Spatially selective hormonal control of RAP2.6L and ANAC071 transcription factors involved in tissue reunion in *Arabidopsis*

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When grafting or wounding disconnects stem tissues, new tissues are generated to restore the lost connection. In this study, the molecular mechanism of such healing was elucidated in injured stems of *Arabidopsis*. Soon after the inflorescence stems were incised, the pith cells started to divide. This process was strongly inhibited by the elimination of cauline leaves, shoot apices, or lateral buds that reduced the indole-3-acetic acid supply. Microarray and quantitative RT-PCR analyses revealed that genes related to cell division, phytohormones, and transcription factors were expressed because of incision. Among them, two plant-specific transcription factor genes, *ANAC071* and *RAP2.6L*, were abundantly expressed. *ANAC071* was expressed at 1–3 d after cutting exclusively in the upper region of the cut gap, with concomitant accumulation of indole-3-acetic acid. In contrast, *RAP2.6L* was expressed at 1 d after cutting exclusively in the lower region, with concomitant deprivation of indole-3-acetic acid. The expression of *ANAC071* and *RAP2.6L* were also promoted by ethylene and jasmonic acid, respectively. In transformants suppressing the function of *RAP2.6L* or *ANAC071*, the division of pith cells was inhibited. Furthermore, the ethylene signaling-defective *ein2* mutant showed incomplete healing. Hence, plant-specific transcription factors differentially expressed around the cut position were essential for tissue reunion of *Arabidopsis* wounded flowering stems and were under opposite control by polar-transported auxin, with modification by the ethylene and jasmonic acid wound-inducible hormones.

regeneration | meristem | stress

Functionally specialized leaves, flowers, and roots of vascular plants are integrated via stems to enable plant responses to changing environments (1). Plant stems provide essential mechanical support of the plant body and deliver nutrients as well as physiological chemical information through vascular bundles (1). Auxin is required to establish the vertical axis and is produced in shoot apices and transported to roots through stele parenchyma or procambial cells with auxin-specific transporters (2). Biotic or biophysical plant environmental stresses often result in wounding that may disintegrate and endanger plant tissues. Cellular responses to wounding involve reconstruction of damaged tissues and also activation of the synthesis of defense-related proteins, including basic pathogenesis-related proteins, as well as wound hormones such as ethylene and jasmonic acid (JA) (3). During reconstruction of damaged tissues, vascular and/or other cells are transformed so as to physiologically connect the existing tissues (4, 5). Such activities have long been observed in grafting techniques, which are advantageous for agriculture and horticulture (6) and also for basic research on systemic physiological events, such as flower induction (7) and hormone actions (8, 9). Low grafting efficiency and tissue incompatibility are often encountered, necessitating an understanding of the healing mechanism underlying grafting. In previous studies on grafting as well as repairing of wounded tissues, much effort has focused on understanding the regeneration of vascular elements, demonstrating the involvement of phytohormones such as auxin and cytokinin, and several NAC (NAM, ATAF1,2 and CUC2) transcription factors (TFs) (4, 10).

Processes reuniting ground tissues such as the cortex and pith in injured regions comprise cell division and successive cell differentiation, although the underlying molecular mechanisms have been less studied than vascular regeneration. Active pectin biosynthesis occurs in cucumber and tomato hypocotyls, and gibberellin (GA), likely produced in and exported from cotyledons, is used for cell division during cortical tissue reunion (11–13). Such regeneration ability has been studied in terms of totipotency in tissue culture, suggesting that TFs are required for regulating gene expression related to regeneration (14, 15). However, the TFs and accompanying molecular processes involved in reuniting ground tissues remain to be determined.

The research reported here aimed to understand the molecular events during the tissue-reunion process in plant stems. To this end, a tissue-reunion inducible system was established by incising *Arabidopsis* inflorescence stems. The incised tissues were investigated by microarray analysis followed by expression/functional analysis of the up-regulated genes. Two plant-specific TF genes, *ANAC071* and *RAP2.6L*, with localized expression in the upper and lower regions of the cut gap, respectively, were required for the division of pith cells in the reunion process. Differential controls of these genes by auxin and the wound-related ethylene and JA hormones are also described.

Results

Histological Time Course of Cell Division During Wounding of the *Arabidopsis* Inflorescence Stems. *Arabidopsis* inflorescence stems were used 7–10 d after bolting. The internodes between the first cauline leaves and rosette leaves, which were no longer capable of cell division and elongation, were incised to half-diameter depth with a microsurgical knife (Fig. S1 *A* and *B*).

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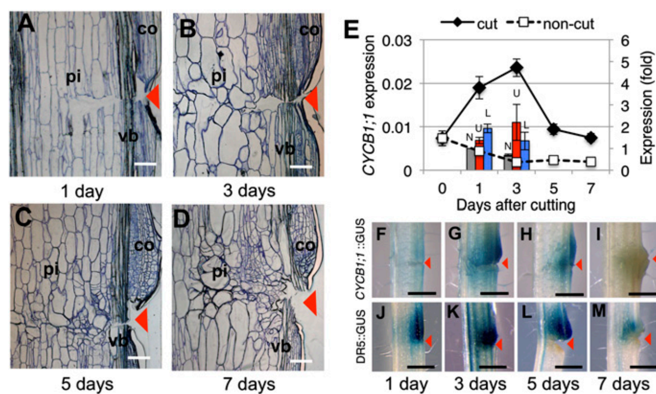

Fig. 1. Process of tissue reunion in the wounded flowering stem of *Arabidopsis*. (*A*–*D*) Light micrographs of the tissue-reunion process. Arrowheads indicate position of the cut. pi, pith; co, cortex; vb, vascular bundle. (*E*) Expression of *Cyclin B1;1*. Each value is the mean ± SE (*n* = 3). Bars show comparative expression between upper (U; red bar) or lower regions (L; blue bar) of cut stem. The noncut (N; gray bar) control was arbitrarily set to 1, and the mean ± SE is shown (*n* = 4). (*F*–*I*) *pCyclin B*::*GUS* plants. (*J*–*M*) *DR5*::*GUS* plants. Photographs were taken at 1 d (*A, F, and J*), 3 d (*B, G, and K*), 5 d (*C, H, and L*), and 7 d (*D, I, and M*) after cutting. (Scale bars, 100 μm in *A*–*D*, 1 mm in *F*–*M*.)

The pith cells around the cut site then began to randomly divide and elongate intrusively toward the cut surface (Fig. 1 *A*–*D*). To obtain an estimate of cell division activity in the wounded stems, quantitative RT-PCR (qRT-PCR) analyses were performed using mitotic cyclin-encoding *cyclin B1;1* (At4g37490), a mitosis marker gene expressed only around the G2/M transition (16) (Fig. 1*E*). The cyclin gene was clearly up-regulated in the wounded region 3 d after cutting, where cell division was initiated in both pith and cortical cells neighboring the wounded regions (Fig. 1*B* and *E*), as was previously observed in wounded cucumber hypocotyls (11). Spatiotemporal histochemical changes in mitotic activity in response to tissue reunion were pursued using the *cyclin B1;1* promoter (designated as *pcyclin B*)::*GUS* transgenic plants (Fig. 1 *F*–*I*). The GUS activity culminated 3 d after cutting (Fig. 1*G*), in agreement with both qRT-PCR analysis (Fig. 1*E*) and morphological observations (Fig. 1 *A*–*D*). The GUS activity was localized in the pith, vascular tissues, and cortex in both the upper and lower regions of the cut gap, although the intensity was much higher in the upper region (Fig. 1*G*).

Roles of Auxin in Tissue Recovery in Incised *Arabidopsis* Stems. The level of auxin was histochemically analyzed using artificial auxin-responsive promoter (*DR5*)::*GUS* transgenic plants (Fig. 1 *J*–*M*). Strong GUS activity occurred at 1–5 d after cutting, with higher intensity in the upper region (Fig. 1 *J*–*L*), indicating that auxin accumulation may have occurred in the upper region immediately after cutting, lasting for 5 d and diminishing, and then tissue reunion completed within almost 7 d (Fig. 1 *L* and *M*). Auxin is derived from either cauline leaves, shoot apices, or lateral buds. Removal of both the shoot apex and lateral buds or only the cauline leaves resulted in minor inhibitory effects (Fig. S1 *E* and *F*), but simultaneous removal of all three severely retarded cell division and *pcyclin B*::GUS activity in the pith tissue (Fig. S1 *D* and *H*). Involvement of auxin in this phenomenon was substantiated by the fact that 10^{-3} M indole-3-acetic acid (IAA) application to the top of the decapitated stem nullified the inhibitory effect of decapitation (Fig. 2*E*). Furthermore, the healing processes in the wound tissue were also inhibited by the application of 10^{-3} M 2,3,5-triiodobenzoic acid (TIBA), an inhibitor for polar auxin transport, to the upper part of the cut gap (Fig. 2*B*). Similar effects were also observed in the polar auxin

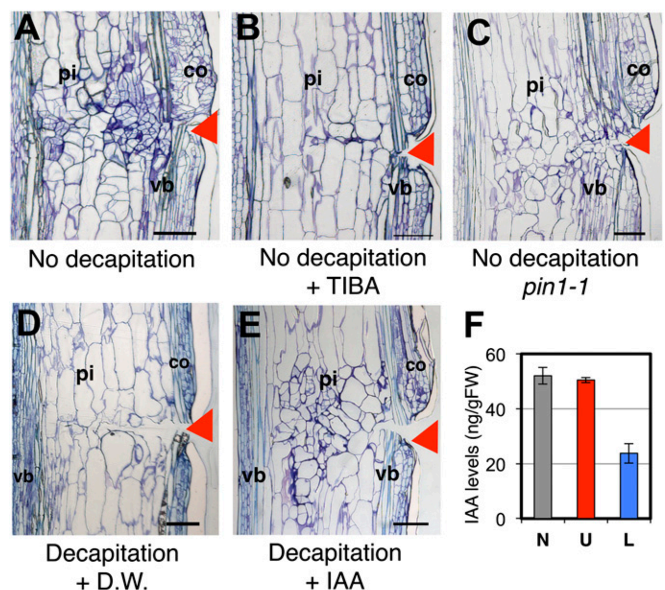

Fig. 2. Roles of auxin on tissue reunion in *Arabidopsis* wounded stems. (*A* and *B*) Control wild type without decapitation. (*C*) *pin1-1*. (*D* and *E*) Decapitated wild type. Lanolin paste containing distilled water (D.W.) (*A* and *D*), 10^{-3} M TIBA (*B*), or 10^{-3} M IAA (*E*) was applied. Arrowheads indicate position of cut. pi, pith; co, cortex; vb, vascular bundle. Images were taken 7 d after stem cutting. (Scale bars in *A*–*E*, 100 μm.) (*F*) Quantification of endogenous IAA in upper (U; red bar), lower (L; blue bar), or noncut region (N; gray bar). Mean ± SE is shown (*n* = 3).

transport mutant *pin1-1* (17) (Fig. 2*C*). In contrast, cortical cell division was activated without regard to the removal of the auxin-delivering tissues (Fig. S1 *C*–*J*).

Finally, the auxin level in the wound tissues was simultaneously measured by evaluating the expression of *IAA5*, an early auxin-inducible gene. Compared with the control, the IAA level was not altered in the upper region but was decreased by 50% in the lower region 1 d after cutting (Fig. 2*F*). Simultaneously, the *IAA5* gene was strongly up-regulated exclusively in the upper region and then strongly reduced, possibly by negative feedback (Fig. S2*A*).

Microarray Analysis of the Tissue-Reunion Process and Gene Expression Profiles of *ANAC071* and *RAP2.6L*. Explants shorter than 5 mm with or without nicks were collected from the same internodes 1, 3, and 5 d after cutting and subjected to an oligonucleotide-based microarray to determine what genes initiate and regulate the tissue-reunion process (Fig. S3 and Dataset S1). Table S1 shows selected genes with expression patterns that correlate with tissue reunion.

The microarray analysis led us to focus on the expression of TFs that were highly up-regulated at an early stage. Two plant-specific TF genes were selected, *ANAC071* (*Arabidopsis* NAC domain containing protein 71; At4g17980) encoding NAC domain-TF, and *RAP2.6L* (At5g13330) encoding an ERF/AP2 -TF (Fig. 3 *A* and *B* and Table S1). *ANAC071* belongs to the NAC domain-TF family known to include 105 predicted NAC proteins in *Arabidopsis* (18), which are involved in the formation of organ primordia and other biological functions, including defense responses (19). *RAP2.6L* belongs to the *APETALA2* (AP2)/ERBP family, which is one of the large families of TFs in *Arabidopsis* involved in many different developmental processes and environmental response events (20, 21). The family is composed of 144 members in *Arabidopsis* and has been divided into five subfamilies: the AP-2 subfamily, RAV subfamily, DREB (A) subfamily, ERF (ethylene response factor; B) subfamily, and others (22).

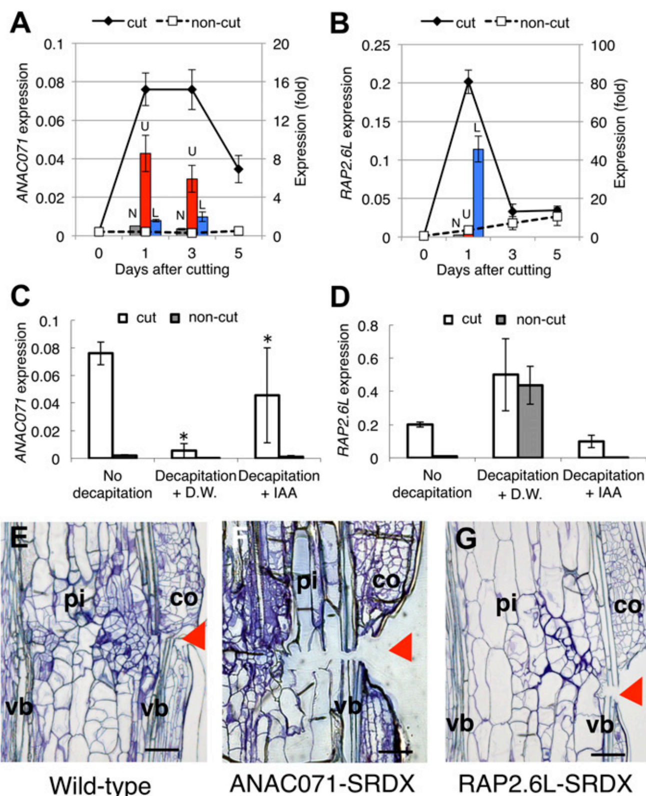

Fig. 3. Gene expression of *ANAC071* and *RAP2.6L* TFs and phenotypes of gene-suppressing transformants. (*A* and *B*) Gene expression of *ANAC071* (*A*) and *RAP2.6L* (*B*). For time-course analysis, the mean ± SE is shown ($n = 3$). Bars show comparative expression between upper (U; red bar) or lower region (L; blue bar) of wounded stem. The noncut (N; gray bar) control was arbitrarily set to 1, and mean ± SE is shown ($n = 4$). (*C* and *D*) Effects of decapitation and IAA application on the expression of *ANAC071* (*C*) or *RAP2.6L* (*D*). Mean ± SE is shown ($n = 3$). *$P < 0.05$ (Fisher's test). (*E*–*G*) Representative phenotype of SRDX transgenic plants. (*E*) Wild type. (*F*) *ANAC071*-SRDX. (*G*) *RAP2.6L*-SRDX. Images were taken 7 d after cutting. Arrowheads indicate position of cut. pi, pith; co, cortex; vb, vascular bundle. (Scale bars in *E*–*G*, 100 μm.)

The *ANAC071* transcript was highly expressed 1–3 d after cutting, and the transcript was very intense in the upper cut region (Fig. 3*A*). Intensive expression of *ANAC071* in the upper region was also observed in *pANAC071*::*GUS* transgenic plants, and the promoter activity was predominantly observed in pith and vascular tissue (Fig. S2*B*). The transcript level was drastically reduced after decapitation but was restored by IAA application (Fig. 3*C*).

The *RAP2.6L* transcript was transiently highly expressed 1 d after cutting, with exclusive expression in the lower region (Fig. 3*B*). The *RAP2.6L* transcript was scarce in the noncut stem (Fig. 3*B*). Decapitation strongly enhanced the level of the *RAP2.6L* transcript in the cut and noncut stems, and this effect was nullified by IAA application (Fig. 3*D*).

Histological Analysis of Transformants Defective in *ANAC071* and *RAP2.6L*. In general, plant TFs constitute a large family that shares highly conserved DNA-binding domains, resulting in much redundancy. Although gene-suppressing transformants are difficult to obtain for such genes (23), this can be overcome by using chimeric repressor silencing technology (CRES-T) (24). To determine the roles of *ANAC071* and *RAP2.6L* in cell division, the gene-suppressing transformants of these genes, *ANAC071*-SRDX and *RAP2.6L*-SRDX, were prepared using the CRES-T method (Fig. S4 *A* and *B*). In *ANAC071*-SRDX, cell division was strongly inhibited but cell elongation was enhanced around the lesion area, resulting in incomplete tissue reunion (Fig. 3 *E* and *F* and Fig. S4 *C* and *D*). In *RAP2.6L*-SRDX, moderate inhibition was observed (Fig. 3 *E* and *G* and Fig. S4 *E* and *F*). Transformants with the plant expression vector alone showed normal reunion.

Involvement of Ethylene in Tissue Reunion and Gene Expression of *RAP2.6L* and *ANAC071*. qRT-PCR analyses revealed that stem cutting promoted the expression of the aminocyclopropane carboxylic acid (ACC) synthase (ACS) gene, *ACS2* (At1g01480), shortly (1 d) after cutting in both the lower and upper regions (Fig. 4*A*). Likewise, nearly equal promoter activity was observed in the upper and lower regions in the *pACS2*::*GUS* transgenic plants 1 d after cutting (Fig. S5*A*). ACS2 is one of the ACC synthases encoded by a multigene family in many plant species and produced in response to various environmental stimuli (25). Decapitation suppressed *ACS2* transcript expression, although such suppression was not restored by exogenous IAA (Fig. S6). It is likely that the *ACS2* gene is spatially regulated by either IAA or injury.

To determine whether ethylene was involved in tissue reunion, the ethylene-insensitive mutant *ein2* was investigated. Five days after cutting the *ein2* stem, cell division was observed only in the cortex neighboring the cut and not in the pith, unlike in the wild-type stem (Fig. S5 *B*–*D*). Complete reunion was not observed up to 7 d after cutting the *ein2* stem (Fig. 4*B*). *ANAC071* expression in the *ein2* cut was significantly lower than in the wild type (Fig. 4*C*), indicating that *ANAC071* was up-regulated by ethylene. In contrast, *RAP2.6L* expression level was approximately two times higher in the *ein2* than in wild type 1 d after cutting (Fig. 4*D*), indicating that *RAP2.6L* was down-regulated by ethylene.

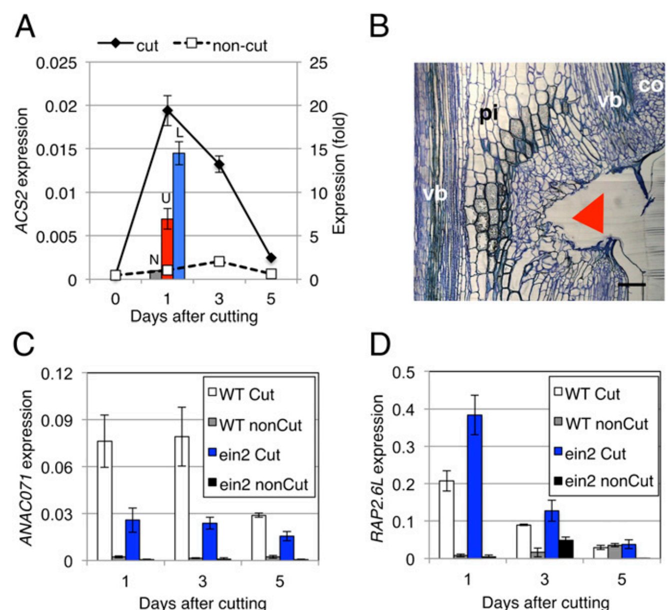

Fig. 4. Involvement of ethylene in tissue reunion and expression of *RAP2.6L* and *ANAC071*. (*A*) Expression of *ACS2*. For time-course analysis, the mean ± SE is shown ($n = 3$). Bars show comparative expression between upper (U; red bar) and lower region (L; blue bar) of cut stem. The noncut (N; gray bar) control was arbitrarily set to 1, and the mean ± SE is shown ($n = 4$). (*B*) Light micrograph of *ein2* cut stem. Images were taken 7 d after cutting. Arrowheads indicate position of cut. pi, pith; co, cortex; vb, vascular bundle. (Scale bar, 100 μm.) The image is a composite to form the complete figure. (*C* and *D*) Relative expression of *ANAC071* (*C*) and *RAP2.6L* (*D*) in cut stem (white bar) or noncut stem (gray bar) of wild type, and cut stem (blue bar) or noncut stem (black bar) of *ein2*. Mean ± SD ($n = 2$) is shown.

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Regulation of *RAP2.6L* by JA. Microarray and qRT-PCR analyses showed that some JA biosynthesis genes were up-regulated during the tissue-reunion process. *LOX2* [encoding lipoxygenase 2 (At3g45140), a JA biosynthesis enzyme] was selected because this gene was exclusively expressed in wounded tissue (Fig. 5*A*) and is known to be wound-inducible (26). *LOX2* expression peaked 1 d after stem cutting, with higher expression in the lower cut region (Fig. 5*A*) and a superimposable expression profile with *RAP2.6L* (Fig. 3*B*). *RAP2.6L* was up-regulated by JA methyl ester upon administration to the intact flowering stem (Fig. 5*B*), in accord with the AtGenExpress microarray database (27).

Discussion

Recovery of Wounded Inflorescence Stems in *Arabidopsis* Is Different from That of Cucumber and Tomato Hypocotyls. As found in our earlier work (11), injured cucumber or tomato hypocotyls never healed when cotyledons were removed. This was due to GA depletion caused by removal of the GA-supplying cotyledons (11, 12). Exogenous GA, but not IAA, can replace the cotyledons. Hypocotyls are embryonic organs consisting of the epidermis, cortex, endodermis, and vascular tissues, but lacking a pith. Its growth requires import of nutrients and hormones from cotyledons because of its juvenility. In cucumber and tomato hypocotyls, injury recovery is accompanied by cell division and elongation mainly in the cortical cells, which characteristically have pectin-rich thick cell walls.

Before the present study, no involvement of GA was found in wound healing because the wounded stems of the GA-deficient *Arabidopsis* mutant *ga3ox1/ga3ox2* recovered with no difficulty. The tissue-reunion mechanism likely changes as developmental stages proceed. *Arabidopsis* inflorescence stem internodes analyzed in this study had no dividing cells and consisted of the epidermis, cortex, endodermis, vascular tissues, and a pith occupying much of the stem center. In the stems, cell proliferation-based

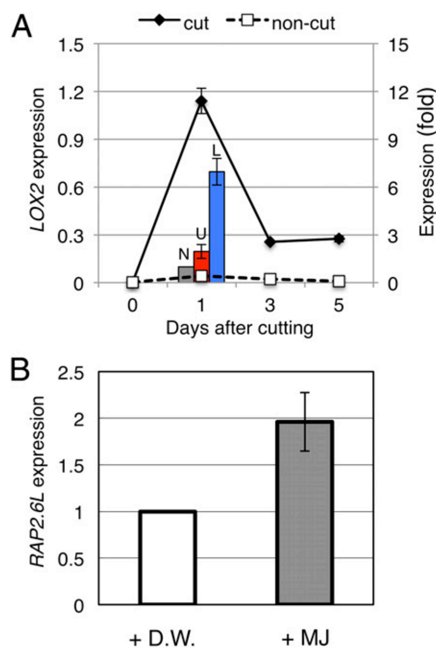

Fig. 5. Regulation of *RAP2.6L* expression by JA. (*A*) Gene expression of *LOX2*. For time-course analysis, the mean ± SE is shown (n = 3). Bars show comparative expression between upper (U; red bar) and lower region (L: blue bar) of cut stem. The noncut stem control (N; gray bar) was arbitrarily set to 1, and the mean ± SE is shown (n = 4). (*B*) Effects of methyl jasmonate (MJ) application on the expression of *RAP2.6L* in the intact flowering stem. The distilled water (D.W.) control was arbitrarily set to 1, and the mean ± SE is shown (n = 3).

reunion of the wounded area proceeded mainly in the pith, in contrast to the events in hypocotyls (Fig. 1). Involvement of auxin in healing the injured stems was substantiated by the fact that auxin-deprived stems, because of TIBA treatment or *pin1-1* mutation, were not capable of wound healing. Interestingly, cortical cell division occurred in both the upper and lower cut stem regions and was not affected by decapitation, auxin transport inhibition (Fig. 2), or GA biosynthesis inhibition, or in GA-deficient mutants.

Upper Cut Region of Wounded Inflorescence Stems Was Healed by *ANAC071*. Microarray analysis followed by qRT-PCR of wounded stems revealed two TF genes, *ANAC071*and *RAP2.6L*, that were strongly up-regulated immediately after artificial wounding. Furthermore, *ANAC071*and *RAP2.6L* were spatially selectively expressed in the upper and lower cut regions, respectively (Fig. 3 *A* and *B*). The requirement of these TFs for pith cell division in tissue reunion was indicated by analyzing TF gene-suppressing transformants (Fig. 3 *E*–*G* and Fig. S4).

ANAC071 was most likely induced by auxin because auxin accumulated in the upper cut region, and *ANAC071* was not present in the auxin-poor lower region. Furthermore, this finding is supported by a previous report that *ANAC071*-TF and related NAM (no apical meristem)-like TFs are up-regulated in auxin-rich callus induction medium (28, 29). Furthermore, auxin and microRNAs may regulate the spatial patterns of the transcription of some NAC family genes, including *CUC1* and *CUC 2* (30). A group of microRNAs was also predicted to target the *Populus* homolog of *ANAC071* (31). The biological functions of NAC-TFs remain to be determined, but several NAC-TFs may be involved in shoot meristem formation, organ boundary specification, and secondary wall thickening (10, 32, 33), together with abscisic acid-mediated stress responses against drought and high salinity (18, 19, 34).

Ethylene, a wound-inducible hormone, was also an important factor activating *ANAC071* because *ANAC071*-TF expression was significantly reduced in the *ein2* mutant (Fig. 4*C*), which had unusual reunion morphology (Fig. 4*B*). ACC, the direct precursor of ethylene, is synthesized by ACSs in response to biotic or abiotic stimuli, and the ACSs are rate-limiting enzymes in ethylene production. Although numerous *ACS* genes are known, *ACS2* was specifically up-regulated in both the upper and lower cut regions in this study (Fig. 4*A*). Decapitation decreased *ACS2* expression in the *Arabidopsis* flowering stem, although this negative effect was not fully compensated after IAA application (Fig. S6*A*). Taken together, the results suggest that auxin activated *ANAC071* and ethylene enhanced *ANAC071* expression in the auxin-rich upper cut region, inducing pith cell division during tissue reunion (Fig. S7).

Lower Cut Region of Wounded Inflorescence Stems Was Healed by *RAP2.6L*-TF. The *RAP2.6L* gene is thought to regulate genes involved in meristem maintenance during shoot regeneration, because T-DNA knockdown of *RAP2.6L* reduced the frequency of shoot development from root explants and hampered the expression of shoot meristem-specific genes (28, 29). In the present study, *RAP2.6L* gene expression appeared in the lower cut region, which had a lower IAA level due to blocked polar auxin transport. It is likely that *RAP2.6L* was up-regulated in response to wounding in a region below the cut where auxin concentrations were low. Furthermore, *RAP2.6L* was not expressed in the IAA-rich upper cut region, indicating that it was down-regulated by IAA. In support of this conclusion, Che et al. (28, 29) clearly showed that *RAP2.6L* is up-regulated in cytokinin-rich, shoot-generating media for *Arabidopsis* root explants but not in auxin-rich, root-inducing media.

LOX2 was also thought to be involved in injury healing because it was up-regulated in the lower cut region immediately after cutting (Fig. 5*A*). *LOX2* is a lipoxygenase participating in JA synthesis that converts linolenic acid to its hydroperoxide.

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The plant hormone JA is a key regulator of plant responses to environmental stresses and biotic challenges (3). *RAP2.6L*-TF expression was enhanced upon application of the JA derivative methyl jasmonate to the flowering stems (Fig. 5*B*). However, decapitation with or without IAA application did not affect the expression of *LOX2* during the reunion process (Fig. S6*B*). It is most likely that this gene is activated by wounding independently of IAA. However, it is not known why *LOX2* was not expressed in the upper cut region. On the other hand, *RAP2.6L* seems to be down-regulated by ethylene because its expression was increased in the *ein2* mutant (Fig. 4*D*). In conclusion, in the lower cut region, *RAP2.6L* was activated because of auxin depletion, and this activation process was positively regulated by JA but negatively regulated by ethylene, resulting in the net incremental increase in *RAP2.6L* expression (Fig. S7), facilitating pith cell division during tissue reunion.

Materials and Methods

Arabidopsis thaliana seeds were germinated and grown in artificial soil under continuous white fluorescent light. After 7–10 d of bolting, stem internodes between the first or second cauline leaves and rosette leaves were cut through half of their diameter with a microsurgical knife (Fig. S1 *A* and *B*), and the plant was then grown for an additional 14 d. Detailed experimental procedures are described in *SI Materials and Methods*.

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