Type-B ARABIDOPSIS RESPONSE REGULATORs Specify the Shoot Stem Cell Niche by Dual Regulation of WUSCHEL

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Plants are known for their capacity to regenerate the whole body through de novo formation of apical meristems from a mass of proliferating cells named callus. Exogenous cytokinin and auxin determine cell fate for the establishment of the stem cell niche, which is the vital step of shoot regeneration, but the underlying mechanisms remain unclear. Here, we show that type-B ARABIDOPSIS RESPONSE REGULATORs (ARRs), critical components of cytokinin signaling, activate the transcription of WUSCHEL (WUS), which encodes a key regulator for maintaining stem cells. In parallel, type-B ARRs inhibit auxin accumulation by repressing the expression of YUCCAs, which encode a key enzyme for auxin biosynthesis, indirectly promoting WUS induction. Both pathways are essential for de novo regeneration of the shoot stem cell niche. In addition, the dual regulation of type-B ARRs on WUS transcription is required for the maintenance of the shoot apical meristem in planta. Thus, our results reveal a long-standing missing link between cytokinin signaling and WUS regulator, and the findings provide critical information for understanding cell fate specification.

INTRODUCTION

Unlike their animal counterparts, the postembryonic development of higher plants depends on the activity of apical meristems residing at each end of the body (Fletcher and Meyerowitz, 2000; Sena et al., 2009; Moreno-Risueno et al., 2015). In the shoot apical meristem (SAM), pluripotent stem cells reside in a specialized microenvironment termed the stem cell niche, which gives rise to the aerial part of the plant (Aichinger et al., 2012; Zhou et al., 2015). The activity of the shoot stem cell niche is tightly controlled by a feedback loop between the homeodomain transcription factor WUSCHEL (WUS) and the small secreted peptide CLAVATA3 (CLV3) in Arabidopsis thaliana (Brand et al., 2000; Schoof et al., 2000). The expression of CLV3 specifically marks the position of stem cells in the central zone (CZ) (Laux, 2003). WUS expression precedes that of CLV3 during embryogenesis and defines the organizing center (OC) beneath the CZ (Aichinger et al., 2012; Galloch et al., 2015). Once produced in the OC cells, WUS proteins move to the CZ to activate CLV3 expression and stem cell specification (Yadav et al., 2011; Daum et al., 2014). Mutation in WUS leads to differentiation of stem cells and loss of the shoot meristem, indicating that this gene is necessary for establishing and maintaining the stem cell niche (Mayer et al., 1998; Aichinger et al., 2012).

Besides the primary shoot meristem, plants are capable of regenerating shoot meristems during their postembryonic development, such as axillary and adventitious shoot meristems (Kerstetter and Hake, 1997; Domagalska and Leyser, 2011; Wang et al., 2014). Six decades ago, Skoog and Miller found that under the induction of exogenous cytokinin and auxin, adventitious shoots could be induced in vitro from a mass of proliferating cells named callus (Skoog and Miller, 1957; Sugimoto et al., 2010; Iwase et al., 2011). This process is defined as de novo shoot regeneration and provides an ideal system for studying the specification of stem cell niche in plants (Duclercq et al., 2011; Iwase et al., 2017). During shoot regeneration, WUS expression promotes cell fate transition from callus cells to OC, which is essential for the specification of the shoot stem cell niche and the subsequent establishment of the shoot meristem (Duclercq et al., 2011; Aichinger et al., 2012; Ikeuchi et al., 2016).

Previous studies have revealed that cytokinin and auxin play critical roles in shoot regeneration (Ikeuchi et al., 2016). Incubation on medium containing a high cytokinin-to-auxin ratio activates the expression of WUS in callus and lateral root primordia and induces the formation of the shoot meristem (Gordon et al., 2007; Chatfield et al., 2013). The expression of Arabidopsis His kinases, which encodes a cytokinin receptor, precedes and subsequently overlaps with that of WUS (Gordon et al., 2009). Mutations in type-B ARABIDOPSIS RESPONSE REGULATORs (ARRs), key regulators of primary cytokinin response genes, result in reduced shoot regeneration (Ishida et al., 2008; Hwang et al., 2012; Hill et al., 2013; Zhang et al., 2015). By contrast, overexpressing type-A ARRs encoding negative regulators of cytokinin signaling suppress shoot formation (Buechel et al., 2010). Moreover, spatial biosynthesis, polar transport, and signaling transduction of auxin are required for shoot regeneration (Gordon et al., 2007; Kareem et al., 2015). Our previous results demonstrated a pattern of auxin and cytokinin essential for shoot meristem induction (Cheng et al., 2013). Cytokinrin response signals were progressively restricted to
the region of future WUS expression due to the spatiotemporal repression of cytokinin biosynthetic genes ISOPENTENYLTRANSFERASE (in Arabidopsis, AtIPTs) by AUXIN RESPONSE FACTOR3 (ARF3). Even though the importance of cytokinin and auxin in shoot regeneration is well known, the underlying molecular mechanisms remain to be elucidated. Particularly, the hormonal regulation of WUS expression is largely unknown.

In this study, we show that cytokinin signaling components type-B ARRs directly activate WUS transcription and repress the expression of auxin biosynthetic genes YUCCAs (YUCs), which in turn indirectly promotes WUS induction. Thus, the dual roles of type-B ARRs on WUS transcription are critical for activating the stem cell program during regeneration. The results of this study provide critical information for understanding the mechanism of cytokinin-regulated shoot regeneration.

RESULTS

ARR1, ARR10, and ARR12 Display Dynamic Expression Patterns during Shoot Regeneration

An Arabidopsis shoot induction system described previously was adopted for the analysis of shoot regeneration in this study (Buechel et al., 2010). First, root explants were pretreated in an auxin-rich callus induction medium (CIM) to generate callus. The callus was then transferred onto a cytokinin-rich shoot induction medium (SIM) to allow shoot induction. It has been shown that ARR1, ARR10, and ARR12 play primary roles in transducing cytokinin signaling (Sakai et al., 2001; Ishida et al., 2008; Hill et al., 2013). Therefore, to investigate how cytokinin regulates WUS expression and shoot regeneration, we first traced the spatiotemporal expression patterns of the three ARR genes using translational reporter lines. ARR signals were hardly detectable at the beginning of SIM incubation. The signals were induced at 2 d on SIM (SIM2) and were enhanced throughout the explant at SIM4. At SIM6, ARR signals were restricted to discrete regions, wherein WUS signals became detectable in a few cells. At SIM8, ARR signals were restricted to the callus protuberance, colocalizing with that of WUS, which marks the regeneration of the stem cell niche. When the shoot meristems were established at SIM12, the signals were located in the central region of the meristem, beneath several layers of the outermost cells and overlapped with that of WUS (Figure 1A). The expression pattern of ARR1 was further confirmed using the transcriptional reporter line and in situ hybridization (Supplemental Figure 1). These results suggest that ARR1, ARR10, and ARR12 function locally in shoot regeneration.

Reestablishment of Shoot Meristem Requires the Function of ARR1, ARR10, and ARR12

Previous studies showed that shoot regeneration capacity was impaired in the arr1 single mutant, as well as its double and triple mutants with arr10 and arr12, indicating that type-B ARRs, which are key regulators in cytokinin signaling, are required for shoot regeneration in Arabidopsis (Ishida et al., 2008; Hill et al., 2013). To determine the roles of ARR in the regeneration of shoots in callus on SIM, the transcription of ARR1, ARR10, and ARR12 needs to be temporally repressed. For this purpose, we used artificial microRNAs (am) driven by an ethanol-inducible promoter to silence the transcripts of ARR1, ARR10, ARR11/12, or ARR1/12 (am-ARR1, am-ARR10, am-ARR11/12, and am-ARR1/12, respectively) (Leibfried et al., 2005; Zhao et al., 2010). The efficiency and specification of these artificial microRNAs were tested by real-time PCR. The results demonstrate that within 12 h of ethanol induction, the transcript levels of the corresponding target ARRs in different transgenic lines were significantly reduced, whereas those of other type-B ARRs, including ARR2, ARR11, ARR13, ARR18, and ARR21 as controls, were not obviously affected (Supplemental Figure 2).

In the wild type, the regenerated shoots in callus were identified at SIM12, and the frequency peaked at SIM22. Under ethanol treatment, regenerated wild-type shoots were observed at SIM14 and reached the peak frequency of regeneration at SIM24 (86.61%) (Supplemental Figure 3), indicating that ethanol has little effect on shoot induction. By contrast, shoot regeneration capacity in the callus expressing artificial microRNAs of ARRs was obviously impaired. Although the am-ARR1 lines showed a slightly reduced regeneration frequency, the am-ARR1/10, am-ARR1/12, or am-ARR10/12 lines gave rise to roots, and shoot regeneration was significantly inhibited. The am-ARR1/10/12 lines showed a more severe phenotype (Supplemental Figure 3).

To further dissect the function of ARR1, ARR10, and ARR12, we silenced their transcription at different stages during shoot formation. As a result, ethanol induction started before restricted WUS expression at SIM4 largely abolished shoot regeneration (frequency of shoot regeneration, 33.18%); instead, roots were regenerated after prolonged incubation (Figures 1B and 1C). However, when ethanol induction was started after the specification of shoot stem cell niche at SIM8 or the establishment of the shoot meristem at SIM12, the percentages of shoot regeneration at SIM8 (77.24%) and SIM12 (81.84%) were similar to those of the nontreated control (85.98%) (Figures 1B and 1C). Once the shoot meristem is determined, low levels of ARR1, ARR10, and ARR12 transcripts are enough to maintain shoot development.

ARR1, ARR10, and ARR12 Control Shoot Regeneration through Regulating WUS Expression

Overlapping expression of ARRs and WUS prompted us to test whether ARRs regulate WUS transcription. We first detected the transcript levels of WUS during shoot regeneration using RT-qPCR and found an obvious reduction in the arr1 10, arr1 12, and arr10 12 double mutants (Figure 2A). We further visualized the expression pattern of WUS using gWUS-GFP reporter lines (Tucker et al., 2008). In control lines, under ethanol treatment, GFP signals were detected and restricted to the callus protuberance in 27.50% callus at SIM10. At SIM14, the signals were observed in the OC of regenerated shoot meristem at a frequency of 31.77% (Figure 2B). However, in the am-ARR1/10/12 transgenic lines, gWUS-GFP signals diminished and shoot meristem formation was abolished in some examined callus. At SIM10 and SIM14, GFP signals were detected in 13.75 and 17.44% of the callus, respectively, but the signals were largely reduced compared with those in control lines. These results indicate that ARR1, ARR10, and ARR12 positively regulate the transcription of WUS (Figure 2B).
To confirm the regulatory roles of ARRs on WUS during shoot regeneration, we performed a 24-h ethanol induction at days 0, 4, 8, and 12 of SIM incubation, respectively, and visualized the expression pattern of gWUS-GFP3 signals. The results show that restricted WUS expression was visible at SIM8 and SIM12 in the nontreated wild type, ethanol-treated wild type, and nontreated am-ARR1/10/12 line (Figure 3); however, silencing of ARR1, ARR10, and ARR12 obviously reduced WUS expression signals (Figure 3). Additionally, RT-qPCR analysis confirmed that a 24-h ethanol treatment significantly reduced the transcript levels of WUS at SIM8 and SIM12 in the am-ARR1/10/12 lines, respectively (Figure 2C).

We next examined whether the regulation of ARRs on WUS is involved in shoot regeneration. For this purpose, we overexpressed WUS in the arr1 12 double mutant and examined the regenerative capacity. All of the examined Pro35S:WUS arr1 12 lines reached 100% of shoot regeneration, indicating that WUS expression is sufficient to rescue the shoot regeneration defects caused by ARR mutations (Figure 4). These results together demonstrate that ARR1, ARR10, and ARR12 are involved in shoot regeneration through regulating WUS expression.

ARR1, ARR10, and ARR12 Directly Activate WUS Transcription

To test whether the activation of ARRs on WUS transcription is direct, we performed chromatin immunoprecipitation (ChIP) analyses. As a result, three fragments (WUS-2, WUS-3, and WUS-4) containing B-type ARR binding element sites in the WUS promoter were strongly enriched at SIM4, SIM8, and SIM12 (Figure 5A). The direct binding of ARRs to the ChIP-positive fragments was examined by electrophoretic mobility shift assays (EMSAs). Two oligonucleotides designated as “Probe a” (–414 to –473 bp upstream of the ATG start codon) and “Probe b” (–559 to –611 bp upstream of the ATG start codon) were biotin labeled. All three ARRs produced clear band shifts with both of these probes (Figure 5B). Moreover, the addition of excess unlabeled competitor probes effectively reduced the amount of shifted bands, indicating that ARR proteins bind specifically to the tested probes. Direct binding was also confirmed by yeast one-hybrid analyses (Figure 5C).

Moreover, the effects of ARR1, ARR10, or ARR12 on the expression of LUC driven by WUS promoter were examined in a protoplast transient expression system. The results showed that coexpression of ARR1, ARR10, or ARR12 significantly enhanced ProWUS:LUC activity (Figure 5D). The activation of WUS transcription by ARR1, ARR10, and ARR12 was further confirmed using a transient expression assay in tobacco (Nicotiana benthamiana) leaves (Supplemental Figure 4). These results indicate that ARR1, ARR10, and ARR12 activate WUS transcription by directly binding to its promoter region.

We next determined whether the ARR binding elements on the WUS promoter contribute to transcriptional regulation during shoot regeneration. For this purpose, we specifically disrupted ARR binding elements within the ChIP-positive fragments (Figure 5A) and examined the transcriptional activation of ARR through a transient expression assay in tobacco leaves. We generated ProWUSm1 by mutating the two most important base pairs
(GATC/T to CTTC/T) in all of the ARR binding elements of WUS-2, WUS-3, and WUS-4 (Sakai et al., 2000). As a result, the transcriptional activation was significantly reduced (Supplemental Figures 5A and 5B). WUS-3 (2706 to 2396 bp upstream of the ATG start codon) largely overlapped with the previously identified region (2726 to 2541 bp upstream of the ATG start codon), which is necessary for the proper expression of WUS (Bäurle and Laux, 2005) (Supplemental Figures 5A and 5B). We thus generated the ProWUSm2 promoter by mutating the 2 bp of ARR binding elements in WUS-3, which demonstrated a similar result with that of ProWUSm1 (Supplemental Figures 5A and 5B). The results indicate that ARR binding elements in WUS-3 played primary roles in ARR-mediated WUS transcription.

We then asked whether a 1-bp mutation is sufficient to disrupt ARR-mediated activation. To confirm this, we generated ProWUSm1 (GATC/T to CATC/T) and ProWUSm2 (GATC/T to GTTC/T) by mutating 1 bp of ARR binding elements in WUS-3. The transcriptional activation by ARR was partially repressed (Supplemental Figures 5A and 5B).

The ProWUSm2 promoter was then used to drive a GFP reporter, and the signals were examined at different stages of SIM incubation. Compared with those of ProWUS:GFP, and in situ hybridization, ProWUSm2:GFP signals were obviously reduced during the SIM incubation (Supplemental Figures 5C and 5D). These results indicate that the ARR binding elements are required for proper expression of WUS during SAM formation.

The Integrated Functions of ARRs Confer the Proper Expression Patterns of YUC1 and YUC4

Our previous work showed that during shoot regeneration, auxin response signals could not be detected in the WUS-expressing region, where cytokinin responses are strong (Cheng et al., 2013). We thus speculated that auxin accumulation was repressed by cytokinin signaling in this region. To test this hypothesis, we examined whether ARRs regulate the expression of YUC genes, which encode key enzymes for auxin biosynthesis. Because our previous results showed that YUC1 and YUC4 play essential roles in shoot regeneration, we detected the expression patterns of both of these genes (Cheng et al., 2013). Indeed, the transcript levels of YUC1 and YUC4, but not YUC2 and YUC6, were obviously increased in arr1 10, arr1 12, and arr10 12 double mutants compared with those in their wild-type counterparts (Figure 6A). Therefore, ARR1, ARR10, and ARR12 negatively regulate YUC1 and YUC4 transcription.

To further dissect the regulation of ARRs on YUC genes, we examined the expression patterns of YUC4 and ARR10 using double reporter lines during shoot regeneration (Figure 6B). At the
early stages of SIM incubation (SIM2 and SIM4), the distribution patterns of YUC4 were similar to those of ARR10, although its signals were weaker than those of ARR10. When ARR10 signals were regionalized, YUC4 signals were substantially decreased in the ARR-expressing regions at SIM6 and were further reduced at SIM8. At SIM12, YUC4 signals were undetectable in the center of the shoot meristem where ARR10 was expressed.

We further compared the spatiotemporal expression patterns of a ProYUC4::YUC4-GFP reporter in the wild type and arr10 12 double mutant explants. In the wild-type explants, GFP signals were progressively confined to a pattern apical and peripheral to the WUS-accumulating region, which was initiated in the region with weak GFP signals at SIM6. When a shoot meristem was formed, YUC4 signals were switched to the region apical to the WUS-expressing area, as previously described (Figure 6C) (Cheng et al., 2013). However, much stronger signals in the arr10 12 double mutant were observed at 0 and 4 d on SIM than in the wild type (Figure 6C). At SIM8, the expression pattern of YUC4 was abolished, and WUS expression was not detected. Instead, the GFP signals were still evenly distributed in the explants and accumulated in the root apical meristem (Figure 6C). At SIM12, strong GFP signals accumulated in the root meristem (Figure 6C). The expression patterns of YUC4 in the arr10 12 double mutant were further confirmed by ProYUC4::GUS expression analysis (Supplemental Figure 6A). Expression of YUC1 in the arr10 12 and arr12 12 double mutants also showed similar patterns to those of YUC4 in the arr10 12 double mutant (Supplemental Figure 6B). Finally, we examined the auxin response in the arr10 12 double mutant using ProDR5::GFP. As expected, GFP signals in the arr10 12 double mutant transgenically expressing ProDR5::GFP exhibited patterns similar to those of YUC1 and YUC4 (Figure 6C). Thus, the results suggest that ARR1, ARR10, and ARR12 repress the expression of YUC1 and YUC4 in the region destined for OC specification, thus restricting the expression of YUC1 and YUC4 to the surrounding region.

**Spatiotemporal Expression of YUC1 and YUC4 Mediated by Type-B ARRs Is Required for Shoot Regeneration**

To further test whether the spatiotemporal regulation of YUC expression is required for shoot regeneration, we analyzed the shoot regeneration capacity in the dominant gain-of-function yuc1 mutant (yuc1D) and the YUC4-overexpressing transgenic lines (YUC4ox) (Zhao et al., 2001; Cheng et al., 2006). The yuc1D and YUC4ox mutants exhibited phenotypes similar to those of the double mutants of arr1, arr10, and arr12, i.e., attenuated shoot regeneration and enhanced adventitious root formation (Figures 7A and 7B). Consistently, the transcript levels of WUS were markedly reduced in transgenic lines overexpressing YUC1 or YUC4 (Supplemental Figure 7A). Furthermore, we detected auxin responses of YUC4ox using a ProDR5::GFP reporter. The GFP signals were spread into the whole explant, suggesting that the locally synthesized auxin functions in a cell-autonomous manner (Supplemental Figure 8).

We examined the shoot regeneration capacity of the yuc1 4 double mutant, and the results showed that explants of the yuc1 4 double mutant gave rise to filiform structures and reduced shoots (Supplemental Figures 9A and 9B). The arr10 12 yuc1 4 quadruple mutant exhibited similar phenotypes to that of the yuc1 4 double mutant (Supplemental Figures 9A and 9B). Comparison of the transcript levels of ARR10 and ARR12 in the wild type and
those in yuc1D and YUC4ox using RT-qPCR did not reveal any obvious differences (Supplemental Figure 9C). These results suggest that YUCs act downstream of the type-B ARRs.

Next, we investigated whether suppression of YUCs in the expressing domains of type-B ARRs is essential for shoot regeneration. We generated transgenic lines expressing YUC4 driven...
by the promoter of ARR10 and examined their capacity for shoot regeneration. Compared with those of the wild-type explants, the frequencies of shoot regeneration in ProARR10:YUC4 lines were obviously decreased (Figures 7A and 7C), indicating that repression of YUC4 in the type-B ARR expression domain is essential for shoot regeneration. We further determined whether repressing YUC expression in the WUS-expressing region is critical for shoot regeneration. To this end, we introduced transgenic lines expressing YUC4 under the WUS promoter. Shoot regeneration in ProWUS:YUC4 lines was severely reduced, demonstrating the importance of repressing YUC expression in the de novo formation of OC (Figures 7A and 7C). The results together indicate that spatial localization of YUC is important for shoot meristem formation.

To test whether YUC1 and YUC4 expression is directly regulated by ARRs, we performed ChIP assays. The results showed that ARR1, ARR10, and ARR12 associate with the YUC4-1 and YUC4-2 fragments of the YUC4 promoter, while ARR1 also associates with the YUC1-5 region of the YUC1 promoter in callus tissue at SIM4, SIM8, and SIM12 (Figure 8A; Supplemental Figures 7B and 7C). EMSA and yeast one-hybrid analyses revealed direct binding of the ARRs to the fragments of the YUC4 promoter (Figures 8B and 8C). Furthermore, we generated a construct containing the GFP gene driven by the mutated YUC4 promoter (ProYUC4m) within which 2 bp of the binding elements by ARR1, ARR10, and ARR12 were mutated (GATC/T to CTTC/T). The ProYUC4m:GFP signal in the wild-type background showed similar patterns to those of ProYUC4:GFP in the arr10 12 double mutant (Figure 8D). Together, these results indicate that ARR1, ARR10, and ARR12 redundantly occupy YUC promoters and suppress their expression and thus prevent the auxin-mediated repression of WUS transcription.

Finally, we analyzed whether direct binding of ARRs on YUC promoters mediated shoot regeneration. For this purpose, we expressed YUC4 under the mutated YUC4 promoter (ProYUC4m) and examined the regeneration capacity of these transgenic lines. As a result, misexpression of YUC4 by these promoters led to an obvious decrease in shoot regeneration percentage (Supplemental Figure 10). The results indicate that the binding elements of ARRs on YUC promoters are critical for shoot regeneration.

ARRs Regulate SAM Maintenance through Regulating WUS Transcription in Planta

To validate the above-described regulatory relationship between WUS and these hormone-related genes in planta, we performed experiments to visualize their expression patterns in the SAM. We first visualized the distribution patterns of ARR1, ARR10, and ARR12 in the SAM and found their signals to be located in the central region of the SAM, overlapping with the OC marked by WUS signals (Supplemental Figure 11A). WUS expression was then detected in the arr10 12 mutant and the am-ARR1/10/12 lines using the gWUS-GFPp reporter. GFP signals were obviously reduced in the arr10 12 mutant compared with those in the wild type (Figures 9A and 9C). In the am-ARR1/10/12 lines,
ethanol treatment for 24 h significantly downregulated WUS expression signals compared with the control lines (Figures 9B and 9C). ChIP assays revealed that ARR1, ARR10, and ARR12 associate with the WUS promoter, respectively (Figure 9D). Thus, three ARRs are required for maintaining WUS expression in the OC through their direct activation of WUS transcription.

We next investigated the effect of ARR1, ARR10, and ARR12 on the expression of YUC4 by visualizing ProYUC4:YUC4-GFP and ProWUS:dsRED reporters (Figure 10A). Signals derived from ProYUC4:YUC4-GFP were located in the L1 cell layer of the wild-type SAM, but expanded to the central region of the SAM in both the arr10 12 and arr1 10 12 mutants. Also, the signals were much stronger in the arr1 10 12 triple mutant than in the arr1 10 12 double mutant (Figure 10A). By contrast, the WUS-expressing region was decreased in the arr10 12 double mutant and was even less in the arr1 10 12 triple mutant, compared with those in the wild type (Figure 10A). Furthermore, ChIP analysis revealed a direct regulation of ARR1, ARR10, and ARR12 on YUC4 expression (Supplemental Figures 11B and 11C), suggesting that the three ARRs repress YUC expression and, thus, auxin accumulation in the OC, which indirectly promotes WUS transcription therein.

We questioned whether the regulation of WUS by the ARRs exerts roles in SAM maintenance. The SAM of the arr1 10 12 triple mutant, yuc1D, YUC4ox, and ProARR10:YUC4 lines was examined. Consistent with the reduced transcript levels of WUS, the SAM size of these lines was decreased compared with that of the wild type (Figures 10B and 10C; Supplemental Figure 12). The arr1 10 12 triple mutant exhibited the greatest magnitude of reduction (Figures 10B and 10C). Further analyses demonstrated that the decrease in SAM size resulted from a reduced cell number (Figure 10C). We thus suggest that defects in ARR1, ARR10, and ARR12 cannot activate WUS transcription. Furthermore, these defects caused ectopic expression of YUC1 or YUC4 in the OC, which might lead to auxin accumulation and subsequent WUS suppression. This fits well with the recent finding that WUS acts as a positive regulator of cell division, and inducible downregulation of WUS expression resulted in a progressive decrease in SAM size (Yadav et al., 2010). Thus, these results suggest that ARR1, ARR10, and ARR12 mediate SAM maintenance through their regulation of WUS transcription.

**DISCUSSION**

**ARR1-, ARR10-, and ARR12-Regulated WUS Expression Plays Critical Roles in Shoot Regeneration**

Double and triple mutants of ARR1, ARR10, and ARR12 could not generate shoots under in vitro culture owing to cytokinin insensitivity (Hill et al., 2013; Ishida et al., 2008). Here, we used ethanol-induced artificial microRNAs to silence the transcription of ARR1, ARR10, and ARR12 at different stages of shoot formation. As a result, the capacity of shoot regeneration was largely reduced in these lines, confirming that these ARRs control shoot regeneration (Figure 1B; Supplemental Figure 3).

A previous study demonstrated that shoot regeneration capacity was largely reduced in the wus-1 mutant (Gordon et al., 2007). To confirm the role of WUS in shoot regeneration, we transferred the WUS gene driven by Pro35S promoter to the arr1 12 mutant and found that overexpressing WUS is sufficient to rescue the shoot regeneration phenotype of the arr1 12 mutant (Figure 4), suggesting that WUS functions in downstream of cytokinin signaling and plays a critical role in shoot regeneration. Since WUS expression is regulated by ARR1, ARR10, and ARR12, we propose that these ARRs control shoot regeneration through regulation of WUS expression.

Cytokinin has been shown to regulate the cell cycle in both cell culture and in planta (Riou-Khamlichi et al., 1999), and it might be involved in shoot formation by controlling cell proliferation. Here, our results indicate that inducible silencing of ARR1, ARR10, and ARR12 significantly reduced shoot regeneration, although callus could be observed (Figure 1B). Overexpressing WUS rescued the defects of the arr mutants (Figure 4). Thus, ARR-mediated WUS expression plays a critical role in shoot regeneration, which may be independent of cell proliferation of callus.

In addition, our results indicated that WUS expression was firstly induced in a few callus cells and then marked the regeneration of the OC. However, this expression pattern is different from previous findings, which showed that WUS is broadly expressed across the callus at the early stage of SIM incubation, but restricted to the center of the shoot meristem later (Gordon et al., 2007; Kareem et al., 2015). To confirm this result, we performed in situ hybridization and found that the two reporters we used mimicked the expression pattern of WUS endogenous mRNA (Figure 1A; Supplemental Figures 5C and 5D).

**ARR-Mediated WUS Transcription Requires RE1- or RE2-Specific Factors**

Cytokinin signaling has been suggested to be a positional cue for WUS expression (Gordon et al., 2009). However, the regulatory pathways between cytokinin receptors and WUS remain to be elucidated. Our results revealed a shortcut, namely, that ARR1, ARR10, and ARR12 could directly bind to the WUS promoter and activate its transcription (Figure 5). A previous study analyzed the
regulatory roles of different regions in the WUS promoter and revealed that the sequences between −726 and −541 bp upstream of the start codon (−600 to −415 bp upstream of the putative transcription start site) are necessary for WUS expression in the stem cell niche of the inflorescence meristem (Bäurle and Laux, 2005). Here, we identified a ChIP-positive fragment (WUS-3,
−706 to −396 bp upstream of the ATG start codon) in the WUS promoter that largely overlapped with this necessary sequence (Figure 5A). Mutating the ARR binding elements within WUS-3 significantly reduced the transcriptional activation by ARR (Supplemental Figures 5A and 5B), indicating the important roles of this fragment in recruiting ARR proteins for transcriptional regulation.

Furthermore, tetrameric tandem repeats of a 57-bp regulatory region (−2586 to −2529 bp upstream of the putative transcription start site) were shown to be sufficient for providing the correct spatial WUS expression pattern in the stem cell niche, and this activity depends on two adjacent short motifs, RE1 and RE2 (Bäurle and Laux, 2005). According to the model envisioned by Bäurle and Laux, we propose a regulatory mechanism for ARR-mediated WUS transcription. In the OC or competent callus cells, ARR1, ARR10, or ARR12 protein is recruited by ARR binding elements within the WUS-3 fragment. Other elements located in WUS-2 and WUS-4 are also involved in the interaction between ARR protein and WUS promoter, but play redundant and minor roles. This is supported by the result that mutating ARR binding elements in WUS-3 or in all three ChIP-positive fragments reduced ARR-mediated activation to similar extents (Supplemental Figures 5A and 5B). Once ARR binds the promoter, it activates WUS transcription by interacting with the previously proposed RE1- or RE2-specific transcription factors. This hypothesis is supported by the fact that ARRs could interact with other proteins via the transactivation domain (Zhang et al., 2015).

ARR1, ARR10, and ARR12 Are Bifunctional Transcription Factors and Play Dual Roles in Regulating WUS Expression

Type-B ARRs have been demonstrated to be transcriptional activators, which directly bind to the promoter region of target genes, such as type-A ARRs, and positively regulate their expression (Hwang et al., 2012). Recent evidence revealed that ARR1 also directly regulates genes whose transcription was repressed by cytokinin (Zhang et al., 2013). Our data show that ARR1, ARR10, and ARR12 exhibit dual functions in transcription regulation and activate the transcription of WUS and repress that of YUCs within the same cell niche. WUS has previously been shown to have a similar dual function (Ikeda et al., 2009). It is possible that different partner proteins interacting with ARRs confer their functions as either transcriptional activators or repressors. Identifying and analyzing these partner proteins will facilitate our understanding of transcription factors with dual roles.

The de novo establishment of shoot meristem depends on the initiation and maintenance of WUS transcription. After the

Figure 9. ARR1, ARR10, and ARR12 Activate WUS Transcription in the SAM.

(A) Expression of gWUS-GFP in the arr1 10 12 mutant. Bars = 50 μm.  
(B) gWUS-GFP signals were significantly downregulated in the am-ARR1/10/12 lines at 24 h after ethanol treatment. Bars = 50 μm.  
(C) The intensity of fluorescence in the arr1 10 12 mutant (upper panel) and am-ARR1/10/12 transgenic lines (lower panel) was measured using ImageJ software. Asterisks denote significant difference compared with wild-type or mock control, as determined by a Student’s t test, with two asterisks denoting P < 0.01.  
(D) ChIP analysis showed that ARR1, ARR10, and ARR12 bind the WUS promoter. The positions of fragments in WUS promoters are shown in Figure 3A. Error bars represent standard deviations of three biological replicates.
type-B ARRs, thus making this region a cytokinin signaling-rich (CSR) one (Figure 11). Meanwhile, ARF3 represses the expression of AtIPTs in the surrounding region, giving rise to an auxin signaling-rich region in a radial pattern encircling the CSR region. Thus, antagonistic regulation between auxin and cytokinin generates the mutually exclusive distribution pattern of the two hormones. In the CSR region, cytokinin functions through ARR1, ARR10, and ARR12 activating the transcription of WUS and ensures a high cytokinin/auxin response ratio in this region by suppressing YUC expression to maintain WUS expression therein (Figure 11). This is supported by a recent study showing that WUS expression is negatively regulated by auxin signaling (Liu et al., 2014). The stable WUS transcription switches callus cells into OC cells, which in turn initiate stem cells through a non-cell-autonomous manner (Yadav et al., 2011; Chatfield et al., 2013; Daum et al., 2014).

Our results demonstrate that WUS signals were colocalized with those of ARR1, ARR10, and ARR12 after the onset of its transcription. The expression regions of ARR1, ARR10, and ARR12 were always larger than the WUS expression region (Figure 1). It is likely that other factors function together with the three ARRs to restrict WUS expression spatially.

It has been shown that root regeneration triggers a program similar to that of embryonic root formation (Etroni et al., 2016). However, the mechanisms underlying shoot regeneration are, at least in part, different from those of embryonic SAM establishment. During shoot regeneration, high levels of cytokinin response signals

Figure 10. Spatiotemporal Expression of YUCs Mediated by Type-B ARRs is Involved in SAM Maintenance.

(A) Expression patterns of ProYUC4:GFP (green) and ProWUS:dsRED (red) reporters in the wild type, arr10 12, and arr 10 12 mutants. Blue signal represents chloroplast autofluorescence. Bars = 50 μm.
(B) and (C) Size and cell number of the SAM in the wild type, arr10 12 mutant, yuc1D, and transgenic lines overexpressing YUC4. Seedlings at 10 d after germination were used for histological analyses in (B). Dashed yellow lines in (B) indicate the boundaries of the SAMs. Histograms in (C) represent width and cell number of SAMs shown in the lower panels of (B). Asterisks denote significant difference compared with the wild type, as determined by a Student’s t test, with two asterisks denoting P < 0.01. Bars = 50 μm.

formation of shoot meristem either from regeneration or embryonic development, proper WUS expression is required to maintain the stem cell niche and subsequently SAM size. Based on their dual function in transcriptional regulation, ARR1, ARR10, and ARR12 directly activated WUS transcription and indirectly promoted its expression by repressing auxin accumulation. Therefore, the dual regulatory roles of ARR1, ARR10, and ARR12 on WUS were critical for the de novo establishment of stem cell niche in vitro and were required for its maintenance in planta.

A Proposed Regulatory Network for de Novo Specification of Shoot Stem Cell Niche Controlled by Cytokinin and Auxin

Pioneer studies demonstrated that cooperation of exogenous cytokinin and auxin induces plant regeneration, which lays an important foundation for wide applications in plant biotechnology and agricultural practices (Duclercq et al., 2011; Ikeuchi et al., 2016). However, the mechanisms underlying this regeneration process are poorly understood. Combining our present results with previous findings (Cheng et al., 2013), we propose a regulatory network for shoot regeneration. During shoot induction, cytokinin signaling in the potential WUS-expressing region directly represses the transcription of YUC1 and YUC4 through

Figure 11. Dual Roles of Type-B ARRs in Regulating WUS Expression and Subsequent Shoot Meristem Formation.

At the early stage of shoot induction, type-B ARRs and YUCs were induced throughout the explant. After 6 d of incubation on SIM, the expression of ARR1, ARR10, and ARR12 was restricted to discrete regions, wherein YUC signals were substantially reduced; moreover, WUS transcription was initiated in a few cells. When ARR signals were restricted to the callus protuberance at SIM8, YUC signals were further reduced there, while WUS signals were enhanced and detected in more cells. At SIM12, ARR1, ARR10, and ARR12 signals were enriched in the center region of the regenerated shoot meristem, where WUS marks the stem cell niche and YUC signals were undetectable. In the region of ARRs expression, the transcription of WUS was activated and maintained, and in parallel, auxin accumulation was inhibited by repressing the expression of YUCs, indirectly promoting WUS induction. Days after the callus was transferred onto SIM are indicated by “D.”
were observed in the progenitor cells of shoot meristem before the onset of WUS expression. By contrast, the signals of the cytokinin response remain undetectable in the prospective SAM until the heart stage of the embryo, which is much later than the initiation of WUS expression (Müller and Sheen, 2008; Cheng et al., 2013; Zürcher et al., 2013). It is likely that cytokinin signaling acts upstream of WUS during shoot regeneration and downstream of WUS in embryonic SAM establishment. Consistently, our results showed that ARR1, ARR10, and ARR12 were critical for shoot regeneration but were not required for SAM formation in plants.

The de novo shoot regeneration may share similar mechanisms with auxillary meristem formation. Both types of meristems are established in the region with low auxin and enriched cytokinin signaling. ARR1 has been shown to be involved in auxillary meristem initiation (Wang et al., 2014). Our results demonstrated a substantial reduction of auxillary meristems in the arr1 10 12 triple mutant (Supplemental Figure 13). It is possible that ARR1, ARR10, and ARR12 regulate auxillary meristem formation through direct activation of WUS expression.

Mutually Exclusive Distribution Pattern of Cytokinin and Auxin Signaling Is Important for Meristem Specification

Out data show that mutually the exclusive distribution pattern of cytokinin and auxin signaling was critical for the specification of the stem cell niche during shoot regeneration. Evidence accumulated in the past few years suggests that the distinct distribution of these two hormones plays pivotal roles in tissue patterning (Chandler and Werr, 2015; Schaller et al., 2015). For instance, in the vascular tissue of Arabidopsis roots, a central localized xylem axis bisects the intervening procambium cells and thus forms a bisymmetric structure (Nieminen et al., 2015; De Rybel et al., 2016). Cytokinin signaling peaks in two bisymmetric procambial cell files, where it regulates the localization of PINFORMED (PIN) efflux carriers and creates an auxin maximum in the adjacent xylem axis. Auxin signaling in turn promotes the expression of the negative regulator of cytokinin signaling AHP6 by ARF5 (Besnard et al., 2014). This mutually exclusive pattern of cytokinin and auxin is required for the specification of the bisymmetric vascular pattern (Bishopp et al., 2011).

Moreover, when the hypophysis cell asymmetically divides and forms the upper lens-shaped cell and the larger basal cell, the cytokinin response of the hypophysis is maintained in the former, whereas the auxin response is detected in the latter (Müller and Sheen, 2008). As a result, the lens-shaped cell gives rise to the QC and the basal cell generates the columella (Laux et al., 2004). During the initiation of the auxillary meristem, an auxin minimum region in the leaf axil is established through PIN-dependent auxin efflux (Wang et al., 2014). In this region, cytokinin perception and signaling are activated to promote the formation of the functional shoot meristem, possibly through inducing WUS expression.

Thus, the mutually exclusive distribution of cytokinin and auxin is widely involved in the specification and function of plant meristems, which might result from the fundamental roles of these two hormones in determining cell fate (Chandler and Werr, 2015). Our study reveals that an interaction between cytokinin and auxin controls shoot regeneration through activating WUS expression, suggesting that inducing the expression of key regulatory genes is critical for cell fate determination.

METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotype Col-0 was used as the wild type in this study except when stated otherwise. Surface-sterilized seeds were plated on half-strength Murashige and Skoog medium containing 0.8% (w/v) agar and 1% (w/v) sucrose (pH 5.7). After vernalization at 4°C for 4 d, seedlings were grown under sterile conditions or in soil at 20 to 22°C, with 16 h of white light (100 μmol m⁻² s⁻¹) and 8 h of dark. The ProWUS:dsRED reporter lines were kindly provided by Elliot M. Meyerowitz (California Institute of Technology) (Gordon et al., 2007). The gWUS-GFP reporter lines were kindly provided by Thomas Laux (University of Freiburg) (Zhang et al., 2013). The arr1 10 double (CS39990), arr1 12 double (CS6981), arr1 10 double (CS39991), and arr1 10 12 triple (CS39992) mutants were obtained from the ABRC. The yuc1D and yuc1 4 double mutants were kindly provided by Yunde Zhao (University of California at San Diego) (Cheng et al., 2006; Zhao et al., 2001). The arr1 10 yuc1 4 quadruple mutant was obtained by crossing arr1 12 with yuc1 4 double mutants.

Plasmid Construction and Plant Transformation

Artificial microRNAs targeting ARR1, ARR11, ARR10, and ARR11/10/12 were designed using the WMD3-Inducible vector (Leibfried et al., 2005; Zhao et al., 2010) to produce the ProAlicAam-ARR1, ProAlicAam-ARR11, ProAlicAam-ARR10, ProAlicAam-ARR10/11/12, and ProAlicAam-ARR11/10/12 constructs. A genomic fragment of 4597 bp containing a 2527-bp sequence upstream of the ATG start codon and the coding region without the stop codon of ARR1 was amplified by PCR from Arabidopsis genomic DNA with the primers pARR1-genomic-F, pARR1-genomic-R, ARR1-cDNA-F, and ARR1-cDNA-R and was recombined into pROKII-GFP to generate the ProARR1:ARR1-GFP expression vector. A 4953-bp genomic fragment containing a 2479-bp region upstream of the ATG start codon and the coding region without the stop codon of ARR1 was PCR amplified using the primers ARR10-genomic-F and ARR10-genomic-R and was recombined into pMDC107 to generate the ProARR10:ARR10-GFP expression vector. The same genomic fragment was amplified with the primers pARR10-mCherry-F and pARR10-mCherry-R and was inserted into the p2300-H2B-mCherry vector digested with SacI and KpnI to generate the ProARR10:ARR10-mCherry vector. A genomic fragment of 5127 bp containing a 2658-bp sequence upstream of the ATG start codon and the coding region without the stop codon of ARR12 was amplified by PCR with the primers ARR12-genomic-F and ARR12-genomic-R and was recombined into pMDC107 to generate the ProARR12:ARR12-GFP expression vector.

For Pro35S:WUS, the 879-bp coding sequence of WUS was amplified by PCR with the primers WUS-CDS-F and WUS-CDS-R and then inserted into the pROKII-GFP vector. For the ProARR10:YUC4 construct, a fragment of 2479 bp upstream of the ATG start codon of ARR10 was amplified from the Arabidopsis genomic DNA with primers pARR10-P-F and pARR10-P-R and inserted into the pCambia1300 vector digested with SacI and BamHI to produce pCambia1300-pARR10.

The full coding sequence of YUC4 was amplified by RT-PCR using total RNA isolated from Arabidopsis seedlings with primers YUC4-cDNA-F and YUC4-cDNA-R and inserted into the pCambia1300-pARR10 digested with Ncol and SalI. All of the expression vectors described above were transformed into the Col-0 wild type using the floral dip method. ProYUC4:YUC4-GFP was described previously (Cheng et al., 2013). ProYUC4:YUC4-GFP was transformed into wild-type plants and these plants were then crossed with ProWUS:dsRED reporter lines to generate ProYUC4:YUC4-GFP, ProWUS:dsRED reporter lines, which were then crossed with the arr1 10 double mutants. The ProDR5-GFP and ProWUS:DsRed reporter lines were described previously (Cheng et al., 2013) and were crossed with arr1 12 double mutants. The
Cytokinin and Auxin Respecify the Stem Cell Niche

ProARR10:ARR10-mCherry lines were crossed with the ProYUC4:YUC4-GFP lines to generate the double reporter lines.

A 2484-bp fragment upstream of the WUS start codon was amplified by PCR from Arabidopsis genomic DNA (ecotype Wassilewskija) with primers pWUS-F and pWUS-R and was introduced into the pGK-3EGFP vector to generate ProWUS:GFP. Mutated WUS promoters were synthesized by Shanghai Sangon Biotechnology Incorporation (Shanghai, China). For ProWUSm1, 2 bp of the ARR binding elements in the ChiP-positive fragments WUS-2, WUS-3, and WUS-4 were replaced (GATC/T changed to CTTC/T). For ProWUSm2, 2 bp of the ARR binding elements in WUS-3 were replaced (GATC/T changed to CTTC/T). For ProWUSm3, 1 bp of the ARR binding elements in WUS-3 was replaced (GATC/T changed to CTAC/T). For ProWUSm4, 1 bp of the ARR binding elements in WUS-3 was replaced (GATC/T changed to CTAC/T). ProWUSm5 was then cloned into the pGK-3EGFP vector to generate ProWUSm5:GFP. ProWUSm6:GFP, and ProWUS:GFP, were examined in the Wassilewskija ecotype background. The ProYUC4m promoter was synthesized by replacing 2 bp of the ARR binding elements in YUC4-1 and YUC4-2 ChiP-positive fragments (GATC/T changed to CTTC/T). ProYUC4m was then cloned into pMDC107 to generate ProYUC4:mGFP. The coding sequence of YUC4 was amplified by PCR using total RNA from Arabidopsis seedlings with primers YUC4-cDNA-F and YUC4-cDNA-R and was introduced into a pROKII-GFP vector to generate pROKII-YUC4-GFP. ProYUC4m was amplified by PCR with primers pYUC4-F and pYUC4-R and was introduced into pROKII-YUC4-GFP to generate the ProYUC4m:YUC4 vector. To analyze the auxin response, ProDR5:GFP reporter lines were crossed with YUC4ox transgenic lines. To analyze the auxin response, ProDR5:GFP reporter lines were crossed with YUC4ox transgenic lines. The sequences of all primers are listed in Supplemental Data Set 1. The sequences of mutated promoters are listed in Supplemental Data Set 2.

The ProARR1-GUS transgenic line was obtained from the ABRC (Mason et al., 2004). The Pro35S:YUC4 (YUC4ox), ProYUC1:GUS, and ProYUC4:GUS transgenic lines were kindly provided by Yun de Zhao (University of California at San Diego) (Cheng et al., 2006). The ProYUC1:GUS transgenic lines were crossed with the arr1 10 and arr1 12 double mutants. The ProYUC4:GUS lines were crossed with the arr10 12 double mutants.

Shoot Regeneration Analysis

Plants were grown for 15 d under sterile conditions as described above. Root explants were cut at 5 to 10 mm from the root tip and incubated on CIM, containing Gamborg’s B5 medium with 2% glucose, 0.5 g/L MES, 0.2 μM kinetin, 2.2 μM 2,4-D, and 0.8% agar. After 6 d in culture on CIM, explants were transferred onto SIM containing Gamborg’s B5 medium with 2% glucose, 0.5 g/L MES, 0.9 μM 3-indoleacetic acid, and 5 μM 2-isopentenyladenine for shoot induction.

For ethanol induction, ethanol was added to the SIM to a final concentration of 0.05% (v/v). For analyses of the frequencies of shoot regeneration, three biological replicates were performed. For each replicate, root explants from more than 100 individuals were used. Different plants were used between distinct replicates. For transgenic plants, separate lines were used in each replicate. Regenerated shoots were defined as described previously (Daimon et al., 2003). The tissues containing a meristem surrounded by three or more leaves or leaf primordia with a phyllotactic pattern were defined as a shoot.

For analyses of stage-specific silencing of ARRs, explants were transferred to SIM media containing 0.05% (v/v) ethanol at 0, 4, 8, or 12 d of SIM incubation, and the shoot regeneration percentages were determined.

The 24-h ethanol induction was performed on gWUS-GFP transgenic explants at days 0, 4, 8, and 12 of SIM incubation. The gWUS-GFP signals were then visualized using confocal microscopy as described below.

For the complementary experiment, Pro35S:WUS was transformed into the arr1 12 double mutant to generate the Pro35S:WUS arr1 12 lines. The shoot regeneration frequency was then determined as above.

Confocal Microscopy

Callus at different days of incubation in SIM that was 5 to 8 mm in diameter was selected using an Olympus SXZ-16 stereomicroscope (Olympus) and cut into sections 1 to 2 mm thick along the longitudinal axis. The sections were then observed, and fluorescent images were captured using a Leica TCS SP5II confocal laser scanning microscope with a 40× oil objective. Multitracking in line scan mode and a 488/561 main dichroic filter were used to image GFP and dsRED together (Heisler et al., 2006). A 561-nm laser line and a 600- to 640-nm band-pass filter were used for dsRED, and a 488-nm laser line and a 505- to 550-nm band-pass filter were used for GFP.

Histochemical GUS Assay

Histochemical GUS assays were performed on transgenic Arabidopsis lines expressing ProARR1:GUS in the wild type, lines expressing ProYUC1:GUS in the wild type, arr1 10 and arr1 12 double mutants, and lines expressing ProYUC4:GUS in the wild type and arr1 10 double mutants. For GUS staining, callus at different days on SIM were harvested and fixed in 90% acetone on ice for 15 min. Each callus was then transferred into GUS staining buffer containing 50 mM NaPO4 (pH 7.2), 2 mM X-gluc (Sigma-Aldrich), 0.5 mM K3Fe(CN)6, and 0.5 mM K4Fe(CN)6 vacuum infiltrated, and incubated at 37°C overnight. The stained callus was photographed using an Olympus SXZ-16 stereomicroscope equipped with an Olympus DP72 digital camera. For anatomical analysis, stained callus was dehydrated for 1 h for each in 70, 80, 90, and 100% ethanol and embedded in paraffin (Sigma-Aldrich). Embossed callus samples were then sectioned at 8 μm, and paraffin was removed by incubation in xylene. Finally, the sections were stained with 0.2% ruthenium red and photographed using an Olympus BX-51 microscope equipped with an Olympus DP71 digital camera.

RT-qPCR

Total RNA was extracted using the TRI reagent (Sigma-Aldrich). The full-length cDNA was generated with the RevertAid first-strand cDNA synthesis kit (Thermo). RT-qPCR was performed on a Chromo4 real-time PCR system (Bio-Rad) using SuperReal PreMix Plus (Tiangen) with genespecific primers. The transcript levels of the genes in each sample were normalized to that of the housekeeping gene TUBULIN2, and the values shown are the mean ± so of three biological replicates. For Figures 2A, 2C, and 6A, callus tissues at different days of SIM incubation were used. Tissues derived from different plants were used in distinct replicates. For Supplemental Figures 2 and 9C, inflorescences of 35-d-old seedlings were used. Tissues used between distinct replicates were generated from different plants. The primers are listed in Supplemental Data Set 1.

In Situ Hybridization

Callus at different days on SIM was collected and fixed in FAA (10% formaldehyde, 5% acetic acid, and 50% alcohol) at 4°C overnight. The fixed tissues were embedded in Paraplast (Sigma-Aldrich) after dehydration and were then sectioned at 8 μm. RNA probes were synthesized and labeled in vitro, and the hybridized signals were detected as previously described (Zhao et al., 2006). Photographs were taken using an Olympus BX-51 microscope equipped with an Olympus DP71 digital camera.

ChiP Assay

ChiP assays were performed using an EZ-ChIP Kit (Upstate) according to the manufacturer’s protocol. Callus tissues incubated for various days on SIM (Figures 5 and 8; Supplemental Figure 7) or shoots (without leaves and cotyledons) from seedlings at 10 d after germination (Figure 9; Supplemental Figure 11) of the ProARR1:ARR1-GFP, ProARR10:ARR10-GFP, and ProARR12:ARR12-GFP transgenic lines were used for ChiP
analyses. For each replicate, 0.3 g of tissue was harvested and cross-linked with 1% (v/v) formaldehyde in GB buffer (0.4 M sucrose, 10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0, and 1 mM PMSF) under a vacuum for 10 min at room temperature. The cross-linking was quenched with 125 mM glycine. The chromatin was then resuspended and sheared by sonication to produce DNA fragments of between 0.2 and 1 kb. The chromatin complexes were immunoprecipitated with anti-GFP antibody (Sigma-Aldrich; lot no. PM1008202, catalog no. SAB5300167). Finally, the precipitated DNA fragments were analyzed using RT-qPCR as described previously (Cheng et al., 2014; Zhou et al., 2009). Tissues derived from different plants were used in distinct biological replicates. The ARR binding elements have been described previously (Sakai et al., 2000; Bhargava A et al., 2013; Kieber and Schaller, 2014). The primers used for qPCR analyses are listed in Supplemental Data Set 1, and the sequences of fragments are listed in Supplemental Data Set 2.

EMSA

The DNA fragments encoding DNA binding domains of ARR1, ARR10, and ARR12 (amino acids 236–299 for ARR1, amino acids 183–235 for ARR10, and amino acids 195–248 for ARR12) were inserted into pGEX-4T-1 vector digested with BamHI and Xhol, which was then expressed in the Escherichia coli BL21 (DE3) cell line to produce GST-tagged ARR protein. The recombinant fusion protein was purified using Glutathione Sepharose 4B (GE Healthcare) following the manufacturer’s instructions. Annealed double-stranded oligonucleotides containing putative binding sequences were labeled with biotin. The LightShift Chemiluminescent EMSA Kit (Thermo) was used for binding reactions. The labeled complex was detected using a Chemiluminescent Nucleic Acid Detection Module (Thermo). The competition experiments were performed with different amounts of nonlabeled oligonucleotides. The mutated competitors in Figures 5B and 8B were generated by replacing two base pairs in the ARR binding sequences are listed in Supplemental Data Sets 1 and 2.

Yeast One-Hybrid Assays

Yeast one-hybrid assays were performed as previously described (Cheng et al., 2013). WUS-3 (−394 to −566 bp upstream of the ATG start codon) and YUC4-2 (−742 to −913 bp upstream of the ATG start codon) fragments were cloned into the pAbAi vector digested with HindIII and KpnI, creating WUS-3-AbAi and YUC4-2-AbAi. WUS-3-AbAi, YUC4-2-AbAi, and pS3-AbAi (positive control; Clontech Laboratories) were linearized by digestion with BbsI prior to transformation of the yeast strain Y1H Gold. The full-length cDNA of ARR1, ARR10, and ARR12 was isolated and cloned into the pDEST-GADT7 activation domain (AD) vector, creating the pAD-ARR1, pAD-ARR10, and pAD-ARR12 plasmid. The p53 sequence was cloned into the pDEST-GADT7 activation domain (AD) vector, creating the pAD-p53 positive control. The pAD-ARR1, pAD-ARR10, and pAD-ARR12 or empty pDEST-GADT7 vector as negative control was subsequently transformed into yeast strains containing WUS-3-AbAi or YUC4-2-AbAi constructs. Activation of the yeast was observed after 3 d on selection plates (synthetic dextrose/-Leu) containing 600 ng mL⁻¹ aureobasidin A. The primers are described in Supplemental Data Set 1. Oligonucleotide sequences are listed in Supplemental Data Set 2.

Transient Expression Assay

The coding sequences of ARR1, ARR10, and ARR12 were cloned into the pGreenII 62-SK vector downstream of the Pro35S promoter and were used as effectors. A 2479-bp fragment upstream of the start codon of WUS or mutated WUS promoters were introduced into the pGreenII 0800-LUC vector upstream of LUC, and these constructs were used as the reporters. The pGreenII 0800-LUC vector harboring the renilla luciferase (REN) gene under the control of the Pro35S promoter was used as the internal control. Protoplast transient expression assays were performed as previously described (Song et al., 2014). Mesophyll protoplast preparation and transfection were performed according to a previously reported method (Yoo et al., 2007). For transient expression assays in tobacco leaves, leaves of Nicotiana benthamiana were transiently transformed and examined as previously described (Guo et al., 2015). Biological replicates represent the results of three independent assays. For transient expression in tobacco leaves, three leaves from different plants were used in one replicate. Protoplasts used in the three replicates were obtained from different plants.

Histological Analyses of the SAM

The SAMs from wild-type and arr1 10 12 mutant seedlings at 14 d after germination were used for αWUS-GFP₃ expression analyses. The intensity of fluorescence was measured using ImageJ software (Cordoba et al., 2016). The shoot meristem width was measured at the maximum width between leaf primordia. ImageJ software was used for measuring shoot meristem width and counting cell number (Maes et al., 2008; Vidal et al., 2010).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative under the following accession numbers: ARR1 (At3g16857), ARR2 (At4g16110), ARR10 (At4g31920), ARR11 (At1g67710), ARR12 (At2g51580), ARR13 (AT2G27070), ARR18 (At5g58080), ARR21 (AT5G07210), WUS (At2g17895), YUC1 (AT4G32540), YUC2 (AT4G13260), YUC4 (AT5G11320), and YUC6 (AT5G25620).

Supplemental Data

- Supplemental Figure 1. Expression Patterns of ARR1 during Shoot Regeneration.
- Supplemental Figure 2. The Effectiveness of Artificial MicroRNAs against ARR1, ARR10, and ARR12.
- Supplemental Figure 3. Defects in ARR1, ARR10, and ARR12 Attenuate Shoot Regeneration.
- Supplemental Figure 4. Transient Expressing ARR1, ARR10, or ARR12 Activates WUS Transcription.
- Supplemental Figure 5. Mutating the ARR Binding Elements in WUS Promoter Region Reduced ARR-Mediated WUS Transcription.
- Supplemental Figure 6. Mutations in ARRs Enhanced the Signals of the pYUC1:GUS and pYUC4:GUS Reporters.
- Supplemental Figure 7. ARR1 Binds the Promoter of YUC1 and Regulates Its Transcription.
- Supplemental Figure 8. Expression Patterns of ProDR5:GFP Were Disturbed in YUCox Lines.
- Supplemental Figure 9. YUCs Act Downstream of ARRs.
- Supplemental Figure 10. Expression of YUC4 under Mutated YUC4 Promoter Reduced Shoot Regeneration.
- Supplemental Figure 11. ARR1, ARR10, and ARR12 Bind the Promoters of YUCs and Regulate Their Expression in the SAM.
- Supplemental Figure 12. Phenotypes of pARR10:TUC4 Transgenic Lines.
- Supplemental Figure 13. ARR1, ARR10, and ARR12 Are Involved in Auxillary Meristem Initiation.
- Supplemental Data Set 1. Primers Used in This Study.
- Supplemental Data Set 2. Oligo Sequences Used in the Yeast One-Hybrid, EMSA, and ChiP Assays.
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AUTHOR CONTRIBUTIONS

X.S.Z., Z.J.C., and Y.L.S. conceived and designed the experiments. W.J.M. and Z.J.C. performed the experiments and data analysis with the help of M.M.Z., X.F.R., Z.W.W., and Y.Y.T. in the laboratory of X.S.Z. X.S.Z. and Y.L.S. wrote the manuscript. X.S.Z. supervised the project.

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REFERENCES


Type-B ARABIDOPSIS RESPONSE REGULATORs Specify the Shoot Stem Cell Niche by Dual Regulation of \textit{WUSCHEL}

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