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Short Communication

ARF6 and *ARF8* contribute to tissue reunion in incised *Arabidopsis* inflorescence stems

Weerasak Pitaksaringkarn[1], Sumie Ishiguro[2], Masashi Asahina[3], Shinobu Satoh[1,*]

[1]Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan; [2]Department of Biological Mechanisms and Functions, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya, Aichi 464-8601, Japan; [3]Department of Biosciences, Teikyo University, Utsunomiya, Tochigi 320-8551, Japan
*E-mail: satoh.shinobu.ga@u.tsukuba.ac.jp Tel & Fax: +81-29-853-4672

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Abstract Previously, we found that auxin and wound-inducible hormones contributed to the control of tissue reunion in both the upper and lower parts of incised *Arabidopsis* stems by inducing the expression of *ANAC071* and *RAP2.6L*, respectively. Here, we reveal how the expression of *ANAC071* and *RAP2.6L* is controlled by auxin. An *arf6 arf8* double mutant exhibited inhibition of cell division in pith tissue 1 week after incision; this was not true of either the single mutants or mutants in other *ARF* genes. *ANAC071* and *RAP2.6L* expression was suppressed in incised stems of the *arf6 arf8* mutant, but *RAP2.6L* expression was enhanced in non-incised stems. In addition, the expression of *DEFECTIVE IN ANTHER DEHISCENCE1* (*DAD1*), which encodes a jasmonic acid biosynthetic enzyme, was induced by the incision in both wild-type and *arf6 arf8* stems, but overall expression level was suppressed in the *arf6 arf8* mutant. We thus propose that auxin accumulation promotes *ANAC071* expression via ARF6 and ARF8 activity in the upper part of incised stems, and that a reduced auxin level induces *RAP2.6L* expression in the lower part of the incision as a result of its release from the suppression caused by the action of ARF6 and ARF8-mediated auxin in non-incised stems. Moreover, auxin signaling via ARF6 and ARF8 is essential for jasmonic acid production, via the induction of *DAD1*, to increase *RAP2.6L* expression during tissue reunion.

Key words: Stem, incision, tissue reunion, auxin, ARF, jasmonic acid.

Stems provide essential support to the plant body by delivering water, nutrients, and chemical information to roots and shoots (Satoh 2006; Kehr and Buhtz 2008). Therefore, stem injuries trigger a defense response and activate repair processes (Reid and Ross 2011). Such processes have found wide application in agricultural grafting, and *Arabidopsis* has been used as a model of grafting at the molecular level (Turnbull et al. 2002; Flaishman et al. 2008). We previously reported that tissue reunion in incised hypocotyls of cucumber and tomato required endogenous gibberellin, as well as manganese, zinc, and boron (Asahina et al. 2002, 2006).

The first internode of the *Arabidopsis* flowering stem has been used in molecular studies of tissue reunion (Asahina et al. 2011) because this internode exhibits less cell division and elongation than does the apex shoot (Suh et al. 2005). Cell division in pith tissue can be induced by the creation of a half incision in the first internode. Such division is inhibited by the elimination of cauline leaves, shoot apices, or lateral buds, which may reduce the supply of indole-3-acetic acid (IAA). Microarray and quantitative RT-PCR analyses have revealed that genes associated with cell division and phytohormone synthesis, and genes encoding transcription factors, are induced after incision (Asahina et al. 2011). In particular, two plant-specific transcription factor genes, *ANAC071* and *RAP2.6L*, were found to be abundantly expressed. *ANAC071* was expressed exclusively in the upper part of the gap 1–3 days after cutting, with concomitant accumulation of IAA. In contrast, *RAP2.6L* was expressed exclusively in the lower part 1 day after cutting, with a concomitant drop in the IAA level. Simultaneously, *LIPOXYGENASE 2* (*LOX2*), encoding a jasmonic acid (JA) biosynthetic enzyme, was expressed in the lower part of the incised stem, and application of methyl jasmonate promoted *RAP2.6L* expression. Pith cell division was inhibited in transformants in which *RAP2.6L* or *ANAC071* functionality was suppressed. Hence, plant-specific transcription factors differentially expressed in different cut regions are essential for tissue reunion after the wounding of flowering stems of *Arabidopsis* and are differentially controlled by polar-transported auxin. Also, JA modifies the response to injury.

Auxin-responsive genes contain auxin-response elements (AuxREs) allowing the specific binding of

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certain transcription factors (ARFs) to promoter regions (Hagen and Guilfoyle 2002; Liscum and Reed 2002). AuxREs are located at –2,654 bp (in the promoter) and at 208 bp (in-frame) in the first exon of *ANAC071*, suggesting that ARFs control *ANAC071* expression during tissue reunion. *Arabidopsis* contains 23 genes encoding ARF proteins. Five ARFs, ARF5-8 and -19, contain glutamine-rich central domains and are thought to be transcriptional activators, whereas the other ARFs are thought to be transcriptional repressors (Guilfoyle and Hagen 2007). The functions of activator-type ARFs have been well-studied. *ARF5* is essential for embryonic, floral, and vascular patterning (Aida et al. 2002; Berleth and Jürgen 1993; Hardtke and Berleth 1998; Hardtke et al. 2004; Przemeck et al. 1996). *ARF7* and *ARF19* overlap in function as revealed by the inhibition of lateral root formation in *arf7 arf19* double mutants (Okushima et al. 2005; Wilmoth et al. 2005). A double mutant in *ARF6* and *ARF8* exhibited defective elongation of the inflorescence stem and floral development, and exhibited decreased JA production (Tabata et al. 2010; Nagpal et al. 2005). Auxin induces the activity of ARFs by binding to a repressor of ARF activity, the Aux/IAA protein, which is subsequently ubiquitinated and degraded by proteasomes in the TRANSPORT INHIBITOR RESISTANT 1 receptor complex (Dharmasiri et al. 2005; Ulmasov et al. 1997).

IAA5, an Aux/IAA protein, was identified in our previous microarray analysis as a gene that was notably upregulated 1 day after incision, compared with the level in non-incised inflorescence stems (Asahina et al. 2011). *IAA5* expression was suppressed in the inflorescence apices of *arf6 arf8* mutants (compared with the wild type) after the application of exogenous auxin and JA (Nagpal et al. 2005). Thus, we selected *ARF6* and *ARF8* as possible candidates for the control of auxin-responsive genes involved in tissue reunion.

We compared the tissue reunion phenotypes of *arf6*, *arf8*, *arf6 arf8*, *arf7 arf19* (activator-type ARFs), and *arf2* (repressor-type ARF as a negative control) mutants with that of the wild-type control 7 days after incision (Figure 1). Seed materials were grown on half-strength MS medium at 22°C under continuous light (32 μmol m^{-2} s^{-1}). Three weeks after germination, plants were transferred to soil and grown under the same conditions until bolting occurred. Stems were fixed to 18-cm lengths of bamboo stake using small pieces of silicon tubing. The first internode of each stem was incised to over half of the diameter using a microsurgery knife (Surgical Specialties Co., Franklin, TN) under a stereomicroscope (S6E; Leica Microsystems Inc., Wetzlar, Germany) and the plants were observed for the next 7 days. The selection of *arf6*, *arf8*, and *arf6 arf8* has been described (Tabata et al. 2010). The *arf7 arf19* (CS24629) and *arf2* (CS24600) mutants were obtained from the Arabidopsis Biological Resource Center (Columbus, OH). Incised stems were fixed overnight in 2.5% [v/v] glutaraldehyde and 1% [w/v] paraformaldehyde in 0.1 M phosphate buffer, pH 6.8–7.4, washed twice in the same phosphate buffer, and dehydrated in a graded series of ethanol baths (30, 50, 70, 90, and 100%; all v/v). Technovit 7100 resin was substituted for ethanol at volume ratios of 3 : 1, 1 : 1, and 1 : 3. The resin-embedded samples were sectioned every 2 μm using an ultra-microtome glass knife (EM-ULTRACUT; Leica Microsystems Inc.) and stained with 0.1% (w/v) toluidine blue O. The extent of tissue reunion 7 days after incision in more than three plants was observed using a light microscope (DMRB; Leica Microsystems Inc.). Typical photographs are shown in Figure 1.

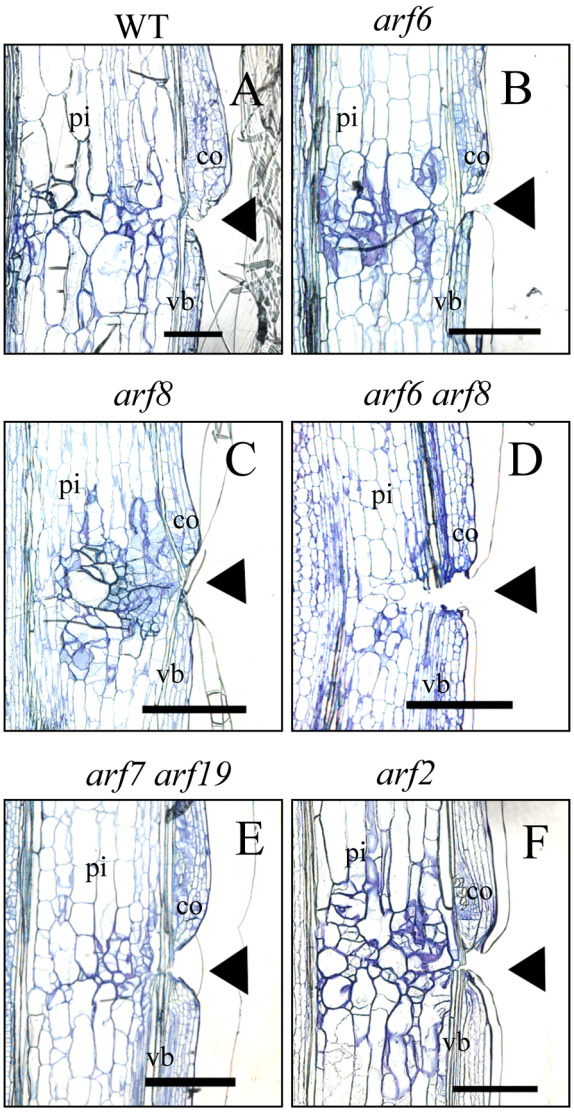

Figure 1. Tissue reunion phenotypes of *ARF* mutants. (A–F). The tissue reunion phenotypes of *arf6*, *arf8*, *arf6 arf8*, *arf7 arf19*, and *arf2* were compared with that of wild-type (WT) plants 7 days after incision. Arrowheads indicate the positions of the incisions. pi, pith; co, cortex; vb, vascular bundle. Bar = 100 μm.

Single mutants in *ARF2*, *ARF6*, and *ARF8*, and the double mutant in *ARF7* and *ARF19*, exhibited pith tissue cell proliferation that did not differ from that in wild type, but proliferation in the *ARF6*/*ARF8* double mutant was strongly inhibited 7 days after incision (Figure 1). These results suggest that *ARF6* and *ARF8* act redundantly to control cellular proliferation in pith tissue after incision.

A microarray analysis revealed that *ARF6* and *ARF8* are expressed at various stages of flower development (Schmid et al. 2005) and a promoter GUS analysis indicated that the expression patterns of *ARF6* and *ARF8* in flowers are similar (Nagpal et al. 2005). In the present study, we used quantitative RT-PCR to measure the expression levels of *ARF6* and *ARF8* in various *Arabidopsis* organs, including incised (1 day after incision) and non-incised stems. Total RNA was extracted using RNeasy® Mini Kits (Qiagen, Hilden, Germany). Complementary DNA was synthesized from 50 μg of RNA using QuantiTect® Reverse Transcription Kits (Qiagen). Expression levels were measured by quantitative RT-PCR using GoTaq® qPCR Master Mix (Promega, Madison, WI) and an analytical platform (model 7000; Applied Biosystems, Foster City, CA). *ACT7* was used as an internal control. All tests were performed in triplicate. The primers used to amplify *ARF6* and *ARF8* have been described elsewhere (Tabata et al. 2010).

ARF6 and *ARF8* were predominantly expressed in flowers, but were also substantially expressed in incised stems at levels significantly higher than in non-incised stems (Figures 2A, B). This suggests that *ARF6* and *ARF8* are involved in tissue reunion in incised stems.

As cell proliferation in pith tissue was inhibited in the *arf6 arf8* mutant, we measured the expression levels of two transcription factors, *ANAC071* and *RAP2.6L* (Figures 3A, B). *ANAC071* levels were reduced in both incised and non-incised stems of the *arf6 arf8* mutant, suggesting that an increased auxin level in the upper part of an incision induces *ANAC071* expression mainly via ARF6 and ARF8 activity. However, some level of *ANAC071* expression was induced in incised stem of *arf6 arf8* compared to non-incised stem indicating involvement of the other unknown transcription factors in this process. In contrast, the *RAP2.6L* level was elevated in non-incised stems of the *arf6 arf8* mutant, consistent with the finding that *RAP2.6L* expression in intact stems is suppressed by auxin (Asahina et al. 2011). However, *RAP2.6L* expression in wild-type plants was enhanced by stem incision. This may be attributable to expression of *RAP2.6L* in the lower part of the incision (Asahina et al. 2011). In this report, *RAP2.6L* expression was not strongly induced in incised stem compared with that in previous work (Asahina et al. 2011) possibly because 1 cm-long stem sections were used for the

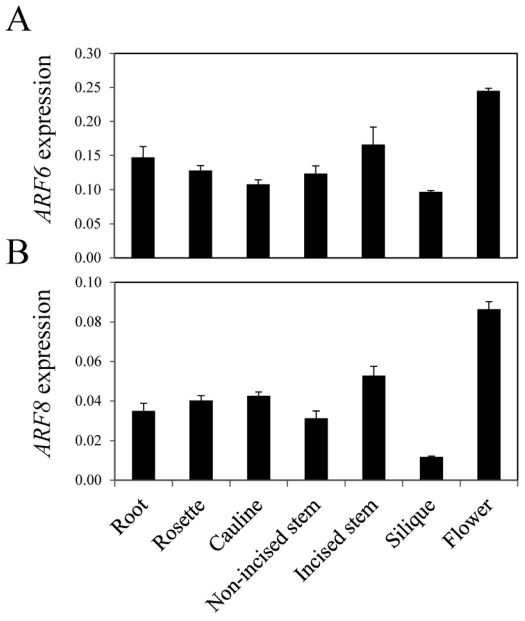

Figure 2. Expression of *ARF6* and *ARF8* in various organs. (A and B). Quantitative RT-PCR was used to measure the expression levels of *ARF6* and *ARF8* in roots, rosette leaves, cauline leaves, non-incised and incised (1 day after incision) inflorescence stems, siliques, and flowers of wild-type plants. *Actin7* was used as an internal control. Error bars show the standard deviation (n=3).

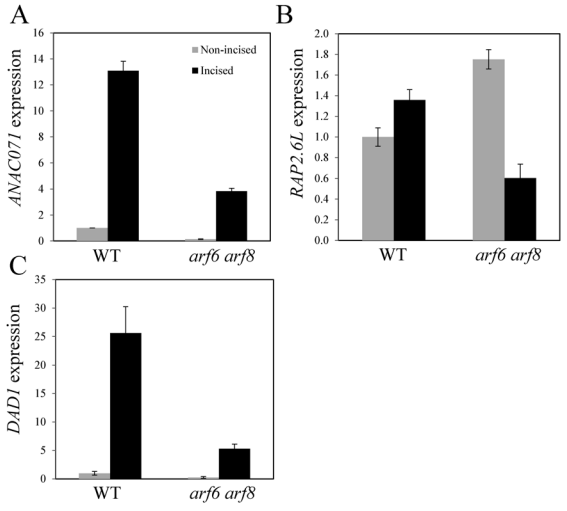

Figure 3. Expression of *ANAC071*, *RAP2.6L*, and *DAD1* in the *arf6 arf8* mutant. (A–C). Quantitative RT-PCR was used to measure the expression levels of *ANAC071*, *RAP2.6L*, and *DAD1* in non-incised and incised inflorescence stems of wild-type (WT) and *arf6 arf8* mutant plants 1 day after incision. Gray and black columns show the expression levels in non-incised and incised stems, respectively. *Actin7* was used as an internal control. Error bars show the standard deviation (n=3).

analysis (0.5 cm-long stem sections were used in the previous work). *RAP2.6L* expression was suppressed in the incised stem of the *arf6 arf8* mutant compared to the wild type. These results initially appear to be inconsistent in terms of auxin action. We had supposed up-regulation of *RAP2.6L* in the incised stem of the *arf6 arf8* mutant compared to that of wild type because its

suppression by auxin is released by *arf6 arf8* mutation. However, *RAP2.6L* is induced by JA, and *LOX2*, an enzyme involved in JA biosynthesis, is abundantly expressed (along with *RAP2.6L*) in the lower part of the incised stem (Asahina et al. 2011). Tabata et al. (2010) reported that in the *arf6 arf8* mutant the JA level was reduced, and, of several JA biosynthetic genes studied, only the expression of *DAD1* was significantly decreased compared to wild type. This shows that *ARF6* and *ARF8* are both required for JA biosynthesis during flower development. Therefore, we used quantitative RT-PCR with primers that were previously described (Tabata et al. 2010), to explore *DAD1* expression further.

In wild-type plants, *DAD1* expression was strongly induced after stem incision. However, *DAD1* expression levels were dramatically decreased in the *arf6 arf8* mutant (Figure 3C). These results suggest that auxin promotes JA production to stimulate *DAD1* expression, and this may explain the observed reduction in *RAP2.6L* expression in the incised stem of the *arf6 arf8* mutant (Figure 3B).

Our results suggest a role for auxin during tissue reunion in incised stems. Auxin that accumulates in the upper part of incised stems is the principal factor promoting *ANAC071* expression. In intact stems, auxin usually suppresses *RAP2.6L* expression, but, after incision, the reduced amount of auxin allows *RAP2.6L* expression in the lower part of the incision. Concomitantly, auxin, even at a reduced level in the lower part of the incision, plays an essential role in JA production by promoting *DAD1* expression, and JA induces *RAP2.6L* expression in the lower part of the incision. Further work on the control of JA biosynthesis in incised stems is required.

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