

ARR5 and ARR6 Mediate Tissue Specific Cross-talk between Auxin and Cytokinin in *Arabidopsis*

Aparna Kakani, Zhaohua Peng

Department of Biochemistry and Molecular Biology, Mississippi State University, Starkville, USA.
Email: zp7@ra.msstate.edu

Received July 23rd, 2011; revised September 1st, 2011; accepted September 10th, 2011.

ABSTRACT

Auxin and cytokinin interaction plays an essential role in a wide range of plant growth and developmental processes. The interaction consequence of the two hormones is highly tissue specific. The molecular mechanisms underlying the tissue specificity are largely unknown. Here we show that the cytokinin signaling pathway key components ARR5 and ARR6 respond to auxin with a highly tissue specific and contrasted pattern in *Arabidopsis* seedlings and calli in the presence of cytokinin. Our results suggest that the two highly homologous but functionally distinct genes, ARR5 and ARR6, play a critical role in mediating tissue specific interaction between auxin and cytokinin.

Keywords: ARR5, ARR6, Auxin, Cytokinin, Callus, *Arabidopsis*, Auxin and Cytokinin Interaction

1. Introduction

The interplay between auxin and cytokinin is essential for plant growth and development. Their interactions includes synergistic, antagonistic and additive, depending on plant species and tissues [1-5]. Apical dominance is a classic example of auxin and cytokinin antagonistic interaction. While auxin produced at the apex represses the outgrowth of lateral buds, cytokinin applied to lateral buds promotes the release of lateral buds from apical dominance. Interestingly, auxin transported from apex promotes lateral root initiation and growth and the cytokinin produced in the root cap stimulates root apical dominance [6]. These observations suggest that interaction between auxin and cytokinin is highly tissue specific.

It has been shown that removal of the endogenous auxin source via decapitation leads to up to 40 fold increase of cytokinin in xylem exudates [7,8]. Studies in *Arabidopsis* demonstrate that auxin mediates a very rapid negative control of the cytokinin pool by mainly suppressing its biosynthesis via the isopentenyladenosine-5'-mono phosphate independent pathway [9]. In addition, auxin has been found to stimulate both oxidative breakdown and glucosylation of active cytokinins in a tissue-dependent manner [1]. On the other hand, an increase in free IAA (active form) has been observed both in cyto-

kinin overproducing lines of *Nicotiana glutinosa* transformed with the bacterial cytokinin biosynthesis gene *ipt* (isopentenyl transferase) and in maize and pea treated with exogenously applied cytokinin [10-12], probably via cytokinin inhibition of enzymes that conjugate free IAA [13]. Meanwhile, it has also been reported that cytokinin overexpression lead to down regulation of the IAA pool in tobacco [14]. The discrepancy among different studies suggests that further investigations are required for a complete picture of the auxin-cytokinin interaction.

The auxin and cytokinin control of cell division in undifferentiated cells presents a good example of synergistic interaction of the two. Studies have shown that auxin increases the expression of a cdc2 class of cyclin-dependent kinases in tobacco pith explants. While the expression of the cdc2 like kinase is induced in response to auxin, its catalytic activity is increased only when the explants are also treated with cytokinin [15]. Cyclin δ 3 is a D cyclin whose expression is highly dependent on cytokinin [16]. It is believed that δ 3 may be the factor required to activate the cdc2 kinase. Therefore, the auxin and cytokinin synergistically control the expression and activity of the cdc2 like kinase, which renders the cell competent for cell division (review, [1]).

Kakani *et al.* [17] recently found that exogenous cytokinin can induce tissue specific up and down regulation

of auxin in *Arabidopsis*. While auxin levels are reduced by exogenous cytokinin in cotyledons, auxin is elevated by exogenous cytokinin in roots and calli. More interestingly, they found that AUX1 plays a critical role in mediating cytokinin stimulated auxin accumulation in young roots and calli. Müller and Sheen reported that cytokinin and auxin interact antagonistically in root stem-cell specification in early embryogenesis [18]. Auxin antagonizes cytokinin signaling by directly activating the repressors of cytokinin signaling, ARR7 and ARR15. However, how cytokinin and auxin interact during shoot stem cell specification was not reported.

Both the auxin and cytokinin signaling pathways involve highly complicated networks that contain a large number of genes. The cytokinin signaling is perceived via a phosphorelay that is similar to the two-component systems used by bacteria for sensing and responding to environmental stimuli (recent review [18,19]). The pathway involves hybrid histidine protein kinases (AHK2, AHK3 and CRE1/WOL/AHK4) as receptors, histidine phosphotransfer proteins (AHPs), and nuclear response regulators (type A-ARRs and type B-ARRs). There are ten type-A ARR proteins (ARR3-ARR9 and ARR15-ARR17) and 11 type-B ARR proteins (ARR1, ARR2, ARR10-ARR14 and ARR18-ARR21) in *Arabidopsis*. The type-A ARRs were originally identified as cytokinin induced genes (review, [19]). At least eight of the ten type-A ARRs are negative regulators of cytokinin signaling with overlapping function. Both gene redundancy and tissue-specific roles have been observed among type-A ARRs in cytokinin response [20-22]. ARR5 and ARR6 share highest homology with each other compared with other members in the same gene family [21]. ARR5 expression was found in the root and shoot meristems in the absence of exogenous cytokinin [23]. In the presence of exogenous cytokinin, the ARR5:GUS expression region was enlarged to include tissues around the shoot meristematic region in the shoot and all tissues in the roots, from the hypocotyl-root junction through the root tip. ARR6:GUS expression was detected in the shoot meristematic region and cotyledon vasculature in young seedlings [21]. Cytokinin treatment resulted in overall higher level expression of ARR6:GUS, including tissues in hypocotyl and root except the root tip [21]. The *arr5* knockout mutant has an altered rosette morphology, which disappeared in *arr5 arr6* double mutant, indicating that ARR5 and ARR6 have opposite functions [21].

Although the essential role of the interplay between auxin and cytokinin in plant growth and development has been well documented, little is known of the underlying molecular mechanisms. Most of the reported studies limited to how the auxin and cytokinin levels modulated each other and in meristem tissue development. In this

study, we report that the cytokinin induction of ARR5 and ARR6 expression is subjected to the regulation of auxin in a tissue and gene specific manner. The distinct auxin regulatory patterns of these two highly homologous genes provide novel insights into the mechanisms underlying auxin and cytokinin interactions, especially the tissue specific interactions of these two hormones.

2. Materials and Methods

2.1. *Arabidopsis* Growth and Callus Induction

Arabidopsis seeds were sterilized and placed on Gamborg's B5 medium (pH 5.7) supplemented with 1% sucrose, 0.9% agar, and 2,4-D (2,4-Dichlorophenoxyacetic acid) and KT (Kinetin) as indicated in the text. After 5 days of cold treatment at 4°C, the Petri dishes were transferred to a 22°C growth chamber for seed germination or callus induction under 16 hrs of light and 8 hrs of dark. The light intensity was 150 mol m⁻²sec⁻² for germination and 50 mol m⁻²sec⁻² for callus induction.

2.2. ARR5:GUS and ARR6:GUS Transgenic Lines

The *Arabidopsis* (Columbia) seeds of ARR5:GUS and ARR6:GUS lines were ordered from *Arabidopsis* Biological Research Center (ABRC) at the Ohio State University [21].

2.3. Histochemical Analyses of GUS Activities

The histochemical stain of GUS was carried out as reported by Sessions *et al.* [24] without sectioning. Briefly, plant materials were stained in GUS staining solution (100 mM Sodium Phosphate at pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 1 mM potassium ferricyanide, 1 mM potassium ferrocyanide and 1 mg/mL of X-Gluc [Gold Bio Technology, Inc.]). The samples were incubated at 37°C overnight after being placed under a vacuum for 10 min in a desiccator. The staining solution was removed and the tissues were cleaned by incubating with several changes of 70% ethanol. The GUS images are acquired using a Zeiss Stemi SV11 (Apo) light Microscope.

3. Results and Discussions

3.1. Auxin Modulates ARR5 and ARR6 Expression with High Tissue Specificity and a Contrast Pattern

In our previous studies, we found that cytokinin can induce auxin redistribution in seedlings and calli and AUX1 plays an essential role in mediating cytokinin induced auxin redistribution [17]. To examine how the cytokinin signaling pathways interact with auxin, we examined the expression of cytokinin pathway genes in response to exogenously applied auxin. ARR5 and ARR6

share highest sequence homology in the *Arabidopsis* genome but may have opposite functions. We found that *ARR5* expression was in the root and shoot meristems of wild-type seedlings as reported [23] (**Figure 1(a)**). In the presence of 0.2 mg/l KT, the *ARR5*:GUS expression was highly induced in roots, from the hypocotyl-root junction through the root tip, and regions surrounding the shoot apical meristem (**Figure 1(b)**). When auxin (2,4-D) was also applied, the *ARR5*:GUS expression in roots were strongly enhanced from the hypocotyl-root junction to root tip (**Figure 1(c)-(f)**). In contrast, *ARR5*:GUS expression in cotyledons, shoot apical meristem, and hypocotyls were suppressed.

When auxin concentration increased to 2.0 mg/l, the *ARR5*:GUS expression was completely shut off in cotyledons and shoot apical meristem regions. But the gene expression in roots was very high.

Interestingly, auxin regulation of *ARR6* expression followed a contrast pattern. In wild-type seedlings, *ARR6*:GUS expression was detected in the shoot apical meristematic region and cotyledon vasculature (**Figure 2(a)**) [21]. Cytokinin treatment elevated the expression of *ARR6*:GUS, particularly in hypocotyls and roots as reported (**Figure 2(b)**). When low auxin (0.05 mg/l and 0.25 mg/l 2,4-D) was applied, *ARR6*:GUS expression was strongly induced in cotyledons, shoot apical meristem region, and hypocotyls. On the other hand, its expression in roots was suppressed starting from the elongation zone. With the increase of 2,4-D, the suppressed region extended to hypocotyl region and root tip in both directions. When 2,4-D reached 2.0 mg/l, *ARR6*:GUS

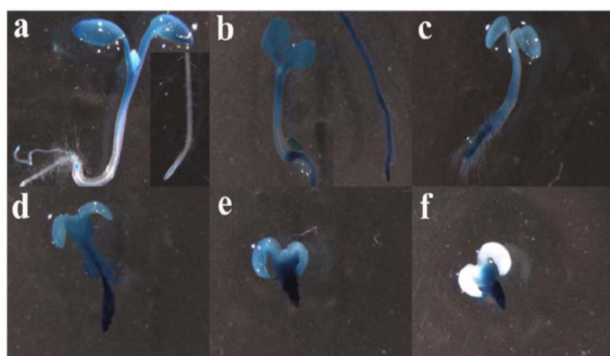


Figure 1. Histochemical assay of *ARR5*:GUS expression in response to cytokinin and auxin induction. The seedlings were germinated in B5 media supplemented with KT and 2, 4-D in concentrations as indicated below. The seedlings were five days old. (a) Wild-type seedling; (b) Seedling grown in medium with 0.2 mg/l KT; (c) Seedling grown in medium with 0.2 mg/l KT and 0.05 mg/l 2,4-D. (d) Seedling grown in medium with 0.2 mg/l KT and 0.25 mg/l 2,4-D; (e) Seedling grown in medium with 0.2 mg/l KT and 1.0 mg/l 2, 4-D; (f) Seedling grown in medium with 0.2 mg/l KT and 2.0 mg/l 2,4-D.

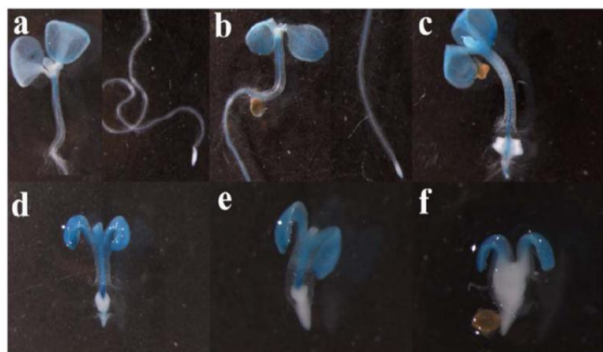


Figure 2. Histochemical assay of *ARR6*:GUS expression in response to cytokinin and auxin induction. The seedlings were germinated in B5 media supplemented with KT and 2, 4-D in concentrations as indicated below. The seedlings were five days old. (a) Wild-type seedling; (b) Seedling grown in medium with 0.2 mg/l KT; (c) Seedling grown in medium with 0.2 mg/l KT and 0.05 mg/l 2,4-D. (d) Seedling grown in medium with 0.2 mg/l KT and 0.25 mg/l 2,4-D; (e) Seedling grown in medium with 0.2 mg/l KT and 1.0 mg/l 2, 4-D; (f) Seedling grown in medium with 0.2 mg/l KT and 2.0 mg/l 2,4-D.

was expressed only in cotyledons. The expression in other tissues was almost completely suppressed. When the *ARR6*:GUS and *ARR5*:GUS expression patterns were compared (compare **Figure 1** with **Figure 2**), it was clear that the expression of *ARR5*:GUS and *ARR6*:GUS followed a reversed pattern with the increase of auxin, suggesting a tissue specific role of these two genes in the interplay between auxin and cytokinin.

While examining the mutant phenotype in *Arabidopsis* [21], it was observed that the reduced rosette size of *ARR5* mutant was not enhanced by the mutation of *ARR6*. Instead, the phenotype disappeared in the double mutant, indicating a different function of these two genes although these two genes have a high level of spatial overlapping in gene expression in cotyledons and hypocotyls in wildtype seedlings. Given that *ARR5* and *ARR6* negatively regulate cytokinin response, our observations suggest that auxin suppress the cytokinin stimuli in plants via modulation of the negative regulators in the cytokinin pathway. The tissue specificity of auxin and cytokinin cross-talk is achieved via differential regulation of the highly homologous genes with different functions.

3.2. Auxin Regulation of *ARR5* and *ARR6* Expression in Calli

To understand auxin and cytokinin interaction in calli, we examined the expression of *ARR5*:GUS and *ARR6*:GUS in calli induced from germinating seeds with different auxin/cytokinin ratios and culturing time. *ARR5*:GUS was highly expressed in calli (**Figure 3**). Some cells, derived from cotyledons, barely expressed *ARR5*

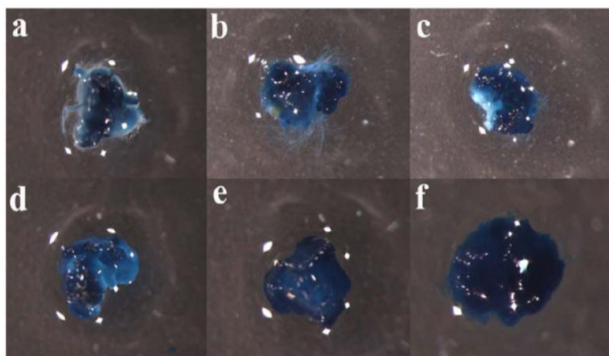


Figure 3. Histochemical assay of ARR5:GUS expression in calli. The calli were induced in B5 media supplemented with KT and 2,4-D in concentrations as indicated below. (a) Four weeks old calli induced by 0.2 mg/l KT and 0.05 mg/l 2,4-D; (b) Four weeks old calli induced by 0.2 mg/l KT and 0.25 mg/l 2,4-D; (c) Four weeks old calli induced by 0.2 mg/l KT and 1.0 mg/l 2,4-D; (d) Four weeks old calli induced by 0.2 mg/l KT and 2.0 mg/l 2,4-D; (e) Six weeks old calli induced by 0.2 mg/l KT and 2.0 mg/l 2,4-D; (f) Eight weeks old calli induced by 0.2 mg/l KT and 2.0 mg/l 2,4-D.

initially when auxin was high. ARR5:GUS became highly expressed across the entire callus after eight weeks of culture. In contrast, the expression of ARR6:GUS in calli was low (**Figure 4**). Although small portion of the tissues, which derived from cotyledons, expressed ARR6:GUS initially, the GUS expression substantially decreased with time. After eight weeks, the ARR6:GUS expression was barely detected in the calli. Our results suggest that ARR5 is highly expressed in calli but ARR6 is silenced in calli.

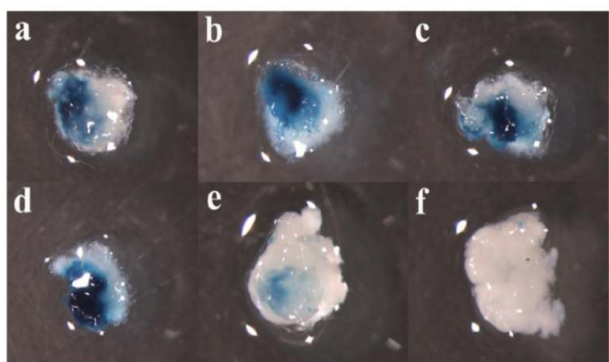


Figure 4. Histochemical assay of ARR6:GUS expression in calli. The calli were induced in B5 media supplemented with KT and 2,4-D in concentrations as indicated below. (a) Four weeks old calli induced by 0.2 mg/l KT and 0.05 mg/l 2,4-D; (b) Four weeks old calli induced by 0.2 mg/l KT and 0.25 mg/l 2,4-D; (c) Four weeks old calli induced by 0.2 mg/l KT and 1.0 mg/l 2,4-D; (d) Four weeks old calli induced by 0.2 mg/l KT and 2.0 mg/l 2,4-D; (e) Six weeks old calli induced by 0.2 mg/l KT and 2.0 mg/l 2,4-D; (f) Eight weeks old calli induced by 0.2 mg/l KT and 2.0 mg/l 2,4-D.

4. Conclusions

The interaction of auxin and cytokinin is essential to plant growth and development. The molecular mechanisms underlying the interaction, particularly the tissue specific response, are still poorly understood. Our results reported here suggest that auxin interacts with the cytokinin pathway by directly regulating the expression of negative regulators ARR5 and ARR6 in the cytokinin signaling pathway. The tissue specific response is achieved by differential regulation of homologous genes with distinct functions.

5. Acknowledgements

We are grateful to Dr. Scott Willard, Dr. Jiaxu Li, Dr. Din-Pow Ma, and Ms Hana Mujahid for a critical reading of this manuscript. This research was approved for publication as Journal Article J-10944 of the MAFES, Mississippi State University.

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