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**Gene Note**

# Characterization of *Arabidopsis* Response Regulator Genes with Regard to Bisphenol A Signaling

Nobuyuki Terouchi,[1,*] Chiaki Kato[2], Natsumi Hosoya[2]

[1] Laboratory of Biological Regulation, Department of Arts and Sciences, Otsuma Women's University, Tokyo 102-0075, Japan; [2] Environmental Information Studies Course, Department of Social Information Studies, School of Social Information Studies, Otsuma Women's University, Tama, Tokyo 206-8540, Japan
E-mail: terouchi@otsuma.ac.jp Tel & Fax: +81-35-275-6065

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**Abstract** *Arabidopsis* response regulators (ARRs) are involved in the His-to-Asp phosphotransfer signal transduction mechanism induced by cytokinins, a class of phytohormones in *Arabidopsis thaliana*. Previous studies have reported ARR mRNA accumulation in response to cytokinins. ARRs consist of 2 families (types A and B). The expression of type-A ARR genes can be induced by cytokinins, while that of type-B genes is constitutive. Recently, we have shown that plant growth induced by the endocrine disruptor bisphenol A (4,4′-isopropylidenediphenol) was similar to that induced by cytokinins. The present study is the first to show that ARR (type-A family; *ARR15* and *ARR16*) mRNA accumulation is influenced by bisphenol A, indicating that bisphenol A is involved in inducing intracellular signal transduction.

**Key words:** *Arabidopsis* response regulator (ARR), *Arabidopsis thaliana*, bisphenol A (4,4'-isopropylidenediphenol), cytokinin (*t*-zeatin), signal transduction.

Bisphenol A is a known endocrine disruptor. It induces estrogenic reactions and promotes cell growth in animals (Krishnan et al. 1993; Steinmetz et al. 1998), but its effects on plants have not yet been examined. Cytokinins, a class of phytohormones, induce growth and shoot differentiation (Mok and Mok 1994; McGaw and Buech 1995). We were the first to show that bisphenol A stimulates cell growth in plants using a cytokinin bioassay system (Terouchi et al. 2004). Bisphenol A may exhibit an activity similar to that of cytokinins.

Studies have reported that cytokinins influence the intracellular signal transduction mechanisms involved in His-Asp phosphorylation (Stock et al. 1989; Appleby et al. 1996). Such a phosphorelay reaction generally involves a sensor kinase (Suzuki et al. 2000; Inoue et al. 2001; Ueguchi et al. 2001) that acts as a cytokinin receptor, such as a His-containing phosphotransfer intermediate (HPT) and response regulators (Appleby et al. 1996; Mizuno 1998). In *Arabidopsis thaliana*, His kinases (AHKs) reportedly function as cytokinin sensors and receptors (Yamada et al. 2001), the latter having a major role during His-to-Asp phosphotransfer (Chang and Stewart 1998; Schaller 2000). *A. thaliana* has 5 genes encoding HPTs (Miyata et al. 1998; Suzuki et al. 1998; Suzuki et al. 2000) and 22 genes encoding *Arabidopsis* response regulators (ARRs) (Brandstatter and Kieber 1998; Imamura et al. 1998; Sakai et al. 1998; Urao et al. 1998; Imamura et al. 1999; Lohrmann et al. 2001; Sakai et al. 2001).

ARR families are classified into basically 2 distinct subtypes (type A includes 10 members and type B and other atypical subtypes, including *ARR22*, consist of 11 members) (D'Agostino and Kieber 1999; Imamura et al. 1999; Kiba et al. 1999). Type-B ARRs induce transcription of type-A ARR genes (Hwang and Sheen 2001; Sakai et al. 2001). Thus, a cytokinin signal response is considered to be involved in the AHK→HPT→ARR phosphorelay pathway. In plants, cytokinin treatment induces expression of type-A ARRs at the level of transcription (Hwang and Sheen 2001; Hutchison and Kieber 2002; Heyl and Schulling 2003; Kakimoto 2003).

We examined whether bisphenol A-dependent signal transduction mechanisms were identical to those of cytokinin and found that bisphenol A induces expression of type-A ARR genes (*ARR15* and *ARR16*).

*A. thaliana* (Colombia; Col) plants were grown on Murashige and Skoog (MS) Gelrite medium for 12 days, and subsequently treated directly with bisphenol A and t-zeatin (cytokinin) by spraying these agents as a fine mist on the plant roots. As cytokinin induces expression of *ARR15* and *ARR16* genes, particularly in roots (Taniguchi et al. 1998; Kiba et al. 2002), we specifically

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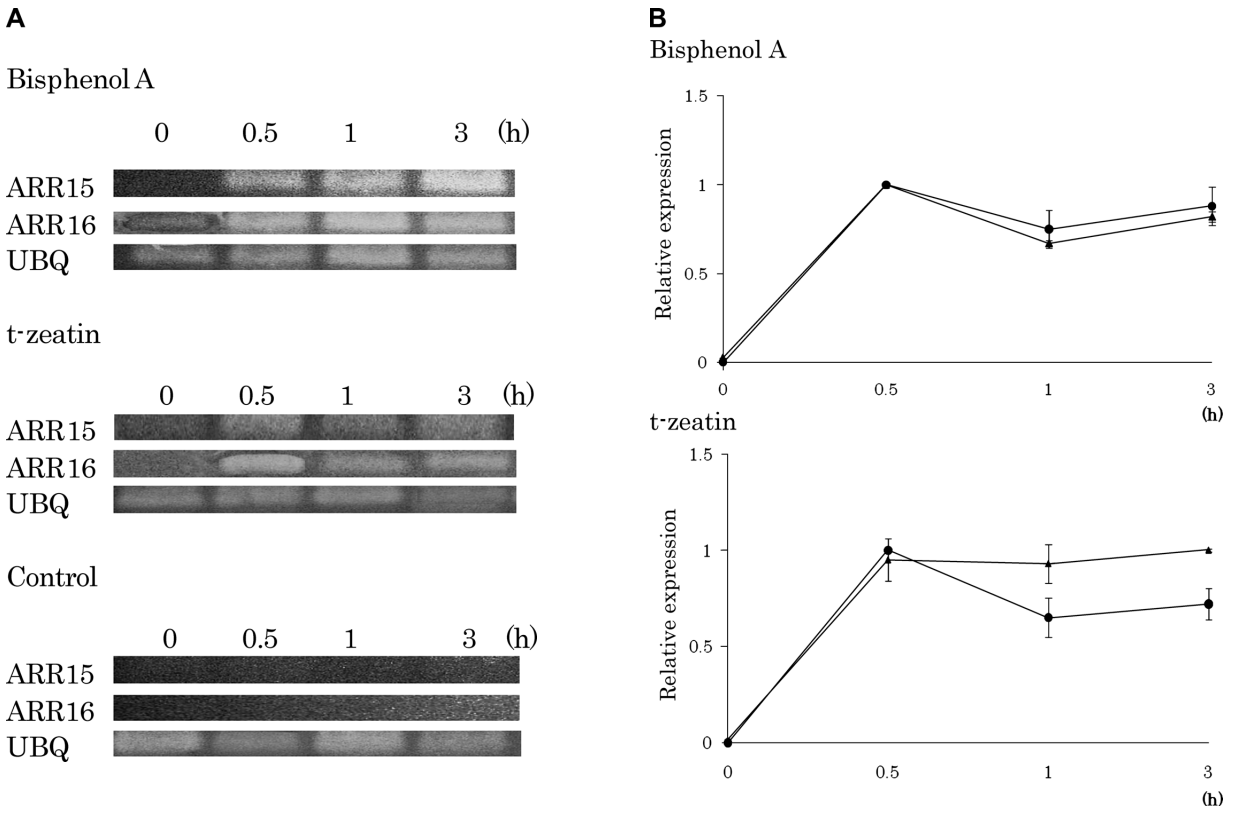


Figure 1. RT-PCR analysis to detect transcription of ARR genes of the type-A family in roots of *Arabidopsis thaliana*. (A) After spraying the roots of the plants with bisphenol A and t-zeatin, mRNA was extracted from the roots at various time intervals and subjected to RT-PCR analysis. Total RNA was extracted from the plants with Isogen (Nippon Gene). RT-PCR was performed with a PrimeScript RT-PCR kit (Takara). The following primers were used for the PCR analysis:

*ARR15*, 5′-ATTCAATGCCGGGACTAACA-3′ and 5′-TTCAGCTTCACCACCTCTC-3′

*ARR16*; 5′-CGTTGAGAGGTTGCTCAAGA-3′ and 5′-CAAGCTTCAAAGGCTTCTC-3′

*UBQ*, 5′-GCGAAGAAGATGATCGAGGT-3′ and 5′-GCGAAGAAGATGATCGAGGT-3′

The thermocycler conditions were 30 s at 94°C, 30 s at 51°C, 14 s at 72°C and a final extension step of 7 min at 72°C (for 32 cycles). The number of PCR cycles for each ARR was determined in order to obtain a linear increase in the product at a concentration of $10^{-1}\,\mu g\,ml^{-1}$. For the control conditions, bisphenol A and *t*-zeatin were replaced with distilled water (DW). The amplified fragments were separated by agarose gel electrophoresis, stained with SYBR Green, and digitally scanned under ultraviolet light. The experiment was repeated three times. (B) Expression levels relative to the most abundant PCR product in the respective reaction set. The relative intensities of the bands were quantified using UN SCAN IT ver. 6.1. software. The *UBQ* gene was used as a reference. The expression profiles were normalized by defining the maximum expression value as 1.0. Error bars show the standard deviation of three PCR experiments. ●, *ARR15*; ▲, *ARR16*.

studied the roots. A concentration of $10^{-1}\,\mu g\,ml^{-1}$ bisphenol A was used to treat the roots, based on our previous observation that a maximum fresh weight of calli was obtained at this treatment concentration (Terouchi et al. 2004). At various intervals (0 to 3 h), RNA was extracted from the roots that were treated with bisphenol A or t-zeatin at various concentrations, and then ARR transcription levels were analyzed using reverse transcription polymerase chain reaction (RT-PCR) with the PrimeScript system (Takara). The PCR conditions included 32 cycles of 30 s at 94°C, 30 s at 51°C, 14 s at 72°C and a final extension step of 7 min at 72°C. The primer sets given in the legend of Fig. 1 for PCR analysis of *ARR15* and *ARR16* were used. RT-PCR showed that expression of both transcripts (*ARR15* and *ARR16*) in the t-zeatin- and bisphenol A-treated roots was markedly enhanced within 30 min (Figure 1A). These findings were also quantitatively confirmed (Figure 1B) by measuring the intensity of the bands using an appropriate internal reference (UBQ).

Next, we examined the effects of bisphenol A and t-zeatin at various concentrations on *ARR15* and *ARR16* expression 30 min after treatment. The *ARR15* and *ARR16* genes were expressed after treatment with both $10^{-1}\,\mu g\,ml^{-1}$ and $1^{-1}\,\mu g\,ml^{-1}$ *t*-zeatin, but only $10^{-1}\,\mu g\,ml^{-1}$ bisphenol A (Figure 2A). Analysis of the relative expression levels of *ARR15 and ARR16* in response to bisphenol A and t-zeatin (Figure 2B) showed increased levels in response to $10^{-1}\,\mu g\,ml^{-1}$ bisphenol A and to $10^{-1}\,\mu g\,ml^{-1}$ and $1\,\mu g\,ml^{-1}$ *t*-zeatin (Figure 2B).

These results showed that bisphenol A affects type-A *ARR15* and *ARR16* genes, suggesting that bisphenol A has a role similar to that of cytokinin as a receptor in the multistep ARR phosphorelay pathway. However, the

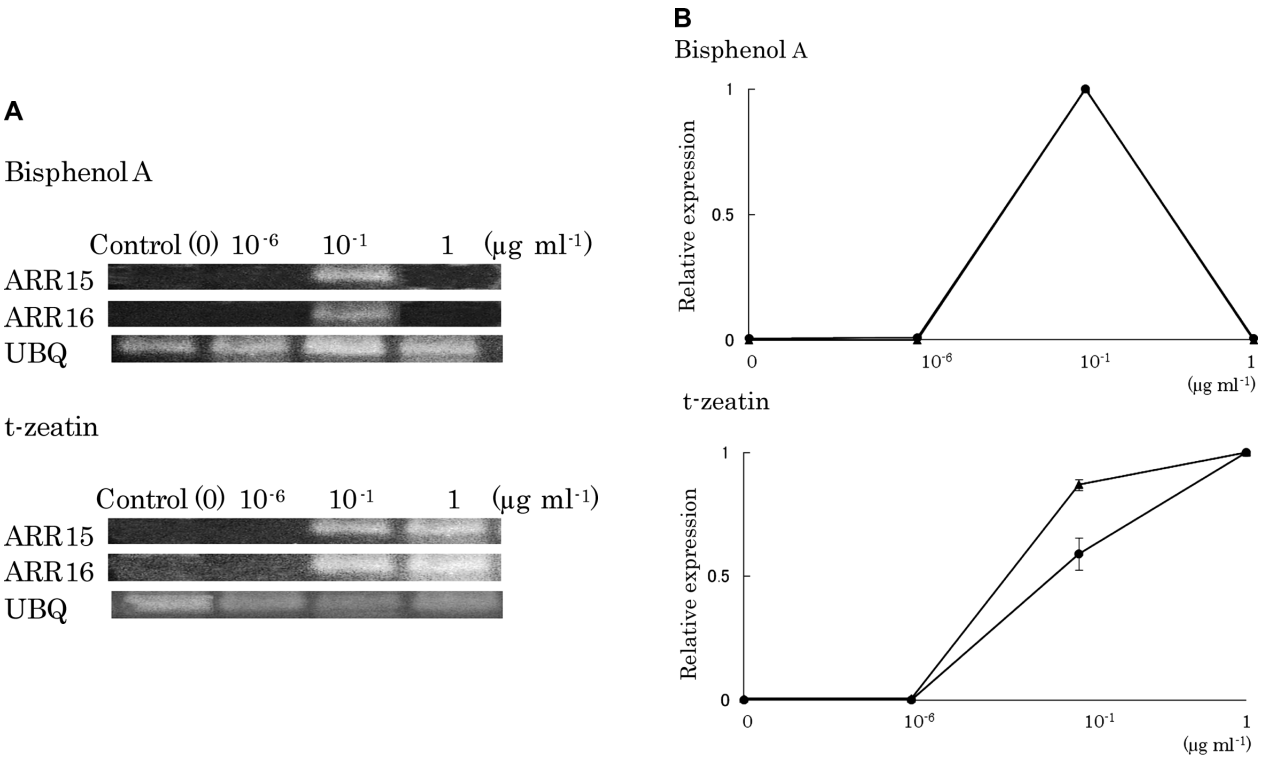


Figure 2. RT-PCR analysis showing transcripts of the ARR gene (type-A family) in roots treated with various concentrations of bisphenol A and t-zeatin 30 min after treatment. (A) Responses of *Arabidopsis* roots to various concentrations of bisphenol A and t-zeatin. The PCR protocol was identical to that described in the legend to Figure 1. (B) Expression levels relative to the most abundant PCR product in the respective reaction set. The relative intensities of the bands were quantified using UN SCAN IT ver. 6.1 software. The expression profiles were normalized by defining the maximum expression value as 1.0. Error bars show the standard deviation of three PCR experiments. ●, *ARR15*; ▲, *ARR16*.

different expression patterns for bisphenol A and t-zeatin (Figure 2) are due to qualitative differences in the receptor or to sensitivity of the receptor to each substrate.

A previous report has determined that His-kinases, AHK2, AHK3, and AHK4, act as primary cytokinin receptors (Yamada et al. 2001); however, the receptor for bisphenol A has yet to be confirmed.We have shown that inhibition of cell growth occurs when is added to soybean callus at high concentration ($1\,\mu g\,ml^{-1}$) (Terouchi et al. 2004). Interestingly, in this study, we found that bisphenol A-induced inhibition seems to correspond with downregulation of *ARR15* and *ARR16* (Figure 2). Moreover, the change in cell growth of callus in response to cytokinin and the change in *ARR15* and *ARR16* expression showed the same tendency (Figure 2). Future studies are ongoing to further investigate this phenomenon.

This is the first study to show bisphenol A plays some role in an intracellular signal transduction mechanism.

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