Expression of *Arabidopsis* response regulator homologs is induced by cytokinins and nitrate

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Abstract We examined cytokinin and nitrate responsiveness in gene expression of five distinct response regulator homologs (ARR3–ARR7) in the leaves of nitrogen-starved *Arabidopsis* plants. The transcripts accumulated after spraying the shoots with *t*-zeatin. The induction of accumulation was highly specific for cytokinins. The transcripts also accumulated by supply of nitrate to the culture medium. These findings suggest that ARRs are involved in inorganic nitrogen signal transduction mediated by cytokinin as in the case of ZmCip1, a response regulator homolog recently identified in maize.

Key words: Response regulator; Nitrogen; Cytokinin; Gene expression; Two-component system; *Arabidopsis thaliana*

1. Introduction

Bacterial two-component systems are phosphotransfer signaling systems for adaptive responses to environments. Two families of signaling transduction proteins, a sensor kinase and a response regulator, have been identified in a wide range of bacterial species and in diverse eukaryotic species including fungi, yeasts and slime molds (for reviews, see [1–3]). In higher plants, small sensor kinases have been identified. *Arabidopsis* ETR1 and ERS and tomato NR (Never-ripe) are sensory histidine kinases that are involved in the ethylene signal transduction pathway [4–7]. CKI1 was identified from *Arabidopsis* and presumed to be a sensor kinase for cytokinin and function in the cytokinin signal transduction pathway [8].

Response regulators have not been found in plants. However, five distinct response regulator homologs, ARR3–ARR7, have been recently identified from *Arabidopsis* [9]. Amino acid sequences of ARRs are similar to that of the orthodox type of *Escherichia coli* response regulator, CheY. Furthermore, ARRs have been shown to function as phospho-accepting response regulators by in vivo and in vitro experiments. However, the stimuli for the response regulators have not been identified.

ZmCip1, another response regulator homolog, recently identified in maize, has been suggested to be involved in N signal transduction mediated by cytokinin [10]. The deduced amino acid sequence of ZmCip1 is highly homologous at the central region to those of ARRs (approximately 60% identity), although the amino- and carboxy-terminal extra sequences are different from each other. Such a structural similarity in the two types of response regulator homologs in plants prompted us to examine the physiological conditions in gene expression of *ARR*s in *Arabidopsis*. We report here the cytokinin- and nitrate-responsive nature of their gene expression.

2. Materials and methods

2.1. Plant material and growth conditions

The Columbia ecotype of *Arabidopsis thaliana* (L.) Heynh. was used in all experiments. Plants were grown hydroponically with MGRL culture medium containing 2 mM $Ca(NO_3)_2$ and 3 mM KNO_3 [11] in a growth chamber at 22°C under fluorescent light at an intensity of 200–250 μE/m^2/s with a photoperiod of 11 h (light)/13 h (dark). The culture medium was renewed every 3 d. For N deprivation, 4-week-old plants were grown for 11 days with MGRL culture medium lacking $Ca(NO_3)_2$ and KNO_3. The ionic balance due to the lack of $Ca(NO_3)_2$ and KNO_3 in the medium was compensated with an equivalent concentration of $CaCl_2$ and KCl. For N recovery, the plants were transferred to MGRL culture medium containing $Ca(NO_3)_2$ and KNO_3.

To examine the specificity of response of *ARR* genes to cytokinins, N-starved plants were grown on 0.4% Gelrite (Wako, Osaka, Japan) plates for 2 weeks in a growth chamber at 22°C under fluorescent light at an intensity of approximately 120 μE/m^2/s. The photoperiod was 16 h (light)/8 h (dark). The Gelrite plates contained a half concentration of Murashige-Skoog salts [12] and 2% sucrose. The source of N nutrient and its concentration was 4.5 mM potassium nitrate. The plants grown under the conditions contained approximately 2% of nitrate in leaves compared to the plants grown under nitrate-sufficient conditions.

2.2. Hormone treatment of plants

Test compounds except ethylene were dissolved in methanol to final concentrations of 100 μM. The solutions were sprayed onto the shoots of the N-starved plants with a fine mist. Ethylene treatment was performed by placing the plants in sealed acryl boxes and injecting 100% ethylene (final concentration 100 μl/l) into the boxes. The boxes were incubated in the growth chamber described earlier. After incubation, leaves were harvested, frozen in liquid N_2, and stored at −80°C until RNA extraction.

2.3. Measurement of nitrate ions

For monitoring nitrate in plants, the rosette leaves were homogenized in 0.1% (w/v) SDS and resultant extracts were centrifuged at 15 000×*g* for 10 min. The supernatants were subjected to nitrate determination with a flow injection analyzer (NOX-1000W, Tokyo Chemical Industry, Tokyo, Japan).

2.4. RNA isolation and Northern analysis

Total RNA was extracted from leaves in the presence of aurintricarboxylic acid as an inhibitor of nucleases [13] followed by lithium chloride precipitation and phenol extraction. The RNA samples were electrophoresed in a 1.2% agarose gel containing formaldehyde [14], and blotted on a nylon membrane (Hybond-N$^+$, Amersham). After fixation, the membranes were hybridized with ^{32}P-labeled probes. The probes were labeled with [α-^{32}P]dCTP using the Multiprime DNA

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Abbreviations: ABA, abscisic acid; BA, benzyladenine; CHS, chalcone synthase; 2,4-D, 2,4-dichlorophenoxyacetic acid; GA_3, gibberellic acid; NR, nitrate reductase

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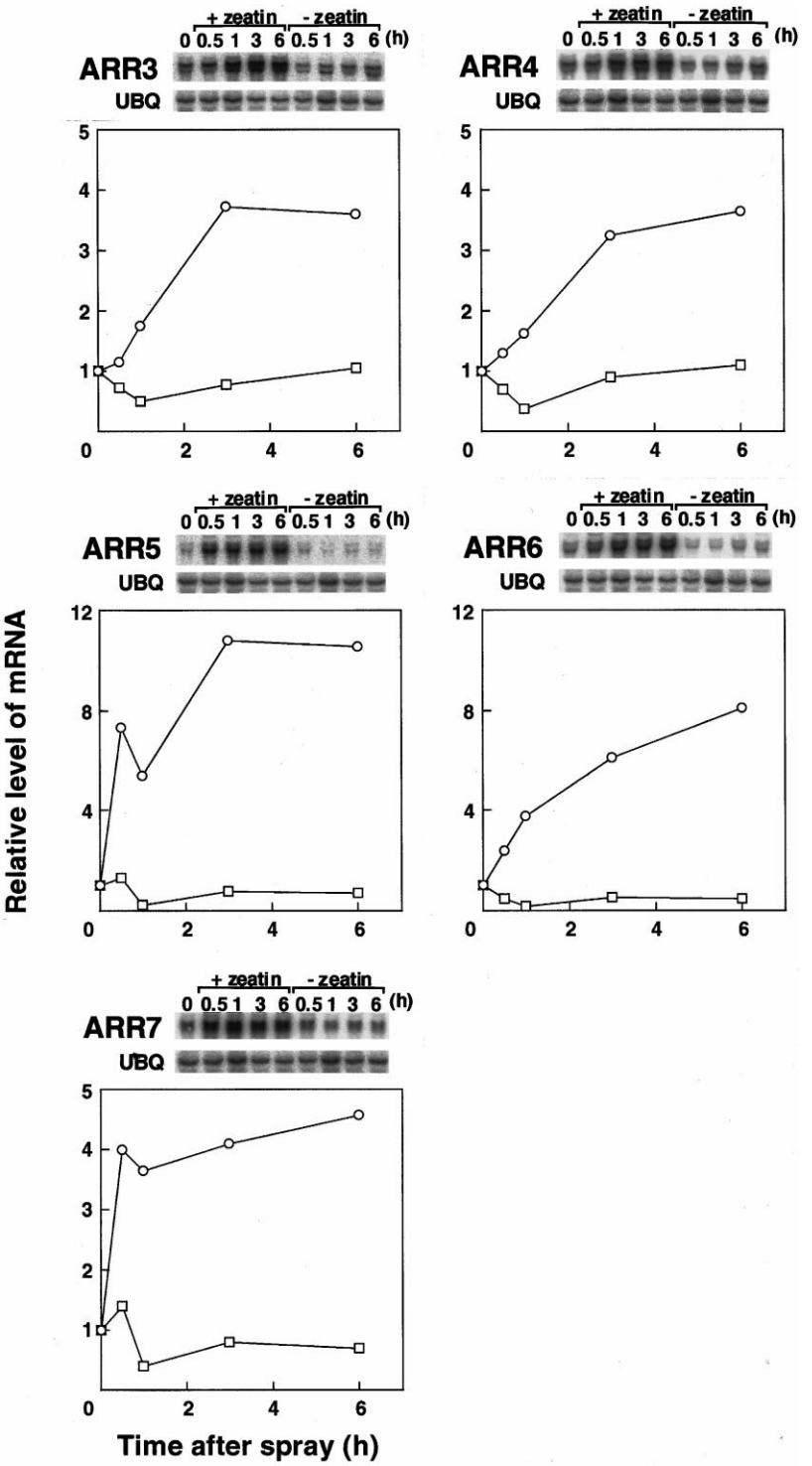

Fig. 1. Induction of accumulation of *ARR* transcripts in the leaves of N-starved *Arabidopsis* by *t*-zeatin. Total RNA (20 μg), prepared from leaves at indicated times after spraying with *t*-zeatin (100 μM) or a 0.2% methanol solution lacking zeatin, was subjected to Northern analysis with the specific gene probes for ARRs and ubiquitin (UBQ). The *ARR* transcript levels in zeatin-treated plants (○) and mock-treated plants (□) were normalized to the ubiquitin content and are shown as relative values taken as one relative unit at zero time.

Labeling System (Amersham). The inserts of *ARR* cDNA clones were used as specific probes [9]. A 3.0-kb *Eco*RI fragment of *Arabidopsis* pAtc46 was used as a probe for NR [15]. A 0.6-kb *Eco*RI/*Xho*I fragment of *Arabidopsis* pCHS3.9 was used as a probe for CHS [16]. Hybridization and washing were performed as described previously [9]. After stripping of the probes, samples were hybridized with a 1.3-kb *Xho*I/*Afl*II fragment of *Arabidopsis UBQ10* cDNA used as a probe for ubiquitin [17]. The hybridized radioactivity of each transcript was quantified by a bioimaging analyzer (BAS 2500, Fujix, Tokyo, Japan) and corrected with that of the largest ubiquitin transcript (approximately 1.6 kb) to standardize the amount of RNA blotted to the membrane.

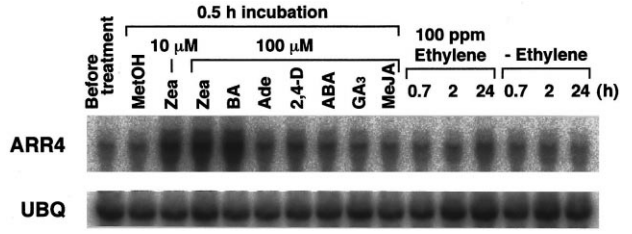

Fig. 2. Responses of *ARR4* gene expression in *Arabidopsis* leaves to phytohormones and related compounds. N-starved plants were sprayed with 0.36% methanol (MetOH), 10 μM *t*-zeatin (Zea), or 100 μM phytohormones: *t*-zeatin (Zea), BA, adenine (Ade), 2,4-D, ABA, GA_3, and methyl jasmonate (MeJA). The sprayed plants were incubated for 0.5 h in the growth chamber. In ethylene treatment, N-starved plants were exposed to ethylene or air for the indicated times. Total RNA (20 μg) was subjected to Northern analysis with the specific gene probes for ARR4 and ubiquitin (UBQ).

3. Results and discussion

To identify the environmental stimuli mediated by ARRs in transduction signaling, we examined the cytokinin responsiveness in the gene expression of *ARR*s in *A. thaliana* plants. The shoots of N-starved plants grown hydroponically were sprayed with a low concentration of methanol solution with or without 100 μM *t*-zeatin. The accumulation of *ARR* transcripts in leaves was induced after spraying with *t*-zeatin, while the levels of transcripts remained unchanged in the leaves sprayed with a solution lacking the hormone (Fig. 1). The induction pattern of accumulation of transcripts appeared to differ with the genes: *ARR5* and *ARR7* responded to *t*-zeatin earlier than *ARR3*, *ARR4* and *ARR6*. The levels of *ARR* transcripts were maintained substantially at least for 6 h after cytokinin treatment. The accumulation of *ARR* transcripts by spraying with *t*-zeatin was also observed in N-sufficient leaves but the extent of induction was low (data not shown).

We chose *ARR4* to further examine the effects of other phytohormones and compounds on the accumulation of transcripts in N-starved plants grown on gel plates. The accumulation of *ARR4* transcripts was sufficiently induced within 30 min after spraying with *t*-zeatin (Fig. 2). BA, another cytokinin, was essentially as effective as *t*-zeatin at the same concentration, but other phytohormones including 2,4-D (auxin), ABA, GA3, and ethylene and methyl jasmonate, a plant growth regulator, were less effective for the accumulation of transcripts. Adenine was also less effective. Similar results were observed in the gene expression of other *ARR*s (data not shown). The results indicate that the effects of *t*-zeatin and BA are due to their function as cytokinins. A substantial number of plant genes are known to be cytokinin-responsive including genes for msr1 (pLS216) [18], hydroxypyruvate reductase [19], CHS and dihydroflavonol reductase [20] and *C4Ppc1* [21]. Compared to these genes, the present results indicate that *ARR*s are early responsive genes to cytokinins similar to maize ZmCip1 [10].

There is an increasing body of evidence suggesting that N is an effector of cytokinin production [22–26]. As an external inorganic N, nitrate has been demonstrated to induce the accumulation of isopentenyladenosine-5′-monophosphate and/or its dephosphorylated form, isopentenyladenosine, in the roots of N-recovering maize plants with a concomitant increase in accumulation of *ZmCip1* transcript in the leaves [10]. To examine the responsiveness of *ARR*s gene expression to inorganic N, we grew plants hydroponically under N-sufficient conditions for 4 weeks and monitored nitrate contents in leaves as a parameter of N availability in plants (Fig. 3A). Nitrate contents in leaves decreased from approximately 110 μmol/g fresh weight to nothing by deletion of nitrate in the culture medium and completely recovered to the initial level after 3 days of supply of nitrate. We then monitored the transcript levels of *ARR*s in leaves during the growth process (Fig. 3B). To confirm the levels of nitrate in leaves available for gene expression, the levels of transcripts for NR [15,27] and CHS [28] which are known to be up- and down-regulated by nitrate, respectively, were examined. By addition of nitrate to N-starved plants, the NR transcript accumulated transiently, while the levels of CHS transcript decreased. In coincidence with the accumulation of NR transcript, the levels of transcripts for five *ARR*s increased by addition of nitrate to N-starved plants. This indicates that *ARR*s are nitrate-responsive in the leaves of *Arabidopsis*.

The above results clearly demonstrate that *ARR*s are cytokinin- and nitrate-responsive genes. This suggests that ARRs are involved, at least in part, in the nitrate signal transduction mediated by cytokinin in *Arabidopsis* as in the case of ZmCip1 in maize. Our results provide further evidence supporting the concept that cytokinins may be a root-to-leaf signal of external inorganic N availability to induce the response regulators in plants. Further investigations on targeting gene(s) of the response regulators and its phosphorelay systems will be necessary for understanding the signaling systems.

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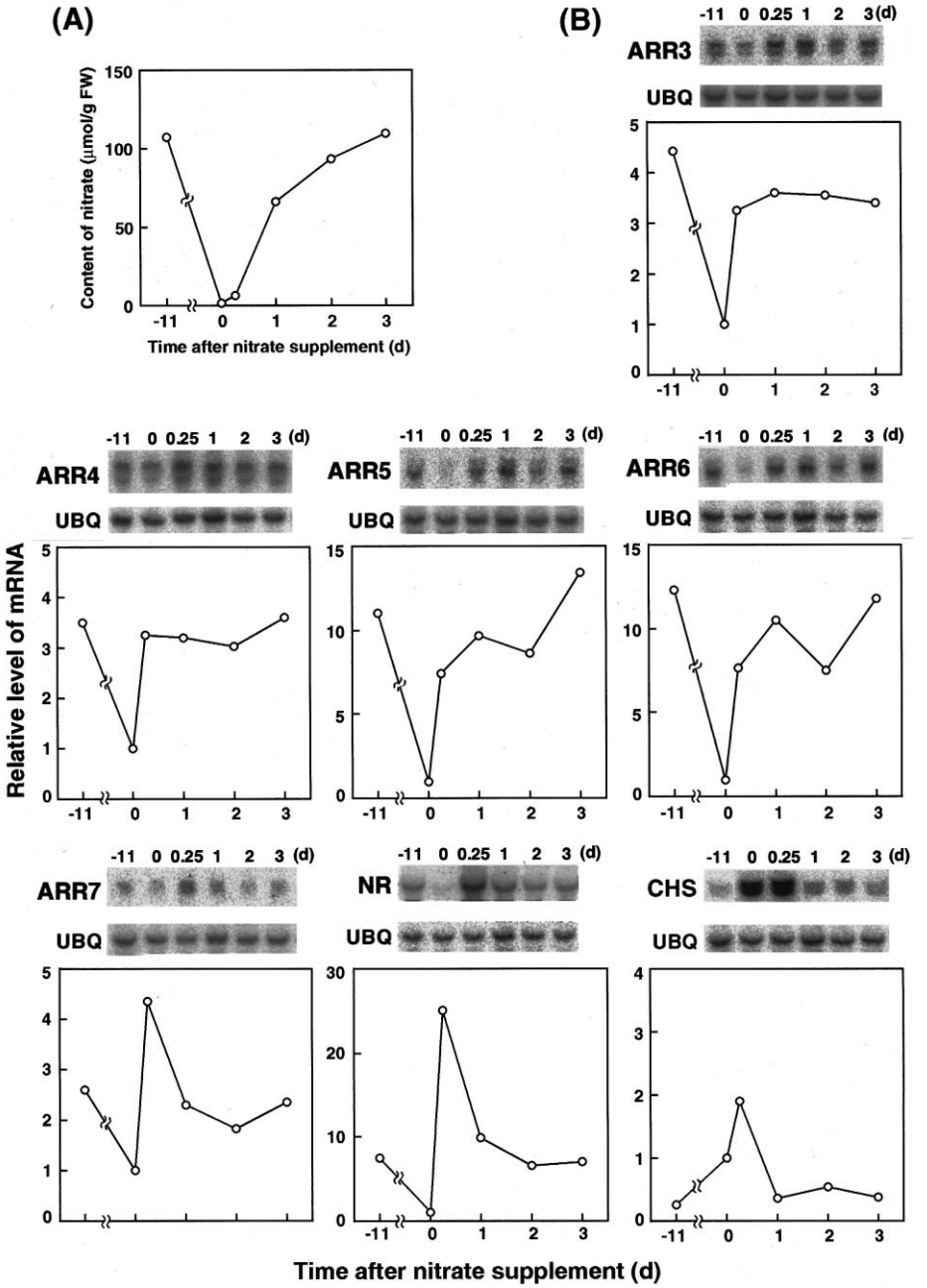

Fig. 3. Changes in the levels of *ARR* transcripts in response to nitrate availability in *Arabidopsis* leaves. A: Changes in nitrate contents in leaves during N starvation and recovery. B: Total RNA (20 μg) was subjected to Northern analysis with the specific gene probes for ARRs, nitrate reductase (NR), chalcone synthase (CHS) and ubiqutin (UBQ). The transcript levels normalized to the ubiquitin content are shown as relative values taken as one relative unit at zero time.

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