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Molecular and Physiological Mechanisms Regulating Tissue Reunion in Incised Plant Tissues

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Interactions among the functionally specialized organs of higher plants ensure that the plant body develops and functions properly in response to changing environmental conditions. When an incision or grafting procedure interrupts the original organ or tissue connection, cell division is induced and tissue reunion occurs to restore physiological connections. Such activities have long been observed in grafting techniques, which are advantageous not only for agriculture and horticulture but also for basic research. To understand how this healing process is controlled and how this process is initiated and regulated at the molecular level, physiological and molecular analyses of tissue reunion have been performed using incised hypocotyls of cucumber (*Cucumis sativus*) and tomato (*Solanum lycopersicum*) and incised flowering stems of *Arabidopsis thaliana*. Our results suggest that leaf gibberellin and microelements from the roots are required for tissue reunion in the cortex of the cucumber and tomato incised hypocotyls. In addition, the wound-inducible hormones ethylene and jasmonic acid contribute to the regulation of the tissue reunion process in the upper and lower parts, respectively, of incised *Arabidopsis* stems. Ethylene and jasmonic acid modulate the expression of *ANAC071* and *RAP2.6L*, respectively, and auxin signaling via *ARF6/8* is essential for the expression of these transcription factors. In this report, we discuss recent findings regarding molecular and physiological mechanisms of the graft union and the tissue reunion process in wounded tissues of plants.

Keywords: tissue reunion, incision, phytohormones, flowering stem, hypocotyl.

Abbreviations: GA; gibberellin A, JA; jasmonic acid, qPCR; quantitative reverse transcription PCR

Footnote: The authors declare that they have no conflict of interest.

Introduction

Spatially and temporally controlled intercellular attachment is essential for the organization of multicellular organisms. In animal cell division, the cells divide by constriction, and just after cell division the daughter cells separate, although the cells may remain adhered depending on their characteristics (Barr and Gruneberg, 2007). In plants, however, each cell is surrounded by a cell wall that defines its shape and fixes its position within tissues (Smith, 2001). In most plants, the cells divide via formation of the cell plate, and daughter cells remain attached just after cytokinesis. In higher plants, however, the cell plate is formed during the last step of cell division to produce two daughter cells that remain attached to each other. Epigenetic re-adhesion of separated cells usually occurs in animal systems, but it rarely occurs in higher plants, such as in carpel fusion during gynoecium development (Walker, 1975; Siegel and Verbeke, 1989; van der Schoot et al., 1995), tissue union during grafting (Kollmann and Glockmann, 1985; Wang and Kollmann, 1996; Richardson et al., 1996; Flaishman et al. 2008), and cell repair in incised tissues. In *Arabidopsis thaliana* mutants, the genes encoding *fiddlehead* (Lolle et al., 1992, 1997; Lolle and Cheung, 1993; Lolle and Pruitt, 1999) and *wax-1* (Jenks et al., 1996) induce the ectopic fusion or adhesion of aerial tissues.

Plants have the ability to undertake defense responses against environmental stresses and biotic attacks as well as by wounding. Wounding threatens plants survival, especially if the injury occurs to the stem, because it alone connects each shoot to the plant (Satoh, 2006). Stobbe et al. (2002) reported that the wound-induced calli of lime trees (*Tilia* sp.) showed subsequent development of a wound tissue comprised of xylem, cambium, and phloem in particular cases. At the cellular level, plants generally respond to wounding by activating defense systems required for healing wounded tissues and protecting them from subsequent pathogen invasion. The physiological responses include the repair and reinforcement of the cell wall and the activation of wound-signaling pathways that induce the production of both local and systemic defense-related proteins, including basic-type pathogenesis-related (PR) proteins and wound-related hormones such as ethylene and jasmonic acid (JA) (Bostock and Stermer, 1989; Leon et al., 2001; Sasaki et al., 2001). When wounding or grafting interrupts the original vascular connection, the formation of new vascular tissues can be induced. Although the molecular mechanisms controlling vascular differentiation are not fully understood, the involvement of phytohormones, such as auxin and cytokinin, in xylem and phloem differentiation has been suggested (Roberts, 1988; Mattsson et al., 1999; Sachs, 2000; Ye, 2002).

In contrast to vascular regeneration and defense responses, the molecular basis of tissue repair,

including tissue reunion in the ground tissues such as the cortex and pith, remains largely unknown (Reid and Ross, 2011). The reunion process in those tissues also includes cell division and cell differentiation to recover the lost tissue (Asahina et al., 2002; 2006; 2011). Although plant cell is known to exert totipotency upon stresses and has been much studied in tissue culture, the underlying molecular mechanisms are beginning to be understood. Several studies suggest that certain transcription factors (TFs) are involved in regulating the expression of many genes that govern the regeneration process (Zimmerman 1993; Sena et al., 2009; Ikeuchi et al., 2013); it is unclear, though, how this healing process is initiated and regulated, including the underlying molecular mechanisms.

In this review, we describe recent findings regarding the molecular and physiological studies of the graft union, and the control mechanisms of tissue reunion in incised tissues, with a specific focus on the involvement of phytohormones.

Molecular and physiological studies of the graft union.

The root and aboveground organs of higher plants are connected by the xylem and phloem of the vascular bundles, and vascular tissues provide the long-distance transport system for water, nutrients and various organic materials within the plant (Jesko, 1989; Satoh et al., 1998; Sakuta and Satoh, 2000, Satoh, 2006). When wounding or grafting interrupts the original vascular connection, the formation of new vascular tissues should be induced. Such activities have long been observed in grafting techniques, which are advantageous for agriculture and horticulture (Davis et al., 2008). Tsukaya et al. (1993) developed the grafting technique used on *Arabidopsis* flowering stems, and grafting experiments using *Arabidopsis* have been performed to study different aspects of plant biology. Flaishman et al. (2008) used homografts and heterografts of *Arabidopsis* inflorescence stems to study the compatibility and ontogeny of graft union formation. Micrografting, a seedling grafting technique, was first reported in *Arabidopsis* by Turnbull et al. (2002) and has been applied to basic research on systemic physiological events, such as flower induction (Corbesier et al., 2007; Notaguchi et al., 2008; Notaguchi et al., 2009) and hormone actions (Matsumoto-Kitano et al., 2008). Yin et al. (2012) developed a simple and effective micrografting method that improved on the previous protocols (Turnbull et al., 2002; Bainbridge et al., 2006).

Previous studies on grafting and the repairing of wounded tissues have demonstrated the involvement of phytohormones, such as auxin and cytokinin in the repairing process of vascular tissues (Sachs, 2000). As related findings, several groups have reported the role of several NAC (NAM, ATAF1, 2 and CUC2) TFs when the vascular elements are regenerated (Kubo et al., 2005; Mitsuda et al., 2005; Yamaguchi et al., 2008). Recently, Yin et al. (2012) performed histochemical and microarray analyses to show gene expression profiles during the grafting of *Arabidopsis* seedlings. Their work addressed the

molecular events underlying graft union development and provided evidence that auxin and wound-inducible hormones, such as ethylene and JA, are involved in the development of the graft union in *Arabidopsis* seedlings, although the gene expression patterns might be different from those of the partially incised stems which will be mentioned in the later sections, depending on the continuity of polarity and auxin polar transport through the non-incised part of the stem.

Differences of tissue participation for the reunion in different plant species.

Tissue reunion of cucumber and tomato hypocotyls.

To investigate the tissue reunion process in the cortex of cut tissues, cucumber and tomato hypocotyls have been transversely cut to half their diameters, and morphological and histochemical analyses were then performed (Asahina et al., 2002). Cell division in the cortex commences 3 days after cutting, and the cortex is nearly fully reunited within 7 days (Fig. 1a). Cell division during tissue reunion is strongly inhibited when the cotyledons were removed from the cucumber seedlings. In Japan, the grafting of cucumber hypocotyls onto squash rootstocks is often performed to prevent damage from soil borne diseases during cultivation (Sato, 1996). Although the exact role of the cotyledons in the formation of the graft union is not understood, cotyledons of the scion and rootstock are preferentially left on the hypocotyl to improve the grafting efficiency. These facts are consistent with our result that tissue reunion in incised cucumber hypocotyls does not occur after the removal of the cotyledons.

This inhibition is reversed by applying gibberellin (GA) to the apical tip of the plants without cotyledons, but not restored by indole-3-acetic acid (Asahina et al., 2002). Supporting this observation, cell division in the cortex is inhibited by the treatment of the cotyledons with uniconazole-P, a GA biosynthesis inhibitor, and this inhibition is also reversed by the simultaneous application of GA. The requirement of GA for tissue reunion in cut hypocotyls is also evident in *gib-1*, the GA-deficient mutant of tomato (Asahina et al., 2002). In contrast to the essential role of the cotyledons, normal tissue reunion in cut hypocotyls is still observed when shoot apices are removed (Asahina et al., 2002). Moreover, normal tissue-reunion occurred in the cortex of the hypocotyl when 10^{-4} M 2,3,5-triiodobenzoic acid (TIBA), an inhibitor of polar auxin transport, was applied to the surface of the shoot above the cut on the hypocotyl. These results indicate that shoot apex-producing auxin is less important in tissue reunion process of hypocotyls in cucumber and tomato seedlings.

To study the effects of cotyledons on the GA content in hypocotyls directly, cotyledons have been removed from seedlings, and endogenous GA levels in the hypocotyls have then been determined (Asahina et al., 2007). Compared with intact seedlings, those without cotyledons contain reduced levels of bioactive GA₄ (Gibberellin A4) and its precursors in the cucumber and tomato hypocotyls. These

results suggest that GA is required for cell division during tissue reunion in the cortex of wounded hypocotyls from cucumber and tomato seedlings and that cotyledons are necessary for maintaining normal GA levels in hypocotyls (Fig. 2).

In the hypocotyls of root-eliminated cucumber seedlings, cell division occurs in the cortex during the tissue reunion process, but intrusive cell elongation and the interdigitation of cortex cells at the cut surface to form a tight tissue connection does not occur, even after 7 days. Experimental results, including the application of particular compounds contained in xylem sap (Sato et al. 2006), suggest that boron, zinc and manganese ions, as well as their uptake from soil to xylem sap, are required for the intrusive cell elongation that occurs during tissue reunion in the cortex of cucumber hypocotyls (Fig. 2; Asahina et al., 2006).

Tissue reunion of Arabidopsis incised stem.

The technique of grafting has been widely used in agriculture, horticulture and the basic studies of plant biology. Apart from the grafting of hypocotyls in *Cucurbita* plants, grafting is usually performed on the stems of fruit trees and *Solanaceae* plants. In *Arabidopsis*, both flowering stem grafting and hypocotyl grafting have been described (Tsukaya et al. 1993; Turnbull et al. 2002). Because *Arabidopsis* seedlings are so small that manipulating their hypocotyls is difficult, we have examined the tissue reunion process in incised flowering stems. When the flowering stem of *Arabidopsis* is incised through half of its diameter, cell division occurs in the pith 3 days after incision (Fig. 1b). However, the process is strongly inhibited by the decapitation of the inflorescence and when the polar transport of auxin is inhibited, either by an inhibitor or in the *pin1* mutant, which affects *PIN-FORMED 1*, a transmembrane protein involved in auxin transport (Asahina et al., 2011). The incised stems of the GA-deficient *Arabidopsis* mutant *ga3ox1/ga3ox2* recover with no difficulty, indicating that GA is not involved in wound healing of stems in *Arabidopsis* (Asahina et al. 2011).

Different hormone requirements for tissue reunion in wounded hypocotyls and stems.

Differences in the hormone requirements for the tissue reunion process between *Arabidopsis* incised flowering stems and cucumber and tomato incised hypocotyls may be due to differences in the tissues and the developmental stages (Table 1). After the incision of cucumber and tomato hypocotyls, cell division and cell elongation occur mainly in the cortex to restore the stem connection, which requires GA for cortex cell division during tissue reunion. The hypocotyl, an embryonic organ, consists of epidermal, cortical, endodermal and vascular tissues, but this is not true of the pith tissue. Its growth is thought to depend on the cotyledons, which supply nutrients and hormones at the early post-germination stage (Reid

and Ross, 2011). In contrast, the *Arabidopsis* flowering stem is a reproductive organ that develops after the juvenile stage and contains epidermal, cortical, endodermal and vascular tissues, as well as the pith, which occupies most of the central part of the stem.

Genetic networks in the tissue reunion process.

A microarray analysis of *Arabidopsis* incised stems revealed differential gene expression levels between cut and uncut stems with intact or decapitated inflorescences and during the tissue reunion process; these findings are summarized in Figure 3. Up-regulated genes included those that are related to cell division and phytohormones, as well as genes that encode TFs, which were predominantly expressed 1 to 3 days after cutting. The expression levels of *INDOLE-3-ACETIC ACID INDUCIBLE 5 (IAA5)*, which encodes an Aux/IAA auxin response protein, *1-AMINO-CYCLOPROPANE-1-CARBOXYLATE SYNTHASE 2 (ACS2)* and *LIPOXYGENASE 2 (LOX2)*, which are involved in ethylene and JA production, respectively, peak at 1 day post-incision.

NAC DOMAIN CONTAINING PROTEIN 71 (ANAC071), which encodes a TF, is predominantly expressed at 1–3 days post-incision, and its expression is promoted by auxin. Auxin-responsive genes contain auxin response elements (AuxREs) in their promoter regions that specifically bind ARFs (Hagen and Guilfoyle, 2002; Liscum and Reed, 2002). In the promoter of *ANAC071*, AuxREs are present at –2654 bp from translational start site. In addition, in *ANAC071-promoter::GUS* transgenic plants, GUS activity was high in the distal region of the incision as well as after indole-3-acetic acid (IAA) application in decapitated plants (Pitaksaringkarn et al., 2014b), suggesting that the auxin may control the expression of *ANAC071* in the tissue reunion process. The *arf6arf8* mutant shows an inhibition of cell division in the pith tissue a week after incision, whereas no inhibition is observed in each single mutant and in the mutants for the other ARFs (Pitaksaringkarn et al., 2014b). Our qPCR analysis indicated that the expression of *ANAC071* decreases in both incised and non-incised stems of the *arf6arf8* mutant (Pitaksaringkarn et al., 2014b). Thus increased auxin levels in the upper part of the incision might induce expression of *ANAC071* with mediation by *ARF6* and *ARF8*.

The expression of *RELATED TO AP2 6L (RAP2.6L)*, a member of the ethylene response factor (ERF) subfamily B-4 of the ERF/AP2 TF family, also peaks at 1 day post-incision and is up-regulated by JA but is suppressed by auxin. The expression level of *RAP2.6L*, another early induced gene by incision, is higher in non-incised stems but is lower in incised stems of the *arf6arf8* double mutants than in those of wild-type plants (Pitaksaringkarn et al., 2014b).

Tabata et al. (2010) reported that, in the *AUXIN RESPONSE FACTOR (ARF)* double mutant *arf6arf8*, the amount of JA is reduced in flower buds, and, of several JA biosynthetic genes studied, only

the expression of *DEFECTIVE ANther DEHISCENCE 1 (DAD1)* is significantly decreased compared with wild type. Our qPCR analyses showed that some of the JA biosynthesis genes are up-regulated and that JA levels are increased during the tissue reunion process (unpublished data). In addition, *DAD1* expression levels were dramatically decreased in the *arf6 arf8* mutant as compared with the wild-type (Pitaksaringkarn et al., 2014). From these results, we hypothesized that auxin signaling via *ARF6/8* is essential for the expression of *ANAC071* in the upper parts of incised stems (Fig. 3a) and that the production of JA, via the induction of *DAD1* (Fig. 3d), induces *RAP2.6L* expression in the lower parts of the incised stems during the tissue reunion process (Pitaksaringkarn et al., 2014).

Ethylene also promotes *ANAC071* expression but suppresses *RAP2.6L* expression. Plants expressing dominant negative form of *RAP2.6L* (*RAP2.6L-SRDX*) or *ANAC071* (*ANAC071-SRDX*) have defects in tissue reunion, and the tissue reunion site in the integral membrane protein *ETHYLENE INSENSITIVE 2 (EIN2)* mutant, *ein2*, has an abnormal morphology. Five days after cutting the *ein2* stem, cell division was observed only in the cortex neighboring the incision and not in the pith, unlike in the wild-type stem. Complete reunion was not observed up to 7 days after cutting the *ein2* stem (Asahina et al., 2011). Although the control mechanisms for the downstream targets of the *ANAC071* and *RAP2.6L* remain unknown, these results indicate that *ANAC071* and *RAP2.6L* are essential for tissue reunion in *Arabidopsis* incised flowering stems but are conversely affected by polar-transported auxin, with fine-tuning by the wound-inducible hormones JA and ethylene (Fig. 3; Asahina et al., 2011).

XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 20 (XTH20) is a selected gene from a microarray analysis that is induced by auxin and is highly expressed in the upper part of cut inflorescence stems, in a manner similar to *ANAC071* (Asahina et al., 2011; Pitaksaringkarn et al., 2014a). Our current analysis indicated that *ANAC071* binds directly to the promoters of *XTH20* and *XTH19* (which is phylogenetically closest to *XTH20*) to induce their expression, and the *xth19xth20* double mutant shows inhibition of pith cell proliferation (Pitaksaringkarn et al., 2014a). Although further research is needed to elucidate how *XTHs* control cell proliferation and/or cytokinesis during tissue reunion, this work revealed that *XTH19* and *XTH20* are directly controlled by *ANAC071*, which regulates the tissue reunion process in *Arabidopsis* incised inflorescence stems.

Wounding promotes callus formation in various parts of *Arabidopsis* seedlings in tissue culture (Iwase et al., 2011; Ikeuchi et al., 2013). Iwase et al. (2011) reported that *WOUND INDUCED DEDIFFERENTIATION 1 (WIND1)*, another AP2/ERF TF gene, is up-regulated in wounded *Arabidopsis* hypocotyls and that *WIND1* promotes cell dedifferentiation, suggesting that *WIND1* proteins, along with other functionally redundant factors, regulates callus formation upon wounding. Iwase et al. (2013) also reported that the *WIND1*-mediated signaling cascade for cell dedifferentiation during callus formation is

conserved in several species of *Brassicaceae* and *Solanaceae* and may be conserved more widely. We found that *WIND1* is not up-regulated during tissue reunion in *Arabidopsis* incised stems at 1, 3, and 5 days after incision (Asahina et al., 2011) and this gene is not responsive to auxin (Iwase et al., 2011). We also found that *WIND1* was not significantly up-regulated at 1 h - 24 h after incision and the incised stems of *WIND1*-SRDX showed normal reunion (unpublished results). This suggests that the different responses or signaling cascades found between the tissue reunion of partially incised inflorescence stems and the wound-induced callus formation of explants may be due to the presence or absence of auxin polar transport along the vertical axis.

Conclusion

The stem is an essential organ that supports plant body structure and subsequent organ development and delivers water, nutrients and chemical information to the roots and shoots of plants (Satoh, 2006; Kehr and Buhtz, 2008). Therefore, as soon as injuries occur to the stem, defense responses and repair processes are activated to protect the plant from further damage and to recover normal physiology (Reid and Ross 2011). Because auxin is produced in the apical meristem and is transported in stems with polarity, its distribution triggers corresponding developmental responses (Sachs, 1991; Friml, 2003; Robert and Friml, 2009) and several physiological responses, including formation of adventitious roots and the breaking of apical dominancy, which are associated with the type of wound and its depth. The wound depth may not disturb a functional route of auxin transportation or may lead to complete interruption of its transportation.

Various compounds and physical factors are involved in the wound signal transduction pathway, and cross-talk between some signaling pathways results in a different pattern of responses to mechanical damage (Che et al., 2007). A detailed analysis of temporal and spatial gene expression levels in cells undergoing tissue reunion will be required for a full understanding of the molecular events during tissue reunion (Fig. 4). How wound-signal transduction is involved in the tissue reunion process, especially in the gene regulatory network, and whether tissue reunion is controlled by similar TFs and plant hormones in different plant species remain to be examined in the future.

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Figure Legends

Figure 1. The tissue reunion process in the incised cucumber hypocotyl and *Arabidopsis* flowering stem. (a) Incised hypocotyl of cucumber. After 7 days of germination, the hypocotyls were incised through half of their diameter transversely 3 cm from the base using a razor blade and the plant was then grown for an additional 7 days. (b) Incised flowering stem of *Arabidopsis*. After 7–10 days of bolting, flowering stem between 1st or 2nd cauline leaf and rosette leaf was incised through half of their diameter with a micro-surgical knife and the plant was then grown for an additional 7 days. Photographs of section were taken from 1 to 7 days after incision. Arrowheads indicate the incision site. pi, pith; co, cortex; vb, vascular bundle. Scale bars: 100 μ m. Microscopy images in (a) are reprinted and modified from Asahina et al. (2002) and in (b) are from Asahina et al. (2011). Detailed experimental procedures are described in previous studies (Asahina et al. 2002; 2011).

Figure 2. Schematic model for tissue reunion in cucumber and tomato incised hypocotyl. Gibberellin (GA) is required for cell division during tissue reunion in the cortex of incised hypocotyls of cucumber and tomato seedlings, and cotyledons are necessary for maintaining normal GA levels in hypocotyl. The microelements boron (B), zinc (Zn) and manganese (Mn) ions from the roots are required for intrusive cell elongation during tissue reunion in the cortex of cucumber hypocotyls.

Figure 3. Schematic model of the phytohormone regulation of *ANAC071* and *RAP2.6L* expression during tissue reunion in *Arabidopsis* incised stem (modified from Asahina et al., 2011). The tissue reunion process is regulated differently in the upper and lower parts of the incision. PIN1 protein is involved in polar transport of auxin in flowering stem. In the upper part, *ANAC071* is up-regulated by IAA (a) and ethylene (b), a wound-inducible hormone. In the lower part, *RAP2.6L*, which was down-regulated by IAA, is activated because of auxin depletion due to blocked polar auxin transport. JA, a key regulator of plant responses to environmental stresses and biotic challenges, also induces *RAP2.6L* expression (c). Auxin signaling via *ARF6/8* is essential for the expression of *ANAC071* and *RAP2.6L* in the upper and lower parts of incised stems, respectively, and the production of JA, via induction of *DAD1* (d) induces *RAP2.6L* expression in the tissue reunion process. The gene expression of *ACS2* and *LOX2/3*, which are involved in ethylene and JA production, respectively, peak at 1 day post-incision.

Figure 4. Schematic model of cellular and molecular events during the tissue reunion process in incised *Arabidopsis* stems.

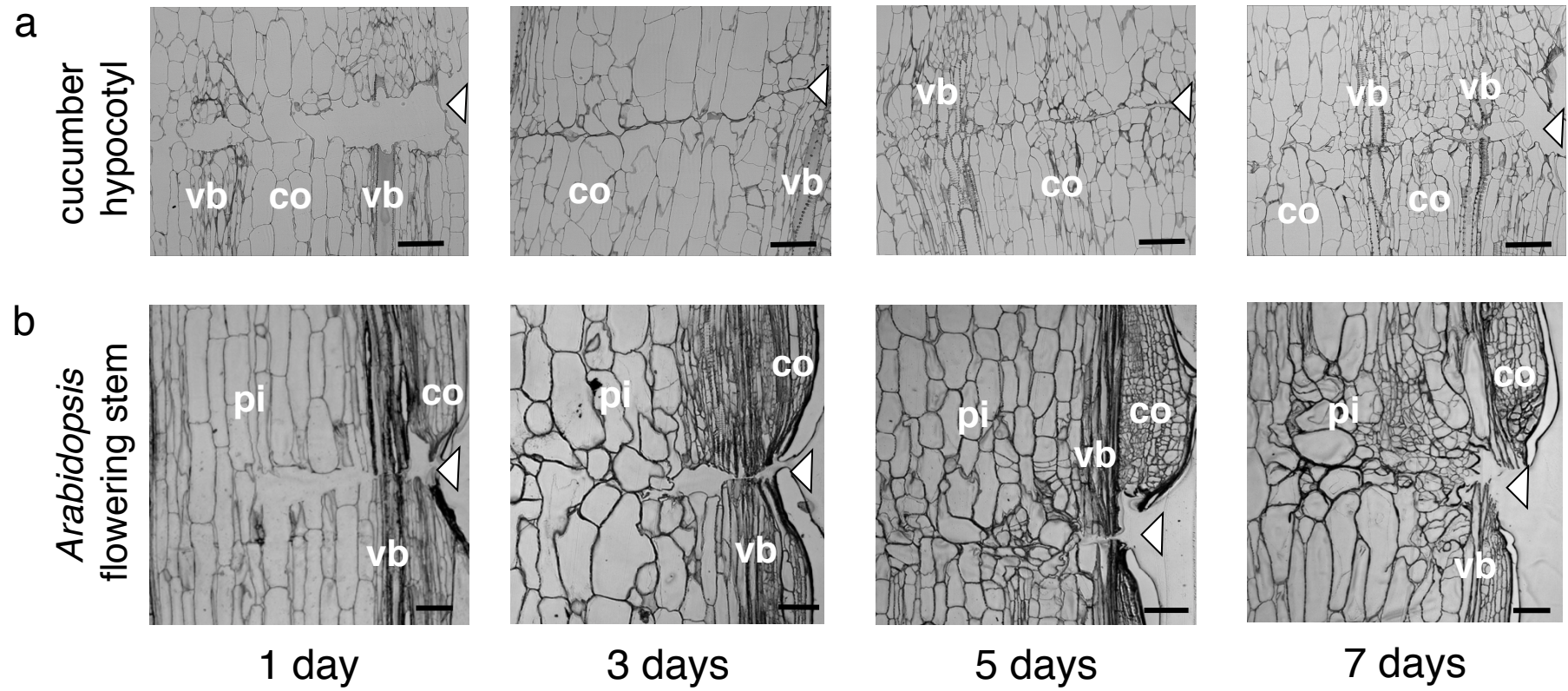


Fig. 1

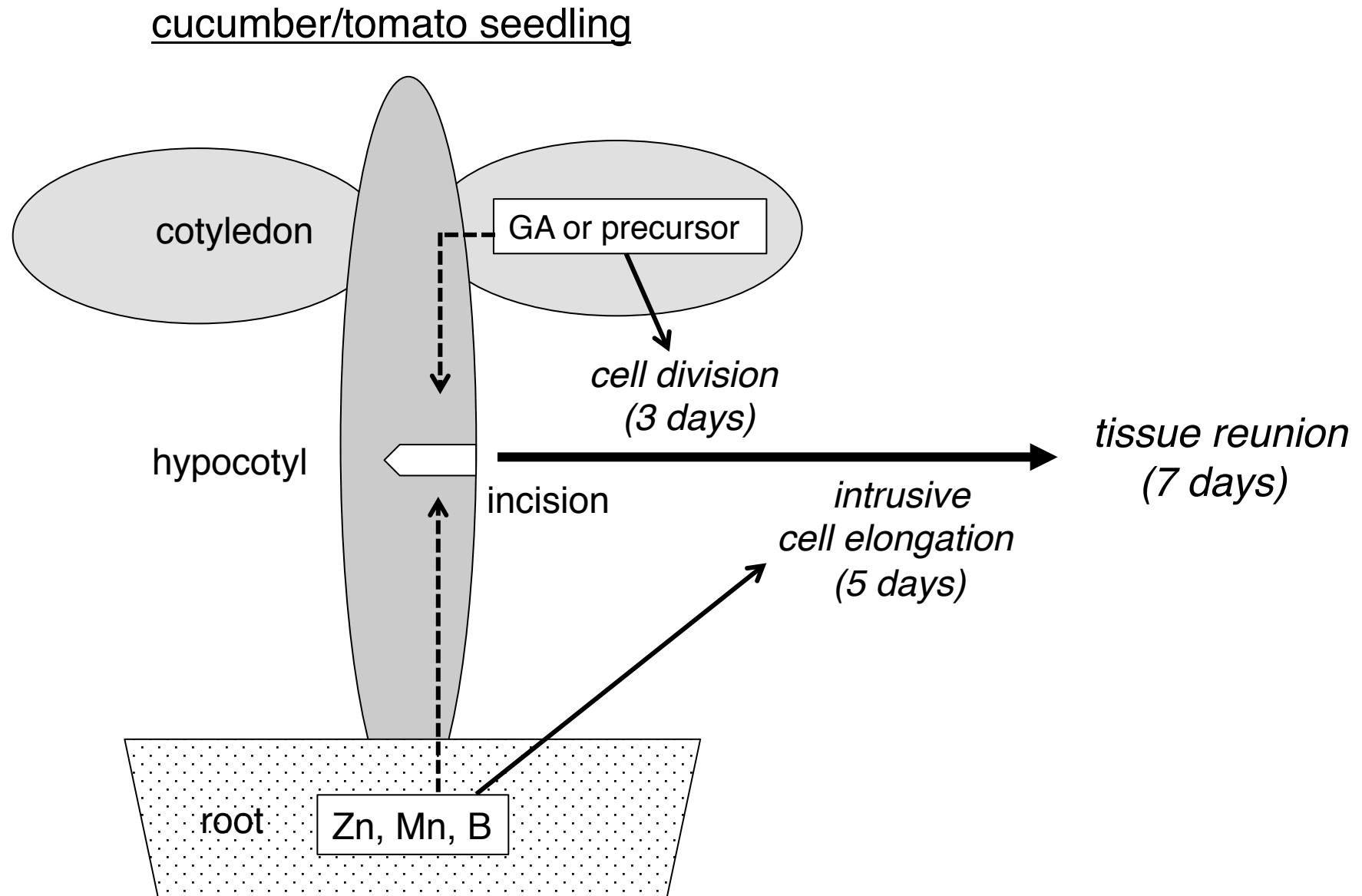


Fig. 2

Arabidopsis stem

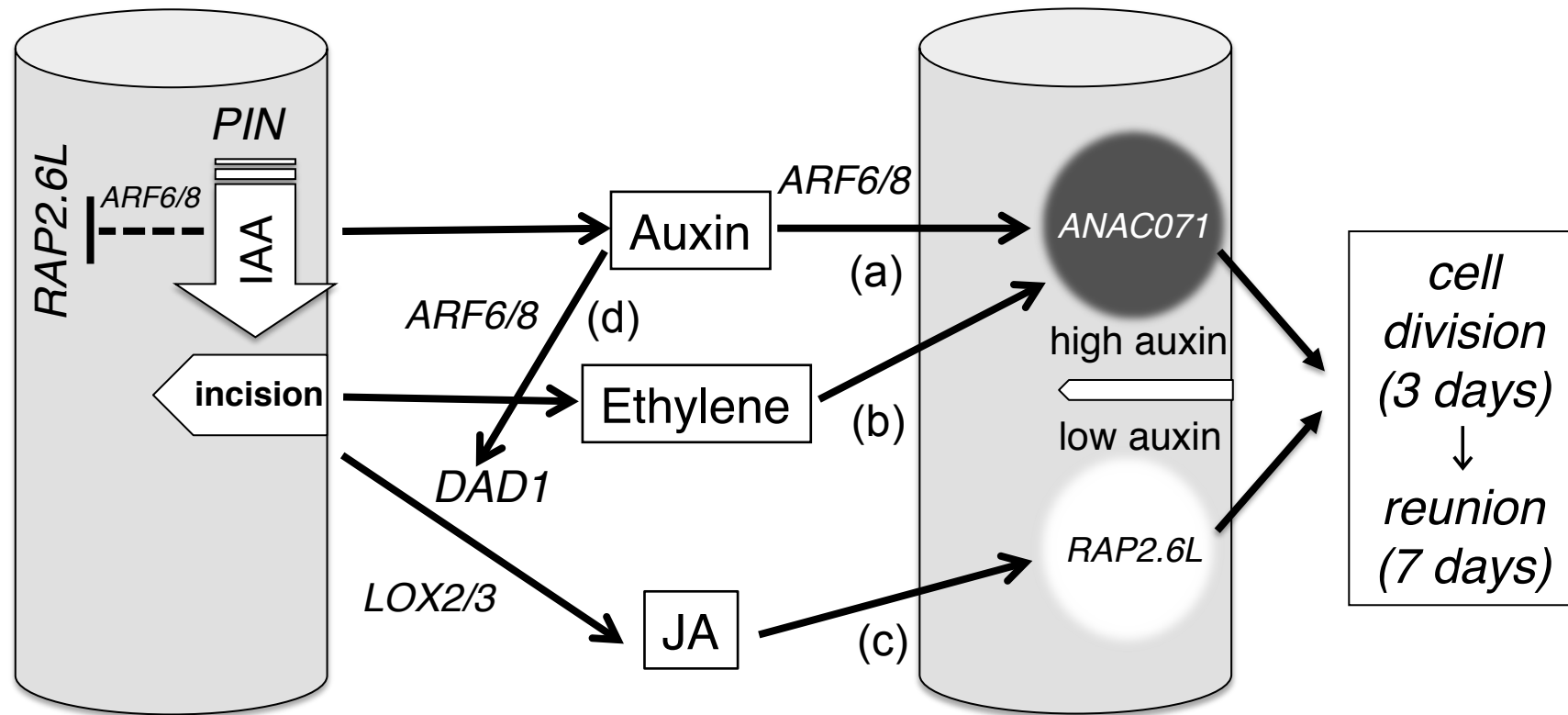


Fig. 3

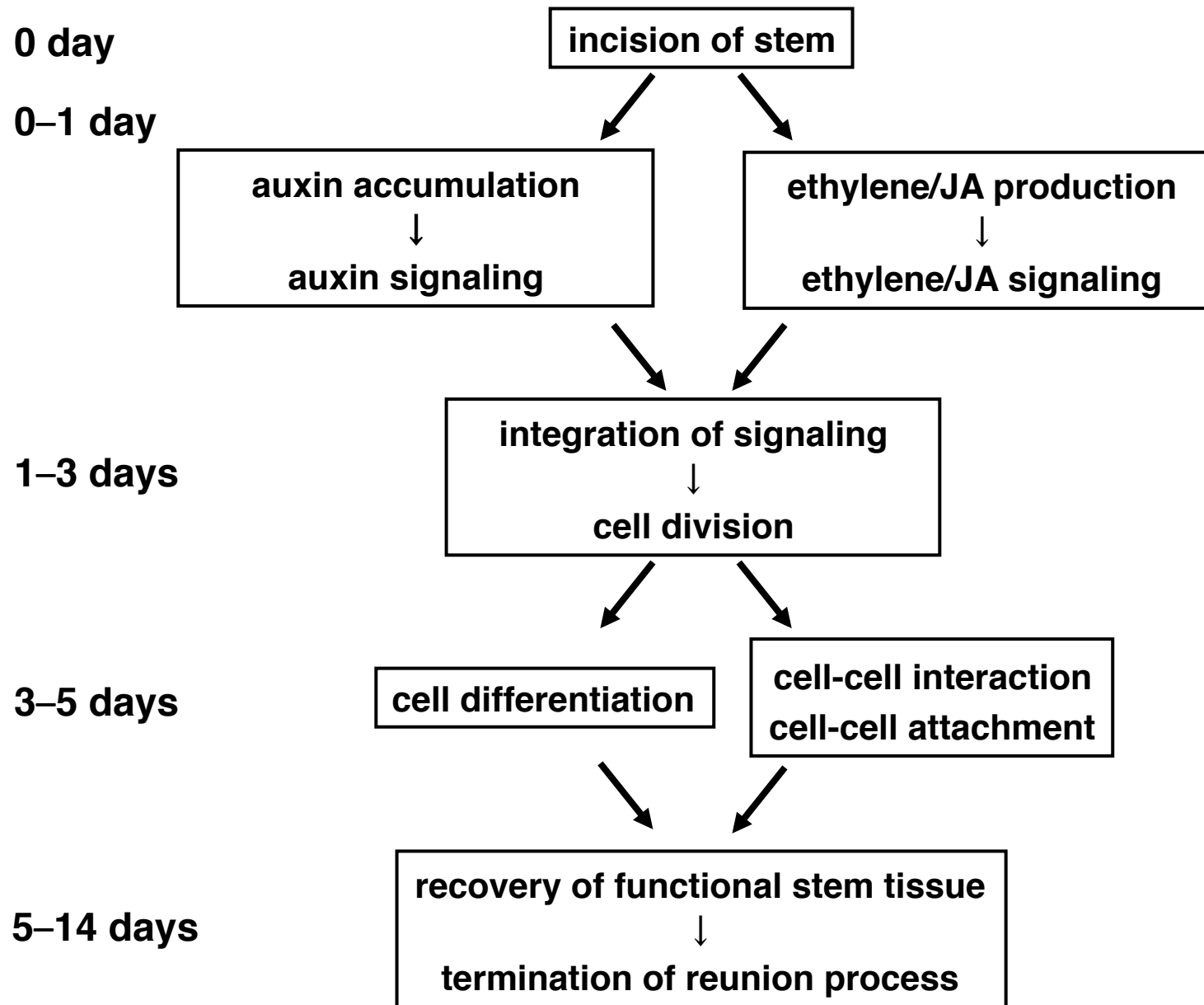


Fig. 4

Table 1. Comparison of tissue-reunion between incised hypocotyl and stem.

	hypocotyl	flowering stem
species	cucumber/tomato	<i>Arabidopsis</i>
stage	seedling (vegetative)	reproductive
reunion tissue (with cell division)	cortex	pith
days for reunion	~7 days	~7–10 days
hormone	GA	auxin, JA, ethylene