grow and reproduce without hosts are facultative parasites. *Phtheirospermum japonicum* (*P. japonicum*) is a model species for facultative root parasitic plants due to its wide range of well-studied hosts, short life cycle, ease of maintenance, and availability of genomic data (Ishida *et al.* 2011; Cui *et al.* 2016). During the infection process, parasitic plants develop a haustorium, an invasive organ, to invade hosts' tissues and form a vascular connection with the hosts (Heidejorgensen & Kuijt 1995). Plant hormones play an important role in the formation of haustoria. During infection or chemical haustoria induction, parasitic plants produce more hormones such as ethylene, gibberellins, abscisic acid, auxin, and cytokinin (Tomilov *et al.* 2005; Zhang *et al.* 2012; Spallek *et al.* 2017).

In *P. japonicum*, an auxin biosynthesis gene *PjYUC3* increases expression in the epidermal cells during haustoria formation (Ishida *et al.* 2016). Silencing of *PjYUC3* by RNAi reduces haustoria number and blocking auxin biosynthesis completely arrests haustoria formation (Ishida *et al.* 2016; Wakatake *et al.* 2020). Despite the extensive agricultural damage the parasitic plants cause (Spallek *et al.* 2013), there is no effective control method to date. Understanding the mechanisms of parasitic plant infection and their interactions with hosts and environments may aid the pest management strategy.

The availability of nutrients in soil is another important environmental factor that influences plant physiology such as root growth, shoot growth, and flowering (Zhang & Forde 2000; Alboresi *et al.* 2005; Castro Marin *et al.* 2011). Although plants can convert light energy and inorganic compounds into chemical energy stored in organic molecules such as sugars and starch, they also require nutrients from soil environments to synthesize cellular components to grow and repair damaged tissues. Essentially, nutrients function as substrates for plants to metabolize into their biomass. Tissue regeneration during tissue culture requires proper amount and ratio of macronutrients including nitrogen, potassium, and phosphorus, and micronutrients such as iron, manganese, and copper (Mengel 2001). Another intriguing aspect of tissue regeneration that is affected by nutrient availability is parasitic plant infection. While reducing most plant growth and development, poor nutrient levels, in contrast, promotes infection of the parasitic plant *Striga* to its host (Yoneyama *et al.* 2007a; Yoneyama *et al.* 2007b; Mwangangi *et al.* 2021). Moreover, soils rich in nutrients reduce parasitic plant growth and infection rate (Cechin & Press 1993; Igbinnosa *et al.* 1996). Phosphorus rich soil reduces growth of parasite *Rhinanthus minor*, while *Phtheirospermum japonicum* fails to infect its host in nutrient rich in *vitro* media (Davies & Graves 2000; Cui *et al.* 2016; Ishida *et al.* 2016; Spallek *et al.* 2017), suggesting that poor nutrient levels may promote parasitic plant infection by reducing host plant fitness and defence while activating the parasite growth (Kokla 2022). The high similarities between parasitic plant infection and graft formation, including vascular connections and auxin signaling, suggest that plant parasitism may also be temperature dependent, and graft formation may *vice versa* be affected by nutrient availability.

2. The aims of the study

The objectives of this study are summarized as follows:

1. To investigate what cell types and tissues are involved during graft regeneration (Paper I).

2. To investigate how plant regeneration adapts to environmental conditions such as temperature and nutrient availability (Paper II and III).

3. Results and discussion

3.1 Cellular requirements for graft formation

During graft formation, plants rely on several cellular processes to aid tissue reconnection. The study of cellular mechanisms of graft formation has been difficult due to technical limitations. In Paper I, we investigated what cell types or tissues are being employed to facilitate the graft healing process with a novel non-invasive approach. Since auxin is crucial for graft formation (Melnyk *et al.* 2015), we miss-expressed a mutant version of *BDL* (Paper I, Fig 1A) to block auxin signaling in each cell type, including epidermis (*pML1*), endodermis (*pCASP1* and *pSCR*), phloem (*pAPL*), phloem precursor (*pSMXL5*), procambium (*pATHB8*, *pPXY*, and *pWOX4*), xylem precursor (*pTMO5*), and xylem pole pericycle (*pXPP*), by employing a recently developed synthetic tool that allows gene expression in an inducible and tissue specific manner (Schürholz *et al.* 2018) (Paper I, Fig 1). Our experiments showed that tissue attachment and callus formation rates were drastically reduced in grafted plants expressing *bdl* in procambium (*pATHB8>>bdl* and *pPXY>>bdl*) (Paper I, Fig 2). These findings suggested that procambial tissues below the graft junction required auxin signaling to form graft junction callus. Since tissue attachment and callus formation were prerequired for the subsequent vascular reconnection, phloem reconnection rates were consistently affected in these grafted plants. To overcome this barrier and test if auxin signaling in procambium directly affected phloem reconnection, we allowed grafted plants to attach and develop callus before inducing *bdl* expression. We then observed a significant reduction in phloem reconnection rates (Paper I, Fig 3), suggesting that the regenerated graft junction callus also required auxin signaling in the procambium to form and

reconnect phloem. In contrast, *bdl* induction in phloem precursors (*pSMXL5>>bdl*) showed normal tissue attachment and callus formation but failed to reconnect phloem (Paper I, Fig 2), indicating the specific role of phloem precursor cells in phloem reconnection. Since blocking auxin signaling in the procambial cells after callus formation did not allow phloem reconnection, we concluded that phloem differentiation occurred within the graft junction callus via procambium cells and subsequently linked the phloem from both sides of the graft junction. Our reasoning is supported by the notion that auxin signaling facilitates procambial cell dividing and differentiating into phloem precursor (Smetana *et al.* 2019). Furthermore, spatial analysis with heterografting showed that auxin responsive procambium on either side of the graft junction was sufficient to rescue tissue adhesion phenotype of mutant plants (Paper I, Fig 4). However, *pATHB-8>>bdl* prevented phloem formation regardless of whether the miss-expression was in the scion or rootstock whereas *pPXY>>bdl* inhibited phloem reconnection when in the scion but not the rootstock (Paper I, Fig 3), most likely due to the different expression domains of *pATHB8* and *pPXY* that drove *bdl* expression in different cell populations within the graft junction callus. Our results additionally suggested that scion and rootstocks may employ different cell types to perceive auxin during graft regeneration. Although previous reports suggest that auxin signaling is more important in the rootstock for phloem reconnection (Melnyk *et al.* 2015; Serivichyaswat *et al.* 2022), a probable explanation for our results is that the synthetic *pOP* promoter implemented for *bdl* expression in our study is conjugated with a cauliflower mosaic virus (CaMV) 35S minimal promoter (Craft *et al.* 2005), whereas the previous reports rely on the native promoters of the mutanted genes, which likely contribute to differences in transcriptional activity. Nonetheless, our results suggested that procambial cells, particularly within *ATHB-8* and *PXY* domains, required auxin signaling for tissue adhesion, callus formation, and phloem reconnection, ultimately highlighting the central role of procambium in graft formation.

During growth and development, plants rely on auxin signaling to promote tissue expansion and cell division (Campanoni & Nick 2005). Conversely, when auxin signaling was blocked in the procambial cells, procambium expansion at the scion graft junction was significantly reduced (Paper I, Fig 5 and Fig 6). Instead, we observed the enlargement of epidermal and cortical

cells in the scion extending to form a callus-like mass toward the graft junction, but without the presence of procambial cells. A previous study has reported the expansion of epidermis and cortex during graft healing (Melnyk *et al.* 2015), but our data suggest that their expansion, without the presence of procambium in the graft junction, was insufficient to facilitate tissue connection (Paper I, Fig 6). Although *bdl* induction in phloem precursors allowed tissue adhesion, no proper vascular connection was formed (Paper I, Fig 6). Furthermore, *bdl* miss-expression also reduced procambial cell division, suggesting that auxin signaling was important for procambial cell proliferation and the subsequent vascular reconnection, consistent with the notion of auxin signaling requirement in the procambium for vascular differentiation (Smetana *et al.* 2019). Although auxin is required for xylem differentiation (Mäkilä *et al.* 2022), xylem reconnection rates were not affected our tested plants (Paper I, Fig 1). This may imply that xylem reconnection may employ auxin signaling through other *Aux/IAA* homologs in Arabidopsis (Luo *et al.* 2018), or require signal magnitudes below the threshold of *bdl* blockage.

A successful graft formation requires a proper and precise coordination of multiple regeneration processes. By employing a novel genetic approach that allows a non-invasive examination of the plant tissues, together with high resolution imaging techniques, we were able to investigate the role of individual cell types during graft formation. Through a series of experiments, we consistently observed that blocking auxin signaling in procambium during graft formation arrested tissue adhesion, callus formation, phloem reconnection, and cell proliferation. Taken together, we confirm that procambium is the key tissue that facilitates tissue adhesion, gives rise to the graft junction tissue, and connects the vasculatures, serving the central role of graft formation.

The horticultural industry heavily relies on grafting for crop propagation and trait improvement. To date, at least 70 commercial fruit crops are produced from grafted plants (Warschefsky *et al.* 2016). With the introduction of automated grafting machines (Fig 1D), billions of plants are being grafted globally (Lee *et al.* 2010a). Nonetheless, climate change is an alarming threat that exposes crops to extreme weather and increasing diseases (Burdon & Zhan 2020). Plant breeding, especially for tree crops, is time consuming but

grafting can readily improve and introduce traits without extensive breeding programs. Moreover, grafting may potentially be used to produce new crop varieties through genome fusion at the graft junction tissues (Fuentes *et al.* 2014). Therefore, a fundamental understanding of grafting mechanisms is tremendously crucial for crop and technique improvement. For the first time, the findings in Paper I present direct evidence confirming that procambium is the important tissue in graft formation. Application of compounds that promote procambial proliferation may improve graft efficiency and potentially overcome graft incompatibility.

3.2 Environmental influences on tissue regeneration

Another aspect of grafting that is worth investigating is how environmental condition such as temperature affects graft development. Grafters have long known that elevating temperature during graft healing can improve graft success rates (Lagerstedt 1982), but the biology behind such enhancement has not been described. In paper II, we aimed to investigate the genetic mechanism by which plants use to accelerate tissue regeneration upon high temperature exposure. Experiments with Arabidopsis grafting showed that elevating recovery temperature from 20°C to 27°C significantly accelerated phloem and xylem connection rates (Paper II, Fig 1). In contrast, reducing recovery temperature to 16°C delayed phloem reconnection rates, suggesting that temperature played a significant role in graft development. Since cambium is associated with graft formation (Paper I), we examined the expression of the cambium-related reporter *pDOF6::Venus* (Smet *et al.* 2019), at the graft junction and observed a significant expression increase with the increasing temperature (Paper II, Fig S3). Morphological analysis of the graft junction revealed that elevated temperatures also increased the size of the scion vascular bundle immediately above the graft junction (Paper I, Fig 1). Moreover, 48 hours of warm recovery after grafting was sufficient for the enhancing effect, but increasing the temperature prior to grafting did not produce a significant enhancement effect on vascular connectivity (Paper II, Fig S3), suggesting that thermo-responsiveness of graft healing occured at an early stage after cutting. To investigate the genetic mechanism of this process, we used a reverse genetics approach and tested various mutant and transgenic genotypes associated with temperature response or auxin signaling (Paper II, Table S1). We found that grafted *pif4* single mutant did

not respond to elevated temperatures (Paper II, Fig 2), suggesting that the *pif4* mutation specifically desensitized temperature responsiveness without perturbing normal grafting dynamics. To investigate the spatial requirement of PIF4, we grafted *pif4* scions to wild-type rootstocks, or *vice versa*, and found that only grafted plants with *pif4* scions did not respond to the elevated temperatures (Paper II, Fig 2). Since cotyledons are important for temperature sensing and thermomorphogenesis (Bellstaedt *et al.* 2019), we furthermore contained the effect of *pif4* within the cotyledons by generating a graft combination whereby *pif4* cotyledons were grafted to the *pSUC2::GFP* scions (for the ease of vascular connectivity detection), and, after healing, the hypocotyls were grafted to wild-type rootstocks. Grafted plants with *pif4* cotyledons did not respond to elevated temperatures, suggesting that PIF4 function in the leaves was sufficient to enhance hypocotyl graft healing. Moreover, PIF4 activates an auxin-biosynthesis gene *YUC8* (Sun *et al.* 2012), and we found that *YUC8* expression was up-regulated by elevated temperatures but down-regulated and non-responsive in the *pif4* mutant (Paper II, Fig 2). Staining of *pYUC8::GUS* also increased with elevated temperatures, particularly in the epidermis, vasculature, and mesophyll, consistent with the expression pattern of PIF4 (Kim et al., 2020). Similar to *pif4* mutants, plants grafted with *yuc2 yuc5 yuc8 yuc9* quadruple mutant (*yucQ*) scions lost the temperature enhancement effect (Paper II, Fig 2). Although *yucQ* mutant carries *yuc2*, *yuc5*, *yuc8*, and *yuc9* mutations, only *YUC8* expression was responsive to warm temperatures (Paper II, Fig S4), suggesting that *YUC8* was responsible for the temperature effect. Our data suggested that *PIF4* and *YUC8* in the cotyledons were required for temperature-induced enhancement of graft formation at hypocotyls. Auxin is known to promote graft formation and wound healing (Asahina *et al.* 2011; Ikeuchi *et al.* 2017; Canher *et al.* 2020; Matosevich *et al.* 2020), so we speculated that auxin may also involve in the temperature enhancement effect. Blocking auxin transport from the cotyledon by applying an auxin transport inhibitor 1-N-naphthylphthalamic acid (NPA) on the petiole significantly reduced the temperature effect (Paper II, Fig 3), suggesting that auxin transported from the cotyledon was crucial for this effect. Warm temperatures also increased auxin response at the graft junction, as observed by fluorescent changes in the auxin responsive *pDR5::GFP* reporter (Ulmasov *et al.* 1997; Friml *et al.* 2003). Our data suggested that the

observed temperature enhancement of graft regeneration at the hypocotyl resulted from the long-distance transport of auxin from the leaf.

Since graft formation comprises multiple regeneration processes (Yin *et al.* 2012; Melnyk *et al.* 2015), we furthered our investigation by examining the temperature effect on tissue adhesion and callus formation. With warm recovery, tissue attachment rates significantly improved in both wild-type plants and *pif4* mutants (Paper II, Fig 4). The size of wound-induced callus was also increased by the elevated temperatures in all the tested genotypes including wild type, *pif4*, *pifQ*, and *yucQ* (Paper II, Fig4, S5), suggesting that temperature enhancement on tissue adhesion and callus formation may rely on genetic pathways other than *PIF4* and YUCCAs.

Plant parasitism and grafting are conceptually similar whereby both processes rely on successful vascular connections (Kokla & Melnyk 2018). Analogous to graft junction callus, parasitic plants develop their invasive structures, called haustoria, connecting xylem, called xylem bridge, to their hosts to withdraw nutrients and water. Like vascular reconnections in grafting, elevated temperatures also enhanced xylem bridge formation from parasite *Phtheirospermum japonicum* (*P. japonicum)* to its host Arabidopsis (Paper II, Fig 4). However, haustoria numbers remained unaffected by warm temperatures, suggesting that the temperature effect was specific to vascular formation. Blocking auxin transport from the parasite shoot using NPA significantly reduced the temperature enhancement on xylem bridge formation (Paper II, Fig 4). Moreover, expression levels of auxin-biosynthesis genes *PjYUC2* and *PjYUC4* were also up-regulated by warm temperatures in parasite shoots but not in roots (Paper II, Fig 4), suggesting that grafting and parasitic plants shared a common feature in temperature sensing in leaves and long-distance transport of auxin to promoting vascular formation in distant tissues. These findings further emphasize the similarity between grafting and plant parasitism.

Another environmental condition that affects plant parasitism is nutrient availability. While high nutrients promote most plant growth and development, environments rich in nutrient reduce parasitic plant grow and virulency (Cechin & Press 1993; Igbinnosa *et al.* 1996). It has been suggested that nutrient increases host fitness and defence mechanism while

lowering host production of parasite germination stimulants (Yoneyama *et al.* 2007a; Yoneyama *et al.* 2007b; Mwangangi *et al.* 2021). We found that haustoria formation in the parasitic plant *P. japonicum* was inhibited in environments rich in nitrate (Paper III, Fig 1). Expression levels of several genes associated with haustoria development were suppressed with nitrate application (Paper III, Fig 2-4). Together with the reduction of ROS accumulation and cell division at the haustorium forming site by nitrate application, these data suggested that nitrogen blocked haustoria formation at an early organ developmental stage (Paper III, Fig 1-4). The hormone abscisic acid increased during nitrate application, and exogenous application of the hormone reduced haustoria formation while blocking abscisic acid biosynthesis partially rescued the phenotype (Paper III, Fig 5, Fig 6). Moreover, transgenic *P. japonicum* roots with their abscisic acid signaling blocked could form haustoria even in the presence of nitrate, overcoming the nitrogen inhibitory effect (Paper III, Fig 6). In many plant species, nitrate application increases cytokinin, a hormone that promotes plant development (Takei *et al.* 2001; Takei *et al.* 2004; Kamada-Nobusada *et al.* 2013; Landrein *et al.* 2018; Mwangangi *et al.* 2021). However, cytokinin levels in *P. japonicum* did not respond to nitrogen (Paper III, Fig 5), suggesting that the haustoria development in response to nitrogen relies on a different mechanism. Although previous studies have reported the increase of abscisic acid levels in other parasitic plants (Kamada-Nobusada *et al.* 2013; Landrein *et al.* 2018), the relationship between nitrogen and abscisic acid in parasitic plants is reported for the first time in Paper III.

Fluctuating environmental conditions have selected plants for high degrees of developmental plasticity. In Paper II, we demonstrated that plant grafting and parasitism share a common developmental program by which temperature sensing in leaves accelerates regeneration rates in distant tissues. The ecological implication of the acceleration in response to rising temperatures may be explained by the resource competition theory (Tilman 1982), predicting that competition between species for limited resources increases selection pressure for resource-acquiring traits. In the temperate zones where the warm period is annually limited, such a trait that optimizes tissue regeneration rates, maximizing light, water, and nutrient acquirement, may confer competition advantages to the species, and is therefore selected. In climates with extended warm periods, the selection pressure for

temperature enhancement may be lower, and we predict that plants in such locations may maintain such traits to lesser degrees. As PIF4 transcription factor is a regulatory hub for high temperature sensing (Wigge 2013) and light signaling (Huq & Quail 2002), our finding in tissue regeneration (Paper II) further emphasized its essential role in plant adaptation for seasonal changes. *PIF4* orthologues of plants from different climate zones may likely be selected by the local temperature and photoperiod conditions and have varying degree of responsiveness, possibly via the ELF3 thermosensory regulation (Jung *et al.* 2020). The resource competition theory may further explain parasitic plants behaviour observed in nutrient rich environments (Paper III). Soils rich in nutrients such as nitrate may lower the degree of resource competition, and the interaction between parasite *P. japonicum* and their hosts therefore become less competitive, resulting in less infective capability. As resource competition drives diversification (Tilman 1982), we speculate that some ecotypes of the parasite *P. japonicum* populating the nutrient rich ecosystems may reduce, or completely lose, the virulency.

4. Future perspectives

This current thesis attempted to elucidate the cellular and genetic mechanisms of tissue regeneration through a series of scientific investigation. In Paper I, we presented direct evidence of procambium as the key tissue for graft formation. We demonstrated that auxin signaling is required for graft-adjacent procambial cells to form a successful graft. Future studies may also investigate how the graft junction cells are reorganized and differentiated into other cell types to complete the healing. It is likely that the graft junction callus may share features with stem-cell organizers of vascular cambium (Smetana *et al.* 2019), but further experiments are needed to confirm this notion, potentially with cell-lineage tracing and molecular genetic studies. Tissue ablation by inducing apoptosis in specific tissues, especially procambium, during graft development will additionally provide a complementary information. Comparative transcriptomic analyses may potentially identify the genes and genetic pathways involved in each regenerative process, from tissue adhesion to xylem reconnection. Furthermore, the inducible tissue-specific auxin resistant materials generated in this study can be readily implemented to study other aspects of plant regeneration and development including would healing, hormone and wound-induced callus formation, plant organogenesis, graft incompatibility, as well as parasitic plant infection.

The work of Paper II described the genetic mechanism of temperature-sensing regeneration. In this paper, we reported that plants perceived warm temperatures in the leaf and accelerate vascular regeneration in the distant tissues though the PIF4-YUCCAs pathway. We demonstrated that this long-distance cell-cell communication relied on auxin signaling. We observed that tissue adhesion and wound-induced callus formation were also promoted by

elevated temperatures, but they rely on different genetic pathways that are yet to be identified by future works. Additionally, we reported that parasitic plant *P. japonicum* also shared this feature in sensing warm temperatures in the leaf and activating distant root infectivity via auxin signaling. Since PIF4 also plays an important role in light signaling (Huq & Quail 2002), future studies may investigate how light affects tissue regeneration. Furthermore, we hypothesize that thermo-activation confers an evolutionary advantage by optimizing plant species in the temperate zones to maximize recovery rates during warm months. To test our hypothesis, future studies could compare the regeneration dynamic of plants across geographical locations.

Paper III is the last paper of this thesis. Here, we investigated how nutrient availability affects plant parasitism. Our findings showed that nitrogen prevented the development of *P. japonicum* haustoria via the hormone abscisic acid. Water availability could potentially be another crucial component for plant parasitism, but how drought impacts parasitism is currently not known. During drought, Arabidopsis rapidly produces abscisic acid to trigger a cascade of osmotic stress responses (Endo *et al.* 2008). The elevated abscisic acid during drought may inhibit haustoria formation, but further experimentation is needed to confirm this. We conducted comparative transcriptomic analysis of haustorium formation during nitrogen application, the molecular mechanisms of how abscisic acid inhibits haustoria development are yet to be described. Perturbing abscisic acid biosynthesis and signaling with genetic tools, such as genome editing or RNAi, could shed some light on these gaps in knowledge.

The work of this thesis confirms that procambium is an important tissue for graft regeneration, refining to our current understanding of graft mechanism. Our findings also align with the notion that procambium is the activator of radial growth through coordinated programs of cell division and differentiation (Esau 1954; Miyashima *et al.* 2019; Smetana *et al.* 2019; Mäkilä *et al.* 2022). In addition, we provide evidence to support the common practice of recovering plants in warm environments and describe a genetic mechanism underlining such temperature-activated healing. Lastly, we demonstrate that the phytohormone abscisic acid regulated the number of haustoria in *P. japonicum* in response to environmental nitrogen levels. This

study provides an explanation for the reported reduction of infection rates in fertilized field (Yoneyama *et al.* 2007a).

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Popular science summary

Plants are resilient organisms with a high adaptation ability. One remarkable example is their exceptional ability to heal and reconnect their damaged tissues after wounding. Since ancient times, gardeners have observed this ability and taken advantage of it by cutting and joining different plant parts together to create a single plant that bears qualities of many; a technique known as grafting. Today, many of our fruits and vegetables are produced from grafted plants. Moreover, several fundamental scientific discoveries in plant biology were made using grafted plants. Despites its importance in horticulture and scientific research, our biological understanding of how plants graft is still lacking. Previous studies have demonstrated that graft healing generally follows the attachment of the damaged tissues, formation of plant stem cells called callus, and reconnection of vascular tissues. However, thorough investigations are often obstructed by the thick, rigid structure of plant tissues and the complexity of the regeneration process. The work of this thesis aimed to use current tools in molecular biology and high-resolution imaging to deepen our knowledge of plant grafting. Through a series of experiments, we discovered that procambium, a plant tissue within the vasculature with an ability to divide and become other cell types, accounted for successful graft healing. Together with cellular responses to the plant hormone auxin, procambial cells multiply and become callus and then vascular vessels, essentially joining the grafted tissues. Furthermore, we demonstrated how environmental conditions such as temperature and nutrient levels affect plant regeneration. Our work showed that plants perceive warm temperatures in the leaf and speed up multiple aspects of tissue regeneration, including graft healing, in distant tissues. Parasitic plants are plants that parasitize other plants by connecting their vasculatures to their host plants to uptake water and nutrients, and we found that the infection

process is also enhanced by warm temperatures. Lastly, we discovered that the parasitic plant *Phtheirospermum japonicum* regulates its infectious levels depending on the levels of nitrogen in the soil via the hormone abscisic acid. Altogether, this thesis highlights the adaptive genetic programs by which plants employ to optimize their developmental responses and maximizing their chance of survival in different environmental conditions including wounding, temperature, and nutrient availability.

Populärvetenskaplig sammanfattning

Växter är motståndskraftiga organismer med hög anpassningsförmåga. Ett anmärkningsvärt exempel är deras exceptionella förmåga att läka och återansluta sina skadade vävnader efter sår. Sedan urminnes tider har trädgårdsmästare observerat denna förmåga och utnyttjat den genom att skära av och sammanfoga olika växtdelar för att skapa en enda växt som bär egenskaper av fler än en individ; en teknik som kallas ympning. Idag produceras många av våra frukter och grönsaker från ympade växter. Dessutom gjordes flera grundläggande vetenskapliga upptäckter inom växtbiologi med hjälp av ympade växter. Trots dess betydelse för trädgårdsodling och vetenskaplig forskning, är vår biologiska förståelse för hur växter ympar fortfarande begränsad. Tidigare studier har visat att lyckad ympanslutning i allmänhet följer vidfästning av de snittade ytorna, bildandet av växtstamceller som kallas kallus och återanslutning av kärlsystemet. Grundliga undersökningar hindras dock ofta av den tjocka, stela strukturen hos växtvävnader och komplexiteten i regenereringsprocessen. Arbetet med denna avhandling syftade till att använda moderna molekylärbiologiska verktyg och högupplöst bildbehandling för att fördjupa vår kunskap om växtympning. Genom en serie experiment upptäckte vi att prokambium, en växtvävnad i kärlsystemet med förmåga att dela sig och bli andra celltyper, stod för framgångsrik ympläkning. Tillsammans med cellulära svar på växthormonet auxin förökar sig prokambiala celler och bildar kallus och sedan kärlsträngar, som i huvudsak förenar de vidhäftade ympvävnaderna. Vidare visade vi hur miljöförhållanden som temperatur och näringsnivåer påverkar förmågan till regeneration. Vårt arbete visade att växter uppfattar varma temperaturer i bladet och påskyndar flera aspekter av vävnadsregenerering i andra delar av växten, inklusive ympläkning. Parasitiska växter är växter som parasiterar andra växter genom att koppla

sina kärlsystem till värdväxtens för att från den ta upp vatten och näringsämnen, och vi fann att infektionsprocessen också förstärks av varma temperaturer. Till sist upptäckte vi att parasitväxten *Phtheirospermum japonicum*, via hormonet abscisinsyra, reglerar sin infektionsaktivitet utifrån vilka nivåer av kväve som finns i jorden. Sammantaget belyser denna avhandling de adaptiva genetiska program som växter använder för optimal utveckling och för maximering av sina chanser att överleva under olika miljöförhållanden, såsom vid skador, olika temperaturförhållanden samt tillgång på näringsämnen.

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RESEARCH REPORT

High temperature perception in leaves promotes vascular regeneration and graft formation in distant tissues

Phanu T. Serivichyaswat[1,*], Kai Bartusch[1,2,3,*], Martina Leso[1], Constance Musseau[1], Akira Iwase[4], Yu Chen[4,5], Keiko Sugimoto[4,5], Marcel Quint[3] and Charles W. Melnyk[1,‡]

ABSTRACT

Cellular regeneration in response to wounding is fundamental to maintain tissue integrity. Various internal factors including hormones and transcription factors mediate healing, but little is known about the role of external factors. To understand how the environment affects regeneration, we investigated the effects of temperature upon the horticulturally relevant process of plant grafting. We found that elevated temperatures accelerated vascular regeneration in *Arabidopsis thaliana* and tomato grafts. Leaves were crucial for this effect, as blocking auxin transport or mutating *PHYTOCHROME INTERACTING FACTOR 4* (*PIF4*) or *YUCCA2/5/8/9* in the cotyledons abolished the temperature enhancement. However, these perturbations did not affect grafting at ambient temperatures, and temperature enhancement of callus formation and tissue adhesion did not require *PIF4*, suggesting leaf-derived auxin specifically enhanced vascular regeneration in response to elevated temperatures. We also found that elevated temperatures accelerated the formation of inter-plant vascular connections between the parasitic plant *Phtheirospermum japonicum* and host *Arabidopsis*, and this effect required shoot-derived auxin from the parasite. Taken together, our results identify a pathway whereby local temperature perception mediates long distance auxin signaling to modify regeneration, grafting and parasitism.

This article has an associated 'The people behind the papers' interview.

KEY WORDS: Grafting, Regeneration, Temperature sensing, Auxin transport, Vascular biology, Parasitic plants, *Arabidopsis thaliana*

INTRODUCTION

Various abiotic and biotic stresses including temperature extremes, herbivory and cutting induce damage that needs to be repaired (Ikeuchi et al., 2019). These stresses are a source of tissue damage, but the environment can also promote regeneration. One notable example is the influence of temperature upon regeneration. Elevated temperatures enhance the formation of stem-cell like tissues, termed callus, that aid the wound healing process (Lee and Seo, 2017). Increased temperatures also improve the horticultural process of plant grafting (Bartusch et al., 2020; Shibuya et al., 2008), which consists of cutting and joining different shoots, known as scions, and roots, known as rootstocks, together to improve stress tolerance and yields (Melnyk and Meyerowitz, 2015; Mudge et al., 2009). At the cut sites, grafts initially form callus (Ikeuchi et al., 2017) that seal the wound, followed by vascular division and differentiation that allows phloem and xylem reconnection (Melnyk et al., 2015). Related processes occur during other forms of wound healing such as when callus forms at the site of cutting or cell layers divide and differentiate to restore tissue integrity after cell ablation (Iwase et al., 2011; Marhava et al., 2019). A common theme to regeneration in plants is the involvement of auxin. Auxin is mainly produced in young leaves (Ljung et al., 2001) and accumulates at the site of injury (Canher et al., 2020) where auxin responses increase (Asahina et al., 2011; Hoermayer et al., 2020; Melnyk et al., 2015). Auxin plays an important role in regenerating the vasculature: disrupting auxin response or auxin transport inhibits graft formation (Matsuoka et al., 2016; Melnyk et al., 2015) and blocks xylem connection formation between parasitic plants and their hosts during the conceptually related process of parasitic plant infection (Ishida et al., 2016; Wakatake et al., 2020).

The success of wound healing at the graft junction depends on internal factors including hormones and the developmental stage, but also on external factors such as light intensity (Bartusch et al., 2020), photoperiod (Marsch-Martínez et al., 2013) and temperature (Turnbull et al., 2002). In *Arabidopsis*, elevated ambient temperature alters growth and developmental traits including elongating hypocotyls, petioles and roots (Quint et al., 2016). The transcription factor *PHYTOCHROME INTERACTING FACTOR 4* (*PIF4*) is the major temperature-signaling hub in aerial tissues (Delker et al., 2014; Koini et al., 2009; Lee et al., 2021). High temperatures deactivate the photoreceptor Phytochrome B (PhyB) and release its suppression of *PIF4*. The PIF4 protein directly upregulates the expression of *YUCCA8* (*YUC8*), a gene associated with auxin biosynthesis (Franklin et al., 2011; Sun et al., 2012). In *Arabidopsis*, high temperatures promote a mobile auxin signal (Bellstaedt et al., 2019) that is activated by epidermal PIF4 in cotyledons (Kim et al., 2020). Cotyledon-produced auxin is then transported via the petioles to the hypocotyl where it causes brassinosteroid-induced cell elongation (Bellstaedt et al., 2019).

Elevating temperatures during graft healing improves grafting success rates in plants including *Arabidopsis* (Bartusch et al., 2020; Turnbull et al., 2002), watermelon (Yang et al., 2016),

[1]Department of Plant Biology, Swedish University of Agricultural Sciences, Ulls grränd 1, 765 51 Uppsala, Sweden. [2]Institute of Molecular Plant Biology, Department of Biology, ETH Zürich, 8092 Zürich, Switzerland. [3]Institute of Agricultural and Nutritional Sciences, Faculty of Natural Sciences III, Martin Luther University Halle-Wittenberg, Betty-Heimann-Str. 5, 06120 Halle (Saale), Germany. [4]RIKEN Center for Sustainable Resource Science, Yokohama 230-0045, Japan. [5]Department of Biological Sciences, Faculty of Science, The University of Tokyo, Tokyo 113-8654, Japan.
*These authors contributed equally to this work

‡Author for correspondence (charles.melnyk@slu.se)

P.T.S., 0000-0002-4927-7727; K.B., 0000-0003-2708-5545; M.L., 0000-0003-4192-8027; C.M., 0000-0003-2393-1767; A.I., 0000-0003-3294-7939; M.Q., 0000-0003-2935-4083; C.W.M., 0000-0003-3251-800X

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eggplant (Shibuya et al., 2007, 2008), walnut (Avanzato and Tamponi, 1988) and tomato (Shibuya et al., 2007). However, the molecular basis for the temperature enhancement of regeneration remains poorly characterized. Here, we investigated the effects of temperature upon various aspects of graft healing including callus formation, tissue attachment and vascular formation and revealed a central role for temperature regulating *PIF4* and *YUC2*/*5*/*8*/*9* in leaves to promote vascular formation in grafted stems. Moreover, pharmacological experiments showed that leaf-derived auxin regulated phloem reconnection at the *Arabidopsis* graft junction and xylem bridge formation between the parasite *Phtheirospermum japonicum* and its host *Arabidopsis thaliana* in a temperature-dependent manner. Taken together, our results suggest a conserved temperature signaling mechanism in leaves regulating vascular regeneration and vascular formation in distant tissues.

RESULTS AND DISCUSSION

Elevated temperatures enhance vascular formation during grafting

As elevating temperatures improves commercial grafting success rates (Lagerstedt, 1982), we tested the effects of temperature upon *in vitro* graft formation in tomato (*Solanum lycopersicum*) and *Arabidopsis*. We applied carboxyfluorescein diacetate (CFDA) to monitor vascular connectivity at the graft junction (Melnyk et al., 2015) (Fig. 1A). Tomatoes grown at 25°C and moved to 30°C immediately after grafting showed significantly faster and higher phloem connection rates compared with those recovered at 20°C (Fig. 1B,C). *Arabidopsis* often grafts faster than tomato (Cui et al., 2021; Melnyk et al., 2015; Yin et al., 2012), so we grafted *Arabidopsis pSUC2::GFP* scions to wild-type rootstocks (Fig. 1A) and observed that after 2 days the phloem connection rate was accelerated by higher recovery temperatures (Fig. 1D; Fig. S1),

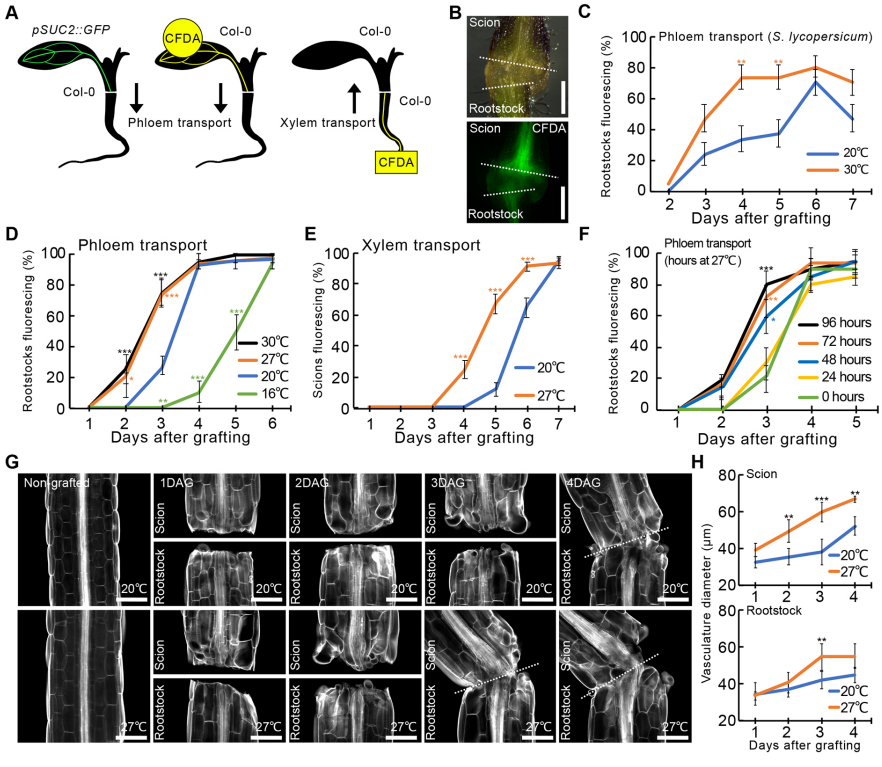

Fig. 1. Elevated temperatures enhance graft formation. (A) Schematic showing phloem and xylem transport measured by appearance of GFP or CFDA fluorescence in the rootstock (phloem) or CFDA fluorescence in the scion (xylem). (B) Movement of CFDA from scion to rootstock of grafted tomato. Dashed lines indicate cut sites. (C) Proportion of grafted tomato that transported CFDA to rootstocks after recovery at 20°C or 30°C [±standard error of a proportion (s.e.p.); n=27 at 1 DAG, n=30 at 2-7 DAG per temperature per time point]. (D) Proportion of grafted *pSUC2::GFP Arabidopsis* scions with fluorescing Col-0 rootstocks (±s.e.p.; n=20 at 16°C, n=60 at 20°C, n=20 at 27°C, n=20 at 30°C). (E) Proportion of grafted *Arabidopsis* that transported CFDA to scions after recovery at 20°C or 27°C (±s.e.p.; n=50 plants per temperature per time point). (F) Proportion of grafted *pSUC2::GFP Arabidopsis* scions with fluorescing Col-0 rootstocks that were recovered at 27°C for the indicated period, then transferred to 20°C recovery (±s.e.p.; n=40 plants per temperature). (G) Longitudinal optical sections of grafted *Arabidopsis* recovered at 20°C and 27°C. Plants are stained with Calcofluor White and dashed lines indicate the cut site. (H) Vasculature diameter including pericycle, cambium, xylem and phloem of grafted *Arabidopsis*, 100 µm from the cut surface and recovered at 20°C or 27°C (mean±s.d.; n=10 plants per temperature per time point). *P<0.05, **P<0.01, ***P<0.001; Fisher's exact test (C-F) or unpaired two-tailed Student's t-test (H) compared with 20°C. Scale bars: 500 µm (B); 100 µm (G).

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similar to tomato grafting. Expression of a reporter gene associated with cambium formation, *pDOF6::erVENUS* (Smet et al., 2019), was also enhanced by elevated temperatures (Fig. S2A,B). Increasing the recovery temperature from 27°C to 30°C did not further promote *Arabidopsis* graft formation, suggesting that 27°C was close to the maximum thermo-induction effect in *Arabidopsis*. In contrast, reducing the recovery temperature to 16°C delayed phloem reconnection (Fig. 1D). Elevated temperatures also increased xylem reconnection rates (Fig. 1E; Fig. S3) and enhanced the size of the regenerating vascular bundle, particularly in the scion (Fig. 1G,H). We next investigated when and for how long elevated temperatures were required to accelerate graft healing and found that 48 h of warm recovery immediately after grafting was sufficient (Fig. 1F). However, providing warm temperatures before grafting (Fig. S2C) had no significant effect upon vascular connectivity (Fig. S2D), suggesting that thermo-responsiveness occurs early after wounding and plays an important role during graft healing.

PIF4 and YUCs are required in the cotyledon for temperature-enhanced vascular formation in the hypocotyl

To better understand how elevated temperatures promoted graft formation, we tested various mutant genotypes associated with temperature response or hormone signaling (Table S1). Most mutants had no effect on temperature enhancement, but the *pif1 pif3 pif4 pif5* quadruple mutant (*pifQ*) and the *pif4* single mutant were exceptional as they did not respond to temperature enhancement at 3 days after grafting (DAG) but had normal grafting dynamics at later time points (Fig. 2A; Fig. S4A), suggesting they specifically affected temperature enhancement. We tested the spatial requirements of *PIF4* by grafting *pif4* scions to wild-type rootstocks, or vice versa, and observed that *pif4* scions did not respond to the elevated recovery temperature, whereas *pif4* rootstocks responded like wild-type (Fig. 2A). The cotyledons play an important role in thermo-sensing (Bellstaedt et al., 2019), so we generated a graft combination whereby the cotyledon of *pif4* was initially grafted to a *pSUC2::GFP* scion and then, after graft healing, a hypocotyl graft was performed to a wild-type rootstock for recovery at 20°C and 27°C. Plants with *pif4* cotyledons did not respond to elevated temperatures (Fig. 2B), indicating that temperature perception via *PIF4* in the leaves was sufficient to accelerate graft healing in the hypocotyl. The auxin-biosynthesis gene *YUC8* is a direct target of PIF4 (Sun et al., 2012) and we found that *YUC8* transcription levels were upregulated in wild-type plants exposed to elevated temperatures, but downregulated and non-responsive in the *pif4* mutant (Fig. 2C). We also observed that *PIF4* transcript levels were not affected in the *yuc2 yuc5 yuc8 yuc9* quadruple mutant (*yucQ*), consistent with PIF4 acting as an activator of *YUC8*. Staining from *pYUC8::GUS* increased in plants grown at 27°C compared with those grown at 20°C and was observed mainly in the epidermis, vasculature and mesophyll (Fig. 2D), consistent with the previously reported expression pattern of *PIF4* (Kim et al., 2020). We tested the *yucQ* mutant in grafting assays and found that plants lost grafting thermo-responsiveness when YUC genes were mutated in the scion (Fig. 2E), but *yucQ* did not affect grafting at later time points, similar to the *pif4* mutant. The *yucQ* genotype carries *yuc2*, *yuc5*, *yuc8* and *yuc9* mutations yet only *YUC8* was responsive to elevated temperatures (Fig. 2C; Fig. S4B), suggesting that *YUC8* might play a central role for the observed

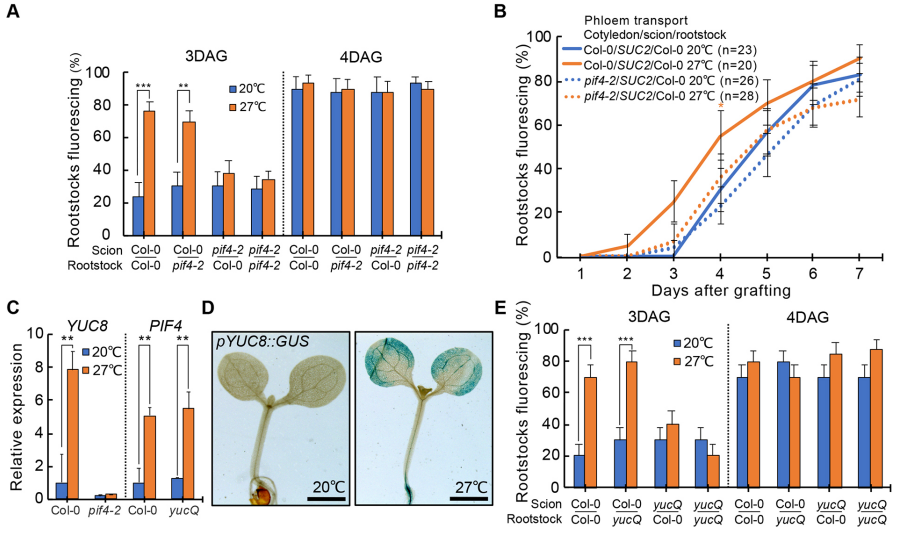

Fig. 2. Temperature-enhanced graft formation requires *PIF4* and YUCs in the cotyledon. (A) Proportion of grafted *pif4-2* or wild-type *Arabidopsis* that transported CFDA to the rootstock 3-4 DAG and recovered at 20°C or 27°C [±standard error of a proportion (s.e.p.); *n*=30 plants per temperature per time point]. (B) Proportion of three genotype *pif4-2* grafts with fluorescent rootstocks (±s.e.p.; *n*=indicated on plot). (C) Relative expression levels of *YUC8* and *PIF4* in Col-0 or *pif4-2* cotyledons after 48 h treatment of 20°C or 27°C (mean±s.d. of three biological replicates). (D) GUS histochemical staining of 8-day-old *pYUC8::GUS* seedlings incubated at 20°C or 27°C for 48 h. Scale bars: 1 mm. (E) Proportion of grafted *yuc2/5/8/9* or wild-type *Arabidopsis* that transported CFDA to the rootstock, recovered at 20°C or 27°C and measured 3-4 DAG (±s.e.p.; *n*=30 plants per temperature per time point). **P*<0.05, ***P*<0.01, ****P*<0.001; Fisher's exact test (A,B,E) or unpaired two-tailed Student's *t*-test (C) compared with 20°C.

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phenotype. We tested whether temperatures affected the expression of vascular-development genes in intact (non-grafted) seedlings but did not detect upregulation (Fig. S4C), suggesting that wounding may be a prerequisite for transcriptional induction. Together, these data indicated a requirement for *PIF4* and YUC genes in the cotyledons for temperature-dependent vascular connectivity in hypocotyl tissues.

Warm temperatures promote vascular formation by enhancing auxin response

As auxin is important for graft formation and wound healing (Asahina et al., 2011; Canher et al., 2020; Ikeuchi et al., 2017; Matosevich et al., 2020), we investigated the role of auxin in temperature enhancement of grafting. We applied an auxin transport inhibitor 1-N-naphthylphthalamic acid (NPA) on the petiole to inhibit the transport of auxin from the cotyledon to the hypocotyl, and observed that NPA-treated plants did not respond to temperature enhancement (Fig. 3A), suggesting that cotyledon-derived auxin is essential for this effect. However, graft dynamics of NPA-treated plants at 20°C were similar to controls at 20°C, suggesting that cotyledon-derived auxin was only relevant for graft formation at elevated temperatures. We next asked whether auxin response at the graft junction was increased by elevated temperatures and found a significant fluorescence increase in the auxin-responsive *pDR5::GFP* reporter (Friml et al., 2003; Ulmasov et al., 1997) with warm temperatures (Fig. 3B). Perturbing auxin response in the rootstock with a dominant negative mutant of *BODENLOS* (*BDL*; also known as *IAA12*) (*bdl-2*) (Hayward et al., 2009) blocked graft formation irrespective of whether grafting was performed at 20°C or 27°C (Fig. 3C). However, when *bdl-2* was present only in the scion, plants grafted like controls at 20°C but were inhibited in elevated temperature responses at 27°C (Fig. 3C). As we previously observed that accelerated graft healing in the hypocotyl was due to temperature perception in the leaves, we asked whether blocking auxin response in the leaves would also affect the thermo-responsiveness of grafting dynamics. Blocking auxin response in the cotyledon by grafting the *bdl-2* cotyledon to a *pSUC2::GFP* scion and wild-type rootstock did not affect temperature enhancement (Fig. 3D), suggesting that *bdl-2* did not play a role in the leaves for temperature enhancement of graft formation and, instead, *bdl-2* had its effect at the region of graft junction formation. Thus, the long-distance transport of, and local response to, an auxin-dependent signal was necessary for temperature to accelerate graft healing.

Temperature-dependent tissue regeneration is widespread

Graft formation involves cell adhesion, callus formation and vascular reconnection (Melnyk et al., 2015; Yin et al., 2012). To test the effects of temperature upon tissue adhesion, we picked up plants 1-2 DAG with forceps (Melnyk et al., 2015) and observed that adhesion rates were significantly increased with the elevated temperatures, but this enhancement was not affected

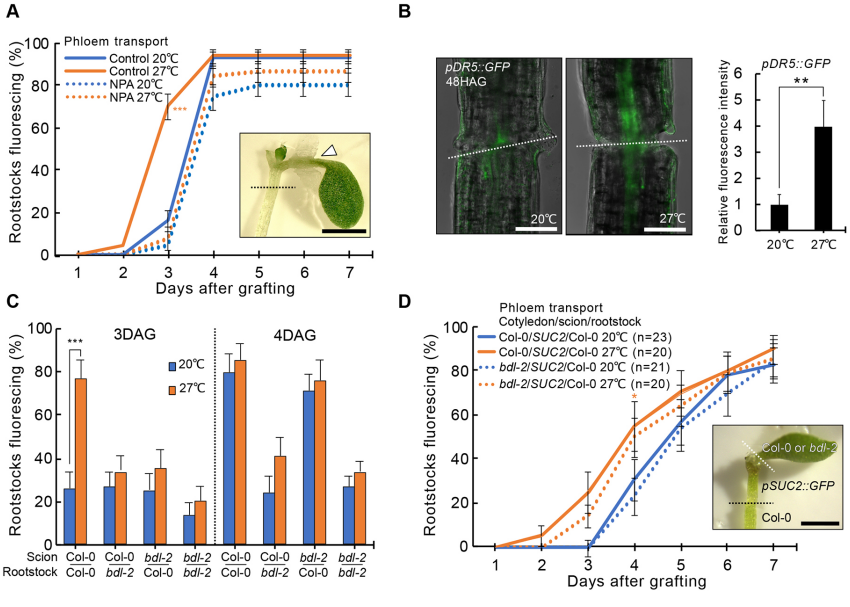

Fig. 3. Temperature promotes graft formation by elevating auxin response. (A) Proportion of grafted *Arabidopsis pSUC2::GFP* scions with fluorescing Col-0 rootstocks after petiole NPA treatments and recovered at 20°C or 27°C [±standard error of a proportion (s.e.p.); *n*=50 plants per treatment]. NPA plaster (arrowhead) and graft junction (dashed line) indicated. (B) Signal intensity of auxin-responsive *pDR5::GFP* signal at the graft junction 48 h after grafting (HAG) recovered at 20°C or 27°C (mean±s.d. of three experiments, each ≥15 plants per temperature treatment). Dashed lines indicate the graft junction. (C) Proportion of grafted *bdl-2* or wild-type *Arabidopsis* that transported CFDA to the rootstock 3-4 DAG and recovered at 20°C or 27°C (±s.e.p.; *n*=30 plants per temperature per time point). (D) Proportion of three genotype grafts with fluorescent rootstocks (±s.e.p.; *n*=indicated on plot). The white and black dashed lines indicate sites of cotyledon and hypocotyl grafting, respectively. **P*<0.05, ***P*<0.01, ****P*<0.001; Fisher's exact test (A,C,D) or unpaired two-tailed Student's *t*-test (B) compared with 20°C. Scale bars: 1 mm (A,D); 100 µm (B).

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in *pif4* mutants (Fig. 4A). To measure callus formation, we used previously described assays (Iwase et al., 2017) and found that elevated temperatures enhanced wound-induced callus formation but this enhancement was not affected in *pif4*, *pifQ*, *yucQ* or *bdl-2* mutants (Fig. 4B,C; Fig. S5A), suggesting that warm temperatures enhanced multiple aspects of wound healing but that phloem enhancement specifically required *PIF4* and *YUC2/5/8/9*.

Parasitic plant infections are conceptually similar to grafting (Kokla and Melnyk, 2018) and their infective structures, haustoria, form xylem connections known as xylem bridges to their hosts to withdraw nutrients. Elevated temperatures can increase haustoria numbers (Rafferty et al., 2019), but we found that elevated temperatures did not affect haustoria number during *A. thaliana* infection by the facultative parasite *P. japonicum* (Fig. S5D). However, we observed that xylem bridge formation was accelerated by elevated temperatures similar to the effect we observed during xylem reconnection at the graft junction (Fig. 1E; Fig. S5E). Warm temperatures also increased the area and length of the haustoria xylem mass adjacent to the parasite root vasculature, the plate xylem (Fig. S5B,C). To investigate a role for leaf-derived auxin, we blocked auxin transport from *P. japonicum* cotyledons using NPA (Fig. S5F). NPA did not affect haustoria number (Fig. 4D), but significantly reduced xylem bridge formation at 27°C but did not affect it at 20°C (Fig. 4E,F). Expression levels of auxin biosynthesis genes *PjYUC2* and *PjYUC4* were upregulated by elevated temperatures in *P. japonicum* shoots but not roots (Fig. 4G; Fig. S5G). Thus, similar to grafting, shoot-derived auxin contributed to vascular formation in basal tissues of *P. japonicum*.

Previous reports have found that elevated temperatures have dramatic effects upon both animal and plant development (Angilletta et al., 2004; Franklin, 2009; Hatfield and Prueger, 2015) and here, we demonstrate that warm temperatures enhanced multiple aspects of wound healing including tissue adhesion, callus formation and vascular regeneration that we could mechanistically separate based on their dependency on *PIF4*. *PIF4* was specifically

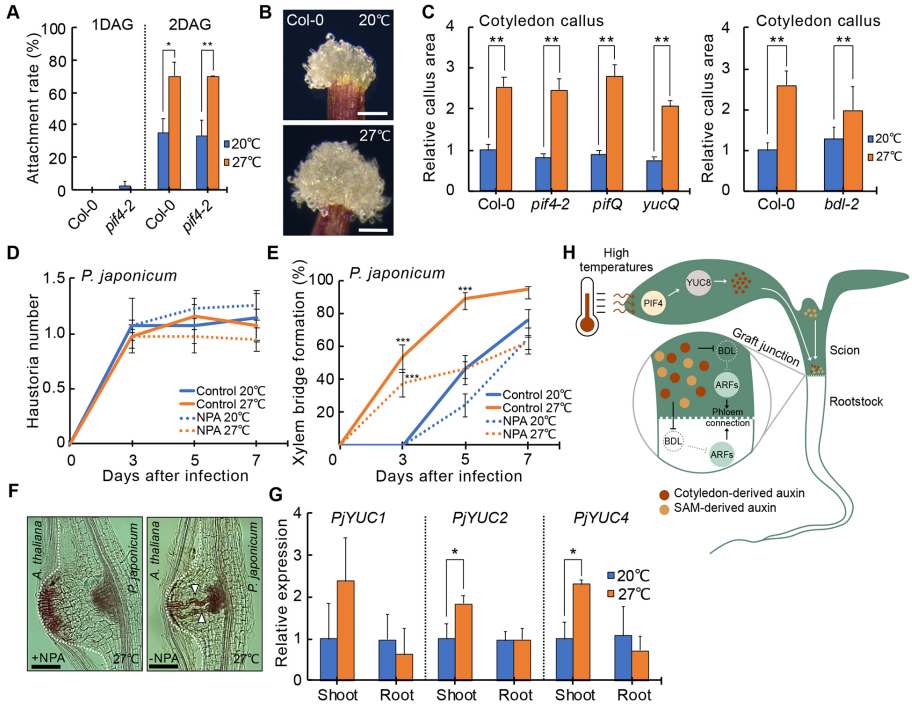

Fig. 4. Temperature-dependent tissue regeneration is widespread. (A) Proportion of grafts attached 1-2 DAG after recovery at 20°C or 27°C [±standard error of a proportion (s.e.p.); *n*=30 plants per temperature per time point]. (B) Callus formation from cut Col-0 petioles at 20°C or 27°C. (C) Callus size 8 days after wounding from various genotypes relative to Col-0 at 20°C (mean±s.d., *n*=60 cotyledons per genotype and temperature). (D) *P. japonicum* haustoria numbers with control or NPA applications at 20°C or 27°C (mean±s.d. from four experiments, each with 20 infections per treatment). (E) Proportion of *P. japonicum* xylem bridge formation with control or NPA applications at 20°C or 27°C (±s.e.p.; *n*=40 infections per treatment). (F) Representative images of haustoria and xylem bridges formed at 7 DPI at 27°C with and without NPA petiole applications. Dashed lines show the interface between *P. japonicum* and *Arabidopsis*. Xylem bridges are indicated by the arrowheads. (G) Relative expression levels of auxin-related genes in *P. japonicum* at 7 DPI in shoots and roots at 20°C or 27°C (mean±s.d. from three experiments). **P*<0.05, ***P*<0.01, ****P*<0.001; Fisher's exact test (A,E) or unpaired two-tailed Student's *t*-test (C,H) compared with 20°C. (H) Proposed model for temperature-dependent vascular regeneration. Elevated temperatures increase PIF4 levels and activate YUC8-mediated auxin production. Auxin moves to the graft junction where it degrades BDL and activates auxin response factors (ARFs) to promote phloem reconnection. SAM, shoot apical meristem. Scale bars: 250 µm (B); 100 µm (F).

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required in cotyledons to promote vascular regeneration, and this protein is known to activate auxin biosynthesis (Franklin et al., 2011), suggesting that transport of cotyledon-derived auxin was sufficient to enhance vascular formation at the graft junction (Fig. 4H). Enhancing auxin response at the graft junction likely enhanced graft healing through the known roles of auxin in promoting xylem differentiation and activating cambium in part via *DOF6* (Fig. S2) (Baima et al., 1995; Miyashima et al., 2019; Ursache et al., 2014), processes that were likely perturbed in the auxin-resistant *bdl-2* scion (Hayward et al., 2009). However, *bdl-2* rootstocks inhibited grafting regardless of temperature, indicating that the rootstock had a different requirement for auxin response and appeared more sensitive to auxin perturbations. Enhanced temperatures also accelerated haustoria development in *P. japonicum*, and this effect was specific to xylem bridge formation but not haustoria initiation. It was previously shown that auxin production is necessary for haustoria formation and that auxin transport drives xylem bridge formation (Wakatake et al., 2020). We extended the role for auxin and found that shoot-derived auxin acted as a long-distance signal to accelerate xylem bridge formation upon elevated temperatures. Our observations in grafted plants and parasitic plants demonstrate a common mechanism by which temperature sensing in leaves changes vascular development and contributes to modifying the rate of vascular regeneration or vascular formation. Such modulations could provide developmental plasticity in response to environmental changes and confer a fitness advantages to accelerate water and photosynthate transport. High temperatures also enhance regeneration of *Hydra* tentacles (Peebles, 1898), zebrafish fins (Boominathan and Ferreira, 2012) and flatworm testes (Wudarski et al., 2019), suggesting the enhancement of regeneration by elevated temperatures is universally relevant and a useful tool to enhance grafting and wound healing.

MATERIALS AND METHODS

Plant materials, growth conditions, and grafting

A. thaliana (L.) ecotype Columbia (Col-0) was used throughout this study unless otherwise indicated. Mutant lines used included *pif4-2* (CS66043), *pifQ* (CS66049), *yucQ* (CS69869), *bdl-2* (Hayward et al., 2009). The previously published transgenic lines include *pSUC2::GFP* (Imlau et al., 1999), *pYUC8::GUS* (Müller-Moulé et al., 2016), *pDR5rev::GFPer* (Friml et al., 2003) and *pDOF6::erVENUS* (Smet et al., 2019). For *in vitro* germination, seeds were surface sterilized with 70% (v/v) ethanol for 10 min, followed by 90% (v/v) ethanol for 10 min. The seeds were then sown and germinated on ½MS media (1% plant agar), pH 5.8. After stratification in the dark at 4°C overnight, the seeds were transferred to 20°C short-day growth conditions (8 h of 140 µmol m^{-2} s^{-1}). *Arabidopsis* grafting was performed on 7-day-old seedlings and carried out according to previously published protocols (Melnyk, 2017a,b), and recovered at 16°C, 20°C, 27°C or 30°C. For the three-segment cotyledon-hypocotyl grafting, cotyledon grafting was first performed when plants were 4 days old (Bartusch et al., 2020), then after 3 days of recovery at 20°C, the attached plants were used for the hypocotyl grafting. GFP or CFDA signals in the rootstocks were observed daily up to 7 DAG. CFDA signals in the scions were observed daily up to 7 DAG.

MoneyMaker tomato (*S. lycopersicum*) seeds were sterilized in 75% bleach solution for 20 min, then rinsed at least five times with sterile water. The seeds were then sown on ½ MS media (1% agar) and germinated at 25°C under short-day conditions (8 h of 140 µmol m^{-2} s^{-1}). Tomato grafting was performed using 7-day-old seedlings. A straight cut was made in the middle of the hypocotyl using a scalpel. Rootstocks and scions were held together within a silicone tube (0.8 mm diameter). The grafted seedlings were transferred on 1% agar media and grown under short-day conditions (8 h of 140 µmol m^{-2} s^{-1}), at either 20°C or 30°C. CFDA signals in the rootstocks were observed daily for 7 DAG.

Phloem and xylem connection assays

Xylem and phloem connections were monitored by the movement of the fluorescent dye CFDA (Thermo Fisher Scientific) across the graft junction. To measure phloem connection, the cotyledon of the *Arabidopsis* grafted plants was wounded with forceps, and then CFDA solution (1 mM) was applied on the surface using a pipette. After 1 h incubation at room temperature, fluorescent signals in the rootstocks were detected. Alternatively, *pSUC2::GFP* (Imlau et al., 1999*)* scions were grafted to wild-type rootstocks, and the GFP signals in the roots were observed daily. For the xylem connection assay, a previously published protocol was modified slightly (Bartusch et al., 2020). In brief, grafted plants with cut root tips were place on a piece of Parafilm, then 1 µl of 1 mM CFDA solution was dropped on the cut site. The signals in the cotyledons were detected after 1 h. For tomato phloem assays, one of the two cotyledons was cut and a drop of CFDA (5 mM CFDA in 1% agar) was applied on the cut site. Seedlings were kept in the dark for at least 2 h. Transversal sections of the hypocotyl (at the shoot-root junction) were made at 2 h and placed on slides to help monitor fluorescence movement. *Arabidopsis* plants and tomato sections were observed under a Leica M205 FA microscope and Leica M205 FCA, with GFP filter to detect CFDA fluorescence in the phloem or xylem. All of the CFDA assays were performed at room temperature. Ungrafted plants were used as controls.

Parasitic plant infection assays

P. japonicum seeds were surface sterilized by washing with 70% ethanol for 20 min, followed by 95% ethanol for 5 min, and sown on ½ MS with 1% sucrose and 0.8% agar. After stratification at 4°C in darkness overnight, the plates were moved to a growth cabinet at 20°C in short-day conditions (8 h of 140 µmol m^{-2} s^{-1}). Four-day-old *P. japonicum* seedlings were transferred to 0.8% water agar for starvation before infection. For the infection, the root of a 5-day-old *Arabidopsis* seedling was aligned to each *P. japonicum* root, and the infection setup was incubated at 20°C or 27°C in short-day conditions (8 h of 140 µmol m^{-2} s^{-1}). At 24 h post infection, swellings on *P. japonicum* root corresponding to early haustoria were marked as day-1 haustoria. The number of haustoria and presence of a xylem bridge were quantified using a Zeiss Axioscope A1 microscope at 3, 5 and 7 days post infection (DPI). Plate xylem area and length were measured on 7 DPI haustoria stained with Safranin-O using a previously published protocol (Spallek et al., 2017).

Histological staining and confocal imaging

Histological staining of GUS was analyzed in *pYUC8::GUS* transgenic seedlings, which were germinated and grown at 20°C for 6 days, then transferred to 27°C, or remained at 20°C, for 48 h. For the staining, seedlings were incubated for 12 h at 37°C with the substrate solution (1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, pH 7.0, 100 mM sodium phosphate buffer, 10 mM Na_2EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide and 0.1% Triton X-100). Stained seedlings were washed with 70% ethanol overnight to remove chlorophyll, and were then photographed using a Leica M205 FA microscope. For confocal microscopy, all images were taken on a Zeiss LSM-780 laser scanning confocal microscope. Graft junction morphology was observed with Calcofluor White staining protocol (Ursache et al., 2018), with 405 nm excitation, 2% laser power, 410-529 nm detection and 210 PMT. Vascular diameter quantifications included cambium, xylem, phloem and pericycle tissues and measured the distance between the pericycle layers encompassing the vascular bundle, 100 µm above the cut site. Samples with GFP and Venus were excited with 488 nm excitation, 10% laser power, 500-524 nm detection and 280 PMT. The images were processed and analyzed using FIJI software (version 2.1.0/1.53c).

NPA treatment assay

The application of the auxin inhibitor NPA plasters on *Arabidopsis* was adapted from a previous study (Bellstaedt et al., 2019). Plants were grown in short-day conditions at 30 µmol m^{-2} s^{-1} to induce longer petioles before grafting, for a more efficient application of NPA plasters. Thin strips of cellulose tissue were soaked in a lukewarm agar solution (1%) with or without 100 µM NPA (Duchefa) and were carefully positioned across

petioles using fine forceps after grafting. For the application of NPA on *P. japonicum*, the NPA plasters were placed on the cotyledons just before the infection assay.

Plant attachment and callus formation assays

For the attachment assays, grafted *Arabidopsis* recovered at 20°C to 27°C were picked up with forceps at the root/hypocotyl junction and scions scored whether they remained attached or fell apart. The petiole callus formation assays was adapted from previously published protocols (Iwase et al., 2017). The explants were incubated at 20°C to 27°C and the area of callus was quantified by ImageJ (version 2.1.0/1.53c). Callus induction was quantified as a percentage of explants with more than one callus cell developing from wound sites.

Gene expression analyses

For *Arabidopsis*, total RNA was extracted from whole seedlings or cotyledons using ROTI Prep RNA MINI (Roth), then subsequently treated with DNase I (New England Biolabs) to eliminate DNA contamination. The cDNA was synthesized with Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific). The transcript levels were measured by quantitative real-time PCR (qPCR) using SYBR-Green master mix (Applied 512 Biosystems) with specific primers (Table S2). The data were normalized against temperature-stable housekeeping gene *PP2A* (Hong et al., 2010). The temperature-stable housekeeping gene *MONENSIN SENSITIVITY1* (*MON1*) transcript levels remained unchanged at 20°C and 27°C in Col-0, *pif4-2* and *yucQ* (Fig. S4D). The relative expression was calculated using the Pfaffl method (Pfaffl, 2001). All reactions were carried out with three biological replicates, each with three technical replicates. For *P. japonicum*, 7 DPI plants were separated from host *Arabidopsis*, and the shoot and root samples were collected. Total RNA extraction, cDNA synthesis and qPCR were performed using the mentioned protocol with *P. japonicum*-specific primers (Table S2). The data were normalized against *PjPP2A*, the homolog of *AtPP2A*.

Statistics

For pairwise comparisons of frequencies, Fisher's exact test was used with the indicated sample sizes. For pairwise comparisons of continuous data, unpaired two-tailed Student's *t*-test was performed.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: P.T.S., K.B., C.W.M.; Methodology: P.T.S., K.B., C.W.M.; Formal analysis: P.T.S., K.B.; Investigation: P.T.S., K.B., M.L., C.M., A.I., Y.C.; Writing - original draft: P.T.S., K.B., C.W.M.; Writing - review & editing: A.I., K.S., M.Q.; Supervision: K.S., M.Q., C.W.M.; Funding acquisition: K.S., M.Q., C.W.M.

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Tissue regeneration is fundamental to maintain tissue integrity. This thesis investigates the genetic and cellular mechanisms of plant during tissue healing and reconnection. We also describe how regeneration programs respond to environmental conditions such as temperatures and nutrient levels. The work of this thesis refines our understanding of plant regeneration and adaptation.

Phanu Theodore Serivichyaswat received his graduate education at the Department of Plant Biology, SLU, Uppsala, Sweden. He obtained his M.Sc. (Molecular Biology) from Korea University, Seoul, South Korea, and B.Sc. (Biosciences) from MFU, Chiang Rai, Thailand.

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