The Type-A Response Regulator, ARR15, Acts as a Negative Regulator in the Cytokinin-Mediated Signal Transduction in *Arabidopsis thaliana*

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The *Arabidopsis thaliana* AHK4 histidine kinase (also known as CRE1 or WOL) acts as a cytokinin signal transducer, presumably, in concert with downstream components, such as histidine-containing phosphotransfer factors (AHPs) and response regulators (ARRs), through the histidine-to-aspartate (His→Asp) phosphorelay. Among 10 members of the type-A ARR family, the cytokinin-induced expression of *ARR15* in roots is selectively impaired in the *crel-1* mutant, which carries a mutation in the *AHK4* gene, suggesting a link between this type-A response regulator and the AHK4-mediated cytokinin signal transduction in roots. To address this issue further, we characterized a T-DNA insertion mutant of *ARR15*, and also constructed transgenic lines (referred to as ARR15-ox) that overexpress the *ARR15* gene in a manner independent of cytokinin. While the T-DNA insertion mutant (arr15-1) showed no apparent phenotype, the cytokinin-independent overexpression of *ARR15* in ARR15-ox plants resulted in a reduced sensitivity toward exogenously applied cytokinin, not only in elongation of roots in plants, but also in green callus formation (or shoot formation) in explants. Cytokinin-induced expressions of certain type-A ARRs were also down-regulated in ARR15-ox plants. These results support the view that ARR15 acts as a repressor that mediates a negative feedback loop in the cytokinin and AHK4-mediated His→Asp phosphorelay.

**Keywords**: *Arabidopsis* — Cytokinin — Phosphorelay — Histidine kinase — Response regulator — Signal transduction.


In *Arabidopsis thaliana*, results of recent intensive studies suggested that histidine-to-aspartate (His→Asp) phosphorelays (Mizuno 1998) are involved in the signal transduction mechanisms underlying propagation of certain environmental stimuli, such as plant hormones (e.g. cytokinin) (for recent reviews, see Hutchison and Kieber 2002, Hwang et al. 2002, Sheen 2002). This model plant has 11 members of the histidine kinase (HK) family, of which five members (e.g. ETR1) act as ethylene receptors (Hua and Meyerowitz 1998, Chang and Stewart 1998). Another HK, AHK4 (also known as CRE1 or WOL), together with its homologs (AHK2 and AHK3), serves as a cytokinin receptor (Mahonen et al. 2000, Inoue et al. 2001, Suzuki et al. 2001, Yamada et al. 2001). Furthermore, this higher plant has five genes each encoding a histidine-containing phosphotransfer (HPT) intermediate of phosphorelay (AHP-series) (Miyata et al. 1998, Suzuki et al. 1998, Suzuki et al. 2001, Suzuki et al. 2002), and 22 genes each encoding a response regulator (ARR-series) (Imamura et al. 1998, Imamura et al. 1999, D'Agostino et al. 2000). Such an AHK→AHP→ARR phosphorelay network appears to be involved in cytokinin signal transduction (Hwang and Sheen 2001, Sakai et al. 2001).

The ARR family members are basically classified into two subtypes (see the reviews cited above). Type-B ARR members appear to serve as DNA-binding transcription factors (Sakai et al. 2000, Lohrmann et al. 2001, Hosoda et al. 2002). Although it is known that the transcripts of type-A ARRs are rapidly induced upon cytokinin treatment of plants, the function of type-A ARRs is less clear (Bradstatter and Kieber 1998, Taniguchi et al. 1998, Kiba et al. 1999). There are two recent reports with regard to the function of type-A ARRs. In the one (Sweere et al. 2001), it was proposed that ARR4 acts as a factor that modulates a red light signaling through interacting with the phytochrome B photoreceptor. In another report (Osakabe et al. 2002), it was suggested that ARR4 and ARR8 are involved in a cytokinin signal transduction, each in a different way that these type-A ARRs somehow function as positive and negatively regulators, respectively. To address the relevant issue more specifically, we here characterized another example, ARR15, based on the following rationale. We previously employed a mutant of the *AHK4* gene (named *crel-1*), which shows a characteristic phenotype with an altered development of the vascular tissues in roots (Inoue et al. 2001). Among 10 members of the type-A ARR family, we found that the expres-
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Signitions of ARR15 and ARR16 in roots were selectively reduced in the cytokinin receptor mutant, suggesting a link between these response regulators and the AHK4-mediated signal transduction in roots (Kiba et al. 2002). To further examine such a putative linkage, we here characterized the function of ARR15, through isolating a T-DNA insertion mutant in the ARR15 gene, and also by constructing transgenic plants that aberrantly express the ARR15 gene in a manner independent of cytokinin.

As demonstrated previously (Kiba et al. 2002), the cytokinin-induced expression of ARR15 in roots was impaired in the cre1-1 background. To confirm this view more directly, we here employed the previously constructed transgenic plants carrying the GUS (β-glucuronidase) gene, fused to the ARR15 promoter, in Columbia (Col-0) wild-type plants (Kiba et al. 2002). The ARR15::GUS recombinant gene contains a 5′ non-coding sequence about 1,500-bp upstream of the inferred ARR15 initiation codon, which was connected to the GUS gene in frame. The resulting plants carrying the single homozygous ARR15::GUS transgene were crossed with the cre1-1 mutation in the Landsberg erecta background (Inoue et al. 2001). As an appropriate reference, the plants carrying the homozygous ARR15::GUS transgene in Col-0 were also crossed with the wild-type Ler. We selected the seeds carrying both the homozygous ARR15::GUS transgene and cre1-1 allele in the Col-0 background. With 3-day-old seedlings of such established ARR15::GUS cre1-1 plants (grown on MS gelrite-plates), together with the appropriate wild-type reference, we examined the expression of ARR15::GUS with and without cytokinin treatment (seedlings were treated by spraying a solution containing 20 μM t-zeatin) (Fig. 1). Histochemical analyses of GUS activities were carried out, as described previously (Jefferson et al. 1987). Representatives of such GUS-stained young seedlings were photographed (Fig. 1). In the stained wild-type seedlings (Fig. 1A), cytokinin-induced GUS activities were evident at a region around root tips (see arrows), as reported previously. This event was more clearly seen in an enlarged picture (Fig. 1A, bottom). In contrast, such cytokinin-induced GUS activities were hardly detected in the roots of cre1-1 plants (Fig. 1B). In the aerial parts of these plants, however, the cytokinin-induced GUS activities were evident in both the wild-type and cre1-1 plants. These results supported the view that the expression of ARR15 in roots is selectively impaired in the cre1-1 background.

To examine the function of ARR15 with special reference to cytokinin actions, a T-DNA insertion mutant of ARR15 in the Col background was isolated. After intensive genome analyses with polymerase chain reaction (PCR) with appropriate primers (denoted by S1 and A1, see Fig. 2A), a transgenic line (named KG15573 in the Kazusa T-DNA insertion bank) was confirmed to carry a T-DNA insertion in the third exon of the ARR15 gene (At1g74890). Multiple T-DNA segments (more than one) appeared to be located at the position between +554 and +555 (the first nucleotide of the inferred initiation ATG codon was taken as +1). This putative mutant allele was designated as arr15-1. T2 seeds of this line were germinated on MS agar-plates, and T3 seeds were collected for five independent plants (P1 to P5), of which two were scored to be hetero-
zygous (P1 and P2), and three homozygous (P3 to P5), with regard to the T-DNA insertion in the \textit{ARR15} gene. From resulting T4 young seedlings, RNA samples were isolated, and then, a reverse transcriptase (RT)-based PCR was carried out (Fig. 2B). Indeed, the transcript of \textit{ARR15} was hardly detected in these putative homozygous \textit{arr15-1} plants.

These T3 seeds carrying the homozygous \textit{arr15-1} allele were germinated and grown, but no obvious phenotype was observed, as compared with the wild-type plants, as far as their visible structures were concerned at any given developmental stage (they set flowers and seeds normally). We then examined these \textit{arr15-1} plants with special reference to cytokinin actions by conducting green callus formation assays with segments of roots (Fig. 2C). Responses of tissue-cultured explants were examined with varied concentrations of cytokinin (t-zeatin) and auxin [2,4-dichlorophenoxyacetic acid (2,4-D)] on appropriate MS agar-plates, as described previously (Imamura et al. 2003). After 35 d incubation, it was shown that both the wild-type and \textit{arr15-1} (P4) explants responded by forming green calli (also by forming shoots, occasionally) in a manner very similar to each other. Essentially the same results were obtained for other \textit{arr15-1} explants (P3 and P5, data not shown). We also examined the effect of cytokinin on root elongation in these mutant plants. The homozygous \textit{arr15-1} seeds (P4) were germinated on MS agar-plates, containing varied concentrations of cytokinin, and their relative root lengths were measured. The results showed that the root elongation of \textit{arr15-1} young seedlings was inhibited by cytokinin in a fashion very similar to that of wild-type seedlings (data not shown).

As noted above, the presumptive loss-of-function mutant of \textit{ARR15} provided us with no hint as to the cellular function of \textit{ARR15}. This may be explained by assuming that there might be another \textit{ARR}(s) that plays a redundant role. To circumvent such a general problem, we constructed a transgenic line that aberrantly expresses the \textit{ARR15} gene in a cytokinin-independent manner. According to a conventional \textit{Agrobacterium}-mediated DNA delivery method (Bechtold et al. 1993), we isolated several independent transgenic lines (T2 seeds), each presumably carrying the 35S-promoter::\textit{ARR15} transgene. In these transgenic plants, the entire \textit{ARR15} coding sequence was placed under the control of the cauliflower mosaic virus (CaMV) 35S promoter so as to be aberrantly (or ectopically) expressed in plants. Two independent transgenic lines of T3 seeds, homozygous and monogenic with regard to the 35S::\textit{ARR15} transgene, were established. These seeds, together with

\begin{figure}
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\caption{Isolation and characterization of a T-DNA insertion mutant of the \textit{ARR15} gene. (A) A schematic representation of the genomic \textit{ARR15} gene (MIPS code, At1g74890), in which rectangles indicate exons [ATG (+1) and TGA (+1121) specify the nucleotide positions of the translation initiation and termination codons, respectively]. In the isolated mutant (referred to as \textit{arr15-1}), a T-DNA segment was found to be located in the third exon, which encodes a portion of the \textit{ARR15} receiver domain containing the phospho-accepting amino acid (aspartate residue, D), as schematically shown. The primers used for RT-mediated PCR analyses are also shown (see arrows denoted by S1 and R1, respectively). (B) Detection of \textit{ARR15}-cDNA for the wild-type (Col-0) and five independent mutant plants each carrying the \textit{arr15-1} allele (P1-P5). Total RNA samples were isolated from 14-day-old plants treated with 20 \textmu M \textit{t}-zeatin for 2 h. They were analyzed by RT-PCR with the primers (S1 and R1). The transcript of \textit{ACT8} was also detected as an internal reference. The PCR products were analyzed by agarose-gel electrophoresis, followed by staining with etidium bromide. Both the cDNA and genomic DNA products were detected, as indicated. (C) Responses of the \textit{arr15-1} mutant plants to cytokinin. Green callus formation assays with explants (root segments) were carried out. Root segments were excised from both the wild-type and \textit{arr15-1} mutant plants, as indicated. They were cultured on plates with MS medium, each containing different concentrations of \textit{t}-zeatin and 2,4-D, as indicated. At 35 d, each representative callus was collected and photographed, after arranged appropriately.}
\end{figure}
wild-type (Col-0) seeds, were germinated on MS agar-plates. The 14-day-old plants (both leaves and roots) were treated with cytokinin (20 \( \mu \text{M} \) t-zeatin) (Kiba et al. 1999). After 30 min treatment, total RNA fractions were prepared, and then they were analyzed by Northern blot hybridization with an \textit{ARR15}-specific DNA probe (Fig. 3).

In the cytokinin-treated wild-type plants, the transcript of \textit{ARR15} was accumulated in response to cytokinin, whereas a larger amount of the corresponding transcript was detected in both the \textit{35S:ARR15} transgenic lines (L1 and L2), regardless of whether or not they were treated with cytokinin (Fig. 3A). These transgenic plants were designated hereafter as ARR15-ox. These ARR15-ox homozygous T3 seeds germinated normally on MS agar-plates (Fig. 3B, 7-day-old). They grew up well even after transfer onto soil, and then they set flowers and seeds normally.

We then examined the phenotypic alteration of ARR15-ox plants with special reference to cytokinin actions. (i) ARR15-ox explants were examined with reference to cytokinin responsiveness in green callus formation of explants, as described previously (Fig. 4). After incubation for 30 d, it was found that both the ARR15-ox transgenic lines were far less sensitive to cytokinin, as compared with wild-type plants, when the green callus and shoot formation from explants (roots) were examined with varied concentrations of t-zeatin and 2,4-D. In these ARR15-ox explants, green callus formation was not significantly induced at any concentrations of t-zeatin, conditions under which wild-type explants vigorously formed green calli and shoots (compare with the results of Fig. 2C). (ii) The inhibition of root elongation by cytokinin was also compared between the ARR15-ox and wild-type plants (Fig. 5). In these experiments, we employed the \textit{cre1-2} mutant as a critical reference, because it was reported that this cytokinin receptor mutant showed a reduced sensitivity to cytokinin in the inhibitory effect on root elongation (Inoue et al. 2001). It was found that both the \textit{cre1-2} and ARR15-ox roots were considerably resistant to the inhibitory effect of varied concentrations of cytokinin \([t\text{-zeatin and benzyl adenine (BA)}]\) (Fig. 5A and 5B, 24-D).
respectively). In other words, both the cre1-2 and ARR15-ox plants showed the same phenotype in this respect. As also shown in Fig. 5C and 5D, the responsiveness of ARR15-ox roots to other hormones [2,4-D, ACC (1-amino-1-cyclopropane-carboxylic acid)] was not significantly different from those of wild-type roots. (iii) Finally, the cytokinin-mediated induction of type-A ARR genes was examined for ARR15-ox plants (Fig. 6). The 14-day-old ARR15-ox plants were treated with cytokinin (20 \mu M t-

Based on the fact that the expressions of the type-A ARR members are exclusively induced by cytokinin at the transcriptional level (Bradstatter and Kieber 1998, Taniguchi et al. 1998), it is believed that these type-A family genes are the downstream targets of the cytokinin-mediated His→Asp phosphorelay signal transduction (Hwang and Sheen 2001, Sakai et al. 2001). Hwang and Sheen (2001) proposed more specifically that a set of type-A ARR members act as transcription repressors that mediate a negative feedback loop in the cytokinin signal transduction. Nevertheless, the function of type-A ARRs remains to be further clarified. To this end, we characterized ARR15 that appears to play a role in concert with AHK4 in roots (Fig. 1), by employing both the T-DNA insertion mutant (arr15-1) and ARR15-ox transgenic plants (Fig. 2, 3).

Unfortunately, the presumptive loss-of-function mutant (arr15-1) provided us with no insight into the cellular function of ARR15 in plants (Fig. 2). This event can be explained by assuming that some other type-A ARRs might play a similar (or redundant) function. This result is not surprising, when considering the fact that pronounced redundancy in the Arabidopsis genome is quite general (Arabidopsis Genome Initiative 2000). Combinatorial genetic analyses of His→Asp phosphorelay components must be required to complement the characterization of a single insertion mutant (e.g. arr15-1) that may or may not display any overt phenotypes. Meanwhile, the results of studies on the ARR15-ox plants provided us with crucial
of a given His→Asp phosphorelay pathway. ARR15 may function as a phospho-AHP phosphatase, thereby inhibiting a flow of phosphoryl group at such an intermediate step of signal transduction. This view is consistent with our previous in vitro observation that a type-A ARR has the ability to dephosphorylate phospho-AHP molecules (Suzuki et al. 1998). In any case, the underlying molecular mechanism remains to be clarified.

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References


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