Differential Expression of Genes for Response Regulators in Response to Cytokinins and Nitrate in Arabidopsis thaliana

Takatoshi Kiba, Mitsutaka Taniguchi¹, Aya Imamura, Chiharu Ueguchi, Takeshi Mizuno and Tatsuo Sugiyama

Department of Biological Mechanisms and Functions, Graduate School of Bioagricultural Science, Nagoya University, Nagoya, 464-8601 Japan

In Arabidopsis thaliana, a number of response regulators are presumably involved in His-Asp phosphorelay signal transduction in response to environmental stimuli, such as phytohormones. Previously, it was shown that expression of a certain set of genes for response regulators are cytokinin- and nitrate-responsive in their mRNA accumulation, under certain growth conditions [Taniguchi et al. (1998) FEBS Lett. 429: 259, Brandstatter and Kieber (1998) Plant Cell 10: 1009]. To answer the critical question of whether or not other response regulator genes, so far identified in Arabidopsis thaliana, are also cytokinin-inducible, here an extended comparative examination was carried out.

It was demonstrated that not all of response regulator genes are necessarily cytokinin-responsive in their transcription. Rather, the members of a certain subfamily (type-A) are cytokinin-responsive, but those belonging to the other (type-B) are not. The presumed nitrate-responsiveness was also assessed for the same set of response regulators, and the analogous view was supported. These results suggest that the two subtypes of response regulators differ from each other, as judged from not only their structural designs, but also the expression profiles of their transcripts in response to plant stimuli.

Key words: Arabidopsis thaliana — His to Asp phosphorelay — Response regulator — Signal transduction.

Widespread His-Asp phosphorelay mechanisms are evolutionary-conserved biological tactics for intracellular signal transduction (for reviews, see Stock et al. 1989, Appleby et al. 1996). Such a phosphorelay is generally made up of "sensor histidine (His)-kinases", "response regulators", and "histidine-containing (HPt) phosphotransmitters" (for reviews, see Perkinson and Kofoid 1992, Mizuno 1998). In the higher plant, Arabidopsis thaliana, results from recent extensive studies suggested that His-Asp phosphorelays may be widely used for propagating external and/or internal stimuli, such as phytohormones (e.g., ethylene and cytokinins) (for a review, see Chang and Stewart 1998). In fact, an inspection of current databases revealed that this plant has, at least, 10 sensor His-kinases, including five ethylene-receptors (ETR1, ETR2, ERS1, ERS2, and EIN4) (Chang et al. 1993, Hua et al. 1995, Hua and Meyerowitz 1998, Sakai et al. 1998), and a putative cytokinin sensor (CKI1) (Kakimoto 1996). A set of Arabidopsis HPt phosphotransmitters were also uncovered very recently (Miyata et al. 1998, Suzuki et al. 1998). With regard to another common signal transducer, response regulators, several independent groups have demonstrated that this plant has a number of genes, each encoding a response regulator (Imamura et al. 1998, Sakai et al. 1998a, Urao et al. 1998). In this higher plant, nonetheless, clarification of the underlying His-Asp phosphotransfer signaling mechanism is at a very early stage.

Imamura et al. (1998) isolated five Arabidopsis cDNAs (named ARR3 to ARR7), each of which encodes a response regulator having a typical phosphoaccepting receiver domain. Urao et al. (1998) reported four cDNAs (ATRR1 to ATRR4), whose predicted products have structural designs very similar to those of ARRs. Subsequently, Sakai et al. (1998a) characterized two more cDNA sequences (named ARR1 and ARR2), each of which encodes a relatively large polypeptide containing a typical receiver domain followed by a large C-terminal extension. Together with these recently-uncovered response regulators, an extensive inspection of current databases revealed that this plant possesses, at least, 14 members of the family of response regulators (Imamura et al. 1999). They were proposed to be classified into two distinct subtypes (7 members in type-A, and 7 in type-B), as judged from their structural designs. Nevertheless, little is known about their biological functions. In this respect, an intriguing recent finding is that expression of some response regulator genes (e.g., ARR3, ARR4/IBC7, ARR5/IBC6, ARR6, and ARR7) are induced upon a treatment of leaves with cytokinins (e.g., t-zeatin and benzyladenine) (Brandstatter and Kieber 1998, Taniguchi et al. 1998). Interestingly, a supplement of nitrate to the plants grown on a nitrogen-starved medium also results in a rapid accumulation of transcripts of the same set of

¹ To whom correspondence should be addressed. E-mail: taniguti @agr.nagoya-u.ac.jp
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cytokinin-responsive genes. Considering the fact that all of these cytokinin-responsive genes, examined so far, appear to belong to the type-A subfamily of response regulators, except for ARR10 (Imamura et al. 1999), a critical question was then arose as to whether or not expression of any other response regulator genes, including members of the type-B subfamily, is affected by such treatments with cytokinins and nitrate. In this study, we extensively address this issue.

To this end, the following new set of response regulator were selected and characterized, in terms of their responsiveness to treatment either with cytokinins or nitrate. They were ATRR3 and ATRR4, representing the type-A subfamily, which were originally identified by Urao et al. (1998), and ARR1 and ARR2, representing the type-B subfamily, which were originally identified by Sakai et al. (1998a). A newly identified B-type response regulator, ARR10, was also included (Imamura et al. 1999).

First, to examine the cytokinin-responsive natures of these selected sets of response regulator genes, we adopted the following experimental conditions, as exactly described previously (Taniguchi et al. 1998). Columbia ecotype of Arabidopsis thaliana (L.) Heynh. was grown hydroponically for four weeks with MGR1 culture medium containing 2 mM Ca(NO\textsubscript{3})\textsubscript{2} and 3 mM KNO\textsubscript{3} (Fujiwara et al. 1992). The plants were transferred to the same fresh medium omitted the N-sources. After 11 days-cultivation, the plants were directly treated with a cytokinin (100 nM of $t$-zeatin) by spraying its fine mist onto the shoots. At time intervals (hour), total RNA fractions were isolated from the cytokinin-treated leaves (Taniguchi et al. 1998). The RNA samples were electrophoresed in 1.2% agarose gel containing formaldehyde, and then blotted onto a nylon membrane (Hybond-N+, Amersham) (Sambrook et al. 1989). These blotted filters were subjected to Northern hybridization analyses, as described previously (Imamura et al. 1998), with each specific DNA probe that was appropriately designed for each response regulator cDNA, ATRR3, ATRR4, ARR1, ARR2, respectively. These DNA probes were prepared by polymerase chain reaction (PCR) with appropriate sets of primers, which were designed according to the cDNA sequences, reported for ATRR3 (GenBank accession no. AB010917), ATRR4 (AB010918), ARR1 (AB016471), and ARR2 (AB016472), respectively. The results of such hybridization analyses were shown in Fig. 1. Note that each hybridized filter was de-probed, and then re-probed with UBQ10 (ubiquitin) cDNA, in order to take an internal and loading control for each result. The results showed that accumulation of the ATRR3 transcript was significantly induced within 60 min, depending on the cytokinin-treatment. The similar event happened on the ATRR4 transcript, although it was less evident. In contrast, both the levels of the ARR1 and ARR2 transcripts were not affected significantly by such treatment with cytokinin.

Since the ARR1 and ARR2 transcripts were not accumulated upon the cytokinin treatment ($t$-zeatin), it was needed to examine another cytokinin (benzyladenine). It was also needed to ask whether or not expression of a set of these type-B response regulators, including ARR10, is affected upon treatment with other phytohormones, such as 2,4-D auxin, abscisic acid, gibberellic acid, jasmonic acid, and ethylene. To this end, further intensive Northern hybridization analyses were carried out. The procedures are essentially the same as those described previously (Taniguchi et al. 1998). As shown in Fig. 2, in each case, no apparent response was observed at the level of their tran

**Fig. 1 Northern hybridization analyses showing transcripts of response regulator genes.** Responses in Arabidopsis leaves to cytokinin-treatment are shown, as indicated. Total RNA fractions were prepared from leaves at the indicated times (hour) after spraying with $t$-zeatin (100 $\mu$M) or a 0.2% methanol solution without $t$-zeatin. They (20 $\mu$g each) were subjected to Northern hybridization analyses with each specific probe indicated (i.e., as type-A response regulator cDNAs, ATRR3, ATRR4, and as type-B response regulator cDNAs, ARR1, ARR2), together with an appropriate probe of ubiquitin (UBQ10) as a loading control.
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Fig. 2 Northern hybridization analyses showing transcripts of response regulator genes. Responses in Arabidopsis leaves to various phytohormones and related compounds are shown, as indicated. N-starved plants were spayed with 0.36% methanol, 10 μM t-zeatin (Zea), or 100 μM each phytohormones: t-Zeatin (Zea), benzyladenine (BA), adenine (Ade), 2,4-dichlorophenoxyacetic acid (2,4-D), abscisic acid (ABA), gibberellic acid (GA), and methyl jasmontate (MeJA). In ethylene treatment, N-starved plants were exposed to ethylene or air for the indicated times in a growth chamber. Total RNA fraction were prepared from leaves of each plant, thus treated. They (20 μg each) were subjected to Northern hybridization analyses with each specific probe indicated (i.e., as type-A response regulator cDNAs, ARR3, and as type-B response regulator cDNAs, ARR1, ARR2, ARR10), together with an appropriate probe of ubiquitin (UBQ10) as a loading control.

Fig. 3 Northern hybridization analyses showing transcripts of response regulator genes. Responses in Arabidopsis leaves to nitrate-treatment are shown, as indicated. Total RNA fractions were prepared from leaves at the indicated times (day) after supplement with a nitrogen source (nitrate) (i.e., during N-starvation and N-recovery). They (20 μg each) were subjected to Northern hybridization analyses with each specific probe indicated (i.e., as type-A response regulator cDNAs, ATRR3, ATRR4; and as type-B response regulator cDNA, ARR1, ARR2, ARR10), together with an appropriate probe of ubiquitin (UBQ10) as loading control.

scripts, under our experimental conditions used. In contrast, the ARR3 (type-A) transcript was accumulated in the cytokinin treated plant leaves (not only by t-zeatin, but also by benzyladenine), as expected (Taniguchi et al. 1998) (not also that ARR3 is not identical to ATRR3, see Imamura et al. 1999).

In the light of previous finding that expression of some response regulator genes (ARR3 to ARR7) respond also to nitrate-supplement to N-starved plants (Taniguchi et al. 1998), here such nitrate-responsiveness was also examined for ATRR3, ATRR4, ARR1, ARR2, ARR10. Plants were grown under essentially the same conditions as those described above (i.e., first in the N-rich medium for 4 weeks, subsequently in the N-deficient medium for 11 d). Then, the grown plants were transferred into the same fresh medium supplemented with 2 mM Ca(NO$_3$)$_2$ and 3 mM KN0$_3$. At time intervals (day), total RNA fractions were prepared from the nitrate-supplemented plant leaves. The samples were analyzed by Northern hybridization analyses with the same sets of probes, as those used above. The results were shown in Fig. 3. The result showed that an accumulation of the ATRR3 transcript was observed transiently and shortly after the nitrate-supplement (6 h), whereas a sustained accumulation was observed for the ATRR4 transcript. In contrast, quite different profiles were observed for ARR1, ARR2, and ARR10, suggesting that their expression seems to be unaffected by such nitrate-supplement (i.e., during the recovery form N-starvation).

As mentioned above, Taniguchi et al. (1998), and Brandstatter and Kieber (1998) demonstrated independently that expression of some response regulator genes (ARR3, ATRR4/IBC7, ATRR5/IBC6, ARR6, and ARR7) are cytokinin-inducible. By confirming this previous finding, here two others (ATRR3 and ATRR4) were also de-
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0.5 h- Incubation

Type-A | ARR3
---|---
Type-B | ARR1 | ARR2 | ARR10

Fig. 2 Northern hybridization analyses showing transcripts of response regulator genes. Responses in Arabidopsis leaves to various phytohormones and related compounds are shown, as indicated. N-starved plants were sprayed with 0.36% methanol, 10 μM τ-zeatin (Zea), or 100 μM each phytohormones: τ-Zeatin (Zea), benzyadenine (BA), adenine (Ade), 2,4-dichlorophenoxyacetic acid (2,4-D), abscisic acid (ABA), gibberellic acid (GA3), and methyl jasmonate (MeJA). In ethylene treatment, N-starved plants were exposed to ethylene or air for the indicated times in a growth chamber. Total RNA fraction were prepared from leaves of each plant, thus treated. They (20 μg each) were subjected to Northern hybridization analyses with each specific probe indicated (i.e., as type-A response regulator cDNAs, ARR3, and as type-B response regulator cDNAs, ARR1, ARR2, ARR10), together with an appropriate probe of ubiquitin (UBQ10) as a loading control.

Fig. 3 Northern hybridization analyses showing transcripts of response regulator genes. Responses in Arabidopsis leaves to nitrate-supplement are shown, as indicated. Total RNA fractions were prepared from leaves at the indicated times (day) after supplement with a nitrogen source (nitrate) (i.e., during N-starvation and N-recovery). They (20 μg each) were subjected to Northern hybridization analyses with each specific probe indicated (i.e., as type-A response regulator cDNAs, ATRR3, ATRR4; and as type-B response regulator cDNA, ARR1, ARR2, ARR10), together with an appropriate probe of ubiquitin (UBQ10) as a loading control.

cytokinin treated plant leaves (not only by τ-zeatin, but also by benzyadenine), as expected (Taniguchi et al. 1998) (not also that ARR3 is not identical to ATRR3, see Imamura et al. 1999).

In the light of previous finding that expression of some response regulator genes (ARR3 to ARR7) respond also to nitrate-supplement to N-starved plants (Taniguchi et al. 1998), here such nitrate-responsiveness was also examined for ATRR3, ATRR4, ARR1, ARR2, ARR10. Plants were grown under essentially the same conditions as those described above (i.e., first in the N-rich medium for 4 weeks, subsequently in the N-deficient medium for 11 d). Then, the grown plants were transferred into the same fresh medium supplemented with 2 mM Ca(NO3)2 and 3 mM KNO3. At time intervals (day), total RNA fractions were prepared from the nitrate-supplemented plant leaves. The samples were analyzed by Northern hybridization analyses with the same sets of probes, as those used above. The results were shown in Fig. 3. The result showed that an accumulation of the ATRR3 transcript was observed transiently and shortly after the nitrate-supplement (6 h), whereas a sustained accumulation was observed for the ATRR4 transcript. In contrast, quite different profiles were observed for ARR1, ARR2, and ARR10, suggesting that their expression seems to be unaffected by such nitrate-supplement (i.e., during the recovery form N-starvation).

As mentioned above, Taniguchi et al. (1998), and Brandstatter and Kieber (1998) demonstrated independently that expression of some response regulator genes (ARR3, ATRR4/IBC7, ATRR5/IBC6, ARR6, and ARR7) are cytokinin-inducible. By confirming this previous finding, here two others (ATRR3 and ATRR4) were also de-
monstrated to be so (Fig. 1). Thus, total 7 members of the family of response regulator genes are now known to be induced at their mRNA levels in response to treatment with cytokinins under certain conditions. Interestingly, Urao et al. (1998) reported that expression of the ATRR1 (identical to ARR4/IBC7) and ATRR2 (identical to ARR5/IBC6) transcripts are induced in response to stress treatments, such as low-temperature (4°C), dehydration and salt treatment (250 mM NaCl), while those of the ATRR3 and ATRR4 genes show no such responses. Together with our results, these suggest that the 7 members belonging to the type-A subfamily may be classified further into certain subgroups, based on their responsiveness to stress treatments. This issue remains to be addressed.

Another important conclusion in this study is that not all of the response regulator genes are necessarily cytokinin-inducible in their transcription. Rather, expression of a certain set of response regulators (ARR1, ARR2, ARR10) were shown to be not cytokinin-inducible. Thus, an interesting fact is that all of the cytokinin-inducible response regulator genes belong to the type-A subfamily, whereas the remaining cytokinin-noninducible ones to the type-B subfamily, as far as the members so far tested are concerned. Thus, it was suggested that these two subtypes of response regulators differ from each other, with regard to not only their structural designs, but also their expression profiles.

It should be finally noted that a link between a cytokinin- and nitrate-responsive natures of a certain response regulator gene was originally reported for ZmCipl of maize (Sakakibara et al. 1998). This cytokinin-inducible maize response regulator gene was suggested to be implicated in an N-signal transduction mediated by cytokinin. In this context, our results in this study is compatible with the idea that an analogous linkage appears to be the case also in Arabidopsis thaliana, because expression of the type-A subfamily of response regulator genes was shown to be nitrate-responsive in a fashion parallel with their cytokinin-responsiveness (Fig. 3). However, clarification of this issue must also await further experimentation.

In short, to extend our earlier reports (Taniguchi et al. 1998, Imamura et al. 1999), here a cytokinin-inducibility was extensively examined for a number of Arabidopsis response regulator genes, and it was demonstrated that not all of them are cytokinin-inducible. Rather, the members of a certain subfamily (type-A) are cytokinin-responsive, but those belonging to the other (type-B) are not. Expression of the type-B subfamily of genes may be constitutive, since none of the tested phytohormones affected their expression (Fig. 2). Although nothing is yet known about the physiological relevance of our findings, they should shed light on the recently-emerging His-Asp phosphorelay signaling pathways that are presumably involved in adaptive responses to phytohormones in higher plants.

This study was supported by Grants-in-Aid (09274101 and 09274102 to TS and TM) for scientific research on a priority area from the Ministry of Education, Science, Sports, and Culture of Japan.

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(Received March 8, 1999; Accepted May 7, 1999)