Pattern of Auxin and Cytokinin Responses for Shoot Meristem Induction Results from Regulation of Cytokinin Biosynthesis by Auxin Response Factor 3

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ABSTRACT

De novo organ regeneration is an excellent biological system for the study of fundamental questions regarding stem cell initiation, cell fate determination, and hormone signaling. Despite the general belief that auxin and cytokinin responses interact to regulate de novo organ regeneration, the molecular mechanisms underlying such a crosstalk are little understood. Here, we showed that spatiotemporal biosynthesis and polar transport resulted in local auxin distribution, which in turn determined the cytokinin response during de novo shoot regeneration. Genetic and pharmacological interference of auxin distribution disrupted the cytokinin response and ATP/ADP ISOPENTENYLTRANSFERASE5 (AtIPT5) expression, affecting stem cell initiation and meristem formation. Transcriptomic data suggested that AUXIN RESPONSE FACTOR3 (ARF3) mediated the auxin response during de novo organ regeneration. Indeed, mutations in ARF3 caused ectopic cytokinin biosynthesis via the mis-expression of AtIPT5 and this disrupted organ regeneration. We further showed that ARF3 directly bound to the promoter of AtIPT5 and negatively regulated AtIPT5 expression. The results from this study thus revealed an auxin-cytokinin crosstalk mechanism involving distinct intermediate signaling components required for de novo stem cell initiation, and shed new light on the mechanisms of organogenesis in planta.

Keywords: de novo organogenesis, gene regulation, auxin, cytokinin, stem cell, Arabidopsis.
INTRODUCTION

Plant cells have an amazing capacity to regenerate organs from differentiated somatic tissues under appropriate culture conditions, a process designated de novo organogenesis. De novo organogenesis consists of two steps. The first step involves the formation of the callus, a mass of undifferentiated pluripotent cells derived from various explant tissues grown on callus induction medium (CIM) that has a high auxin/cytokinin ratio. The second involves stem cell initiation, pattern establishment and organ regeneration. Depending on the auxin/cytokinin ratios of the induction medium, either shoots or roots can be regenerated (Skoog and Miller, 1957; Bhojwani and Razdan, 1996; Che et al., 2002).

Shoot formation is the most studied de novo organogenesis process. Because the shoot meristem gives rise to all aerial parts of the plant body, de novo shoot formation is widely used in agricultural biotechnology to propagate plants. In addition, de novo shoot formation is highly controlled, and can thus serve as an excellent experimental system to study fundamental biological processes such as stem cell initiation, cell fate determination, cell differentiation, and hormonal crosstalk (Che et al., 2006; Birnbaum and Sánchez, 2008).

The formation of the de novo shoot meristem involves a similar degree of patterning and cell organization to that of the embryonic shoot apical meristem (SAM) (Mayer et al., 1998; Gordon et al., 2007). The SAM consists of three distinct cell zones, the central zone (CZ), the peripheral zone (PZ) and the rib zone (RZ) (Gifford and Corson, 1971; Steeves and Sussex, 1989). At the top of the SAM, the CZ contains stem cells, descendents of which are either displaced to the PZ and may undergo differentiation to form specific organs or to the RZ to form stem tissues. In addition to a similar cell organization, a common group of regulatory proteins controls the establishment of the shoot meristem both during embryogenesis and de novo organ formation. The expression of WUSCHEL (WUS) is the earliest event to mark stem cell initiation in
Arabidopsis. WUS-expressing cells in the organizing center establish and maintain stem cell populations within the CZ of the embryonic shoot meristem (Laux et al., 1996; Mayer et al., 1998; Schoof et al., 2000; Weigel and Jürgens, 2002). Ectopic expression of WUS is sufficient to induce somatic embryo formation in Arabidopsis (Zuo et al., 2002). Similarly, spatiotemporal WUS expression is critical for the establishment of the meristem during de novo shoot formation (Gordon et al., 2007).

Apart from cell organization and a few regulatory proteins such as WUS, little is known about the mechanisms that regulate stem cell initiation and meristem formation during de novo shoot regeneration. Different ratios of exogenous auxin and cytokinin determine cell fates in the callus, indicating the importance of these ratios and the potential crosstalk between these two hormones in pattern formation during organ regeneration. Indeed, previous results have shown that the cytokinin response is critical for de novo stem cell initiation and shoot meristem establishment in Arabidopsis (Gordon et al., 2007; Su et al., 2009; Cheng et al., 2010). Mutations of the cytokinin receptor gene ARABIDOPSIS HIS KINASE4 (AHK4) or type-A ARABIDOPSIS RESPONSE REGULATOR7 (ARR7) and ARR15 affect de novo shoot formation of Arabidopsis (Buechel et al., 2009). A strong cytokinin response initiated by AHK4 promotes the expression of WUS, which is sufficient to induce the formation of the shoot meristem (Gordon et al., 2009). Interestingly, exogenous auxin increases the expression of AHK4 during callus formation, while exogenous cytokinin regulates the expression of auxin efflux carriers PINFORMEDs (PINs) and auxin biosynthetic genes YUCCAs (YUCs) in the callus (Ruzicka et al., 2009; Jones et al., 2010). In addition, auxin controls cytokinin response through the negative regulation of ARR7 and ARR15 by ARF5/MONOPTEROS to maintain SAM (Zhao et al., 2010). The auxin and cytokinin responses transiently and antagonistically interact during early embryogenesis (Müller and Sheen, 2008), suggesting an extensive crosstalk between these two hormones during organogenesis.

In our current study, we showed that a spatiotemporal auxin gradient, established
through its coordinated local biosynthesis and polar transport, regulated the spatial cytokinin response during de novo shoot induction. Such an auxin-cytokinin pattern was critical for spatial WUS induction, shoot meristem establishment, and subsequent shoot regeneration. We further showed that the spatial auxin-cytokinin crosstalk was determined by the negative regulation of ISOPENTENYLTRANSFERASE genes (in Arabidopsis thaliana, AtIPT) via the auxin signaling component AUXIN RESPONSE FACTOR 3 (ARF3). Our results thus reveal an auxin-cytokinin crosstalk for shoot meristem induction that involves novel intermediate signaling components.

RESULTS

Mutually Exclusive Distribution of Auxin and Cytokinin Responses during Stem Cell Initiation and Meristem Formation

Previously, we induced callus formation from plant pistils in an auxin-rich CIM, and then transferred the calli onto a cytokinin-rich shoot induction medium (SIM) for shoot induction (Cheng et al., 2010), and we also suggested that the expression of WUS/CLAVATA (CLV3) marked stem cell initiation and shoot meristem formation is developmentally regulated during these processes (Su et al., 2010). Therefore, we studied response of endogenous auxin during stem cell initiation and meristem formation using DR5::GFP; pWUS::DsRed-N7 reporter lines. GFP signals were detected uniformly at the edge region of the non-induced callus (SIM0, Fig. 1, A-C). However, these signals progressively translocated to a restrictive region of the outermost cell layers following SIM induction for two days (SIM2), when stem cell initiation, as indicated by WUS expression, had not yet started (Fig. 1, D-F). SIM induction for four days (SIM4) caused relocalization of GFP signals to a “ring”, i.e. a circular region apical and peripheral to the region of high WUS expression (Fig. 1, G-N). Formation of shoot meristem by SIM incubation for six days (SIM6) accompanied the high GFP signals switch to the region immediately apical to the WUS expression
domain (Fig. 1, O-R). These results show that the distribution pattern of the auxin response is mutually exclusive of \( WUS \) expression during stem cell induction and shoot meristem formation.

To evaluate the dynamic distribution of the endogenous cytokinin response during stem cell initiation and meristem formation, we used \( TCS::GFP, pWUS::DsRed-N7 \) reporter lines. \( TCS \) is a synthetic cytokinin response promoter (Müller and Sheen; 2008). The cytokinin response was distributed to regions corresponding to that of the auxin response in SIM0 (Fig. 2, A-C). However, the cytokinin response adopted a progressively restrictive pattern under SIM induction (Fig. 2, D-F), substantially overlapping with the region of \( WUS \) expression in SIM4 (Fig. 2, G-M). A strong cytokinin response was detected in a group of cells within the pro-meristem overlapping with the \( WUS \) expression region at SIM6 (Fig. 2, N-Q). These results indicate that the distribution pattern of the cytokinin response overlapped with that of \( WUS \) expression during stem cell initiation and shoot meristem formation.

To verify the mutually separate distribution of auxin and cytokinin responses during stem cell initiation and shoot meristem formation, we performed double labeling using reporter lines expressing both \( TCS::GFP \) and \( DR5rev::3XVENUS-N7 \). The cytokinin and auxin responses merged well at the edge region at SIM0 (Supplemental Fig. S1A), as shown by separate labeling experiments (Fig. 1, A-C; Fig. 2, A-C). However, SIM4 resulted in translocation of the \( DR5rev \) signals to the “auxin ring” region, whereas the cytokinin response was restricted to the center of the “auxin ring” (Supplemental Fig. S1, B and C) where \( WUS \) expression was also detected (Fig. 2, G-I). These results show that the auxin response is distinct from the cytokinin response, which is associated with the \( WUS \) expression pattern during stem cell initiation and shoot meristem induction, demonstrating a mutually exclusive distribution of the auxin and cytokinin responses during these processes.
Auxin Transport Plays Important Roles in de Novo Shoot Regeneration

Because the regional auxin response could be the result of local auxin biosynthesis or dynamic auxin transport, we studied these two aspects during de novo shoot formation. A major pathway leading to auxin biosynthesis is mediated by the YUCs (Cheng et al., 2006). Using genome-wide transcriptional and qRT-PCR analysis, we found that the transcriptional levels of \textit{YUC1} and \textit{YUC4} among the \textit{YUC} members were significantly enhanced during shoot induction (Fig. 3A; Li et al., 2011; ArrayExpress accession: E-MEXP-3120). Furthermore, we observed that SIM4 greatly induced the promoter activity of the \textit{pYUC1::GUS} and \textit{pYUC4::GUS} reporter lines for which GUS activity was restricted to the future shoot initiation sites by SIM4, whereas no GUS activity was detected on SIM0 (Fig. 3, B and C). Time lapse analysis of reporter lines containing both \textit{pYUC4::GFP} and \textit{pWUS::DsRed-N7} showed a similar dynamic distribution of \textit{YUC4} promoter activity to that of the auxin response, neither of which overlapped with the \textit{WUS} expressing region on SIM4 (Fig. 3, E-G and Fig. 1, G-N). These results suggest that \textit{YUC1} and \textit{YUC4}-mediated auxin biosynthesis contributes to the distribution of the auxin response.

With the exception of auxin biosynthesis, polar auxin transport via auxin efflux carrier PINs may also contribute to the spatially restricted auxin distribution (Wiśniewska et al., 2006). Indeed, although PIN1 did not show polarized membrane localization at SIM0 (Supplemental Fig. S2, A-C), SIM incubation for 1 day (SIM1) induced its polarization (Supplemental Fig. S2, D-F). Prolonged incubation on SIM resulted in a more restricted and polarized localization pattern (Supplemental Fig. S2, G-I). At SIM4, PIN1 was detected in the outermost cell layer of the region apical to that of the \textit{WUS} expression domain where stem cells would be initiated (Supplemental Fig. S2, J-Q). At SIM6, PIN1 became accumulated in the cells of layer 1 of pro-meristem (Supplemental Fig. S2, R-U). To provide further evidence whether PIN1-mediated polar auxin transport is critical for the auxin response during stem cell initiation and shoot meristem formation, we undertook pharmacological analysis using
N-1-naphthylphthalamic acid (NPA), an inhibitor of polar auxin transport (Lomax et al., 1995). The application of NPA at SIM4 disrupted the spatiotemporal auxin response and WUS expression (Fig. 1, S-U) in a manner that resembled the situation in the non-induced callus (Fig. 1, A-C).

The spatiotemporal expression of both the auxin biosynthetic and auxin transport pathways correlated well with the distribution of the auxin response during de novo shoot regeneration. To test whether this correlation is biologically relevant, we analyzed the efficiencies of the de novo shoot regeneration of plants whose auxin biosynthesis or polar transport mechanisms had been genetically disrupted. As shown in Table I, mutations of both YUC1 and YUC4 caused significantly lower regeneration frequencies than wild type or YUC single mutants (Table I, Supplemental Table S1). The frequency of shoot regeneration was significantly reduced by expressing antisense PIN1 (Table I). These results indicate that the genetic disruption of either auxin biosynthesis or polar auxin transport suppresses shoot regeneration, further indicating the importance of the auxin response during de novo shoot regeneration.

The Spatiotemporal Biosynthesis of Cytokinin Relies on Polar Auxin Transport

Because the cytokinin response, as indicated by TCS::GFP reporter signals, showed a spatially restricted distribution in the callus following SIM incubation (Fig. 2, A-M), we wondered whether this resulted from cytokinin biosynthesis. To test this possibility, we analyzed the expression of the cytokinin biosynthetic gene AtIPTs by qRT-PCR. The expression of AtIPT3, AtIPT5 and AtIPT7 were upregulated in SIM incubation (Fig. 4A). Using the pAtIPT5::GUS reporter line, we detected the expression of AtIPT5 at the whole edge of the non-induced callus (Fig. 4, B and F). Incubation on SIM caused a gradual disappearance of the AtIPT5 signals in all regions except those of the future pro-meristems (Fig. 4, C, D and G). Eventually, the AtIPT5 signals were restricted to the pro-meristem region (Fig. 4, E and H). In addition, mutations of the AtIPTs significantly reduced the frequencies of shoot regeneration. Shoot regeneration
frequencies of the atipt5 atipt7 double mutants and the atipt5 atipt7 atipt3 triple mutants were much lower than those of the wild type or single mutants (Table I). These molecular and genetic analyses indicate the importance of AtIPT-dependent cytokinin biosynthesis during de novo shoot regeneration.

Because the cytokinin response was spatially correlated with the auxin response (Fig. 1, A-C; Fig. 2, A-C), we tested whether interrupting auxin response pathway could affect the dynamic cytokinin response. For this hypothesis, we disrupted the polar auxin transport of the TCS::GFP reporter lines either genetically by down-regulating PIN1 expression or pharmacologically by applying NPA. Both PIN1 down-regulation or NPA treatment abolished the dynamic distribution of cytokinin response initiated by SIM induction (Fig. 2, R-W). NPA treatment also abolished SIM-induced WUS expression (Fig. 2, R-T). In addition, NPA treatment abolished the translocation of AtIPT5 expression during SIM induction (Fig. 4, I-K). These data indicate that the distribution of the cytokinin response and the cytokinin biosynthesis depends on polar auxin transport, and by inference, on an intact auxin response.

**ARF3 Mediates the Auxin Response during de Novo Shoot Regeneration**

Genetic and pharmacological evidence has revealed the importance of the auxin response during de novo shoot regeneration. To evaluate whether there are changes at the auxin level during de novo shoot regeneration, we assayed the endogenous auxin concentration during shoot induction. The auxin level was significantly higher in the calli of SIM4 when stem cell initiation and meristem formation has commenced than that of SIM0 when only non-differentiating pluripotent cells were present (Supplemental Fig. S3). The increased auxin level was correlated with an increased expression of auxin biosynthetic genes (Fig. 3A).

The increased endogenous auxin level following SIM induction prompted us to identify the auxin response genes that might function in de novo shoot regeneration. To this end, we performed genome-wide transcriptomic analysis using the Affymetrix
Arabidopsis ATH1 Genome Arrays (Cheng et al., 2010; Li et al., 2011; Supplemental Table S2; ArrayExpress accession: E-MEXP-3120). Among the genes whose transcriptional changed from SIM0 to SIM4/SIM6 were more than 1.5 fold, we identified several ARFs (Supplemental Table S2). However, ARF3 was the only gene in our dataset that appeared to be upregulated by SIM incubation in a previous transcriptomic screen when the roots were used as explants (Che et al., 2006).

Exogenous auxin was found to upregulate the ARF3 expression (Supplemental Fig. S4). To elucidate the expression pattern of ARF3 during shoot regeneration, we performed in situ hybridization. The ARF signals were distributed evenly at the edge region of the SIM0 callus (Fig. 5A). SIM2 caused a spatial restriction of ARF3 expression (Fig. 5B). Progressive translocation of ARF3 expression by SIM incubation resulted in a pattern (Fig. 5C-D) similar to that of the auxin response (Fig. 1, G-N). Finally, high ARF3 expression was detected in the shoot meristem (Fig. 5E). These results show that the ARF3 expression profile parallels the dynamic auxin response distribution during shoot meristem formation.

To test whether ARF3 mediates the auxin response during de novo shoot regeneration, we adopted a reverse genetic approach. Using two mutant alleles for ARF3, arf3/ett-1 a null mutant and ett-2 a weak mutant (Sessions et al., 1997; Sohlberg et al., 2006), we first compared the frequencies of de novo shoot regeneration of the mutants versus wild type. As shown in Table I, mutations of ARF3 caused a significant reduction of shoot regeneration. Consistent with the properties of the two ARF3 mutants, the strong allele ett-1 hardly regenerated any shoots whilst the weak allele ett-2 showed some shoot regeneration capacity, although much reduced compared with the wild type (Table I; Fig. 5, F-H). We also determined the frequency of shoot regeneration in the ett-1 atipt5-1 double mutant. The result showed that the double mutant with 12.85%±2.56% was slightly lower than that in the ett-1 single mutant with 16.67%±1.06% (Supplemental Fig. S5). Because ARF3 is considered to be a transcriptional repressor (Guilfoyle and Hagen, 2007), we further tested the shoot
regeneration frequencies of other ARFs that also serve as transcriptional repressors. However, none of the other repressor ARFs affected the frequencies of de novo shoot regeneration (Table I; Supplemental Table S1), consistent with our transcriptomic data in which none of the other repressor ARFs showed significant transcriptional changes (Supplemental Table S2). These results indicate that ARF3 is a key mediator of the auxin response during de novo shoot regeneration.

**ARF3 Negatively Regulates Spatiotemporal AtIPT Expression**

Disrupting polar auxin transport abolished the dynamic cytokinin response induced by SIM (Fig. 2, R-W), suggesting that the SIM-induced cytokinin response is dependent on auxin signaling. Because ARF3 mediates the auxin response to SIM induction and the dynamic cytokinin response results from AtIPT expression, we used several approaches to test whether these two pathways converge.

First, we analyzed the GUS pattern of pAtIPT5::GUS reporter lines either in wild type or in the arf3/ett-2 mutant background to detect possible changes in AtIPT5 expression. We did not observe any difference in the GUS staining patterns at SIM0 between wild type and the arf3 mutants (Fig. 6, A and C). However, GUS signals were found to be restricted to the future pro-meristem region by SIM4 in wild type (Fig. 6B) but to be insensitive to SIM induction in the arf3 mutants (Fig. 6D). In addition, the transcript levels of AtIPT5 were increased in the arf3 single mutants compared with the wild type lines under SIM induction (Fig. 7). These data suggest that ARF3 negatively regulate AtIPT5 expression in high auxin response regions.

Second, we evaluated the effects of mutating the auxin response elements (AuxREs) within the promoter sequence of AtIPT5 and found that they rendered this gene insensitive to SIM induction in the wild type background. AuxREs, including three TGTCTC elements and 13 TGTCNN elements (Ulmasov et al., 1999a), were identified within a 2.2 Kb-promoter region of AtIPT5 (Fig. 6E; Supplemental Table S3). We generated a mutant version of the AtIPT5 promoter (AtIPT5m) that contained point
mutations in several AuxREs potentially abolishing ARF binding (Fig. 6E). The GUS activity of the \textit{pAtIPT5m::GUS} reporter line showed a similar uniform distribution pattern (Fig. 6F) to that of \textit{pAtIPT5::GUS} reporter line at SIM0 (Fig. 6A). However, the GUS activity of \textit{pAtIPT5m::GUS} reporter line failed to respond to SIM induction and the spatial restriction of the reporter activity normally observed in the \textit{AtIPT5::GUS} line did not occur (Fig. 6G).

To test whether ARF3 directly binds to the promoter of \textit{AtIPT5}, we performed yeast one-hybrid analysis, surface plasmon resonance (SPR) measurements and electrophoretic mobility shift assays (EMSA). The yeast one-hybrid experiments showed growth of the yeast on selection medium, suggesting positive binding of ARF3 to the \textit{AtIPT5} promoter (Fig. 8A). Furthermore, ARF3 proteins were produced by \textit{in vitro} transcription and translation, and used in SPR and EMSA analysis (Supplemental Table S3). Elevated Resonance Unit (RU) values were detected over time (Fig. 8B). In EMSA experiments using biotin-labeled 26-bp oligos (-155 to -130) covering two AuxREs, a clear ARF3-dependent mobility shift was identified (Fig. 8C). The wild type oligos could compete for binding of the ARF3 proteins whereas the mutated oligos for the AuxREs could not (Fig. 8C), indicating that ARF3 proteins directly bind to the promoter region of \textit{AtIPT5} to regulate its expression (Supplemental Table S3).

To next determine whether ARF3 directly associates with the promoter sequence of \textit{AtIPT5} \textit{in vivo}, we performed chromatin immunoprecipitation (ChIP) assays using the \textit{pARF3::ARF3tasiR-GUS} transgenic lines (Col, Marin \textit{et al}., 2010). As shown in Fig. 8E, compared with the Mouse IgG mock control, the calli sampled from \textit{pARF3::ARF3tasiR-GUS} transgenic lines showed a strong enrichment of fragment “a” (covering -155 to -130 in EMSA) but not of fragment “b” (negative control) in the promoter region (Fig. 8, D and E). Fragment “a” was moderately enriched in calli sampled from \textit{pMP (ARF5)::MP-GFP} transgenic plants and was not enriched at all in \textit{35S:6myc-ARF8} transgenic calli (Fig. 8E). These results indicate that ARF3 proteins directly binds to the promoter of \textit{AtIPT5} \textit{in vivo}.
DISCUSSION

Plant tissues could regenerate shoots or somatic embryos in vitro, two processes that could be mechanistically distinct (Verdeil et al., 2007). Shoot regeneration reflects cell pluripotency, while somatic embryo induction is the full expression of totipotency (Atta et al., 2008; Verdeil et al., 2007). Previously, we showed that auxin response signals did not accumulate at the edge region of embryonic callus before somatic embryo induction (Su et al., 2009). However, strong signals were evenly distributed at the edge region of callus before shoot meristem induction (Fig. 1, A-C). This difference implies that although auxin plays important roles in both processes, organ regeneration and somatic embryogenesis are regulated by different mechanisms.

Auxin has been proposed to be a morphogen in planta (Dubrovsky et al., 2008) due to its versatility in plant development (Vanneste and Friml, 2009), including meristem formation and embryogenesis (Reinhardt et al., 2000; Heisler et al., 2005; Müller and Sheen, 2008). Auxin signaling extensively interplays with the signal responses of other phytohormones, such as cytokinin, brassinosteroid, ethylene, etc (Ruzicka et al., 2009; Jones et al., 2010; Depuydt and Hardtke, 2011; Willige et al., 2011). Exogenous auxin represses local cytokinin biosynthesis in the nodal stem of pea through PsIPT1 and PsIPT2 (Tanaka et al., 2006). Cytokinin biosynthesis during organogenesis was also found to be negatively regulated by auxin treatment (Nordstrom et al., 2004). In developing root and shoot tissues, ectopic biosynthesis of cytokinin causes a rapid increase in auxin biosynthesis (Jones et al., 2010). Cytokinin also alters auxin responses through the transcriptional regulation of auxin signaling and transport-related genes (Dello Ioio et al., 2008; Ruzicka et al., 2009). However, the underlying molecular mechanisms between auxin and cytokinin interaction during shoot induction remains unknown.

We showed here that auxin response controlled the spatiotemporal distribution of
cytokinin biosynthesis through the negative regulation of AtIPT expression by ARF3 during de novo shoot regeneration. Previous studies have reported that auxin biosynthesis (Cheng et al., 2007; Zhao, 2008) and polar transport (Wiśniewska et al., 2006) can occur spatiotemporally. As a result, auxin signaling is location-sensitive (Weijers et al., 2006; Schlereth et al., 2010). We also showed that the auxin response was promoted by exogenous cytokinin through local auxin biosynthesis and polar auxin transport, resulting in the formation of the “auxin ring” within the callus where cytokinin biosynthesis was inhibited. The spatiotemporal biosynthesis of cytokinin could then be sensed by AHK4 to promote local WUS expression, leading to stem cell initiation and meristem formation (Gordon et al., 2009; Su et al., 2011). Our present results thus reveal a novel molecular mechanism that underlies auxin-cytokinin crosstalk. Such a crosstalk plays a critical role in stem cell initiation and meristem formation during de novo shoot regeneration.

Some crucial questions remain to be addressed. First, it is necessary to point out that ARF3 is an atypical ARF, lacking two domains for dimerization with the Aux/IAAs whereas dimerization with and the auxin-induced degradation of Aux/IAAs is the standard auxin sensing mechanism for ARFs (Ulmasov et al., 1999a; Guilfoyle and Hagen, 2007). Hence, ARF3 either senses auxin signals through cofactors as reported previously (Ulmasov et al., 1999a; Pfluger and Zambryski, 2004), or utilizes a yet-to-be-identified mechanism for auxin sensing. Second, it has been shown that activator-type ARFs and repressor-type ARFs can bind to the same AuxRE elements, and thus regulate the same target genes (Ulmasov et al., 1999b; Vernoux et al., 2011). The ARF activator-to-repressor ratio is critical for the stability of gene expression during auxin response (Vernoux et al., 2011). Our results showed that both the repressor ARF3 and the activator ARF5 bound to the promoter of AtIPT5 (Fig 6E). This result put forward an interesting possibility that ARF3 and ARF5 antagonistically regulated the expression of AtIPT5, a possibility worthy of further analysis. Third, de novo stem cell initiation and meristem formation are induced by SIM containing high cytokinin and
low auxin (Skoog and Miller, 1957). High cytokinin levels could regulate the expression of auxin biosynthetic gene YUCs or polar auxin transporter PINs, as reported in planta (Ruzicka et al., 2009; Jones et al., 2010). However, except for ARF3 whose expression was induced by exogenous cytokinin, none other target genes transcriptionally responding to cytokinin in callus was identified. Genes identified by SIM induction in our transcriptomic data might contain such candidates.

Taken together, we propose that the specific pattern of auxin-controlled cytokinin distribution determine the developmental fate of pluripotent cells during the de novo SAM formation. Auxin biosynthesis and transport mediates local auxin response, which defines the spatial distribution of cytokinin. Such a crosstalk is mediated through the negative regulation of AtIPTs by ARF3 possibly together with other ARFs. This spatiotemporal distribution of cytokinin is essential for WUS induction, which controls meristem formation (Gordon et al., 2009). Our study thus provides new and important findings on the molecular mechanisms underlying de novo shoot regeneration.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotypes Wassilewskija (Ws) or Columbia (Col) were used in this study. Marker lines of the Col and Landsberg (Ler) ecotypes were crossed with wild-type Ws for at least four generations to clear the genetic background. The origins and ecotypes of the transgenic lines and mutants were as follows: pWUS::DsRed-N7, DR5rev::3XVENUS-N7 (Ler, Gordon et al., 2007); DR5rev::GFP, pPIN1::PIN1-GFP (Col, Xu et al., 2006); pAtIPT5::GUS (Ws, Atta et al., 2009); pARF3::ARF3tasiR-GUS (Col, Marin et al., 2010); pMP (ARF5)::MP-GFP (Col, Schlereth et al., 2010); pYUC1::GUS, pYUC4::GUS and the single mutants yuc1, yuc2, yuc4, yuc6 and the double mutants yuc1 yuc4 (Col, Cheng et al., 2007); the single mutants arf4-1 (Col, Pekker et al., 2005); the single mutants atipt5-1 (Ws), atipt7-1 (Col), the double
mutants atipt5-1 atipt7-1 and the triple mutants atipt3-2 atipt5-1 atipt7-1 (Miyawaki et al., 2006); antisense PIN1 vectors (Ws, Su et al., 2009). The single mutants arf3 (ett-2, CS8555, Ws ecotype), arf1-2 (CS24598, Col ecotype) and arf9-1 (CS24609, Col ecotype) were obtained from ABRC. The arf3 (ett-1, Ws ecotype) single mutants were kindly provided by Sohlberg et al., (2006). The TCS::GFP construct (Müller and Sheen, 2008) was previously transformed into wild-type (Ws) plants that harbor pWUS::DsRed-N7 using the Agrobacterium-mediated floral dip method (Clough and Bent, 1998).

Seeds were sterilized and plated on 0.8% (w/v) agar solid medium (1/2-strength MS, 0.5% sucrose (w/v), at pH 5.7; Murashige and Skoog, 1962). After cold treatment to overcome dormancy, they were cultivated under sterile conditions (light intensity 40 μM photons m⁻² sec⁻¹, 20-22°C with a 16 h light/ 8 h dark cycle) for approximately 35 days as explants.

**In vitro Culture and Shoot Induction**

*In vitro* culture and shoot induction using pistils as explants were performed according to Cheng et al., (2010).

**Construction of GFP and GUS Reporters**

For the pYUC4::GFP construct, a DNA fragment containing a 2873 bp sequence upstream of the translational start codon of YUC4 was subcloned in the pBI121-GFP vector. The primers YUC4p-F and YUC4p-R are listed in Supplemental Table S4. For the pAtIPT5m::GUS construct, a DNA fragment containing 2,019 bp of mutated sequence upstream of the translational start codon of AtIPT5 was subcloned in the pBI121-GUS vector. The primers PAtIPT5-F, PAtIPT5-R, PAtIPT5A-F, PAtIPT5A-R, PAtIPT5B-F, PAtIPT5B-R, PAtIPT5C-F, PAtIPT5C-R, PAtIPT5D-F, and PAtIPT5D-R are also listed in Supplemental Table S4.
GUS Assays

GUS assays were performed as described previously by Cheng et al., (2010).

**In situ Hybridization**

Plant tissues were fixed in FAA (10% formaldehyde : 5% acetic acid : 50% alcohol) overnight at 4°C. After dehydration, the fixed tissues were embedded in paraplast (Sigma) and sectioned at 8 μm. Antisense and sense RNA probes were used for hybridization according to a detailed process previously described by Zhao et al., (2006). The sequences of the ARF3-F and ARF3-R probes are listed in Supplemental Table S4.

**Confocal Microscopy**

Approximately 100 calli were imaged to determine the patterns of each marker at different time points after induction. Light-yellowish calli of approximately 3 to 5 mm in diameter were selected under an Olympus JM dissecting microscope and then cut into 1 to 2 mm sections along the longitudinal axis of the callus. These sections were observed and all fluorescence images were captured using a confocal laser scanning microscope (Zeiss 510 Meta CLSM with 10×air, 20×air, 40×oil and 63×oil objectives). For co-labeling by VENUS and GFP, multitracking in frame mode was used. VENUS excitation was performed using a 514 nm laser line in conjunction with a 530 to 600 band-pass filter. GFP excitation was performed using a 488 nm laser line and collected using a 545 secondary dichroic in conjunction with a 505 to 530 band-pass filter. The specific sets of filters used for each marker were similar to those described earlier by Gordon et al., (2007) and Su et al., (2009).

**Chemical Treatments**

NPA (10 mM; Sigma) and estradiol (10 mM; Sigma) stock solutions were prepared in dimethylsulphoxide (DMSO) and added to SIM at a final concentration of 50 μM and
10 μM, respectively. Calli were transferred onto fresh medium with estradiol every two days. An equal volume of DMSO was added to the medium in the control experiments.

**qRT-PCR Analysis**

The primers used in the qRT-PCR analysis are listed in Supplemental Table S4. qRT-PCR reactions were performed for each cDNA dilution using the SYBR Green Master mix according to the manufacturer’s protocol (Bio-Rad Laboratories). For all samples, cDNAs were normalized using *TUBULIN2* and *ACTIN2*, and the measurements were carried out in three biological replicates. The comparative CT method, means and standard deviations, was used to calculate and analyze the results (Schmittgen and Livak, 2008)

**DNA Microarray Analysis**

Total RNAs were isolated from each of the following frozen tissue types: the calli induced on SIM for 0, 4, and 6 days. RNA purification, biotin-labeling of cRNA, and chip hybridizations were performed by the Affymetrix custom service (CapitalBio, Beijing, China). Three biological replicates of each tissue type were analyzed.

An Affymetrix GeneChip Scanner 3000 was used to scan signals from microarray images. GeneChip Operating Software Version 1.4 (GCOS 1.4) was used to produce MAS4.0 signals and presence-absence calls. Normalization was performed separately for each chip to avoid the introduction of dependencies among biological replications by using dChip 2006 software. Significance Analysis of Microarrays software 2.10 was employed to identify differentially expressed genes between different tissues using fold change ≥ 1.5 and q value < 0.05 as cutoffs.

**Yeast One-hybrid Analysis**

Yeast One-Hybrid Analysis was performed using a kit (Clontech Laboratories, Catalog #630491) according to the manufacturer’s protocol. Fragments of *AtIPT5* (-24
and the *AtIPT5* mutant sequence (*AtIPT5m*) were cloned into the *HindIII/KpnI* sites of pAbAi, creating pAtIPT5-AbAi and pAtIPT5m-AbAi, respectively. Each of the plasmids pAtIPT5-AbAi, pAtIPT5m-AbAi and p53-AbAi was linearized by digestion with *BbsI* prior to transformation of the yeast strain Y1HG0ld. The p53-AbAi construct is a yeast reporter vector that serves as a positive control in the kit (Clontech Laboratories, Catalog #630491). The full-length cDNA of *ARF3* was isolated and cloned into the pGADT7 AD vector creating the pAD-ARF3 plasmid. The pAD-ARF3 or empty pGADT7 AD vectors as negative controls were subsequently transformed into the yeast strain containing the pAtIPT5-AbAi or pAtIPT5m-AbAi constructs. Activation of the yeast was observed after three days on selection plates (SD/-Leu) containing 100 ng/mL Aureobasidin A (AbA). The primers for ARF cDNA (ARF3-S and ARF3-X) are described in Supplemental Table S4.

**SPR Measurements**

SPR measurements were performed using a BIAcore-2000 (Pharmacia) at 25°C. The biotin-labeled promoter of *AtIPT5* was immobilized on a sensor chip SA. Dialyzed samples containing ARF3 protein were used as the mobile phase partner, injected at a 20 µL min⁻¹ flow rate. The SPR signal in resonance units (RU) was used as a measure of its interaction and kinetics. Data sets were analyzed using CLAMPFIT 8.0 (Axon Instruments).

**EMSA**

Wild-type and mutated oligonucleotides (oligos) were commercially synthesized as single stranded (ss) DNA. The wild-type oligo sequence corresponds to the -130 to -155 region in the *AtIPT5* promoter. The mutated oligo differed from the wild-type in that GAGACA (-143 to -137) had been replaced by TCTCTT. To generate double-stranded oligos, equal amounts of complementary single stranded oligos were mixed, boiled for 2 min, and slowly cooled down to 25°C. For the binding reaction, the LightShift
Chemiluminescent EMSA kit (Pierce) was used. For competition experiments, different amounts of non-labeled wild-type and mutated double-stranded oligos were used for the binding reaction.

**ChIP Assays**

The immunoprecipitation of bound chromatin was performed using a ChIP kit (Upstate Catalog#17-371) in accordance with the manufacturer’s protocol. Calli of the pARF3::ARF3tasiR-GUS (Marin et al., 2010) transgenic plants, pMP (ARF5)::MP-GFP (Heisler et al., 2005), 35S::6myc-ARF8 and wild type plants were induced on SIM for four days and fixed with 1% (v/v) formaldehyde in GB buffer under a vacuum for 10 min at room temperature. Glycine was then used to quench unreacted formaldehyde under vacuum for 5 min and the tissues were ground in liquid nitrogen. Chromatin was then isolated from the tissues, resuspended in SDS lysis buffer with protease inhibitors, and sonicated to achieve an average DNA size of between 0.2 and 1 kb. Next, the chromatin extract was cleared by centrifugation. The ChIP protocol from the kit (Catalog#17-371), and antibodies against GUS, GFP and MYC (Sigma) were then used to obtain purified DNA that was subsequently analyzed in triplicate by qRT-PCR. Mouse IgG was used as a mock control. For the 35S::6myc-ARF8 construct, the ARF8 CDS fragment was amplified and then cloned into the BamHI and SacI sites of the myc-pBA vector. The fold enrichment of the specific chromatin fragment was normalized to the expression levels of the UBQ10 amplicon and was calculated for each amplicon using the following equation: $2^{(\text{C}_{\text{AtIPT5 MOCK}} - \text{C}_{\text{AtIPT5 ChIP}})/2 - (\text{C}_{\text{UBQ10 MOCK}} - \text{C}_{\text{UBQ10 ChIP}})}$. The primers used to amplify ARF8 cDNA (ARF8-L-myc and ARF8-R-myc), AtIPT5 promoter DNA (fragment a, +39 to -359 and fragment b, -850 to -1133), AtIPT5p-F, AtIPT5p-R, AtIPT5p-F’ and AtIPT5p-R’ and UBQ10 (UBQ10-5 and UBQ10-3) are listed in Supplemental Table S3.

**ACCESSION NUMBERS**
Sequence data generated from the experiments described in this article can be found in the *Arabidopsis* Genome Initiative or GenBank/EMBL databases under accession numbers: *TUB2* (AT5G62690), *WUS* (AT2G17950), *PIN1* (AT1G73590), *YUC1* (AT4G32540), *YUC2* (AT4G13260), *YUC4* (AT4G32540), *YUC6* (AT5G25620), *ARF3* (AT2G24765), *AtIPT1* (AT1G68460), *AtIPT3* (AT3G63110), *AtIPT4* (AT4G24650), *AtIPT5* (AT5G19040), *AtIPT6* (AT1G25410), *AtIPT7* (AT3G23630), *AtIPT8* (AT3G19160), *AtIPT9* (AT5G20040).

**Supplemental Data**

**Supplemental Figure S1.** Redistribution of auxin and cytokinin responses within the callus on SIM incubation.

**Supplemental Figure S2.** Localization of PIN1 within the callus during shoot induction.

**Supplemental Figure S3.** Free IAA levels were increased in calli after being transferred from CIM onto SIM for 4 days compared with those in the non-induced calli (calli on SIM for 0 day).

**Supplemental Figure S4.** Relative expression of *ARF3* responds to auxin by qRT-PCR.

**Supplemental Figure S5.** Shoot regeneration was significantly inhibited in the *ett-1 atipt5-1* double mutant.

**Supplemental Table S1.** Frequencies of shoot regeneration in the indicated mutants using roots as explants.
**Supplemental Table S2.** The expression levels of *ARF3* are significantly increased in calli after the transfer onto SIM for 4 days and 6 days, which is determined by the Affymetrix Arabidopsis ATH1 Genome (Arrays ArrayExpress Accession: E-MEXP-3120).

**Supplemental Table S3.** Oligo sequences and primers used in the yeast one-hybrid, EMSA, SPR and ChIPs assays.

**Supplemental Table S4.** Primers used in this study.

**ACKNOWLEDGEMENTS**

We thank all who generously provided plant materials or constructs as listed in the METHODS section. We also thank Dr. Sharman O’Neill (Department of Plant Biology, College of Biological Sciences, University of California, Davis, CA 95616, USA) for critical reading of the manuscript. This research was supported by grants from the National Natural Science Foundation (NNSF) of China (90917015 and 90717006), and the Ministry of Science and Technology (MOST) of China (2007CB948200 and 2007CB947600).

**REFERENCES**


regeneration in plants and animals. Cell **132**: 697-710.


FIGURE LEGENDS

Figure 1. Regional establishment of auxin responses relative to *WUS* expression.

(A-C) *DR5rev::GFP* signals (green) in the edge region of the non-induced callus (SIM for 0 day; 94.3%, n=122). (D-F) Regional distribution of *DR5rev::GFP* signals in the calli grown on SIM for 2 days (83.3%, n=108). (G-J) Auxin distribution indicated by the *DR5rev::GFP* signals and *WUS* expression indicated by the *pWUS::DsRed-N7* signal (magenta) in calli grown on SIM for 4 days (81.5%, n=127). (K-N) Traverse view of the callus grown on SIM for 4 days (80.9% n=110). (O-R) *DR5rev::GFP* and *WUS* signals accumulate in the pro-meristem of calli grown on SIM for 6 days (80.0%, n=100). (S-U) Auxin distribution and *WUS* expression in the calli grown on NPA-containing SIM for 4 days (86.8%, n=152). Bright-field (B, E, I, and T) and merged images of GFP (green) and DsRed (magenta) channels (J, N, and R) are shown. The Chlorophyll autofluorescence are in blue (M, N, Q and R). Bars=100 µm.

Figure 2. Regional establishment of cytokinin responses relative to *WUS* expression.

(A-C) *TCS::GFP* signals (green) in the edge of the non-induced callus (94.5%, n=127). (D-F) *TCS::GFP* signals beginning to distribute regionally in the calli grown on SIM for 2 days (85.9%, n=128). (G-I) Regional distribution of the *TCS::GFP* signals co-localizes with the *pWUS::DsRed-N7* signal (magenta) in the calli grown on SIM for 4 days (89.2%, n=222). (J-M) Traverse views of the calli grown on SIM for 4 days. (N-Q) *TCS::GFP* and *WUS* signals accumulated in the pro-meristem of calli grown on SIM for 6 days (80.0% n=110). (R-T) Cytokinin response (*TCS::GFP*) and *WUS* (*pWUS::DsRed-N7*) expression in the callus grown on NPA-containing SIM for 4 days. (U-W) Cytokinin responses (*TCS::GFP*) in the calli transformed with antisense *PIN1* driven by an estrogen receptor-based transactivator, XVE. The calli were incubated on estrogen-containing SIM for 4 days (82.8%, n=116). (A-I) and (N-W) are longitudinal sections of calli. Bright-field (B, E, H, S, and V) and merged images of GFP (green) and DsRed (magenta) channels (I, M and Q) are shown. The Chlorophyll autofluorescence
are in blue (L, M, P and Q). Bars=100 µm.

**Figure 3.** Spatiotemporal expression of auxin biosynthetic genes during shoot induction.

(A) qRT-PCR analysis of *YUC* expression level in non-induced calli and in calli grown on SIM for 4 days. (B) *pYUC1::GUS* signals in non-induced calli (91.7%, n=121), and in calli grown on SIM for 4 days (84.0%, n=156) or 6 days (81.5%, n=162). (C) *pYUC4::GUS* signals in the non-induced callus (85.9%, n=198), and in the calli grown on SIM for 4 days (85.6%, n=167) or 6 days (83.3%, n=150). (D-K) Localization of *pYUC4::GFP* (green) and *pWUS::DsRed-N7* (magenta) signals in the non-induced callus (D, 90.2%, n=122), and in the calli grown on SIM for 4 days (E-G, 84.4%, n=166) or 6 days (H-K, 80.3%, n=152). (D-K) are longitudinal sections of calli. Bright-field (D and F) and merged images of GFP (green) and DsRed (magenta) channels (G and K) are shown. The Chlorophyll autofluorescence are in blue (J and K). Bars=1 mm (B, C), 100 µm (D, G and K).

**Figure 4.** Expression of cytokinin biosynthetic genes *AtIPTs* within the callus during shoot induction and following the NPA treatment.

(A) Expression levels of *AtIPTs* in the non-induced calli, and in calli grown on SIM for 4 days determined by qRT-PCR. (B) *pAtIPT5::GUS* signals in the non-induced callus (85.9%, n=199). (C-E) Regional distribution of *pAtIPT5::GUS* signals in calli grown on SIM for 2 days (C, 90.5%, n=199), 4 days (D, 89.5%, n=190) or 6 days (E, 87.9%, n=182). (F-H) *AtIPT5* expression patterns in the non-induced callus (F, 90.9%, n=209), and in calli grown on SIM for 4 days (G, 89.5%, n=190) or 6 days (H, 87.9%, n=182). (I-K) *pAtIPT5::GUS* signals distributed uniformly at the edge region of the calli grown on SIM for 0 days (I, 85.2% n=88), 4 days (J, 83.3% n=66) and 6 days (K, 84.9% n=63) when treated with NPA, respectively. (F-K) are longitudinal sections of calli. PM, pro-meristem. Bars=700 µm (B-E), 200 µm (F, G, I and J) or 50 µm (H).
Figure 5. Spatiotemporal expression of ARF3 and its mutational effects on shoot formation.

(A-E) *In situ* hybridization analyses showing the spatial expression of ARF3 in the non-induced callus (A, 87.5% n=112), or in calli grown on SIM for 2 days (B, 80.5%, n=123), 4 days (C, 75.4%, n=130), 6 days (D, 72.7%, n=110) or 8 days (E, 73.2%, n=123). (F) Regenerated shoots from wild-type calli grown on SIM for 18 days (89.9%, n=99). (G) The *arf3*/*ett2* mutant callus grown on SIM for 18 days showing no shoot regeneration. (H) The *arf3*/*ett2* mutant callus grown on SIM for 18 days with a few regenerated shoots (34.5%, n=200). SAM shoot apical meristem, CL cauline leaf. Arrows indicate regenerated shoots and arrowheads indicate ARF3 signals. (A-E) are longitudinal sections of calli. Bars=100 µm (A-C), 50 µm (D-E) and 1 mm (F-H).

Figure 6. AuxRE-dependent ectopic expression of *AtIPT5* in *arf3*.

(A-B) *pAtIPT5::GUS* signals in the wild-type callus induced on SIM for 0 day (A, 89.5%, n=190) or 4 days (B, 85.9%, n=199). (C-D) GUS signals in the calli of the *arf3* mutant grown on SIM for 0 days (C, 82.7%, n=168) or 4 days (D, 80.4%, n=148). (E) Schematic illustration of the *AtIPT5* promoter. TGTCTC and TGTCNN on both the sense and antisense strand are indicated by blue and green bars, respectively; red asterisks denote point mutations (TGTCTC-TGGC). (F-G) *pAtIPT5m::GUS* signals in the non-induced callus (F, 88.8% n=179) or in the calli grown on SIM for 4 days (G, 85.4%, n=198). (A-D; F-G) are longitudinal sections of calli. Bars=80 µm.

Figure 7. *AtIPT* expression is enhanced in the *arf3* mutant.

(A-C) qRT-PCR analyses showed that the expression of *AtIPT3*, *AtIPT5* and *AtIPT7* were enhanced in *arf3* mutant. *AtIPT5* is the most significantly enhanced under these conditions.
Figure 8. ARF3 directly binds to the promoter of AtIPT5.

(A) Yeast One-Hybrid analysis revealing the direct interaction between ARF3 and the AtIPT5 promoter. Yeast strains containing the PAtIPT5 promoter-AbAi or PAtIPT5m promoter-AbAi constructs were grown on media under selective (SD/-Leu, +100 ng/mL Aureobasidin A or AbA) or non-selective (SD/-Leu, -AbA) conditions. Full-length ARF3 cDNAs fused to pGADT7 AD are indicated on the plates, and the empty pGADT7 AD vector was used as a negative control. The p53-AbAi vector was used as a positive control in the kit (Clontech Laboratories, Catalog #630491). (B) Interaction between ARF3 and the AtIPT5 promoter (red line) was determined by SPR analysis. The AtIPT5m promoter was introduced as a negative control (blue line). (C) EMSA analysis showing the interaction between ARF3 and the AtIPT5 promoter. The retarded DNA-protein complex was competed using either wild-type probe or the mutated probes at a 5×, 25× and 50× molar excess. (D) The AuxREs TGTCTC and TGTCNN on both sense and antisense strands of the AtIPT5 promoter are indicated as blue or green bars, respectively. Red lines indicate fragments amplified in (E). Fragment “a” (-356 to +39) includes the sequence used in the EMSA experiments (-155 to -130), and fragment “b” (-1133 to -850) was used as a negative control. (E) Enrichment of specific regions of the AtIPT5 promoter (fragments “a” and “b”) using anti-GUS, anti-GFP, and anti-MYC antibodies in pARF3::ARF3tasiR-GUS, pMP (ARF5)::MP-GFP, and 35S::6myc-ARF8 transgenic plants, respectively. Mouse IgG was used as a mock control. The fold enrichments of specific regions (fragments “a” and “b”) were detected by qRT-PCR analysis after normalization to the unrelated UBQ10 control sequence. Means were calculated from three biological replicates, and each biological sample was examined using three PCR technical replicates.
Table I. Mutations in auxin and cytokinin-related genes alter the rate of *Arabidopsis* shoot regeneration *in vitro* using pistils as explants.

<table>
<thead>
<tr>
<th>Mutants and Antisense PIN1</th>
<th>Regeneration Frequency$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type (ecotype Col)</td>
<td>24.36±1.11%$^a$</td>
</tr>
<tr>
<td><em>yuc1</em> single mutant (ecotype Col)</td>
<td>23.98±1.22%$^a$</td>
</tr>
<tr>
<td><em>yuc2</em> single mutant (ecotype Col)</td>
<td>22.65±1.13%$^a$</td>
</tr>
<tr>
<td><em>yuc4</em> single mutant (ecotype Col)</td>
<td>22.68±1.60%$^a$</td>
</tr>
<tr>
<td><em>yuc6</em> single mutant (ecotype Col)</td>
<td>23.57±1.24%$^a$</td>
</tr>
<tr>
<td><em>yuc1 yuc4</em> double mutant (ecotype Col)</td>
<td>9.21±0.89%$^b$</td>
</tr>
<tr>
<td><em>arf1</em> single mutant (ecotype Col)</td>
<td>23.26±1.91%$^a$</td>
</tr>
<tr>
<td>Wild Type (ecotype Ws)</td>
<td>88.26±2.13%$^a$</td>
</tr>
<tr>
<td><em>arf3/ett-1</em> single mutant (ecotype Ws)</td>
<td>7.90±1.14%$^b$</td>
</tr>
<tr>
<td><em>arf3/ett-2</em> single mutant (ecotype Ws)</td>
<td>35.58±2.07%$^b$</td>
</tr>
<tr>
<td>antisense PIN1 cDNA (ecotype Ws)</td>
<td>7.63±1.46%$^b$</td>
</tr>
<tr>
<td><em>atipt5</em> single mutant (ecotype Ws)</td>
<td>79.86±1.14%$^a$</td>
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<tr>
<td>Wild Type (ecotype Col×Ws)</td>
<td>80.86±1.34%$^a$</td>
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<tr>
<td><em>atipt5,7</em> double mutant (ecotype Col×Ws)</td>
<td>43.34±2.02%$^b$</td>
</tr>
<tr>
<td><em>atipt3,5,7</em> triple mutant (ecotype Col×Ws)</td>
<td>6.44±1.30%$^b$</td>
</tr>
</tbody>
</table>

$^a$Not significantly different from wild type (Student’s t test, P > 0.05).

$^b$Significantly different from wild type (Student’s t test, P < 0.01).

$^c$For the measurement shoot regeneration frequencies from wild type and mutant calli, calli cultured on SIM for 14 days were used. Data are the mean values from three sets of biological replicates. In each replicate, at least 100 calli were examined.
Figure 1. Regional establishment of auxin responses relative to WUS expression.

(A-C) DR5rev::GFP signals (green) in the edge region of the non-induced callus
(SIM for 0 day; 94.3%, n=122). (D-F) Regional distribution of DR5rev::GFP signals in the calli grown on SIM for 2 days (83.3%, n=108). (G-J) Auxin distribution indicated by the DR5rev::GFP signals and WUS expression indicated by the pWUS::DsRed-N7 signal (magenta) in calli grown on SIM for 4 days (81.5%, n=127). (K-N) Traverse view of the callus grown on SIM for 4 days (80.9% n=110). (O-R) DR5rev::GFP and WUS signals accumulate in the pro-meristem of calli grown on SIM for 6 days (80.0%, n=100). (S-U) Auxin distribution and WUS expression in the calli grown on NPA-containing SIM for 4 days (86.8%, n=152). Bright-field (B, E, I, and T) and merged images of GFP (green) and DsRed (magenta) channels (J, N, and R) are shown. The Chlorophyll autofluorescence are in blue (M, N, Q and R). Bars=100 µm.
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(A) qRT-PCR analysis of YUC expression level in non-induced calli and in calli grown on SIM for 4 days. (B) pYUC1::GUS signals in non-induced calli (91.7%, n=121), and in calli grown on SIM for 4 days (84.0%, n=156) or 6 days (81.5%, n=162). (C) pYUC4::GUS signals in the non-induced callus (85.9%, n=198), and in the calli grown on SIM for 4 days (85.6%, n=167) or 6 days (83.3%, n=150). (D-K) Localization of pYUC4::GFP (green) and pWUS::DsRed-N7 (magenta) signals in the non-induced callus (D, 90.2%, n=122), and in the calli grown on SIM for 4 days (E-G, 84.4%, n=166) or 6 days (H-K, 80.3%, n=152). (D-K) are longitudinal sections of calli. Bright-field (D and F) and merged images of GFP (green) and DsRed (magenta) channels (G and K) are shown. The Chlorophyll autofluorescence are in blue (J and K). Bars=1 mm (B, C), 100 µm (D, G and K).
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(A) Expression levels of *AtIPTs* in the non-induced calli, and in calli grown on SIM for 4 days determined by qRT-PCR. (B) *pAtIPT5::GUS* signals in the non-induced callus (85.9%, n=199). (C-E) Regional distribution of *pAtIPT5::GUS* signals in calli grown on SIM for 2 days (C, 90.5%, n=199), 4 days (D, 89.5%, n=190) or 6 days (E, 87.9%, n=182). (F-H) *AtIPT5* expression patterns in the non-induced callus (F, 90.9%, n=209), and in calli grown on SIM for 4 days (G, 89.5%, n=190) or 6 days (H, 87.9%, n=182). (I-K) *pAtIPT5::GUS* signals distributed uniformly at the edge region of the calli grown on SIM for 0 days (I, 85.2% n=88), 4 days (J, 83.3% n=66) and 6 days (K,
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(A-E) In situ hybridization analyses showing the spatial expression of ARF3 in the non-induced callus (A, 87.5% n=112), or in calli grown on SIM for 2 days (B, 80.5%, n=123), 4 days (C, 75.4%, n=130), 6 days (D, 72.7%, n=110) or 8 days (E, 73.2%, n=123). (F) Regenerated shoots from wild-type calli grown on SIM for 18 days (89.9%, n=99). (G) The arf3/ett2 mutant callus grown on SIM for 18 days showing no shoot regeneration. (H) The arf3/ett2 mutant callus grown on SIM for 18 days with a few regenerated shoots (34.5% n=200). SAM shoot apical meristem, CL cauline leaf.
Arrows indicate regenerated shoots and arrowheads indicate \( ARF3 \) signals. (A-E) are longitudinal sections of calli. Bars=100 µm (A-C), 50 µm (D-E) and 1 mm (F-H).
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(A-B) *pAtIPT5::GUS* signals in the wild-type callus induced on SIM for 0 day (A, 89.5%, *n*=190) or 4 days (B, 85.9%, *n*=199). (C-D) GUS signals in the calli of the *arf3* mutant grown on SIM for 0 days (C, 82.7%, *n*=168) or 4 days (D, 80.4%, *n*=148). (E) Schematic illustration of the *AtIPT5* promoter. TGTCTC and TGTCNN on both the sense and antisense strand are indicated by blue and green bars, respectively; red asterisks denote point mutations (TGTCTC-TGGC). (F-G) *pAtIPT5m::GUS* signals in the non-induced callus (F, 88.8% *n*=179) or in the calli grown on SIM for 4 days (G, 85.4%, *n*=198). (A-D; F-G) are longitudinal sections of calli. Bars=80 µm.
Figure 7. *AtIPT* expression is enhanced in the *arf3* mutant.

(A-C) qRT-PCR analyses showed that the expression of *AtIPT3*, *AtIPT5* and *AtIPT7* were enhanced in *arf3* mutant. *AtIPT5* is the most significantly enhanced under these conditions.
Figure 8. ARF3 directly binds to the promoter of *AtIPT5*. (A) Yeast One-Hybrid analysis revealing the direct interaction between ARF3 and the *AtIPT5* promoter. Yeast strains containing the PAtIPT5 promoter-AbAi or PAtIPT5m promoter-AbAi constructs were grown on media under selective (SD/-Leu, +100 ng/mL Aureobasidin A or AbA) or non-selective (SD/-Leu, -AbA) conditions. Full-length ARF3 cDNAs fused to pGADT7 AD are indicated on the plates, and the empty pGADT7 AD vector was used as a negative control. The p53-AbAi vector was used as a positive control in the kit (Clontech Laboratories, Catalog #630491). (B) Interaction between ARF3 and the *AtIPT5* promoter (red line) was determined by SPR analysis. The *AtIPT5m* promoter was introduced as a negative control (blue line). (C) EMSA analysis showing the interaction between ARF3 and the *AtIPT5* promoter. The
retarded DNA-protein complex was competed using either wild-type probe or the mutated probes at a 5×, 25× and 50× molar excess. (D) The AuxREs TGTCTC and TGTCNN on both sense and antisense strands of the AtIPT5 promoter are indicated as blue or green bars, respectively. Red lines indicate fragments amplified in (E). Fragment “a” (-356 to +39) includes the sequence used in the EMSA experiments (-155 to -130), and fragment “b” (-1133 to -850) was used as a negative control. (E) Enrichment of specific regions of the AtIPT5 promoter (fragments “a” and “b”) using anti-GUS, anti-GFP, and anti-MYC antibodies in pARF3::ARF3tasiR-GUS, pMP (ARF5)::MP-GFP, and 35S::6myc-ARF8 transgenic plants, respectively. Mouse IgG was used as a mock control. The fold enrichments of specific regions (fragments “a” and “b”) were detected by qRT-PCR analysis after normalization to the unrelated UBQ10 control sequence. Means were calculated from three biological replicates, and each biological sample was examined using three PCR technical replicates.