Studies on the Molecular Mechanism of Tissue Reunion in *Arabidopsis* Incised Inflorescence Stem

January 2014

Weerasak PITAKSARINGKARN

Studies on the Molecular Mechanism of Tissue Reunion in *Arabidopsis* Incised Inflorescence Stem

A Dissertation Submitted to

the Graduate School of Life and Environmental Sciences,

the University of Tsukuba

in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy in Science

(Doctoral Program in Integrative Environmental Science)

Weerasak PITAKSARINGKARN

ABBREVIATIONS

ANAC071, ARABIDOPSIS NAC DOMAIN CONTAINING PROTEIN 71

ARF, AUXIN RESPONSE FACTOR

cDNA, complementary deoxyribonucleic acid

D.W., sterile distilled water

GUS, β-glucuronidase

IAA, indole-3-acetic acid

Km^r, Kanamycin resistance gene

MS medium, Murashige and Skoog' s medium

NAC, NAM, (no apical meristem), ATAF, (Arabidopsis transcription factor activator), CUC, (cup-shaped cotyledon)

PCR, polymerase chain reaction

PIN1, PIN FORMED 1

QRT-PCR, quantitative reverse transcription-polymerase chain reaction

RT-PCR, reverse transcription–PCR

SRDX, EAR motif repression domain

35S pro, cauliflower mosaic virus 35S promoter

T-DNA, transfer DNA

TIBA, 2, 3,5-triiodobenzoic acid

T35S, terminator of 35S promoter

X-Gluc, 5-bromo-4-choloro-3-indolyl-β-D-glucuronide

XTH, xyloglucan endotransglucosylase/hydrolase

ABSTRACT

Tissue reunion is known to be a natural phenomenon in plant which occurs during wounding or grafting. To re-connect the vascular system and protect themselves from pathogen infection and survive under environmental stress, plants have developed some mechanisms to repair wounded tissues. In order to understand the tissue reunion process in molecular level, morphological and gene expression analysis were performed using the *Arabidopsis* cut flowering stem. In morphological study of wild type plant, the cell division in tissue reunion process has been found to start in 3 days after cutting and almost complete in 14 days after incision. Microarray analysis showed up-regulation of the genes related to cell division, transcription, cell wall metabolism and phytohormone, and differential gene expression between incised and not-incised inflorescence stems during tissue reunion process was observed. In this study, I focused on the genes encoding transcription factor, hormone signaling and cell wall modification enzymes.

Previously, auxin and wound-inducible hormones were revealed to contribute to the control of tissue reunion process in upper and lower part of incised stem by inducing the expression of *ANAC071* and *RAP2.6L*, respectively.

In this thesis, I show how *ANAC071* and *RAP2.6L* expressions are controlled by auxin. *arf6arf8* double mutant showed inhibition of cell division in pith tissue after a week of incision, while no inhibition was observed in each single mutant and in the mutants for the other *ARFs*. The expression of *ANAC071* and *RAP2.6L* were suppressed in incised stem of *arf6arf8*, but *RAP2.6L* expression increased in non-incised stem of *arf6arf8*. In addition, expression of *DAD1* (DEFECTIVE IN ANTHER DEHISCENCE1), the gene for a jasmonic acid biosynthesis enzyme, was induced by the incision in wild type, but was strongly suppressed in *arf6arf8*. From these results, the hypothesis was proposed that accumulation of auxin promotes *ANAC071* expression via ARF6 and ARF8 in upper part of incised stem and decreased auxin level induces *RAP2.6L* expression in lower part of incision by release from the suppression of *RAP2.6L* by auxin via ARF6 and ARF8 in non-incised stem. Moreover, auxin signaling via ARF6 and ARF8 is essential for the production of jasmonic acid via induction of *DAD1* to induce *RAP2.6L* expression in tissue reunion process.

Auxin was thought to be the main factor controlling this process, and next I revealed that *ANAC071*, a transcription factor gene, was up-regulated in the

upper region of the incised stem. *XTH20* and the phylogenetically close *XTH19* were up-regulated in the upper part of the incised stem, similar to *ANAC071*. *XTH19* was also expressed in the lower incision region after 3 days or after auxin application to the decapitated stem. Horizontal positioning of the plant with the incised side up resulted in decreased *DR5:GUS*, *ANAC071*, *XTH20*, and *XTH19* expression and reduced pith cell proliferation. In incised stems of Pro_{35S}*:ANAC071-SRDX* plants, expression of *XTH20* and *XTH19* was substantially and moderately decreased, respectively. *XTH20* and *XTH19* expression and pith cell proliferation were suppressed in *anac071* plants and were increased in Pro_{35S}*:ANAC071* plants. Furthermore, ANAC071 bound to the *XTH20* and *XTH19* promoters to induce their expression, with the *xth19xth20* double mutant showing inhibition of pith cell proliferation. This is the first study revealing *XTH20* and *XTH19* induction by auxin via ANAC071 in the upper part of an incised stem and their involvement in cell proliferation in the tissue reunion process.

My research clarified the functions of genes for transcription factor, hormone signaling and cell wall related enzymes in tissue reunion process.

INTRODUCTION

Injuries caused by wind, herbivores, and chewing insects are one of the critical environmental factors affecting plant survival. Therefore, plants have developed primary physical barriers such as cuticle, bark, and thorns. Defense responses are activated by wound response hormones like ethylene and jasmonic acid to prevent further damage when injuries occur (León et al., 2001). Unlike other organs, stems provide essential structural support and deliver nutrients, water, and physiological and chemical information between organs through vascular tissues (Satoh, 2006; Kehr and Buhtz, 2008). Therefore, damaged stems need to be repaired as soon as possible to recover their physiological functions. This ability has long been used for agricultural grafting. The mechanism of the tissue reunion process has been explored to improve grafting efficiency and overcome tissue incompatibility. Turnbull et al. (2002) developed micro-grafting in young *Arabidopsis* seedlings to study long-distance signaling pathways including flowering, systemic acquired resistance, and abiotic stress responses. In mature *Arabidopsis*, homografts and heterografts were used as a model system to study the compatibility and ontogeny of graft union formation (Flaishman et al., 2008).

Although regeneration of vascular tissue in damaged stems has been widely observed (Flaishman et al., 2003), adherence between incised or separated tissues is also required for the tissue repair process. Auxin is required to heal the hypocotyl graft between the scion and root stock in *Arabidopsis* (Yin et al., 2012). Analysis of partially incised hypocotyls of cucumber and tomato also indicates the importance of endogenous gibberellin (GA) and Mn, Zn, and B mineral elements for repairing incised hypocotyls (Asahina et al., 2002; Asahina et al., 2006). Microarray analysis of *Arabidopsis* inflorescence stems, which was used to better understand the molecular basis of tissue repair, showed the up-regulation of genes involved in cell division, cell wall modification, and phytohormone synthesis and signaling (Asahina et al., 2011). According to analyses of the *pin1-1* mutant and decapitated plants with or without indole acetic acid (IAA) application, auxin was shown to (i) be a key factor controlling cell division in pith tissue during the repair of incised *Arabidopsis* stems, and (ii) induce expression of *ANAC071*, a NAM, ATAF1/2, CUC2 (NAC)-type transcription factor, in the distal region of the incision (Asahina et al., 2011). The NAC domain was originally characterized from consensus sequences from

petunia NAM and from *Arabidopsis* ATAF1, ATAF2, and CUC2 (Olsen et al., 2005). NAC family genes are plant-specific transcription factors and are expressed in various developmental stages and tissues (Kubo et al., 2005; Mitsuda et al., 2005). The control of downstream target genes by NAC has been demonstrated in dehydration stress responses (Tran et al., 2004), secondary wall biosynthesis (Zhong et al., 2007) and defense responses in *Arabidopsis* (Bu et al., 2008)**.**

Asahina et al., (2011) revealed 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. Application of methyl jasmonate, the expression of *RAP2.6L* was promoted. In transformants suppressing the function of *RAP2.6L* or *ANAC071*, the division of pith cells was inhibited. 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 jasmonic acid.

Theologically, auxin responsive genes should contain auxin-response elements (AuxREs:TGTCTC) for specificity binding of ARFs in promoter region. In promoter of *ANAC071*, AuxREs was found in -2654 bp and in frame exon 208 bp which is possibility that ARFs may possibly control expression of *ANAC071* in tissue reunion process while not appear in promoter of *RAP2.6L* (Hagen and Guilfoyle, 2002; Liscum and Reed, 2002). *Arabidopsis* has 23 genes encoding ARF proteins. Five *Arabidopsis* ARFs, *ARF5-8* and *19*, contain a glutamine-rich central domain and are thought to be transcriptional activators, whereas the others are thought to be transcriptional repressors (Guilfoyle and Hagen, 2007). The functions of each ARFs activator-type have previously been characterized. *ARF5* is essential for embryonic, flower and vascular patterning (Aida et al., 2002; Berleth and Jürgen, 1993; Hardtke and Berleth, 1998; Hardtke et al., 2004; Przemeck et al., 1996). The Overlapped function of *ARF7* and *ARF19* was demonstrated as inhibition of lateral root formation in *arf7arf19* (Okushima et al., 2005; Wilmoth et al., 2005). Double mutant of *ARF6* and

ARF8 inhibited inflorescence stem elongation and flower development with decreased jasmonic acid production (Tabata et al., 2010; Nagpal et al., 2005). The presence of auxin promotes activity of ARFs by ubiquitinated and degraded of its repressor through Aux/IAA protein TRANSPORT INHIBITOR RESISTANT1 (TIR1) receptor complex which is F-box protein (Ulmasov et al., 1997; Dharmasiri et al., 2005).

IAA5 encoding an Aux/IAA protein was highly up-regulated after 1 day of incision in compare with non-incised inflorescence stem revealed by our previous microarray analysis (Asahina et al., 2011). The expression of *IAA5* decreased in inflorescence apices of *arf6arf8* in compare with wild type after application of exogenous auxin and jasmonic acid (Nagpal et al., 2005). According to this evidence, I selected *ARF6* and *ARF8* for the first candidate genes which may control auxin response gene in tissue reunion process.

I explored how auxin controls tissue reunion process via ARFs by using phenotype and expression examination. The results showed strong inhibition of cell division in pith tissue of *arf6arf8*, not in that of either *arf6* or *arf8* was observed. The result suggests the functional redundancy of *ARF6* and *ARF8* in

tissue reunion process. Moreover, expression of both *ANAC071* and *RAP2.6L* were suppressed in incised stem of *arf6arf8*. These results indicate that tissue reunion process is controlled by ARF6 and ARF8 dependent auxin signaling.

Pro$_{35S}$*:ANAC071-SRDX* transgenic plants showed that *ANAC071* controls cell proliferation in pith tissue after an incision is made (Asahina et al., 2011). However, the control of downstream targets by *ANAC071* remains unknown. The target genes should be included in the up-regulated genes reported in our previous microarray analysis. Indeed, *XTH20* (xyloglucan endotransglucosylase/hydrolases20) was up-regulated in the microarray analysis of the tissue reunion process (Asahina et al., 2011). The *XTH* family is implicated as having a principal role in the construction and restructuring of xyloglucan crosslinking within the cellulose/xyloglucan framework by catalyzing molecular grafting and/or hydrolysis of xyloglucans. XTHs are also involved in various physiological processes, mainly by participating in cell enlargement (Nishitani and Tominaga, 1992; Okazawa et al., 1993; Fry, 2004). *XTH20* is induced by auxin in roots (Vissenberg et al., 2005; Osato et al., 2006), and here same as *ANAC071* in decapitated plant with IAA application *XTH20*

was also induced. This indicates that *XTH20* may be a downstream target of *ANAC071* via auxin flow in incised stem.

I analyzed the molecular basis of the early repair process in partially incised stems through induction of specific transcription factors by auxin in the distal region of the incision. Auxin-induced ANAC071 controlled the expression of two targeted downstream genes, *XTH20* and *XTH19*, which were redundantly involved in cell proliferation in pith tissue. The results indicate the involvement of XTH in cell proliferation in plant tissue.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The *Arabidopsis* T-DNA insertion mutants *arf7arf19* (CS24629), *arf2* (CS24600), *anac071* (SALK_012841), *xth20* (SALK_066758), and *xth19* (GABI Kat 369E06) were obtained from the Arabidopsis Biological Resource Center at Ohio State University. The homozygous lines were selected by PCR using specific primers (Table 1), and expression levels of the relevant genes were confirmed as compared with wild-type.

To generate the transgenic plants, the amplified fragments were cloned into pENTER-D topo vector (Invitrogen; pENTR™ Direction TOPO® Cloning Kits) (see Table 1 for primer sequences). The inserts from entry clones were then integrated into binary vectors pKGWFS7 for promoter:GUS and pK2GW7 for over-expression using LR Clonase (Invitrogen), and the constructed plasmids were used for *Agrobacterium*-mediated transformation of *Arabidopsis* (Col-0). Transformants were selected by kanamycin, and the expression levels of the relevant genes were confirmed as compared with wild-type plants.

Arabidopsis seed materials were vernalized in sterilized distilled water at 4°C for 3 days and then germinated on half-strength MS medium at 22°C under

continuous light conditions (32 μmol m^{-2} s^{-1}). After 3 weeks of germination, the plants were transferred to soil and grown under the same conditions until bolting.

To generate incised plants, the first internode of the inflorescence stem was incised to half of its diameter under a stereomicroscope using a microsurgery knife (Surgical Specialties Co.) and grown for an additional 7 days under conditions as described (Asahina et al., 2011).

Microscopic Analysis

Incised inflorescence stem samples were immersed overnight in fixative solution (2.5% [v/v] glutaraldehyde and 1% [w/v] paraformaldehyde in 0.1 M phosphate buffer, pH 6.8–7.4). After fixation, samples were washed twice in the same buffer, dehydrated with an ethanol series, and embedded in Technovit 7100 resin (Heraeus Kulzer). Tissue sections (2.0 μm thick) were prepared using an ultra-microtome glass knife (Reichert EM-ULTRACUT, Leica). The sections were stained with 0.1% (w/v) toluidine blue O for 10 min. The tissue reunion region was observed with a light microscope (DMRB, Leica).

Decapitation and Application of Phytohormone

After each plant bolted, the distal region of the first internode was removed, and 10^{-3} M IAA or distilled water as a mixture with lanolin (1:1, v/v) was immediately applied. The remainder of the first internode was incised 24 h after application as described in Asahina et al., (2011).

Histochemical Staining with 5-Bromo-4-Chloro-3-indolyl-β-*D*-Glucuronide (X-Gluc)

Plant tissues were softened with 90% (v/v) acetone on ice for 20 min and then immersed in staining solution [100 mM phosphate buffer (pH 7), 10 mM EDTA, 1 mM $K_3Fe(CN)_6$, 1 mM $K_4Fe(CN)_6 \cdot 3H_2O$, 0.1% (w/v) TritonX-100, and 0.1 M X-Glu] under vacuum for 30 min. The plant tissue samples were then incubated at 37°C for 8 h (Jefferson, 1987) and cleared with 70% (v/v) ethanol for approximately 12 h. Expression positions were observed using a stereomicroscope (Leica MCSLIII).

RNA Preparation and Quantitative RT-PCR

Total RNA was extracted from *Arabidopsis* incised and non-incised flowering stems by RNAqueous RNA isolation kit with plant RNA Isolation aid (Ambion). Complementary DNA was synthesized from total RNA (50 μg) by the QuantiTect Reverse Transcription Kit (Qiagen). Real-time PCR using TaqMan technology or SYBR Green I reagents (Qiagen) was performed using cDNA as a template on a sequence detector system (model 7000; Applied Biosystems, Foster, CA) with specific primers (Table 1). To normalize and confirm expression levels among various stages of incision treatment, *ACT7* was used as an internal standard with three biological replications per sample.

EMSA

The coding region of *ANAC071* was amplified by PCR using specific primers (*Eco*RI sense and *Sal*I antisense; see primer sequences in Table 1). The PCR product was digested with *Eco*RI and *Sal*I and introduced into *Eco*RI- and *Sal*I-digested pCold ProS2 (Takara). The His-tag fusion protein was produced in *E. coli* (Origami (DE3); Novagen) and purified using His-Accept resin (Nacalai Tesque Inc.). Oligonucleotides (see primer sequences in Figure 9C and 9D)

were labeled with biotin using Biotin 3’ End DNA Labeling Kit (Thermo Fisher Scientific). To make double-stranded biotin-labeled probes, the complementary oligonucleotide pairs were annealed at 95°C for 5 min and slowly cooled to room temperature overnight. Double-stranded unlabeled oligonucleotides were made using the same method. The labeled probes were incubated for 1 h at 28°C with 2 μg of purified His-ANAC071 protein using the Light Shift Chemiluminescent EMSA kit (Thermo Fisher Scientific) and were supplemented with 25 μg/mL poly (dI-dC), 0.1 μg/mL BSA, 2 mM EDTA, and 1% (w/v) NP-40. The protein-probe mixtures were separated in 6% (w/v) polyacrylamide native gels in 0.5× TBE buffer and transferred to Biodyne membrane (PALL Gelman Laboratory). Migration of the biotin-labeled probes was visualized with the Chemiluminescent Nucleic Acid kit (Thermo Fisher Scientific) and a detection module (LAS-4000 mini biomolecular imager; Fujifilm).

RESULTS

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

I 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. 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 2). These results suggest that *ARF6* and *ARF8* act redundantly to control cellular proliferation in pith tissue after incision.

Expression of *ARF6* and *ARF8* in *Arabidopsis* incised inflorescence stem

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, I 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. *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 (Figure 3). This suggests that *ARF6* and *ARF8* are involved in tissue reunion in incised stems.

ARF6 **and** ***ARF8*** **control expression of** ***ANAC071, RAP2.6L*** **and JA biosynthesis gene in** ***Arabidopsis*** **incised inflorescence stem**

The expressions of two selected TFs from previous microarray analysis, *ANAC071* and *RAP2.6L* (Asahina et al., 2011), were observed in non-incised and incised stem of wild type and *arf6arf8* 1 day after incision. *ANAC071* expression showed strongly decrease in both non-incised and incised stem of *arf6arf8* (Figure 4A). *RAP2.6L* expression showed increase in non-incised but, decrease in incised stem of *arf6arf8* (Figure 4B). Further, expression of *DAD1*, one of JA biosynthesis genes and strongly reduced in flower bud of *arf6arf8* (Tabata et al. 2010), was observed. *DAD1* showed strongly decrease expression both non-incised and incised stem of *arf6arf8* (Figure 4C). These results

indicated that *ARF6* and *ARF8* control expression of *ANAC071* and *RAP2.6L* via JA biosynthesis gene.

Spatial and Temporal Expression of *ANAC071, XTH20,* and *XTH19* in Incised *Arabidopsis* Inflorescence Stems

The expression levels of *ANAC071*, *XTH20*, and *XTH19* which is phylogenetically closest to *XTH20* in various organs of *Arabidopsis* were determined by quantitative RT-PCR. These genes were highly expressed in incised inflorescence stems. *XTH20* and *XTH19* were also highly expressed in roots, but the concomitant expression of *ANAC071* was observed only in incised stems (Figure 5A, 5B, and 5C). The tissue-specific expression of *ANAC071*, *XTH20*, and *XTH19* was examined in more detail using promoter-reporter markers. The 1.5-kb upstream region of each gene was fused with the β-glucuronidase (GUS) reporter gene and introduced into *Arabidopsis*. $Pro_{ANAC071}$*:GUS* was highly expressed in the distal region 1 and 3 days after the incision was made (Figure 5D and 5E), confirming our previous quantitative RT-PCR results showing that *ANAC071* is specifically expressed in the distal

region of incised inflorescence stems (Asahina et al., 2011). The expression of *XTH20* and *XTH19* was higher in the distal than proximal regions of the incision at 1 day (Figure 5P, and 5Q), but expression of *XTH19* was similar in both regions at 3 and 5 days (Figure 5Q). In Pro_{XTH20}*:GUS* and Pro_{XTH19}*:GUS* transgenic plants, GUS activity was higher in the distal than the proximal region for Pro_{XTH20}*:GUS* (Figure 5H-5K), but expression was similar in both regions for Pro_{XTH19}*:GUS* (Figure 5H–5O). *XTH19* was also expressed in the siliques and flowers of intact plants (Figure 5C). These results indicated that the expression of *ANAC071* and the expression of *XTH20* are highly correlated.

Induction of *ANAC071*, *XTH20*, and *XTH19* by Auxin in *Arabidopsis* Incised Inflorescence Stems

The expression of $Pro_{ANAC071}$*:GUS* in response to auxin was higher in the distal part of the incision (Figure 5D-5G). The expression of *XTH20* and *XTH19* was also induced by IAA application (Figure 6A and 6B). In Pro_{XTH20}*:GUS* transgenic plants, GUS activity was high in the distal region of the incision after IAA application, which was similar to the pattern with $Pro_{ANAC071}$*:GUS* (Figure

6D and 6F). In *Pro$_{XTH19}$:GUS* transgenic plants, GUS activity was detected in both the distal and proximal regions of the incision, but the level of *Pro$_{XTH19}$:GUS* was higher in the distal region (Figure 6H). These results indicated that *ANAC071*, *XTH20*, and *XTH19* are auxin-inducible genes in incised *Arabidopsis* stems and that the expression of *XTH20* is more well correlated with that of auxin-induced *ANAC071* than is *XTH19*.

Effect of Gravity on Tissue Repair in *Arabidopsis* Incised Inflorescence Stems

Auxin is a gravity-responsive hormone, and thus the effect of the gravity response on the tissue repair process was examined. After incision of the stems, the plants were incubated in a horizontal position with the incision site facing either downward or upward, in the same or opposite direction as the gravity vector, respectively. Pith cell number 7 days after the incision was made did not increase at the cut surface for those plants grown with an upward-facing incision (Figure 7A–7D). In addition, GUS activity in *Pro$_{ANAC071}$:GUS*, *Pro$_{XTH20}$:GUS*, *Pro$_{XTH19}$:GUS*, and *Pro$_{DR5}$:GUS* (to monitor the auxin response)

transgenic plants decreased when the incision sites were upward facing as compared with downward-facing incisions and control (i.e. vertical) stems (Figure 7E–7P). All of these results indicated a good correlation between the auxin response and *ANAC071*, *XTH20*, and *XTH19* expression.

Control of the Expression of *XTH20* and *XTH19* by *ANAC071* in Incised *Arabidopsis* Stems

As described above, the expression of *ANAC071*, *XTH20*, and *XTH19* was correlated with the incision response (Figure 5) and with the auxin response (Figures 6 and 7). Thus, *ANAC071* is a key auxin-response factor that controls tissue reunion (Asahina et al., 2011). Hence, the expression levels of *XTH20* and *XTH19* were examined in incised stems of Pro_{35S}*:ANAC071-SRDX* transgenic plants to determine whether *XTH20* and *XTH19* were controlled by *ANAC071*. The expression level of *XTH20* in incised stems significantly decreased in Pro_{35S}*:ANAC071-SRDX* transgenic plants as compared with the wild-type incised plants (Figure 8A). Moreover, the expression level of *XTH19* in incised stems of Pro_{35S}*:ANAC071-SRDX* transgenic plants moderately decreased as

compared with the wild-type incised plants (Figure 8B). Furthermore, decreased expression of *XTH20* and *XTH19* in *anac071* T-DNA insertion mutant and increased expression in *Pro$_{35S}$:ANAC071* transgenic plants were observed as compared with wild type (Figure 8C and 8D). These results indicated that *XTH20* and *XTH19* may be downstream genes under the control of *ANAC071* in incised *Arabidopsis* stems.–

Binding of ANAC071 to *XTH20* and *XTH19* Promoters

The *Arabidopsis* NAC protein family targets the recognition sequence CATGT and the core binding site CACG to bind with promoters of downstream genes (Bu et al., 2008). The promoters of *XTH20* and *XTH19* at –923 bp and –102 bp upstream from their respective coding sequences (Figure 9A and 9B) had sequences similar to the recognition site (CATG) and core binding site (CACC) (Figure 9C and 9D). Hence, I examined whether ANAC071 binds to the promoters of *XTH20* and *XTH19* using an electrophoretic mobility shift assay (EMSA). Purified His-ANAC071 fusion protein was incubated with labeled DNA probes, and an EMSA was performed. A high-affinity DNA-protein

complex was detected (Figure 9E and 9F; lane 2 arrowhead), and DNA-binding was reduced by the addition of an excess of unlabeled oligonucleotide probes corresponding to the *XTH20* or *XTH19* promoter, which acted as specific competitors (Figure 9E and 9F; lane 3 and 4). These results indicated that ANAC071 was likely to control *XTH20* and *XTH19* expression by binding to each promoter.

ANAC071, *XTH19*, and *XTH20* Control Cell Proliferation in Pith Tissue of Incised *Arabidopsis* Stems

Because *XTH20* and *XTH19* were controlled by ANAC071, the tissue reunion phenotype was examined in the *anac071* mutant and Pro_{35S}*:ANAC071* transgenic plants, as well as in the *xth19* and *xth20* mutants. The *anac071* mutant had fewer pith cells as compared with wild type (Figure 10B and 10I), as was previously described for Pro_{35S}*:ANAC071-SRDX* transgenic plants (Asahina et al., 2011). Introduction of Pro_{35S}*:ANAC071* into the *anac071* background restored cell division in pith tissue (Figure 10C and 10I). Moreover, two lines of Pro_{35S}*:ANAC071* transgenic plants showed higher cell numbers in

pith tissue than in wild-type control plants (Figures 10A, 10D, 10E, and 10I) as well as slight increase in rosette leaf number, length and width (Figure 11). These results suggested that *ANAC071* controls the tissue reunion process by controlling cell proliferation in the pith tissue.

To study the function of *XTH20* in the tissue reunion process, I examined the *xth20* T-DNA insertion mutant. In the *xth20* single mutant 7 days after the incision was made, the number of pith cells was not significantly different as compared with wild type (Figure 10A, 10F, and 10I). I hypothesized that *XTH20* may act redundantly with *XTH19*. The single mutant of *XTH19* had similar pith cell numbers as wild-type control plants (Figure 10A, 10G, and 10I). Furthermore, I generated the *xth20xth19* mutant, which exhibited fewer pith cells 7 days after the incision was made as compared with wild type (Figure 10H and 10I). In addition, the primary root length of *xth20xth19* was significantly decreased as compared with that of *xth20*, *xth19*, and wild-type plants 5 and 10 days after sowing (*t*-test, $P < 0.05$) (Figure 12A and 12B), which may be related to the expression of *XTH20* and *XTH19* in intact roots (Figure 5B and 5C). These results support our hypothesis that *XTH20* and *XTH19* are

functionally redundant and are involved in the cell proliferation of pith tissue during the tissue reunion process.

DISCUSSION

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 (Figure 3A, 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, I measured the expression levels of two transcription factors, *ANAC071* and *RAP2.6L* (Figure 4A, B). *ANAC071* expression 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 thesis, *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 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. I 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, I 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 4C). 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 4B).

My results suggest a role of 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.

Auxin is produced in the apical meristem and is transported to all plant organs (Robert and Friml, 2009). Auxin plays an important role in cell

proliferation and vascular differentiation (Berleth et al., 2000; Flaishman et al., 2003; Teala et al., 2006). Histochemical analysis of *Pro$_{DR5}$:GUS* transformants, which can monitor auxin accumulation, showed high GUS activity in the distal region of the incision 3 days after the incision was made (Figure 7E). Tissue reunion is strongly inhibited by decapitation or TIBA (an inhibitor of auxin polar transport) application to wild-type plants and on *pin1-1* mutants, whereas the application of 10^{-3} M IAA to decapitated plants leads to the recovery of the reunion process (Asahina et al., 2011). These results indicate that auxin is a main factor in the regulation of tissue repair in the *Arabidopsis* incised inflorescence stem. Xie et al. (2000) reported that *NAC1* is inducible by auxin and promotes lateral root formation. Previous studies suggest that some *XTH* genes, including *XTH20* and *XTH19*, are regulated by auxin in the roots (Vissenberg et al., 2005; Osato et al., 2006). Application of auxin to decapitated stems increased both *XTH20* and *XTH19* expression (Figure 6A and 6B), and the direction of gravity controlled their expression (Figure 7L and 7O) as well as the expression of *ANAC071* (Figure 7I). However, *XTH19* was also expressed in the proximal region of the incision (Figure 5M, 5Q, Figure 6H) and was

constitutively expressed in the silique and flower (Figure 5C). Thus *ANAC071*, *XTH20*, and *XTH19* were induced by auxin in the tissue reunion process, but *XTH19* may also be controlled by other factors.

The plant-specific NAC transcription factor family, with 105 members predicted in the *Arabidopsis* genome (Ooka et al., 2003), is important for various physiological processes such as lateral root formation, auxin signaling, the defense response to abiotic stress (Olsen et al., 2005), xylem formation (Kubo et al., 2005), and thickening of the secondary wall (Mitsuda et al., 2005). Previous work reported the use of *Pro$_{35S}$:ANAC071-SDRX* to show that *ANAC071* controls the tissue reunion process in incised inflorescence stems (Asahina et al., 2011). Here I showed that the expression levels of *ANAC071* in the *anac071* mutant and in *Pro$_{35S}$:ANAC071* transgenic plants and the expression of *Pro$_{35S}$:ANAC071-SRDX* were correlated with the expression of *XTH20* and *XTH19* (Figure 8A–8D). The *anac071* mutant showed strong inhibition of the reunion process (Figure 10B and 10I). Complementation of *anac071* with *ANAC071* also showed recovery of cell proliferation in pith tissue (Figure 10C and 10I). Moreover, wild-type plants that over-expressed *ANAC071*

had more cell proliferation than did wild-type control plants (Figure 10A, 10D, 10E and 10I). These results suggested that *ANAC071* is an important transcription factor that controls its downstream genes and pith cell proliferation in the tissue reunion process.

The expression of *XTH20* and *XTH19* in both incised and non-incised tissue decreased in *Pro$_{35S}$:ANAC071-SRDX* plants (Figure 8A and 8B). The expression of *XTH20* and *XTH19* decreased in *anac071* plants, particularly 3 days after the incision was made, but substantially increased in *Pro$_{35S}$:ANAC071* transgenic plants (Figure 8C and 8D). These results suggested that *ANAC071* controls the expression of *XTH20* and *XTH19* in the tissue reunion process. Bu et al. (2008) demonstrated the direct binding of ANAC019 to the *VSP1* promoter, which contains the recognition site (CATGT) and core binding site (CACG) for the NAC protein family. The promoters of *XTH20* and *XTH19* also contain a similar recognition site (CATGT and CATG) and core binding site (CACC), which differs from the usual core binding site (CACG) for NAC proteins (Figure 9C and 9D). EMSA assays revealed that the ANAC071 protein bound to the promoters of *XTH20* and *XTH19* and that the CACC sequence was

important for recognition by ANAC071 (Figure 9E and 9F). The expression pattern of *XTH20* (Figure 5I and 5P) was different from that of *XTH19* (Figure 5M and 5Q), suggesting that some unknown factor(s) participates in the expression of *XTH19* in the proximal region of the incised stem.

XTH20 was selected from a previous microarray analysis (Asahina et al., 2011). The function of XTHs is to split and reconnect xyloglucan molecules in plant cell walls, thereby affecting cell enlargement (Nishitani and Tominaga, 1992; Okazawa et al., 1993; Fry, 2004). Hyodo et al. (2003) suggested the involvement of *XTH9* in cell elongation in the flowering stem. Rose et al. (2002) revealed the gene structure and phylogenetic tree of the *Arabidopsis XTH* family, showing that *XTH20* and *XTH19* are close homologs. Vissenberg et al. (2005) reported the differential expression of *XTH17*, *XTH18*, *XTH19*, and *XTH20*, which have highly conserved sequences in the promoter region. In my experiment, the *xth20* single mutant did not show an inhibition of the reunion process (Figure 10F). Thus, *XTH20* may act redundantly with other genes that regulate this process. Therefore, I made a double mutant of *XTH20* and *XTH19*. *xth20xth19* showed strong inhibition of the reunion process (Figure 10H) and a

lower pith cell number (Figure 10I). In addition, primary root elongation in *xth20xth19* was also inhibited (Figure 12A and 12B). These results indicated that *XTH20* and *XTH19* act redundantly to control cell proliferation in the reunion process as well as primary root elongation. As far as I know, this is the first report showing that XTH is involved in cell proliferation in plant tissue.

During cytokinesis, the nascent cell wall produces a cell plate that fuses to the pre-existing cell wall of the mother cell. This process involves the restructuring of xyloglucan cross-links in the cellulose/xyloglucan framework, possibly including a role for XTH20 and XTH19. Yokoyama and Nishitani (2001) and Yokoyama et al. (2004) have also hypothesized a function for XTHs in cell plate formation during cytokinesis by showing the localization of XTH in the cell plates of BY-2 cells. During cell plate formation, xyloglucan molecules that are produced and transported from the Golgi apparatus may be required as materials for cell plates and nascent cell walls (Moore and Staehelin, 1988). The molecular grafting between xyloglucan molecules by XTHs may be involved in the generation of high-molecular-weight xyloglucan chains in the cell plate, and this may regulate rheological properties of the cell plate (Yokoyama et al., 2004).

Arabidopsis XTH9 is expressed in the meristematic region where cell division actively occurs (Hyodo et al., 2003), which supports data presented in this report. Future research will elucidate how XTHs control cell proliferation and/or cytokinesis by modulating the xyloglucan molecules and the cellulose/xyloglucan framework in the cell plate and/or cell wall.

REFERENCES

Aida M., Vernoux T., Furutani M., Traas J., and Tasaka M. (2002) Roles of *PIN-FORMED1* and *MONOPTEROS* in pattern formation of the apical region of the *Arabidopsis* embryo. *Development*, **129**, 3965-3974.

Asahina, M., Azuma, K., Pitaksaringkarn, W., Yamazaki, T., Mitsuda, N., Ohme-Takagi, M., Yamaguchi, S., Kamiya, Y., Okada, K., Nishimura, T., Koshiba, T., Yokota, T., Kamada, H., and Satoh, S. (2011) Spatially selective hormonal control of RAP2.6L and ANAC071 transcription factors involved in tissue reunion in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA*, **108**, 16128–16132.

Asahina, M., Iwai, H., Kikuchi, A., Yamaguchi, S., Kamiya, Y., Kamada, H., and Satoh, S. (2002) Gibberellin produced in the cotyledon is required for cell division during tissue reunion in the cortex of cut cucumber and tomato hypocotyls. *Plant Physiol*., **129**, 201–210.

Asahina, M., Gocho, Y., Kamada, H., and Satoh, S. (2006) Involvement of inorganic elements in tissue reunion in the hypocotyl cortex of *Cucumis sativus*. *J. Plant Res*., **119**, 337–342.

Berleth T. and Jurgens G. (1993) The role of the *monopteros* gene in organising the basal body region of the *Arabidopsis* embryo. *Development*, **53**, 1366-1376.

Berleth, T., Mattsson, J., and Hardtke, C.S. (2000) Vascular continuity and auxin signals. *Trends Plant Sci*., **5**, 387–393.

Bu, Q., Jiang, H., Li, C.-B., Zhai, Q., Zhang, J., Wu, X., Sun, J., Xie, Q., and Li, C. (2008) Role of the *Arabidopsis thaliana* NAC transcription factors ANAC019 and ANAC055 in regulating jasmonic acid-signaled defense responses. *Cell Res*., **18**, 756–767.

Dharmasiri N., Dharmasiri S., and Estelle M. (2005) The F-box protein *TIR1* is an auxin receptor. *Nature*, **435**, 441-445.

Flaishman, M. a., Loginovsky, K., Golobowich, S., and Lev-Yadun, S. (2008) *Arabidopsis thaliana* as a model system for graft union development in homografts and heterografts. *J. Plant growth regul.*, **27**, 231–239.

Flaishman, M. a., Loginovsky, K., and Lev-Yadun, S. (2003) Regenerative xylem in inflorescence stems of *Arabidopsis thaliana*. *J. Plant Growth Regul*., **22**, 253–258.

Fry, S.C. (2004). Primary cell wall metabolism : tracking the careers of wall polymers in living plant cells. *New Phytol*., **161**, 641–675.

Fukaki, H., Fujisawa, H., and Tasaka, M. (1996) Gravitropic response of inflorescence stems in *Arabidopsis thaliana*. *Plant Physiol*., **110**, 933–943.

Guilfoyle T. J. and Hagen G. (2007) Auxin response factors. *Curr Opin Plant Biol*., **10**, 453-460.

Hagen G. and Guilfoyle T. (2002) Auxin-responsive gene expression: genes, promoters and regulatory factors. *Plant Mol Biol*., **49**, 373-385.

Hardtke C. S. and Berleth T. (1998) The *Arabidopsis* gene *MONOPTEROS* encodes a transcription factor mediating embryo axis formation and vascular development. *EMBO J*., **17**, 1405-1411.

Hardtke C. S., Ckurshumova W., Vidaurre D. P., Singh S. A., Stamatiou G., Tiwari S. B., Hagen G., Guilfoyle T. J., and Berleth T. (2004) Overlapping and non-redundant functions of the Arabidopsis auxin response factors MONOPTEROS and NONPHOTOTROPIC HYPOCOTYL 4. *Development*, **131**, 1089-1100.

Hyodo, H., Yamakawa, S., Takeda, Y., Tsuduki, M., Yokota, A., Nishitani,

K., and Kohchi, T. (2003) Active gene expression of a xyloglucan endotransglucosylase/hydrolase gene, XTH9, in inflorescence apices is related to cell elongation in *Arabidopsis thaliana*. *Plant Mol. Biol*., **52**, 473–482.

Jefferson, R.A. (1987) Assaying chimeric genes in plants: The GUS gene fusion system**.** *Plant Mol. Biol.*, **5**, 387–405.

Kehr, J. and Buhtz, A. (2008) Long distance transport and movement of RNA through the phloem. *J. Exp. Bot*., **59**, 85–92.

Kubo, M., Udagawa, M., Nishikubo, N., Horiguchi, G., Yamaguchi, M., Ito, J., Mimura, T., Fukuda, H., and Demura, T. (2005) Transcription switches for protoxylem and metaxylem vessel formation. *Genes Dev*., **19**, 1855–1860.

León, J., Rojo, E., and Sánchez-Serrano, J.J. (2001) Wound signalling in plants. *J. Exp. Bot.*, **52**, 1–9.

Liscum E. and Reed J. W. (2002) Genetics of Aux/IAA and ARF action in plant growth and development. *Plant Mol* Biol., **49**, 387-400.

Mitsuda, N., Seki, M., Shinozaki, K., and Ohme-takagi, M. (2005) The NAC transcription factors NST1 and NST2 of *Arabidopsis* regulate secondary wall thickenings and are required for anther dehiscence. *Plant Cell*, **17**, 2993–3006.

Moore, P.J. and Staehelin, L.A. (1988) Immunogold localization of the cell-wall-matrix polysaccharides rhamnogalacturonan I and xyloglucan during cell expansion and cytokinesis in Trifolium pratense L .; implication for secretory pathways. *Planta*, **174**, 433–445.

Nagpal P., Ellis C. M., Weber H., Ploense S. E., Barkawi L. S., Guilfoyle T. J., Hagen G., Alonso J. M., Cohen J. D., Farmer E. E., Ecker J. R., and Reed J. W. (2005) Auxin response factors ARF6 and ARF8 promote jasmonic acid production and flower maturation. *Development*, **132**, 4107-4118.

Nishitani, K. and Tominaga, R. (1992) Endo-xyloglucan transferase, a novel class of glycosyltransferase that catalyzes transfer of a segment of xyloglucan molecule to another xyloglucan molecule. *J. Biol. Chem.*, **267**, 21058–21064.

Okushima Y., Overvoorde P. J., Arima K., Alonso J. M., Chan A., Chang C., Ecker J. R., Hughes B., Lui A., Nguyen D., Onodera C., Quach H., Smith A., Yu G., and Theologis A. (2005) Functional genomic analysis of the AUXIN RESPONSE FACTOR gene family members in Arabidopsis thaliana: unique and overlapping functions of ARF7 and ARF19. *Plant Cell*., **17**, 444-463.

Okazawa, K., Sato, Y., Nakagawa, T., Asada, K., Kato, I., Tomita, E., and Nishitani, K. (1993) Molecular cloning and cDNA sequencing of endoxyloglucan transferase, a novel class of glycosyltransferase that mediates molecular grafting between matrix polysaccharides in plant cell walls. *J. Biol. Chem.*, **268**, 25364–25368.

Olsen, A.N., Ernst, H. a, Leggio, L. Lo, and Skriver, K. (2005) NAC transcription factors: structurally distinct, functionally diverse. *Trends Plant Sci*., **10**, 79–87.

Ooka, H., Satoh, K., Doi, K., Nagata, T., Otomo, Y., Murakami, K., Matsubara, K., Osato, N., Kawai, J., Carninci, P., Hayashizaki, Y., Suzuki, K., Kojima, K., Takahara, Y., Yamamoto, K., and Kikuchi, S. (2003) Comprehensive analysis of NAC family genes in *Oryza sativa* and *Arabidopsis thaliana*. *DNA Res*., **247**, 239–247.

Osato, Y., Yokoyama, R., and Nishitani, K. (2006) A principal role for AtXTH18 in Arabidopsis thaliana root growth: a functional analysis using RNAi plants. *J. Plant Res*., **119**, 153–162.

Przemeck G. K., Mattsson J., Hardtke C. S., Sung Z. R., and Berleth T.

(1996) Studies on the role of the *Arabidopsis* gene *MONOPTEROS* in vascular development and plant cell axialization. *Planta*, **200**, 229-237.

Reid J. B. and Ross J. J. (2011) Regulation of tissue repair in plants. *Proc Natl Acad Sci USA*., **108**, 17241-17242.

Robert, H.S. and Friml, J. (2009) Auxin and other signals on the move in plants. *Nature Chem Biol*., **5**, 325–332.

Rose, J.K.C., Braam, J., Fry, S.C., and Nishitani, K. (2002) The XTH family of enzymes involved in xyloglucan endotransglucosylation and endohydrolysis: current perspectives and a new unifying nomenclature. *Plant Cell Physiol*., **43**, 1421–1435.

Satoh, S. (2006) Organic substances in xylem sap delivered to above-ground organs by the roots. *J. Plant Res*., **119**, 179–187.

Schmid M., Davison T. S., Henz S. R., Pape U. J., Demar M., Vingron M., Schölkopf B., Weigel D., and Lohmann J. U. (2005) A gene expression map of *Arabidopsis thaliana* development. *Nat Genet*., **37**, 501-506.

Suh M. C., Samuels A. L., Jetter R., Kunst L., Pollard M., Ohlrogge J., and Beisson F. (2005) Cuticular lipid composition, surface structure, and gene expression in *Arabidopsis* stem epidermis. *Plant Physiol*., **139**, 1649-1665.

Tabata R., Ikezaki M., Fujibe T., Aida M., Tian C. E., Ueno Y., Yamamoto K. T., Machida Y., Nakamura K., and Ishiguro S. (2010) *Arabidopsis* auxin response factor6 and 8 regulate jasmonic acid biosynthesis and floral organ development via repression of class 1 *KNOX* genes. *Plant Cell Physiol*., **51**, 164-175.

Tran, L.P., Nakashima, K., Sakuma, Y., Simpson, S.D., Fujita, Y., Maruyama, K., Fujita, M., Seki, M., Shinozaki, K., and Yamaguchi-shinozaki, K. (2004) Isolation and Functional Analysis of Arabidopsis Stress-Inducible NAC Transcription Factors That Bind to a Drought-Responsive cis-Element in the *early responsive to dehydration stress* 1 Promoter. *Plant cell*, **16**, 2481–2498.

Turnbull C. G. N., Booker J. P., and Leyser H. M. O. (2002) Micrografting techniques for testing long-distance signaling in *Arabidopsis*. *Plant J*., **32**, 255-262.

Teale, W.D., Paponov, I. a, and Palme, K. (2006) Auxin in action: signalling, transport and the control of plant growth and development. *Nat. Rev. Mol. Cell Biol.*, **7**, 847–859.

Turnbull, C.G.N., Booker, J.P., and Leyser, H.M.O. (2002) Micrografting techniques for testing long-distance signalling in Arabidopsis. *Plant J.*, **32**, 255–262.

Ulmasov T., Murfett J., Hagen G., and Guilfoyle T. J. (1997) Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. *Plant Cell*, **9**, 1963-1971.

Vissenberg, K., Oyama, M., Osato, Y., Yokoyama, R., Verbelen, J.-P., and Nishitani, K. (2005) Differential expression of AtXTH17, AtXTH18, AtXTH19 and AtXTH20 genes in Arabidopsis roots. Physiological roles in specification in cell wall construction. *Plant Cell Physiol.*, **46**, 192–200.

Wilmoth J C, Wang S, Tiwari S B, Joshi A D, Hagen G, Guilfoyle T J, Alonso J M, Ecker J R, and Reed J W (2005) NPH4/ARF7 and ARF19 promote leaf expansion and auxin-induced lateral root formation. *Plant J.*, **43**, 118-130.

Xie, Q., Frugis, G., Colgan, D., and Chua, NH., (2000) Arabidopsis NAC1 transduces auxin signal downstream of TIR1 to promote lateral root development. *Genes Dev*., **14**, 3024–3036.

Yin, H., Yan, B., Sun, J., Jia, P., Zhang, Z., Yan, X., Chai, J., Ren, Z., and Zheng, G. (2012) Graft-union development : a delicate process that involves cell – cell communication between scion and stock for local auxin accumulation. *J. Exp. Bot*., **63**, 4219–4232.

Yokoyama, R. and Nishitani, K. (2001) Endoxyloglucan transferase is localized both in the cell plate and in the secretory pathway destined for the apoplast in tobacco cells. *Plant Cell Physiol*., **42**, 292–300.

Yokoyama, R., Tanaka, D., Fujino, T., Itoh, T., and Nishitani, K. (2004) Cell wall dynamics in tobacco BY-2 cells. Biotechnology in Agriculture and Forestry **53**, Edited by Nagata et al., pp. 217–230, Springer-Verlag Berlin Heidelberg.

Zhong, R., Richardson, E. a, and Ye, Z.-H. (2007) The MYB46 transcription factor is a direct target of SND1 and regulates secondary wall biosynthesis in Arabidopsis. *Plant cell*, **19**, 2776–2792.

FIGURES AND TABLES

Figure 1. Photographs and illustration of how *Arabidopsis* flowering stem was cut

Arabidopsis was used as a plant model to study tissue reunion process (A). The first inter node of flowering stem was cut with microsurgery knife (B and C). The cutting treatment was done by cut half of its diameter (D).

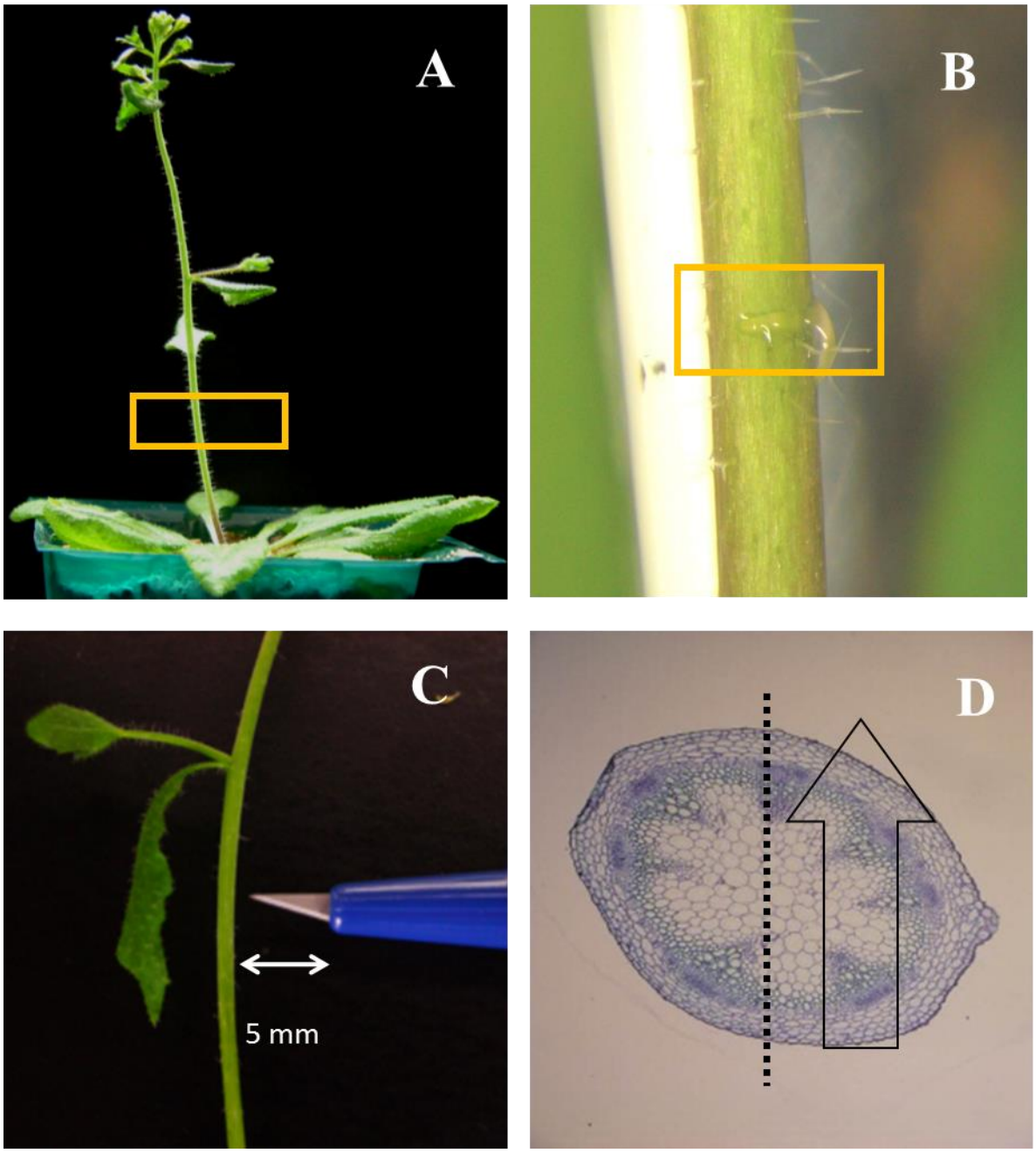

A
B
C
5 mm
D

Figure 2. Tissue reunion phenotypes of the mutants for *ARFs*

(A)-(F) Tissue reunion phenotypes of *arf6*, *arf8*, *arf6arf8*, *arf7arf19* and *arf2* in compared with wild type were observed 7 days after incision. Arrowheads indicate the position of the incision. pi, pith; co, cortex; vb, vascular bundle. Bar=100 μm.

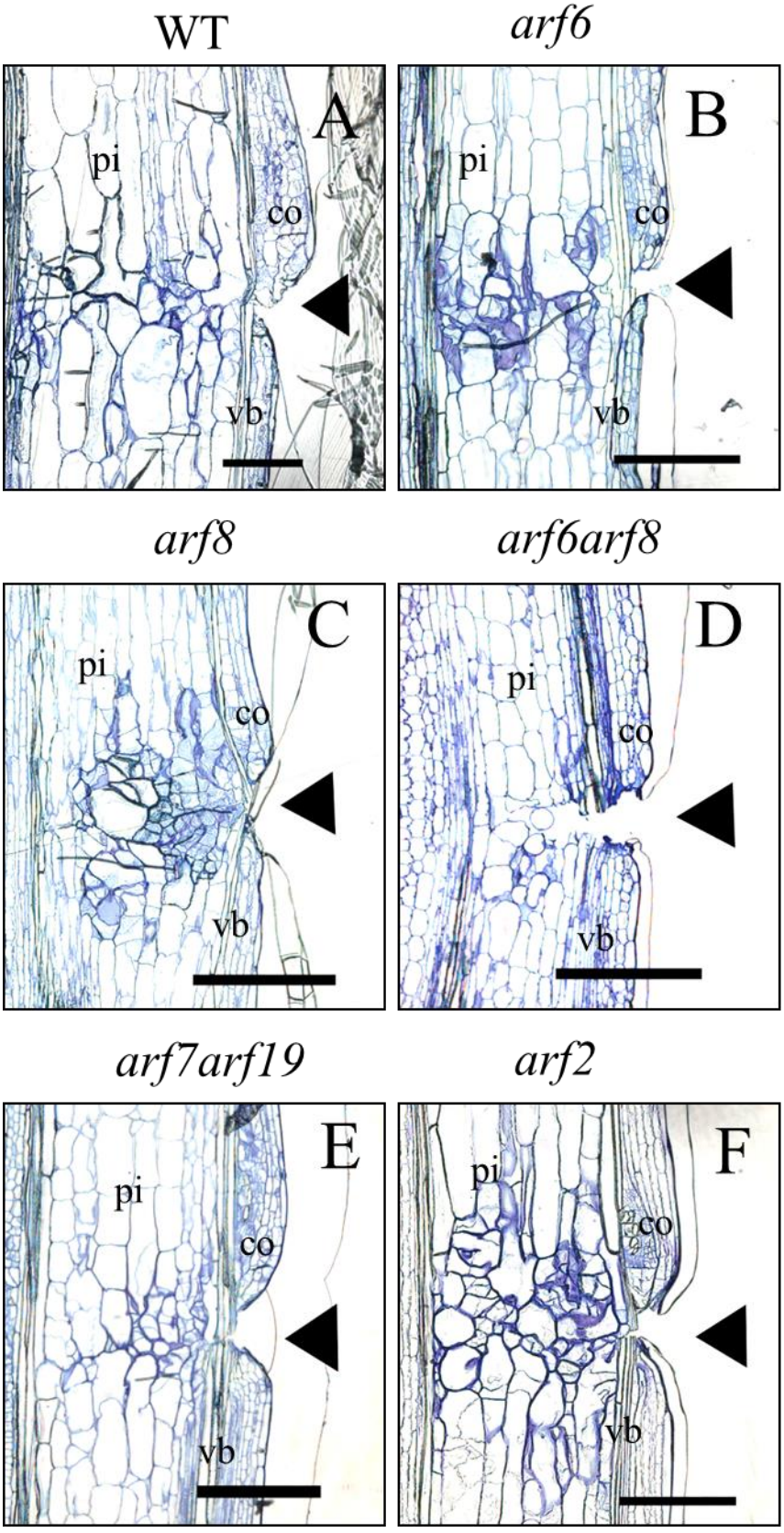

WT
A
pi
co
vb
arf6
B
pi
co
vb
arf8
C
pi
co
vb
arf6arf8
D
pi
co
vb
arf7arf19
E
pi
co
vb
arf2
F
pi
co
vb

Figure 3. Expression of *ARF6* and *ARF8* in various organs.

(A and B) The expression of *ARF6* and *ARF8* were examined by quantitative RT-PCR in root, rosette leaves, cauline leaves, non-incised, incised (one day after incision) inflorescence stem, silique and flower, in wild type plants. *Actin7* was used as an internal control. Error bars represent ± SD (*n*=3).

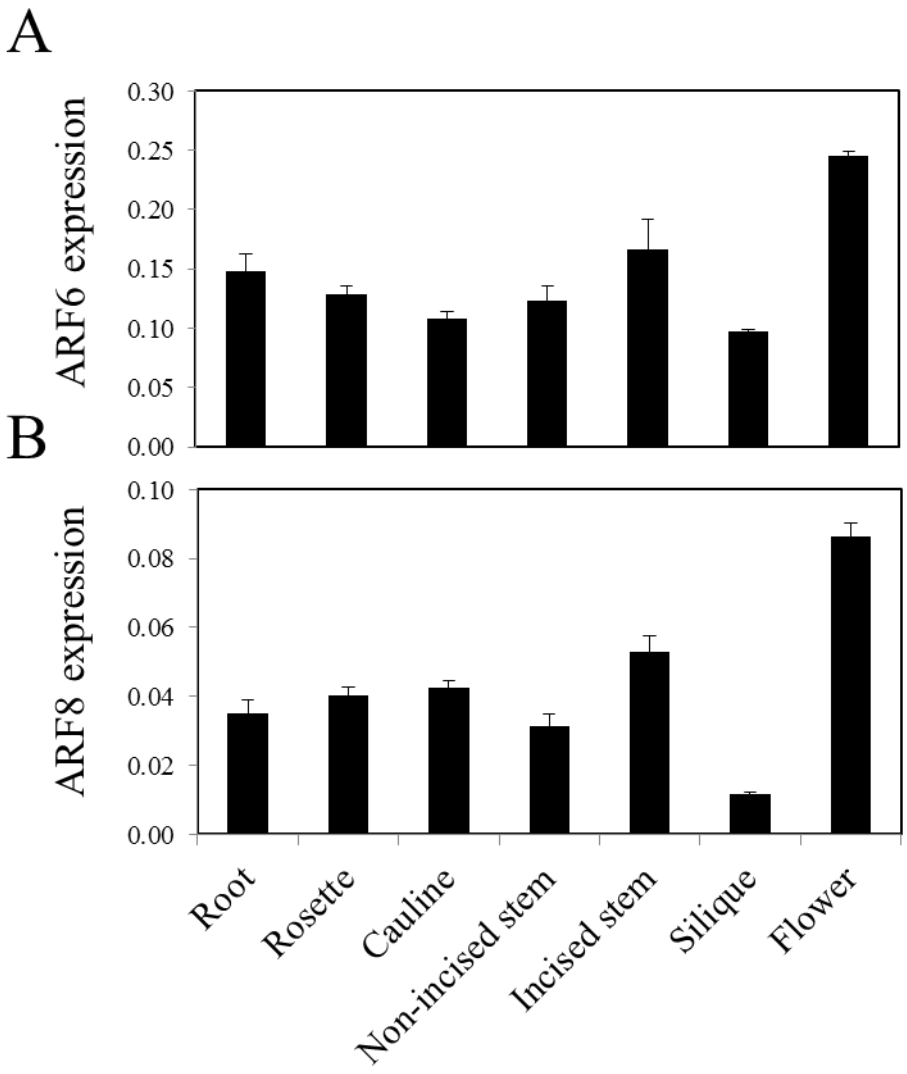

A
ARF6 expression
0.30
0.25
0.20
0.15
0.10
0.05
0.00
B
ARF8 expression
0.10
0.08
0.06
0.04
0.02
0.00
Root
Rosette
Cauline
Non-incised stem
Incised stem
Silique
Flower

Figure 4. The expression of *ANAC071*, *RAP2.6L* and *DAD1*in *arf6arf8*.

(A-C) Expression of *ANAC071*, *RAP2.6L* and *DAD1* were examined in non-incised and incised inflorescence stem of wild type and *arf6arf8* 1 day after incision by quantitative RT-PCT. Gray and black column represent the expression of non-incised and incised stem, respectively. *Actin7* was used as an internal control. Error bars represent ± SD (*n*=3).

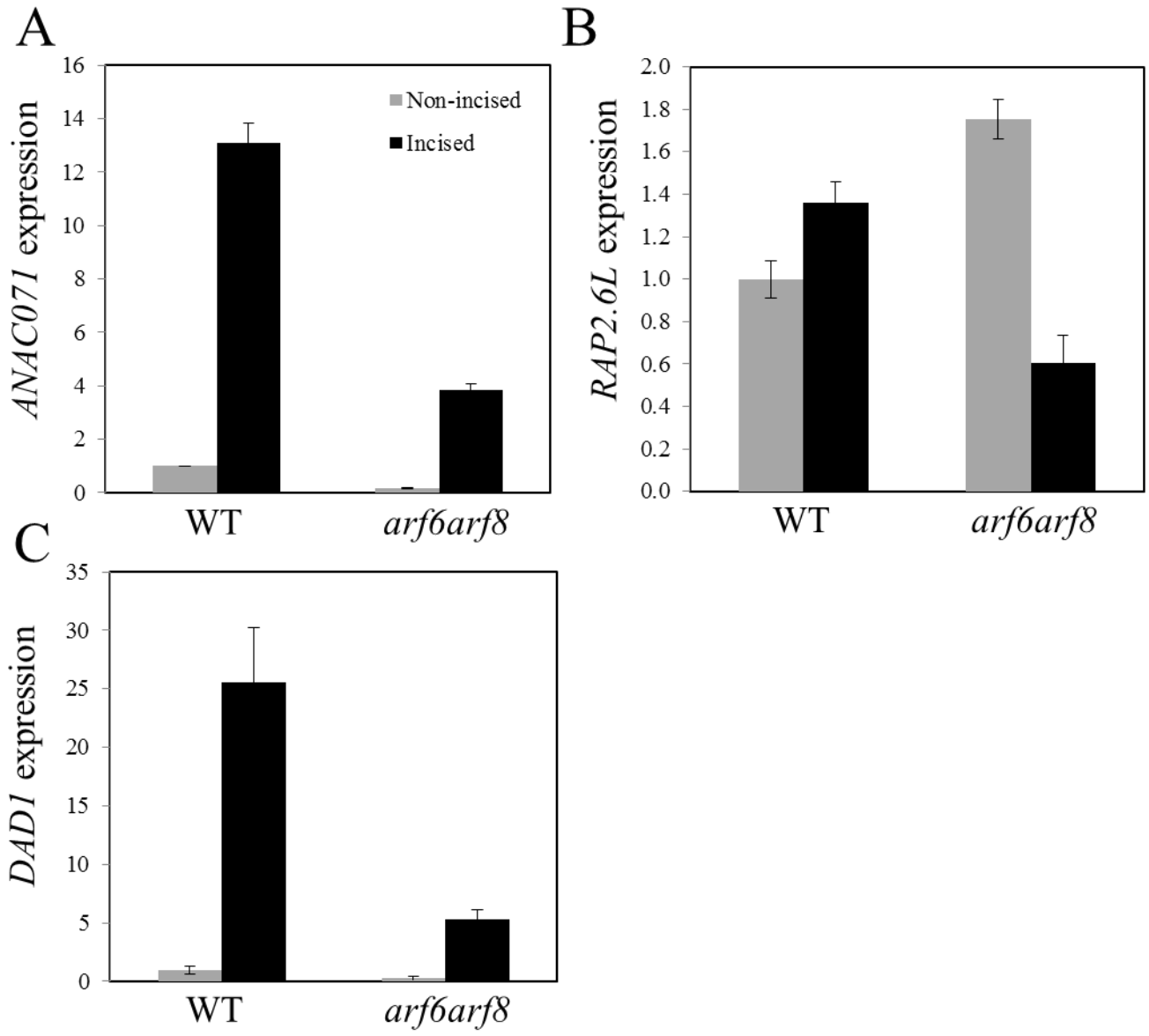

A
ANAC071 expression
16
14
12
10
8
6
4
2
0
Non-incised
Incised
WT
arf6arf8
B
RAP2.6L expression
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
WT
arf6arf8
C
DAD1 expression
35
30
25
20
15
10
5
0
WT
arf6arf8

Figure 5. Tissue-specific expression of *ANAC071*, *XTH20*, and *XTH19*.

(A–C) The expression of *ANAC071* **(A)**, *XTH20* **(B)**, and *XTH19* **(C)** was examined by quantitative RT-PCR in roots, rosette leaves, cauline leaves, incised and non-incised inflorescence stems, siliques, and flowers in wild-type plants. *Actin7* was used as an internal control.

(D–O) Promoter activity of *ANAC071*, *XTH20*, and *XTH19*. GUS activities were detected in incised inflorescence stems of $Pro_{ANAC071}$*:GUS*, Pro_{XTH20}*:GUS*, and Pro_{XTH19}*:GUS* transgenic plants. The photographs were taken 1 day (D, H, and L), 3 days (E, I, and M), 5 days (F, J, and N), and 7 days (G, K, and O) after the incision was made (indicated by arrowhead). Bar = 0.5 mm.

(P) and **(Q)** The expression of *XTH20* and *XTH19* in non-incised and distal and proximal regions of incised inflorescence stems at 1, 3, and 5 days after the incision was made. Mean ± SD (n = 3).

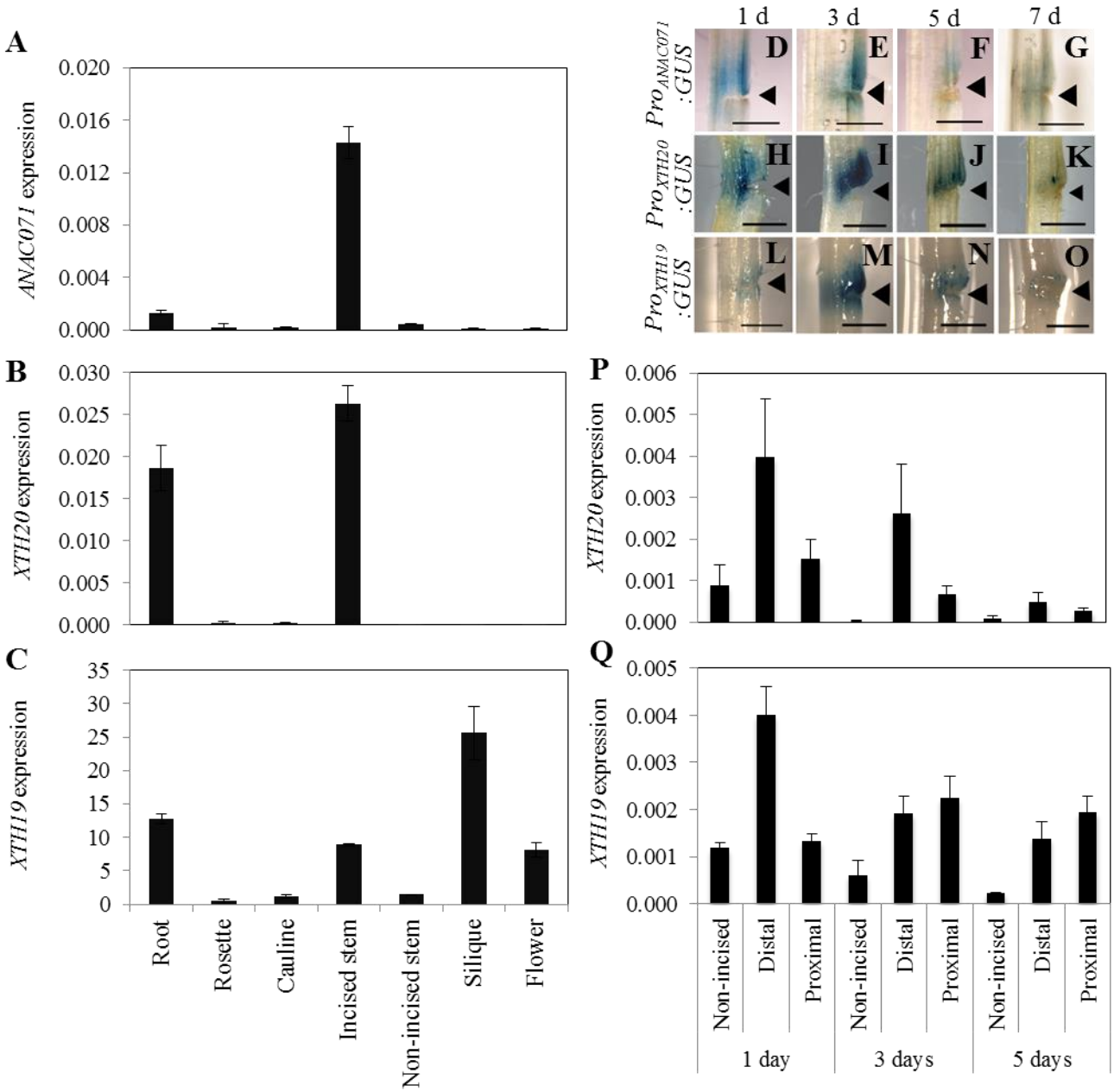

A
ANAC071 expression
0.020
0.016
0.012
0.008
0.004
0.000
1 d
3 d
5 d
7 d
Pro ANAC071 :GUS
D
E
F
G
Pro XTH20 :GUS
H
I
J
K
Pro XTH19 :GUS
L
M
N
O
B
XTH20 expression
0.030
0.025
0.020
0.015
0.010
0.005
0.000
P
XTH20 expression
0.006
0.005
0.004
0.003
0.002
0.001
0.000
C
XTH19 expression
35
30
25
20
15
10
5
0
Root
Rosette
Cauline
Incised stem
Non-incised stem
Silique
Flower
Q
XTH19 expression
0.005
0.004
0.003
0.002
0.001
0.000
Non-incised
Distal
Proximal
Non-incised
Distal
Proximal
Non-incised
Distal
Proximal
1 day
3 days
5 days

Figure 6. Effect of auxin on the induction of *ANAC071*, *XTH20*, and *XTH19* in the tissue reunion process.

(A) and **(B)** Expression levels of *XTH20* **(A)** and *XTH19* **(B)** in incised (black bar) and non-incised (gray bar) stems. The expression levels of *XTH20* and *XTH19* were examined by quantitative RT-PCR in non-decapitated plants and in decapitated plants 1 day after incision was made 1 day the application of distilled water (D.W.) or 10^{-3} M IAA at the top of the cut stems. Mean ± SD (*n* = 3).

(C–H) Promoter activity of *ANAC071*, *XTH20*, and *XTH19*. GUS activity was detected 1 day after an incision was made in inflorescence stems of $Pro_{ANAC071}$*:GUS*, Pro_{XTH20}*:GUS*, and Pro_{XTH19}*:GUS* transgenic plants after decapitation followed by application of distilled water (D.W.) **(C, E, and G)** or 10^{-3} M IAA **(D, F, and H)**. Bar = 0.5 mm.

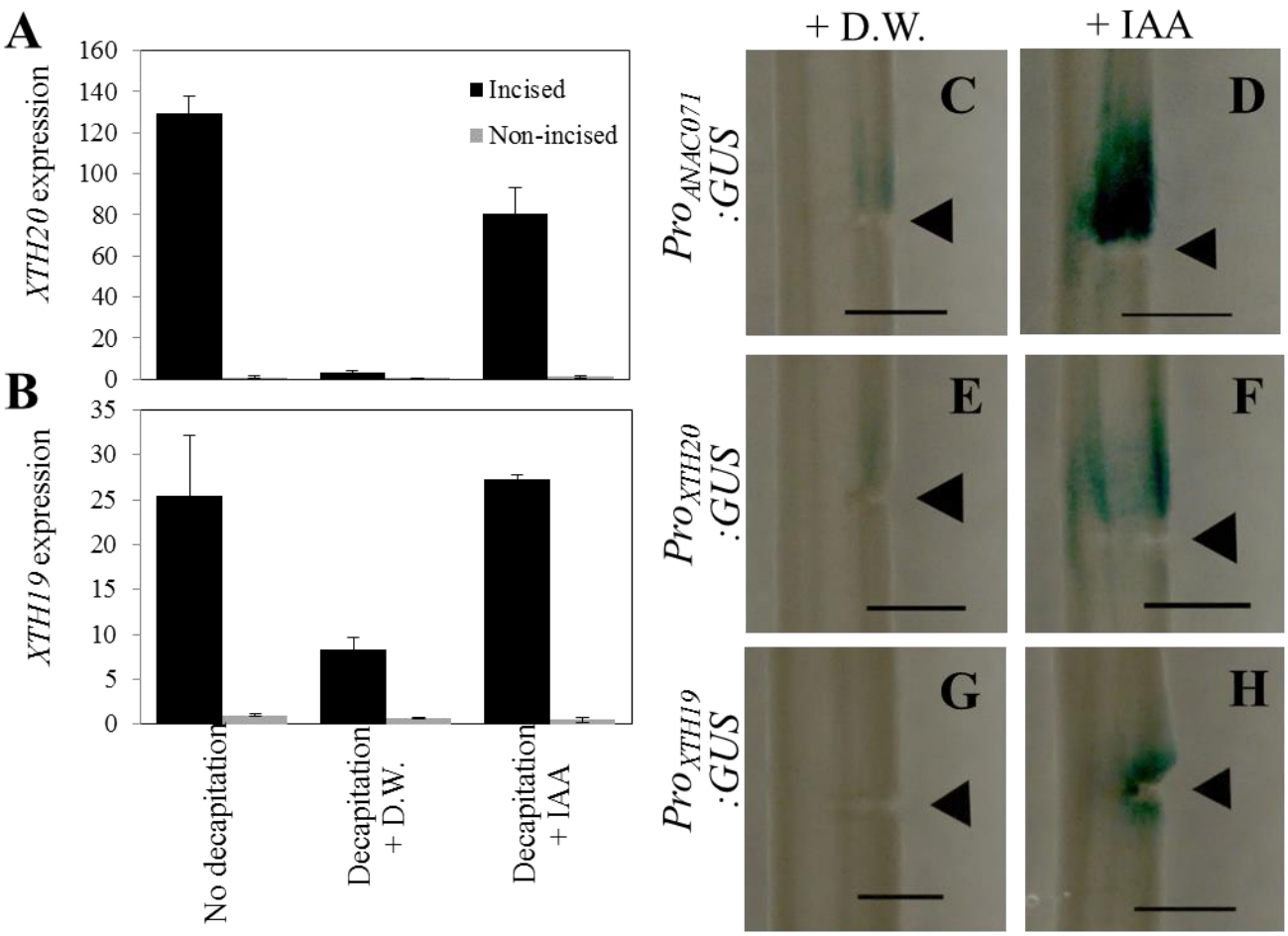

A
XTH20 expression
160
140
120
100
80
60
40
20
0
Incised
Non-incised
B
XTH19 expression
35
30
25
20
15
10
5
0
No decapitation
Decapitation + D.W.
Decapitation + IAA
+ D.W.
+ IAA
Pro ANAC071 :GUS
Pro XTH20 :GUS
Pro XTH19 :GUS
C
D
E
F
G
H

Figure 7. Effects of gravity on the tissue reunion process.

(A–C) Tissue reunion phenotypes observed 7 days after an incision was made in plants grown vertically **(A)** or horizontally with upward-facing **(B)** and downward-facing **(C)** incisions. Arrowheads indicate the position of the incision. g, gravity; pi, pith; co, cortex; vb, vascular bundle. Bar = 100 μm. **(D)** Cell density in pith tissues calculated from sectioned samples as grown in **(A–C)**. Mean ± SD (n = 7). *P < 0.05 (t-test). (Calculation is described in Figure 14).

(E–P) Promoter activity of *DR5*, *ANAC071*, *XTH20*, and *XTH19*. GUS activity was detected in incised inflorescence stems of Pro_{DR5}*:GUS*, $Pro_{ANAC071}$*:GUS*, Pro_{XTH20}*:GUS*, and Pro_{XTH19}*:GUS* transgenic plants 3 days after an incision was made. Immediately after the incision was made, plants were oriented vertically as a control **(E, H, K, and N)** or horizontally with upward-facing **(F, I, L, and O)** or downward-facing **(G, J, M, and P)** incisions. Bar = 0.5 mm.

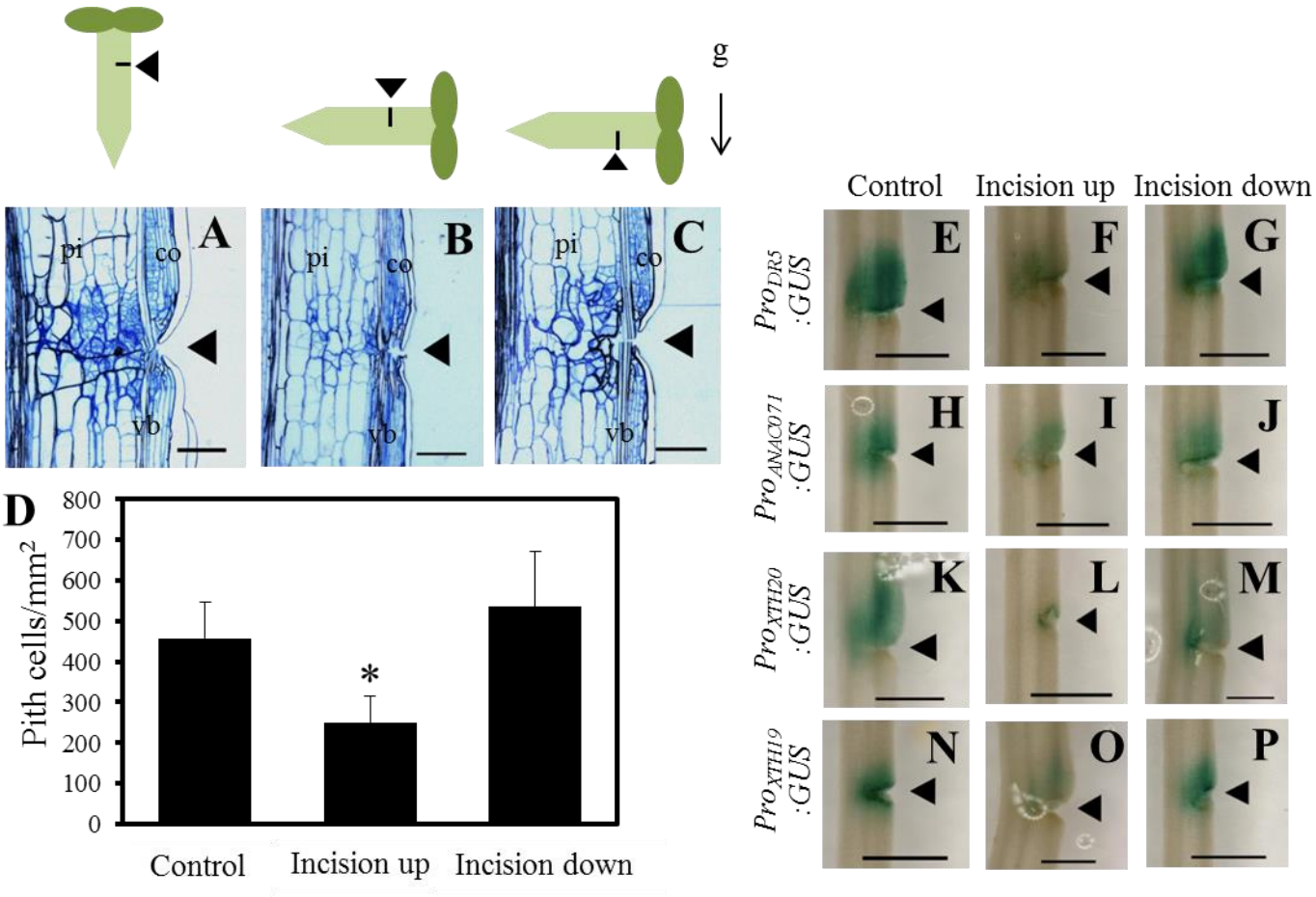

g
A
B
C
pi
co
vb
D
Pith cells/mm2
800
700
600
500
400
300
200
100
0
*
Control
Incision up
Incision down
Control
Incision up
Incision down
ProDR5:GUS
ProANAC071:GUS
ProXTH20:GUS
ProXTH19:GUS
E
F
G
H
I
J
K
L
M
N
O
P

Figure 8. Control of the expression of *XTH20* and *XTH19* by *ANAC071* during the tissue reunion process.

(A) and **(B)** Expression of *XTH20* **(A)** and *XTH19* **(B)** was examined by quantitative RT-PCR in inflorescence stems of wild-type (incised = blue; non-incised = red) and Pro_{35S}*:ANAC071-SRDX* transgenic (incised = green; non-incised = purple) plants 0–7 days after the incision was made. Mean ± SD (*n* = 3).

(C) and **(D)** Quantitative RT-PCR analysis of *XTH20* **(C)** and *XTH19* **(D)** in incised (black) and non-incised (gray) inflorescence stems of wild-type, *anac071*, and Pro_{35S}*:ANAC071* transgenic plants 3 days after the incision was made. Mean ± SD (*n* = 3).

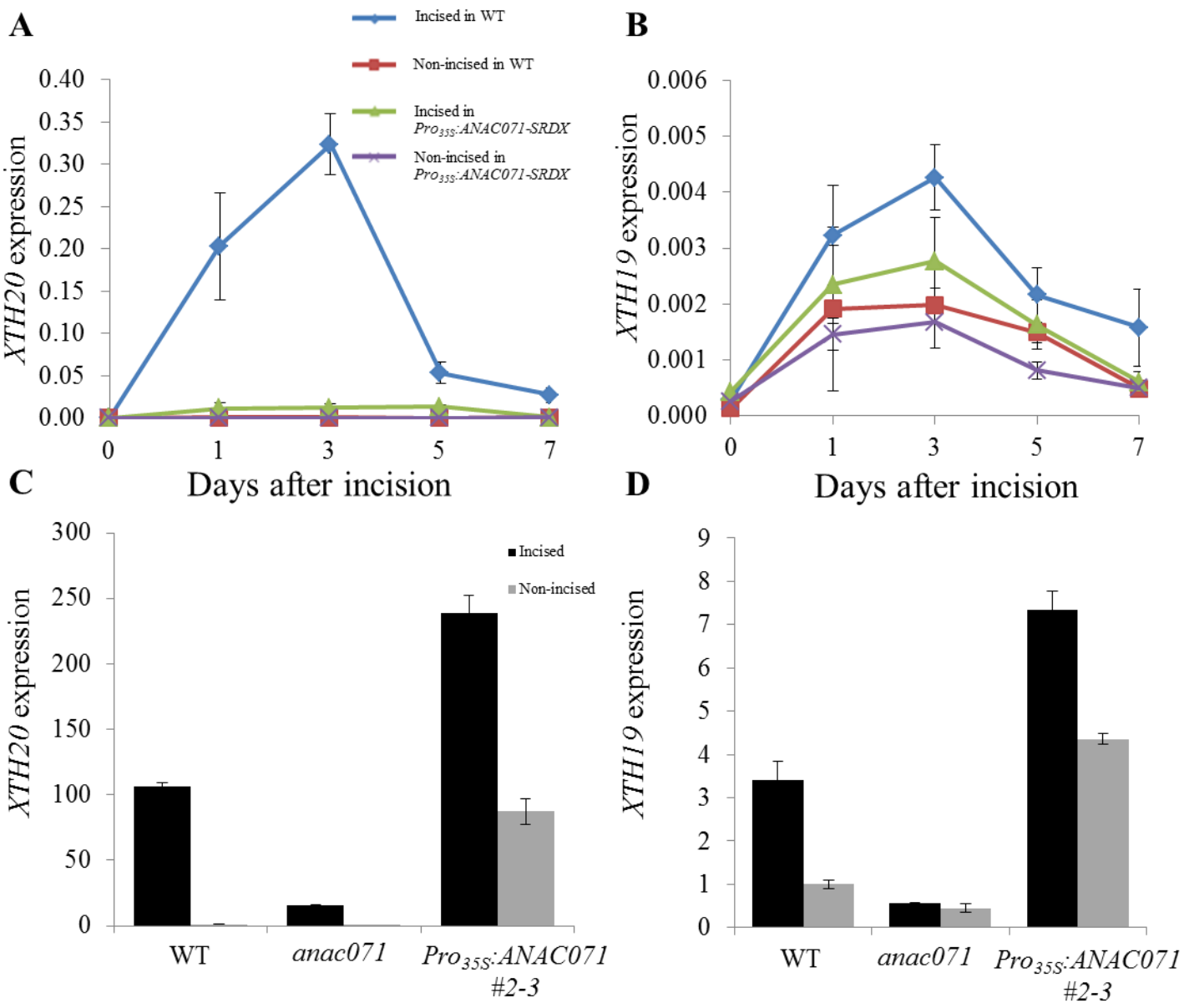

A
Incised in WT
Non-incised in WT
Incised in Pro35S:ANAC071-SRDX
Non-incised in Pro35S:ANAC071-SRDX
XTH20 expression
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
1
3
5
7
Days after incision
B
XTH19 expression
0.006
0.005
0.004
0.003
0.002
0.001
0.000
0
1
3
5
7
Days after incision
C
Incised
Non-incised
XTH20 expression
300
250
200
150
100
50
0
WT
anac071
Pro35S:ANAC071 #2-3
D
XTH19 expression
9
8
7
6
5
4
3
2
1
0
WT
anac071
Pro35S:ANAC071 #2-3

Figure 9. Binding of ANAC071 to *XTH20* and *XTH19* promoters.

(A) and (**B**) Illustration of selected oligonucleotides containing the *cis*-elements of the NAC recognition site (CATGT and CATG) and a presumed core binding site (CACC) in the promoters of *XTH20* **(A)** (–923 bp) and *XTH19* (**B)** (–102 bp). Black boxes indicate exons.

(C) and (**D)** Oligonucleotides used in the EMSA. The probes for *XTH20* **(C)** and *XTH19* (**D)** (pXTH20 and pXTH19, respectively) contained the *cis*-element of the NAC recognition site (CATGT and CATG) and the presumed core binding site (CACC) in the *XTH20* and *XTH19* promoter regions.

(E) and (**F)** The EMSA showed binding of His-ANAC071 to the CATGT, CACC, and CATG motifs in *XTH20* **(E)** and *XTH19* (**F)** promoters. Protein-DNA complexes were detected when His-ANAC071 fusion protein was incubated with labeled pXTH20 or pXTH19 probes (lane 2). Unlabeled pXTH20 or pXTH19 (lanes 3 and 4) were used for 100-fold (lanes 3) and 200-fold (lanes 4) excess levels of competitors. Arrowheads indicate the position of the protein-DNA complex.

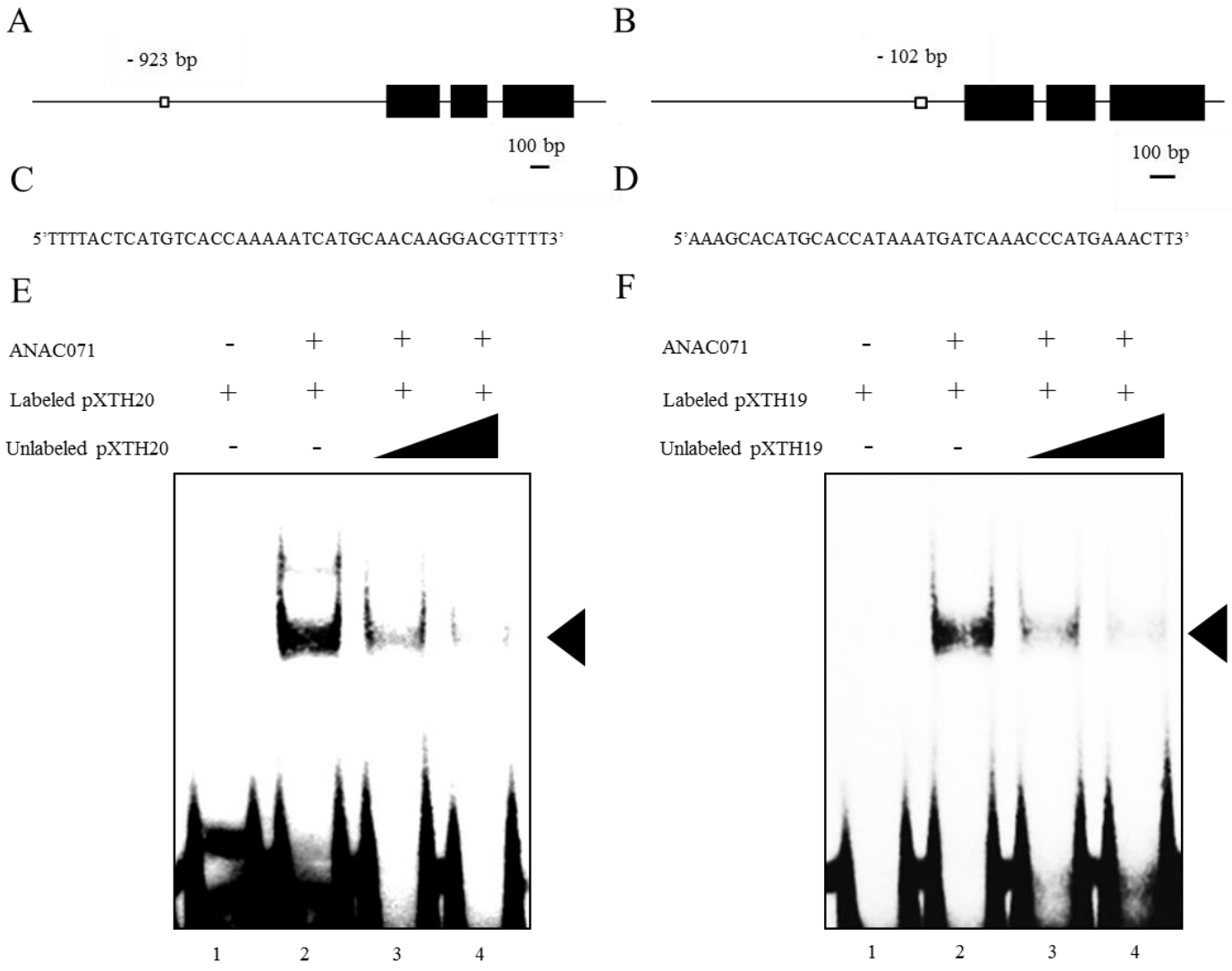
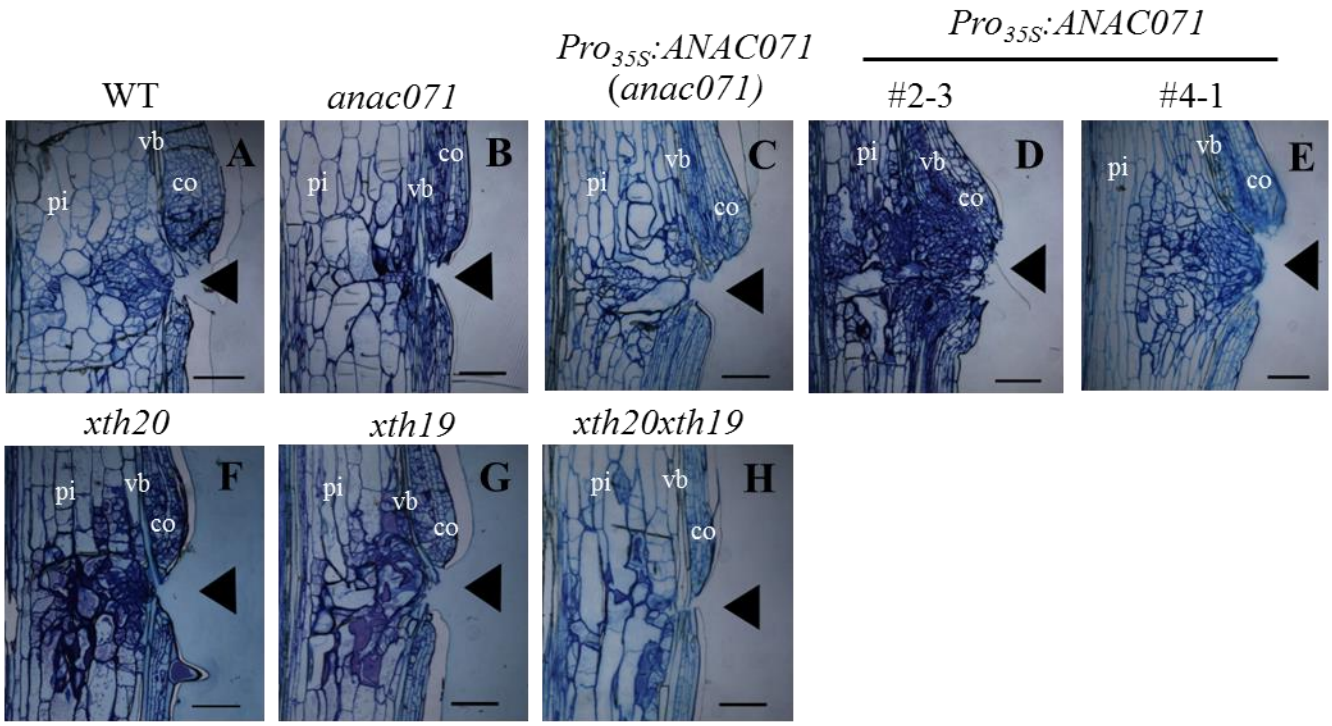

A
- 923 bp
100 bp
B
- 102 bp
100 bp
C
5'TTTTACTCATGTCACCAAAAATCATGCAACAAGGACGTTTT3'
D
5'AAAGCACATGCACCATAAATGATCAAACCCATGAAACTT3'
E
ANAC071 - + + +
Labeled pXTH20 + + + +
Unlabeled pXTH20 - -
1 2 3 4
F
ANAC071 - + + +
Labeled pXTH19 + + + +
Unlabeled pXTH19 - -
1 2 3 4

Figure 10. Phenotypes of *anac071*, *xth20*, and *xth19* single mutants and the *xth20xth19* double mutant and the effect of *ANAC071* overexpression on cell division in pith tissue.

(A–H) Tissue reunion phenotype observed 7 days after the incision was made. *Pro_{35S}:ANAC071* was introduced into *anac071* mutant (*Pro_{35S}:ANAC071* (*anac071*)) or into wild type two lines (*Pro_{35S}:ANAC071* #2-3 and #4-1). Arrowheads indicate the position of the incision. pi, pith; co, cortex; vb, vascular bundle; WT, wild type. Bar = 100 μm.

(I) Cell density in pith tissue was calculated from sectioned samples. Mean ± SD (n = 7). *P < 0.05 (t-test compared with WT).


WT
anac071
Pro35S:ANAC071 (anac071)
Pro35S:ANAC071
#2-3
#4-1
A
B
C
D
E
pi
vb
co
xth20
xth19
xth20xth19
F
G
H

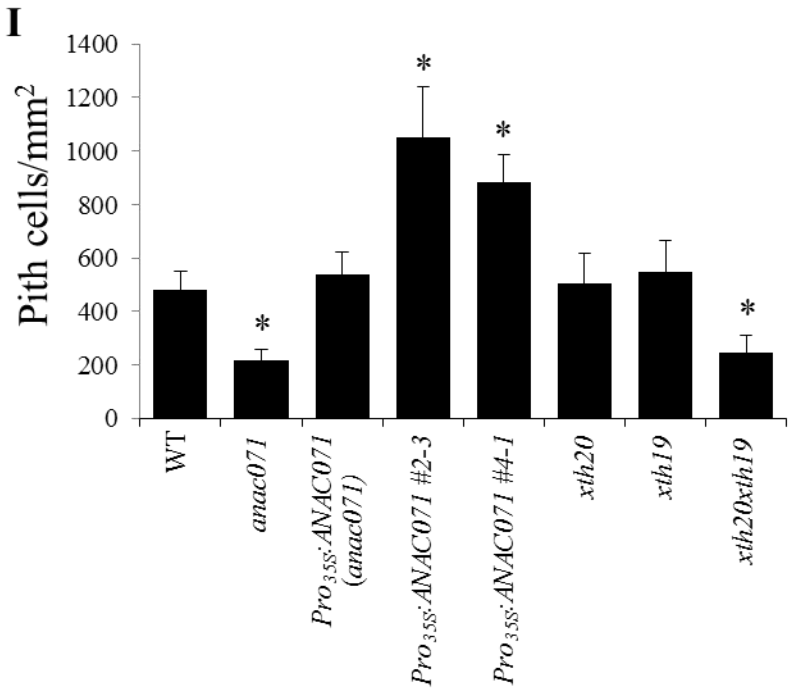

I
Pith cells/mm2
1400
1200
1000
800
600
400
200
0
*
WT
anac071
Pro35S:ANAC071 (anac071)
Pro35S:ANAC071 #2-3
Pro35S:ANAC071 #4-1
xth20
xth19
xth20xth19

Figure 11. Morphology of plants.

(A) Photograph of wild-type, *anac071*, *Pro$_{35S}$:ANAC071* transgenic wild-type lines (#2-3 and #4-1), *Pro$_{35S}$:ANAC071* in the *anac071* background, *xth20* and *xth19* single mutants, and the *xth19xth20* double mutant (from left to right). Bar = 5 cm.

(B) Rosette leaf number in mature plants (3 weeks after transfer to soil). Mean ± SD (n = 12)

(C) Leaf length (black bar) and width (gray bar) of the sixth rosette leaf. Mean ± SD (n = 12)

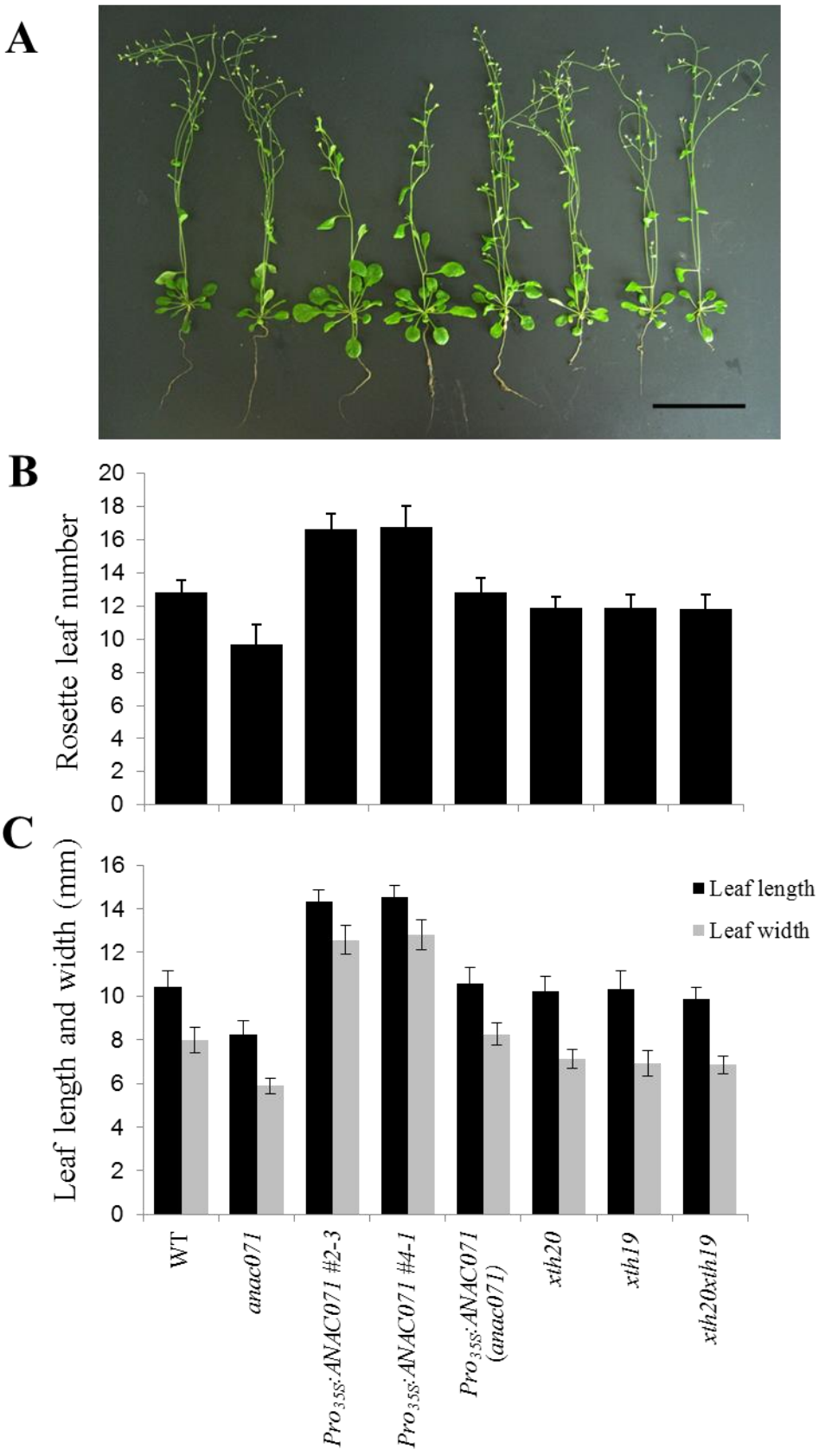
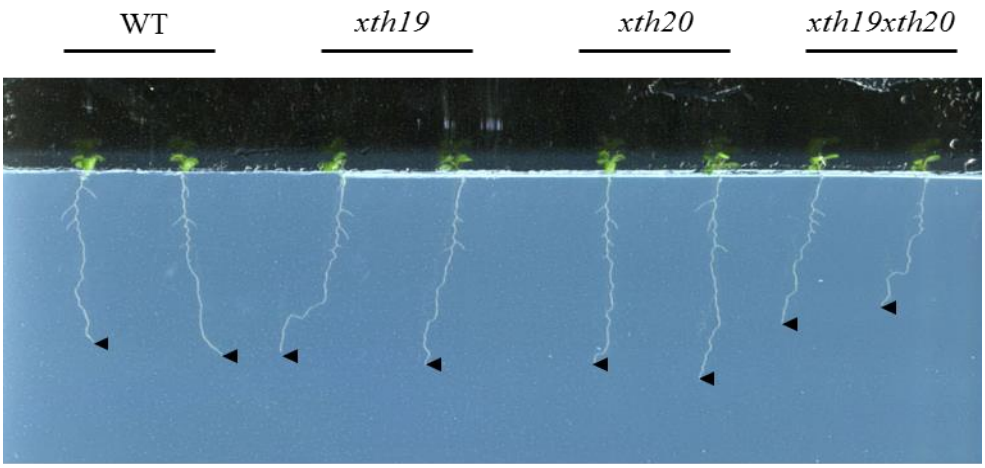

A
B
Rosette leaf number
20
18
16
14
12
10
8
6
4
2
0
C
Leaf length and width (mm)
16
14
12
10
8
6
4
2
0
Leaf length
Leaf width
WT
anac071
Pro35S:ANAC071 #2-3
Pro35S:ANAC071 #4-1
Pro35S:ANAC071 (anac071)
xth20
xth19
xth20xth19

Figure 12. Primary root length of the *xth* mutants.

(A) Ten days after sowing plants in half-strength MS medium. Arrowheads indicate root tips.

(B) Primary root length of wild-type (white circle), *xth19* (white diamond), *xth20* (black square), and *xth19xth20* (black diamond) plants 5, 10, and 15 days after sowing. Mean ± SD (n = 12).

A


WT
xth19
xth20
xth19xth20

B

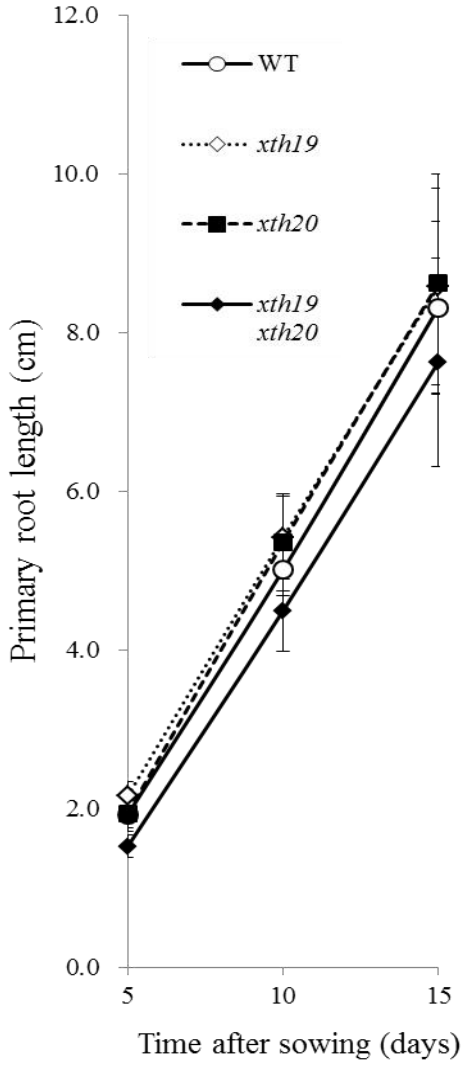

12.0
10.0
8.0
6.0
4.0
2.0
0.0
Primary root length (cm)
5
10
15
Time after sowing (days)
WT
xth19
xth20
xth19
xth20

Figure 13. Tissue reunion phenotypes at 1, 3, and 5 days after the incision was made.

(A–X) Tissue reunion phenotype of wild type **(A, I, Q)**, *anac071* **(B, J, R)**, *Pro$_{35S}$:ANAC071* in the *anac071* background **(C, K, S)**, *Pro$_{35S}$:ANAC071* transgenic lines #2-3 **(D, L, T)** and #4-1 **(E, M, U)**, *xth20* **(F, N, V)** and *xth19* **(G, O, W)** single mutants, and the *xth19xth20* double mutant **(H, P, X)** 1 day **(A–H)**, 3 days **(I–P)**, and 5 days **(Q–X)** after incision. Arrowheads indicate positions of the incision. pi, pith; co, cortex; vb, vascular bundle. Bar = 100 μm. Three plants for each genotype were examined, and representative photographs are shown.

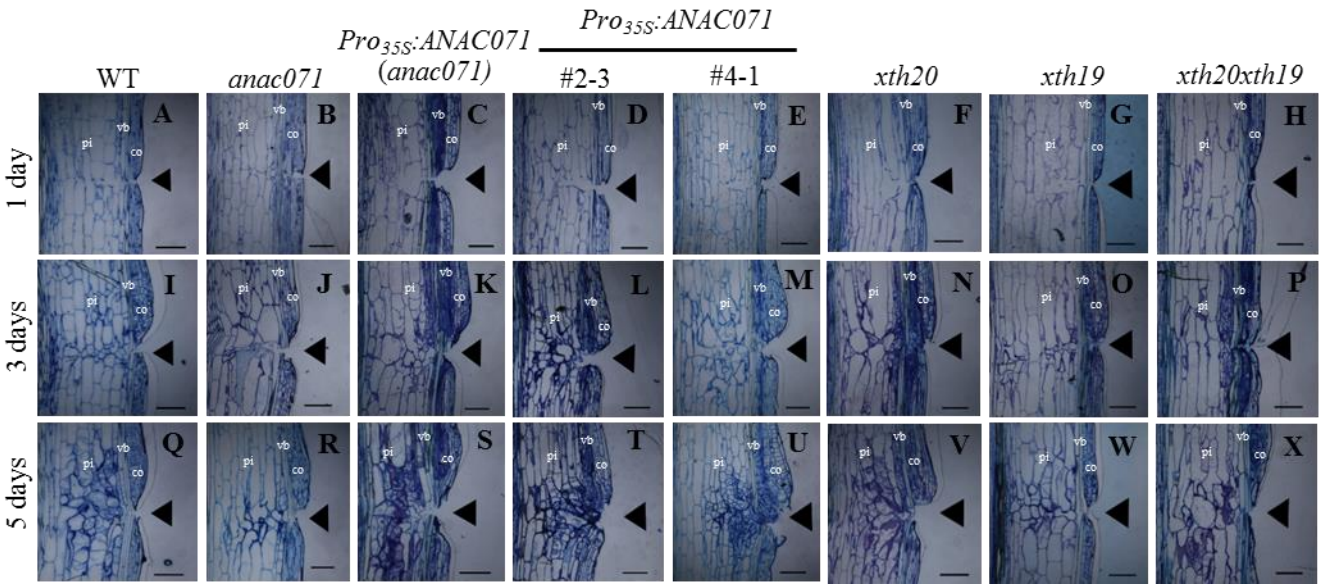
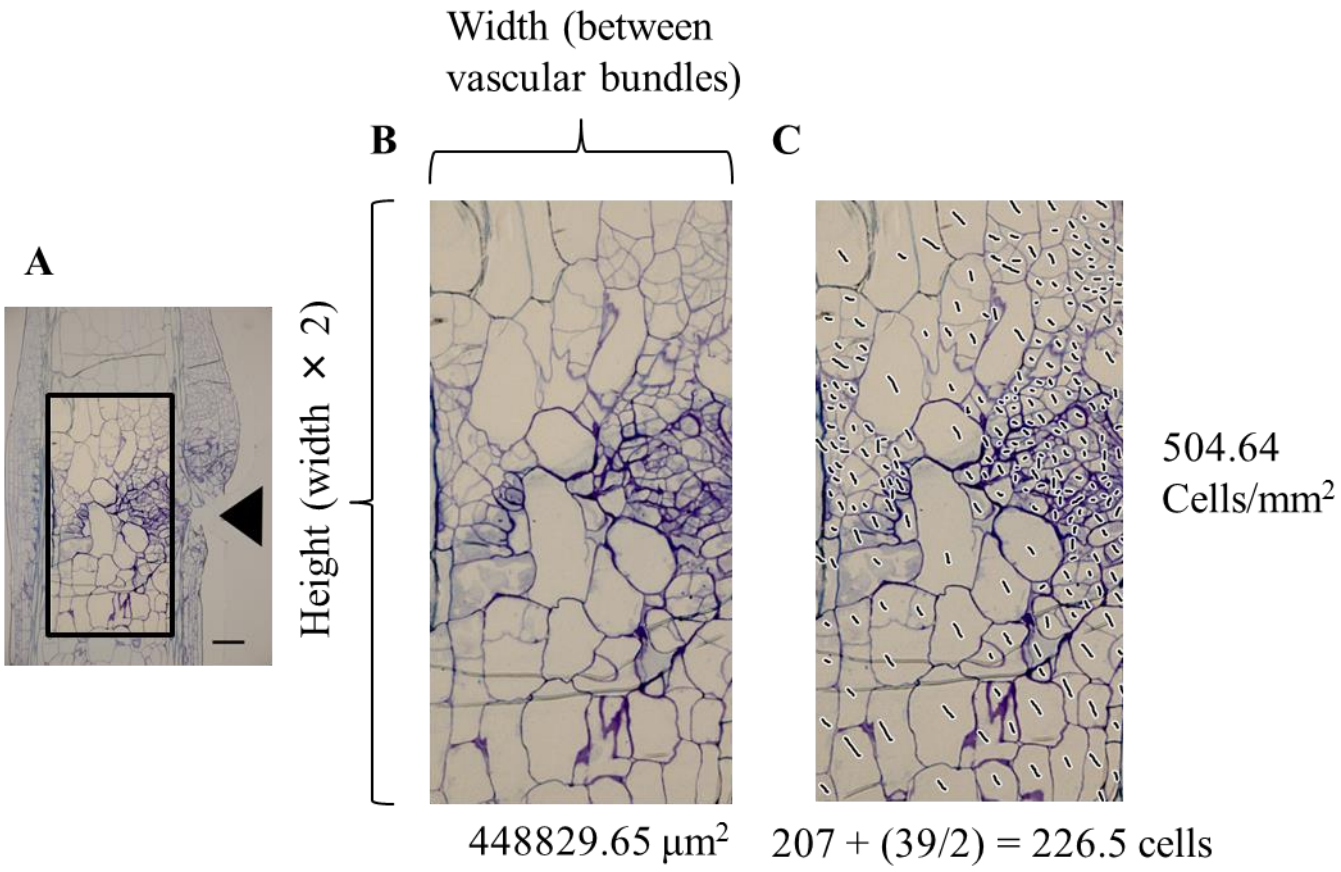

Pro35S:ANAC071
Pro35S:ANAC071
WT
anac071
(anac071)
#2-3
#4-1
xth20
xth19
xth20xth19
1 day
3 days
5 days
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X
vb
pi
co

Figure 14. Method for calculation of cell density.

(A) Selected rectangle of pith tissue around the incision site for cell counting, with width (between vascular bundles) and height (width × 2). Bar = 100 μm.

(B) The selected area was measured with ImageJ software.

(C) Average cell number was counted manually based on the formula shown. The partial cells refer to those that were touching an edge of the rectangle.



$$\text{Average cell number} = \left\{\text{Number of complete cells}\right\} + \left\{\frac{\text{Number of partial cells}}{2}\right\}$$

Table 1. List of primers used in this research.

Name of primer	DNA sequence (5' to 3')
Plasmid construction	
Pro$_{ANAC071}$:GUS	
F pANAC071	CACCGAAATCGTCCAGCCATAATTGG
R pANAC071	TGCAAAATTCACACTTGTAGCTTGC
Pro$_{35S}$:ANAC071	
F OX-ANAC071	CACCATGGGAGTTCATGTTTGCCTC
R OX-ANAC071	CTAAGAACGAACCAACATTTCTTGT
Detection of T-DNA insertion	
F-*anac071* (SALK_012841C)	CGCATGTTATAGCTGGAT
R-*anac071* (SALK_012841C)	CTAAGAACGAACCAACATTTCTTGT
F-*xth20* (SALK_066758)	GGATGAAATAGATTTTGAGTT
R-*xth20* (SALK_066758)	CAGTGCAATAGTTGTAGACC
F-*xth19* (GK369E06.01)	ATGAAGTCTTTTACGTTCTTG
R-*xth19* (GK369E06.01)	GACTTTCCATTAACCCATAC
LBb1	GCGTGGACCGCTTGCTGCAACT
LB1-GABI	CTTTCTTTTTCTCCATATTGACCATCA
Primer for qRT-PCR analysis	
F ANAC071	CCTCTCCTTGTCGCGATGAA
R ANAC071	ATGCTTGAAGAGTCGTTTGTAGTAGAAG
F XTH20	TAATCACGTGGCATTGTCTTTGTA
R XTH20	AATTTATTTAGCGCAAATACTCAGCTAG
F XTH19	TGCAGCTAAATGATTGATTCTTTGAT
R XTH19	CCATTGAGTTACAAAGACAACGCAA
F ARF6	GCTACAAACAGCCGATTCACC
R ARF6	GCATCCTAAACCGCATACCAA
F ARF8	TCCTTGGTGATGACCCATGGGAGTC
R ARF8	CCACTGCCTTCTCCATGATCTCCCA
F RAP2.6L	GAGGAAGCTGCTTTAGCCTATGA
R RAP2.6L	TGGACCCGTTCAGGGAAGT
F DAD1	TAAGGAGCTTCGGCTGAGTAGCCGT
R DAD1	GAGAACTCTCCGAGCTGTTTCTCTG
F ACT7	CAGTGTCTGGATCGGAGGAT
R ACT7	TGAACAATCGATGGACCTGA
Primer for EMSA	
ANAC071-His-tag construction	
F ANAC071-pCold	GGAGGCCAGTGAATTCATGGGGAGTTCATGTTTGCCTC
R ANAC071-pCold	TCATCTGCAGCTCGAGCTAAGAACGAACCAACATTTCTTGT

Acknowledgements

This is a great chance of my life to come to Japan and participate in Plant Physiology Lab, University of Tsukuba. It opens my vision to see how Japanese scientists work study and plant physiology. These all things will not happen without the kindness of my supervisor, Professor S. Satoh. Thank you for all of his suggestions and discussions through the course of my research.

I wish to express my thanks to Professor M. Ono and Dr. K. Sage-Ono for construction training which improved my technique in molecular works.

Special thanks to Associate Professor K. Miura for his kindness collaboration and invaluable discussion.

Thank you for many suggestions and comments during the course of my research which gratefully supporting my research due to Associate Professor H. Iwai, Assistant Professor J. Furukawa and Associate Professor T. Mizuguchi. I also would like to thanks Professor M. Ohme-Takagi, and Professor K. Nishitani for their kindness seeds material.

Special thanks for all of members in Plant Physiology laboratory, University of Tsukuba for wonderful encouragement.