In Arabidopsis thaliana, Histidine-to-Aspartate (His→Asp) phosphorelay is a paradigm of a signaling system that is considered to be involved in response to plant hormones, including ethylene and cytokinin. In the current framework of His→Asp phosphorelay in this higher plant, the type-B ARR (response regulator) family members appear to act as DNA-binding transcriptional regulators. Although Arabidopsis thaliana has 11 type-B ARR family members, except for ARR1 and ARR2, no biological information is available with regard to others. As the main objective of this study, we characterized another example, ARR11, in terms of not only its in vitro biochemical properties, but also its biological activity in plants. In plants, the ARR11 gene was expressed predominantly in roots. In vitro, ARR11 showed the ability to acquire a phosphoryl group from a histidine-containing phosphotransfer intermediate (AHP), and also it showed the ability to recognize a specific nucleotide sequence, GGATT. These in vitro results supported the view that ARR11 is indeed a DNA-binding transcription factor, the ability of which is most likely modulated by phosphorylation in its receiver domain. In vivo, when a C-terminal DNA-binding domain lacking the N-terminal phospho-accepting (or receiver) domain was aberrantly expressed, the resulting transgenic plants showed characteristic anomalies during development of apical parts. The observed anomalies included “unusual proliferation of tissues in cotyledons” and “outgrowth of adventitious shoots near cotyledons”. These results with regard to the functions of ARR11 are mainly discussed in comparison with those of the previously characterized type-B response regulators.

Keywords: Arabidopsis — Response regulators — Signal transduction — Cytokinin.

Abbreviations: AHK, Arabidopsis histidine kinase; AHP, Arabidopsis histidine-containing phosphotransfer factor; ARR, Arabidopsis response regulator.

Introduction

In Arabidopsis thaliana, results of recent intensive studies suggested that Histidine-to-Aspartate (His→Asp) phosphorelays are involved in the signal transduction mechanisms underlying propagation of certain environmental stimuli, such as plant hormones (e.g. ethylene and cytokinin) (for recent extensive reviews, see Chang and Stewart 1998, Hutchison and Kieber 2002, Hwang et al. 2002, Sheen 2002, Schaller et al. 2002, and references therein). This model plant has 11 histidine (His)-kinases (HK) family members, of which five members (e.g. ETR1) have been demonstrated to be ethylene receptors (Chang et al. 1993, Hua and Meyerowitz 1998). Recently, it was shown that another HK (AHK4, also known as CRE1 or WOL), together with its homologs (AHK2 and AHK3), acts as a cytokinin-receptor (Inoue et al. 2001, Suzuki et al. 2001, Ueguchi et al. 2001, Yamada et al. 2001). Furthermore, this higher plant has five genes each encoding an Hpt intermediate of phosphorelay (AHP-series) (Suzuki et al. 1998, Suzuki et al. 2000), and 22 genes each encoding a response regulator (ARR-series) (Imamura et al. 1998, and also see the reviews cited above). The members of the ARR family are basically classified into two distinct subtypes [type-A, 10 members; type-B, 11, and another atypical one (ARR22, MIPS protein code At3g04280)], based on their structural designs and cytokinin-inducible expression properties of their transcripts (Imamura et al. 1999, Kiba et al. 1999). One can thus envisage that a complex and multistep AHK→AHP→ARR phosphorelay network must be implicated in a variety of biological events in this higher plant. Indeed, two recent papers provided further evidence that such a view is highly plausible, by showing that a type-B ARR functions as a transcriptional activator for a type-A ARR gene. Sakai et al. (2001) demonstrated in plants that ARR1/ARR2 activate the ARR6 gene in a cytokinin-dependent manner. Hwang and Sheen (2001) demonstrated that ARR1/ARR2/ARR10 activate the transcription of ARR4/5/6/7 in an AHK4 and/or CKI1-dependent manner in response to cytokinin in protoplasts. They also proposed that the type-A ARR members act as repressors, which mediate a negative feedback loop in the cytokinin signaling (Hwang and Sheen 2001). When the fact that Arabidopsis thaliana has 11 HKs, 5 AHPs, and 22 ARRs was considered, a simple question arose as to which HK functions in concert with which ARR. Another question is where, when and how they play their biological roles in plants. To clarify these problems, we must characterize each His→Asp phosphorelay component, one by one. To this

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end, we here focus on the type-B ARR family members that are assumed to function as transcriptional regulators, considering that among the 11 type-B ARR members, so far only a few instances (ARR1, ARR2 and ARR10) have been characterized in vivo and in vitro (Imamura et al. 1999, Imamura et al. 2001, Lohrmann et al. 1999, Lohrmann et al. 2001, Sakai et al. 1998, Sakai et al. 2000, Sakai et al. 2001). We here chose ARR11 as another example, and then it was extensively characterized with reference to its in vitro and in vivo properties, in comparison with those reported previously for other type-B ARRs, such as ARR1 (Sakai et al. 2001).

Results and Discussion

 ARR11 as a member of the type-B ARR family

The type-B ARR family members include ARR1 (MIPS protein code, At3g16855/At3g16857), ARR2 (At4g16110), ARR10 (At4g31920), ARR11 (At1g67710), ARR12 (At2g25180), ARR13 (At2g27070), ARR14 (At2g01760), ARR18 (At5g58080), ARR19 (At1g49190), ARR20 (At3g2670) and ARR21 (At5g07210). Among these, the corresponding cDNAs have previously been isolated for ARR1, ARR2 (or APR5), ARR10 (or APR4) and ARR11 (or APR3), respectively (Imamura et al. 1999, Lohrmann et al. 1999, Sakai et al. 1998). In a strict sense, therefore, others are hypothetical. However, we recently succeeded in isolating cDNAs each corresponding to ARR12, ARR14, ARR18, ARR19, ARR20 and ARR21 (our unpublished results). To understand the common and/or specific functions of these type-B ARR family members, we must characterize these ARRs one by one, in detail and in comparison with each other.

To gain a quick hint as to this issue, a non-rooted neighbor-joining phylogenetic tree was constructed with the about 120 amino acid sequences of the phospho-accepting receiver domains (Fig. 1A), which are commonly found in the N-terminal portions of the type-B ARR family members (Fig. 1B). These ARRs also share with another common motif of about 60 amino acids, which are preceded by the receiver domain (Fig. 1B). This common motif is generally referred to as the “GARP-motif”, which is widespread in a number of plant transcription factors, including maize GOLDEN2, ARRs and Chlamidomonas PSR1 (Riechmann et al. 2000). Recently, the three dimensional structure of the ARR10 GARP-motif has been determined, demonstrating that this is indeed a sequence-specific DNA-binding motif with a helix-turn-helix structure (Hosoda et al. 2002). This is well consistent with the idea that the type-B ARR family members function as transcriptional regulators. Considering these facts, the results of phylogenetic analysis revealed that the type-B ARR family members are further classified into several sub-groups in a pair-wise manner (e.g. ARR1/ARR2, ARR10/ARR12, ARR13/ARR21, ARR19/ARR20) (Fig. 1A). ARR11, ARR14 and ARR18 appear to be exceptions (Fig. 1A). It is worth mentioning that the same view was induced from another phylogenetic tree, which was constructed with the amino acid sequences of common GARP-motifs (data not shown). Based on these considerations, we here chose ARR11 to examine its properties in comparison with the ARR1/ARR2 pair, which are so far the best-characterized type-B family members.

Expression profile of ARR11 transcript

We first isolated the corresponding cDNA of ARR11 by mean of RT-PCR (reverse transcriptase-based polymerase chain reaction) with appropriate primers. These PCR primers were designed according to the predicted ORF (open-reading-frame) of ARR11 in the current Arabidopsis database (MIPS protein code, At1g67710) (http://mips.gsf.de/proj/thal/db/index.html). The determined nucleotide sequence for the isolated cDNA showed that the deduced amino acid sequence is not exactly the same as that proposed in the database, in that the proposed one...
contains an extra stretch of 13 amino acids (SKAVKFFPVSCLA) at the middle. This is simply due to an alternative prediction of the exon–intron junction in the database. We used the isolated cDNA as a representative transcript of \( \text{ARR11} \), which encodes a polypeptide of 521 amino acids. With this cDNA as a probe, Northern blot hybridization analyses were carried out for RNA samples from different organs of \( \text{Arabidopsis} \) plants (Fig. 2). The \( \text{ARR11} \) mRNA was detected predominantly in roots, although a significant level of transcript was found in leaves. This expression profile of \( \text{ARR11} \) is similar to that reported for \( \text{ARR1} \) (Sakai et al. 1998).

Biochemical properties of \( \text{ARR11} \)

Although the deduced \( \text{ARR11} \) amino acid sequence contains a typical receiver domain (see Fig. 1B), this fact does not necessarily mean that \( \text{ARR11} \) has the ability to undergo phosphorylation. To examine this, a truncated \( \text{ARR11} \) polypeptide was purified with an \( \text{Escherichia coli} \) expression system (Fig. 3A, left panel). The purified polypeptide, designated as \( \text{ARR11-RB} \), extends from the first methionine to Asn456 (see Fig. 1B). As a possible phosphodonor toward \( \text{ARR11} \), the \( \text{AHP2} \) protein was also purified (Fig. 3A, left panel), and then it was radioactively phosphorylated with an \( \text{E. coli} \) membrane system (Fig. 3A, right panel), as described previously (Suzuki et al. 1998). \( \text{AHP2} \) is a representative of the five \( \text{AHP} \) family members, which are assumed to act as intermediates of His→Asp phosphorelay (or presumed phospho-donors toward \( \text{ARRs} \)) (Hwang and Sheen 2001, Suzuki et al. 2002). The radioactively phosphorylated \( \text{AHP2} \) polypeptide was incubated with the purified \( \text{ARR11-RB} \) polypeptide. The result showed that \( \text{ARR11-RB} \) rapidly acquired a radioactive phosphoryl group form \( \text{AHP2} \) (Fig. 3A, right panel).

The isolated \( \text{ARR11-RB} \) polypeptide also contains a GARP-motif, which is assumed to act as a DNA-recognition domain, as mentioned above. The purified polypeptide was used for an in vitro DNA-binding gel shift assay (Fig. 3B). The radioactively labeled DNA probes were 34-bp oligonucleotides (5'-cgaacgtaattactGGAATgtcctggataactg-3'), which contain a GGATT-core sequence and its mutated variants. The experimental rationale here was that these probes are exactly the same as those used for the in vitro binding assays for \( \text{ARR1/ARR2} \), conducted previously by Sakai et al. (2000). This in vitro result showed that \( \text{ARR11-RB} \) preferentially binds to GGATT (Fig. 3B). If this sequence was changed to any one of the mutated variants, the apparent DNA-binding affinity of \( \text{ARR11-RB} \) was markedly reduced. It is worth noting that both \( \text{ARR1} \) and \( \text{ARR2} \) bind more preferably to AGATT, rather than GGATT (Sakai et al. 2000), while \( \text{ARR11-RB} \) showed relatively poor ability to recognize AGATT (Fig. 3B).

**Fig. 2** Northern blot hybridization showing that \( \text{ARR11} \) is expressed predominantly in roots. Northern blot hybridization was carried out with a specific DNA probe for \( \text{ARR11} \). RNA fractions were prepared from the different organs of plants indicated. The \( \text{UBQ10} \) transcript was also detected as an internal and loading reference.

**Fig. 3** Characterization of the in vitro properties of \( \text{ARR11} \). (A) Isolation of the \( \text{AHP2} \) and \( \text{ARR11-RB} \) polypeptides and characterization of their in vitro phosphorelay activity. A set of polypeptides used in this study was purified (\( \text{AHP2} \) and \( \text{ARR11-RB} \), see Fig. 1). They were analyzed by SDS-PAGE, followed by staining with Coomassie Brilliant Blue (left panel). Lane 1, \( \text{AHPs} \) (2 \( \mu \)g); lane 2, \( \text{ARR11-RB} \) (3 \( \mu \)g). In vitro analyses of phosphotransfer between the purified \( \text{AHP2} \) and \( \text{ARR11-RB} \) polypeptides were carried out (right panel). Radioactively phosphorylated \( \text{AHP2} \) were purified (lane, 0 min), and then the isolated \( \text{ARR11-RB} \) polypeptide was added (see the arrow). At intervals (min), the samples were analyzed by SDS-PAGE, followed by autoradiography. The last lane (10 min) is an appropriate reference without addition of \( \text{ARR11-RB} \). (B) The purified \( \text{ARR11-RB} \) polypeptide was subjected to DNA binding gel-shift assays using synthetic \( ^{32} \text{P} \)-labeled DNA 34-mers containing the core GGATT sequence or the mutated variants, as indicated. Details are given in Materials and Methods.
These results as to the biochemical properties of ARR11 demonstrated that this response regulator displays both the in vitro abilities as to phosphorylation and DNA-binding. One can thus envisage that ARR11 is a DNA-binding transcription factor, the ability of which is most likely modulated by phosphorylation in its receiver domain.

Construction of transgenic plants aberrantly expressing each one of two functional domains of ARR11

We then attempted the construction of transgenic plants, which aberrantly express either the N-terminal phospho-accepting receiver domain (designated as ARR11-R; see Fig. 1B) or the C-terminal DNA-binding domain (designated as ARR11-C; also see Fig. 1B). The experimental rationale of this study is as follows. If the receiver domain alone was expressed in plants (designated tentatively as ARR11-R overexpressing plants, ARR11-R-ox), an endogenous His→Asp phosphorelay pathway might be perturbed by this artificial phospho-accepting molecule, which would potentially act as an absorber of a phosphoryl group. This presumptive event would result in a dominant-negative effect on an intrinsic His→Asp phosphorelay pathway in plants. On the other hand, if the DNA-binding domain was specifically expressed in plants (designated tentatively as ARR11-C overexpressing plants, ARR11-C-ox), the resulting truncated DNA-binding transcriptional regulator would function in a manner independent of a His→Asp phosphorelay in plants. This presumptive event would result in a dominant-positive effect on an intrinsic His→Asp phosphorelay pathway in plants. In fact, such transgenic plants have already been characterized with ARR1 (Sakai et al. 2001). In the previous report, the C-terminal DNA-binding domain of ARR1 (named ARR1-ADDK) was expressed in transgenic plants (named ARR1-ADDK-ox), demonstrating that such transgenic plants exhibited a striking phenotype with special reference to cytokinin-responses. We thus wanted to compare ARR11-C-ox plants with ARR1-ADDK-ox plants in terms of their resulting phenotypes, and wanted to ask the critical question of whether or not the events reported for ARR1-ADDK-ox plants are specific (or characteristic) to this particular response regulator.

According to a conventional Agrobacterium-mediated DNA delivery method (Bechtold et al. 1993), we isolated several independent transgenic lines (T2 seeds), each presumably carrying either 35S-promoter::ARR11-R or 35S-promoter::ARR11-C transgene. In these transgenic plants, each appropriately portion of the ARR11 coding sequence was placed under control of the cauliflower mosaic virus (CaMV) 35S promoter so as to be aberrantly (or ectopically) expressed in plants (see Fig. 1). ARR11-R was designed so as to specify a polypeptide extending from Met1 to Asn130, whereas ARR11-C was constructed so as to encompass the amino acid sequence from Asn130 to the C-terminal Asn520. In each case, these resulting transgenic plants (six independent lines of T2 seeds for each), together with wild-type plants (Columbia ecotype), were germinated on MS agar-plates (Fig. 4A, each representative is shown). These young seedlings of ARR11-R-ox and ARR11-C-ox displayed normal morphologies indistinguishable from those of wild-type plants. RNA samples were prepared from a whole plant, and then they were separated on agarose gel. Northern blot hybridization with a probe specific for the ARR11 coding sequence was carried out for the presumed ARR11-R-ox (B) and ARR11-C-ox (C) plants, respectively. The UBQ10 transcript was also detected as an internal and loading reference.

ARR11-C-ox plants show anomalies in development

These established ARR11-R-ox and ARR11-C-ox transgenic plants (T2 seeds) were germinated on MS agar-plates
containing hygromycin. Wild-type (Col.) plants containing the hygromycin-resistant HPT transgene were used as an appropriate reference. As mentioned above, their young seedlings appeared to be normal, as far as the apparent morphologies of apical parts and roots were concerned (see Fig. 4A). After being transferred into soil, ARR11-R-ox as well as wild-type plants normally grew up. The resulting adult plants flowered and set seeds. In the case of ARR11-C-ox plants, however, certain morphological alterations became apparent, after being grown for about 3 weeks on MS agar-plates (Fig. 5, panels b–e), as compared with wild-type plants (panel a). The observed phenotypic alterations were an abnormal development (or aberrant proliferation) of cotyledons (panels b and c), and an appearance of leaf-like structures arising from a junction of cotyledons and leaf-petioles (panels d and e). These leaf-like structures appear to be the outgrowth of adventitious shoots. Such events were commonly observed for most of the hygromycin-resistant seedlings (>80%), and for all the six independent ARR11-C-ox transgenic lines, although the details of such events varied slightly from one line to another (also one plant to another). In some instances (but not all), several adventitious shoots also appeared on the adaxial surface of cotyledons, suggesting de novo generation of secondary meristem on cotyledons of ARR11-C-ox plants (Fig. 5, panel f). The aberrantly proliferated cotyledons began bleaching and showing a

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**Fig. 5** ARR11-C-ox plants show morphological anomalies during development. The wild-type (Col.) and ARR11-C-ox transgenic plants were grown on MS-based agar-plates for 21 d. Pictures were taken to highlight the morphological anomalies, observed for ARR11-C-ox plants: (a) wild-type (Col.); (b) ARR11-C-ox (line 11); (c) ARR11-C-ox (line 17); (d) ARR11-C-ox (line 11); (e) ARR11-C-ox (line 17); (f) ARR11-C-ox (line 17).

**Fig. 6** Anomalously developed cotyledons, commonly observed in ARR11-C-ox plants. Plants were grown, as indicated in Fig. 5. A transverse section of wild-type cotyledon was observed under a microscope (panel a). An anomalous cotyledon of ARR11-C-ox (line 17) was detached (panel b), and its transverse section was also observed under a microscope (panel c). Note that, as compared with the wild-type cotyledon (panel a), much larger cells were seen in the mutant cotyledon, and anomalously developed large vascular tissues were also apparent (panel c). The abbreviations used are: ad, adaxial; ab, abaxial; Va, vascular tissues.
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concave structure (or a curled structure toward the adaxial side) (Fig. 5). Such aberrant proliferations of cotyledons were more apparent, when a transverse section of cotyledons was observed under a microscopy (Fig. 6). As compared with wild-type cotyledons (panel a), much larger cells were seen in the mutant cotyledons, and anomalously developed large vascular tissues were also apparent (panels c).

It should be noted here that we could detect no apparent alteration with regard to root development of ARR11-C-ox transgenic plants, as far as they were examined on MS agar-plates, as shown in Fig. 4A as an example. Therefore, we did not examine ARR11-C-ox any further, with special reference to root development. Nevertheless, this issue remains to be addressed more carefully in the light of the previous findings that the mutational lesions (named *wol* and *crel-1*) of the *AHK4* gene encoding a cytokinin receptor His-kinase result in a remarkable alteration in the development of root vascular tissues (Mahonen et al. 2000, Inoue et al. 2001, Kiba et al. 2002). In any event, it was clearly shown that ARR11-C-ox transgenic plants display a striking anomaly in the development of apical parts, namely, “unusual proliferation of tissues in cotyledons” and “outgrowth of adventitious shoots near cotyledons”.

**Fate of ARR11-C-ox seedlings**

To further examine the fate of such ARR11-C-ox plants, they were transferred into soil, and they were grown until they flowered (Fig. 7). The morphology of such adult ARR11-C-ox plants was apparently bushy with more inflorescences (panel b), as compared with wild-type plants (panel a). This is probably due to the outgrowth of axillary inflorescences. The structures of flowers of ARR11-C-ox plants (panel c) were seemingly indistinguishable from those of wild-type plants (data not shown), but the resulting carpels (or siliquae) were occasionally unusual (panels d and e). We harvested the resulting T3 seeds form these ARR11-C-ox plants (both the heterozygous and homozygous seeds with respect to the ARR11-C-ox transgenes). Essentially the same phenotypes were repeatedly observed for the seedlings from the heterozygous T3 seeds. Curiously, however, the seedlings from the homozygous T3 seeds tended to show less severe phenotypes, due to an unknown reason. The ARR11-C-ox plants may be genetically unstable, particularly when the transgene occurs in the homozygous state. In any event, it was suggested that the aberrant expression of the C-terminal domain of ARR11 containing the DNA-binding GARP-motif caused an aberrant development in certain organs and/or tissues in transgenic plants.

**Properties of ARR11-C-ox explants with special reference to cytokinin-action**

By conducting callus-formation assays with segments of hypocotyls, we then examined responses (i.e. green callus formation) of tissue-cultured explants to varied concentrations of cytokinin (*t*-zeatin) and auxin (2,4-dichlorophenoxyacetic acid, 2,4-D) (Fig. 8), as described previously (Kakimoto 1996). The results showed that the segments of hypocotyls from ARR11-C-ox transgenic explants (line 19, see the second panel) responded more sensitively by forming green callus (also by forming shoots, occasionally) to a lower concentration of *t*-zeatin, than wild-type explants did (see the first panel). In order to demonstrate this event more convincingly, other lines of ARR11-C-ox were also examined under the same conditions (see the third and forth panels, respectively), showing essentially the same results. Responsiveness of ARR11-R-ox explants on this assay was more or less the same as that seen for wild-type explants (data not shown). These results suggested that ARR11-C-ox explants exhibited an altered property as to the responsiveness to cytokinin-action (and/or auxin-action).

**Implications**

In this study, the in vitro and in vivo properties of the ARR11 response regulator were examined, in the hope of gaining further insight into the His→Asp phosphorelay signal transduction in *Arabidopsis thaliana*. The results provided us with several implications, as follows.
The in vitro results of this study provided evidence that supports the following view. ARR11 is one of the 11 type-B response regulator members, which presumably functions as a DNA-binding transcriptional regulator, whose activity appears to be modulated through phosphorylation of its intrinsic receiver domain. It was demonstrated here that ARR11 acquires a phosphoryl group from AHP2. Nevertheless, we do not know anything about the specificity of such a phosphoryl-transfer reaction in plants. The real in vivo partner of ARR11 may be other AHPs. Clarification of this problem must await further experimentation. The recognition sequence of ARR11 is GGATT, which is very similar to that recognized by ARR1. However, ARR1 binds more preferably to AGATT in vitro (Sakai et al. 2000). We also showed previously that ARR10 binds to the same AGATT sequence very efficiently (Hosoda et al. 2002). In any case, these in vitro events are not surprising because all these response regulators each have a highly homologous GARP motif that has been demonstrated recently to be a DNA-recognition helix-turn-helix module (Hosoda et al. 2002). This suggests that other type-B ARR members also most likely recognize the same and/or similar short oligonucleotide sequence(s). Solely based on such a common recognition sequence, therefore, we may not be able to formally predict any specific target gene for each type-B ARR in silico. These views, learnt from ARR11 in this study, must be kept in mind to understand the in vivo function of each type-B ARR.

Another insight was gained from the in vivo characterization of ARR11 with an artificial transgenic plant. An aberrant expression of the C-terminal DNA-binding domain of ARR11 resulted in remarkable anomalies in plant development of apical parts. ARR11-ox transgenic plants showed the phenotypes of “unusual proliferation of tissues in cotyledons” and “outgrowth of adventitious shoots near cotyledons”, suggesting an important biological role of this response regulator in plant development. Although the observed events may be an artificial and indirect action of APRR11-C-ox, this observation is at least consistent with the idea that the C-terminal DNA-binding domain of ARR11 serves as a transcriptional activator, the activity of which is negatively regulated through the phosphorylation of its N-terminal receiver domain. In any event, it should be emphasized that these phenotypic alterations in ARR11-C-ox plants are very similar to those observed previously for ARR1-ADDK-ox plants, in which the C-terminal DNA-binding domain is expressed in a similar manner (Sakai et al. 2001). The in vivo results of ARR1-ADDK-ox plants have been explained by proposing that ARR1 is implicated in a signal transduction in response to cytokinin. By analogy, one can envisage that ARR11 also implicated in a cytokinin-mediated signaling pathway. This view is supported by the results of this study from the callus-formation assays with segments of ARR11-C-ox explants (hypocotyls). Assuming that a given AHK histidine-kinase acts as an upstream cytokinin-receptor, we thus propose that, not only ARR1, but also ARR11 is also implicated in a cytokinin signaling pathway.

Arabidopsis thaliana has 11 type-B response regulators. However, their in vivo and in vitro natures have been characterized only for ARR1 (and partially in ARR2 and ARR10) (Imamura et al. 1999, Lohrmann et al. 1999, Lohrmann et al. 2001, Sakai et al. 1998, Sakai et al. 2000), thus providing an enormous challenge for us in the future. In this study, as a first step, we extensively characterized ARR11 as another example in the comparison with ARR1. The results revealed that the emerged in vivo and in vitro properties of ARR11 were very
Similar to those reported previously for ARR1, as mentioned above. Thus, an important lesson of this study is that the previously proposed scenario for ARR1 is not characteristic (or unique) to this particular type-B response regulator. Rather, it may be suggested that both ARR11 and ARR1 might exert redundant functions in plants, presumably, in cytokinin-mediated phosphorylation pathways. This is consistent with the fact that a T-DNA insertion mutant (arr1-1) shows no severe phenotype in plant development (the arr1-1 mutant plants were slightly resistant to the inhibitory effect of cytokinin on root elongation) (Sakai et al. 2001). In short, to clarify the biological function of each remaining type-B ARR member by genetic means, the important and intriguing lessons of this study must be kept in mind.

Finally, it would be worth considering at the molecular level as to how the aberrant (or ectopic) expression of ARR11-C results in the observed anomalies in plant development in ARR11-C-ox plants, in comparison with in the case of ARR1-C. Results in the observed anomalies in plant development in ARR11-C-ox plants. In contrast to the case of ARR1-ΔDDK-ox plants, it was demonstrated that at least these ARR genes were not necessarily hyperactivated in ARR11-C-ox plants (the result of ARR16 was not shown). Note that a high level of the ARR11-C transcript was indeed detected in these ARR11-C-ox transgenic plants tested (Fig. 9). Assuming that both ARR1 and ARR11 are involved in an early step in the cytokinin signal transduction pathway, therefore, one can learn from this study that ARR1 and ARR11 might activate each distinct target gene(s) in response to cytokinin. Most importantly, with each of type-B ARRs, identification of more crucial target genes, other than type-A ARR genes, must await further intensive studies to understand the whole view of the His→Asp phosphorylation signal transduction pathway in response to cytokinin, providing an enormous future challenge.

Materials and Methods

Arabidopsis and related materials

The Columbia ecotype of Arabidopsis thaliana (L.) Heynh. was used in most experiments. Plants were grown with 16 h light / 8 h dark fluorescent illumination at 22°C on soil or under continuous light on agar plates containing MS salts and 2% sucrose, unless otherwise noted, as described previously (Taniguchi et al. 1998).

Escherichia coli and related materials

E. coli K-12 strain DZ225 (F−, ΔenvZ, lacU169, araD139, rpsL, relA, flgB, thi4), carrying the plasmid-borne multicopy arcB gene, was used to prepare cytoplasmic membranes. E. coli cells were grown in Luria-broth, and the urea-treated cytoplasmic membranes purified, as described previously (Suzuki et al. 1998). The cytoplasmic membranes were used to phosphorylate the Arabidopsis AHP2 phosphotransmitter protein in vitro, as described previously. E. coli strain BL21(DE3) was used as a host for preparations of Arabidopsis ARR11-RB polypeptides, as described previously (Suzuki et al. 1998).

DNA sequencing

Sequencing of double-stranded DNA on plasmids was carried out with an automated DNA sequencer (Model 373A) (Applied Biosystems), with the recommended sequencing kits according to the manufacturer’s instructions.

PCR amplification

With appropriate pairs of primers, polymerase chain reaction (PCR) was carried out to prepare DNA segments. The conditions were primarily 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min, 25 cycles (Thermal Cycler 480, Takara Shuzo). A PCR kit was used according to the supplier’s instructions.

Plasmid construction

For overproduction of the ARR11 polypeptides, plasmids pET-ARR11-RB were constructed as follows: the ARR11-RB coding sequence was amplified from the corresponding cDNA clone by using appropriate pairs of PCR primers (see below). The amplified DNA segment was cloned into an E. coli expression vector, pET22b (+) (Novagen), so as to be placed under the T7 phage promoter. The ARR11-RB coding sequence thus cloned has an initial methionine codon from the vector and a C-terminally extended histidine-tag. The
PCR primers used are: 5'-GAGGCATAATGACTAGTAGGACAGG and 5'-CACAGGATCTTATCTTTACTTGGTCT.

Purification of ARR11-RB with Ni-Column

E. coli BL21 cells carrying pET-ARR11-RB were grown in M9-glucose medium containing 0.2% casamino acids and 15% sucrose. A cleared cell lysate was obtained by use of French Pressure Cell Press (AMINCO, IL, U.S.A.). This sample was applied onto a Ni column with the rapid affinity purification pET4His-Tag system (Novagen).

Other details were described previously (Imamura et al. 1998, Imamura et al. 1999).

Preparation of RNA and Northern blot hybridization

Total RNA was isolated from appropriate organs of Arabidopsis plants by the phenol-sodium dodecyl sulfate (SDS) method (Taniguchi et al. 1998). For Northern blot hybridization, RNA was separated in agarose gels (1%) containing 2.2 M formaldehyde, then transferred to Hybond-N+ nylon membranes. The fixed membranes were hybridized with 32P-labeled DNA fragments in 6x standard saline phosphate and EDTA (1x SSPE 0.18 M NaCl, 10 mM phosphate, 1 mM EDTA, pH 7.4), 5x Denhardt’s solution, and 0.5% SDS buffer containing 10% dextran sulfate and 100 μg/ml salmon sperm DNA, at 65°C for 18 h. The membranes were washed twice with 2x SSPE and 0.1% SDS for 15 min at room temperature, twice with 2x SSPE and 0.1% SDS for 30 min at 65°C, and then with 0.2x SSPE and 0.1% SDS for 30 min at 65°C. The washed membranes were exposed and analyzed with BAS-2000II (Fuji Photo Film).

In vitro phosphotransfer experiment

The ARR11-RB and AHP2 polypeptides were purified, as described previously (Suzuki et al. 1998). Urea-treated membranes (10 μg of protein) were incubated with the purified AHP2 polypeptide (4 μg) at 37°C in the presence of 0.05 mM [γ-32P]ATP (10,000 cpm pmol-1), 50 mM KCl and 5 mM MgCl2, in TEDG buffer (Tris-HCl, EDTA, DTT, glycerol). The reaction mixture was immediately applied onto a Sephadex G-75 column previously equilibrated with TEDG buffer. Fractions containing the labeled AHPs, which were eluted with TEDG buffer (30 μl) containing 50 mM KCl and 7 mM MgCl2. After incubation, the samples were immediately subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by autoradiography, as described above (Imamura et al. 1999).

DNA-binding gel shift assay

Purified ARR10-RB was incubated with appropriate 32P-labeled 34-bp DNA fragments, and the resulting samples analyzed on a 6% polyacrylamide gel (30 : 0.5, monomer : bis). The running buffer comprised 40 mM Tris-acetate (pH 7.4), 5 mM Na-acetate and 1 mM EDTA. DNA fragments in the gels were detected by autoradiography. Other details were described previously (Hosoda et al. 2002).

Construction of ARR11-R-ox and ARR11-C-ox transgenic plants

To construct ARR11-R-ox and ARR11-C-ox, respectively, each corresponding coding sequence of the ARR11 was isolated by PCR, then were inserted into the pSK1 vector at the XhoI site downstream of the CaMV 35S promoter, to yield pSK1-ARR11-R and pSK1-ARR11-C. This construct was transformed into Agrobacterium tumefaciens strain EHA101, and then wild-type Arabidopsis plants (Col.) were transformed by vacuum infiltration procedures, as described previously (Bechtold et al. 1993). Transgenic lines segregating the hygromycin resistance as a single locus were used in further analyses, as described in the text. The PCR primers used for these experiments were: ARR11R-ox, 5'-GGGCTTGAAGAGAAAGC- GC, and 5'-GGGCGGCGCTCATAGAGCCTCTTCTCT, ARR11C- ox, 5'-GTTCATATGAGGAGGCTGTGATCGAAGGG and 5'-CCTTATAGATTAAGATGAAATAAACCTTGGTCT.

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References


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