Possible His to Asp phosphorelay signaling in an *Arabidopsis* two-component system

Takeshi Urao[a], Shinichi Miyata[a], Kazuko Yamaguchi-Shinozaki[a,*], Kazuo Shinozaki[b]

[a] *Biological Resources Division, Japan International Research Center for Agricultural Science (JIRCAS), Ministry of Agriculture, Forestry and Fisheries, 1-2 Ohwashi, Tsukuba, Ibaraki 305-8686, Japan,*

[b] *Laboratory of Plant Molecular Biology, Institute of Physical and Chemical Research (RIKEN), Tsukuba Life Science Center, 3-1-1 Koyadai, Tsukuba, Ibaraki 305-0074, Japan*

Received 15 June 2000; accepted 23 June 2000

Edited by Gianni Cesareni

Abstract We have so far cloned a cDNA encoding a hybrid-type histidine kinase (ATHK1), three cDNAs encoding phosphorelay intermediates (ATHP1–3), and four cDNAs encoding response regulators (ATRR1–4) from *Arabidopsis thaliana*. To determine which molecules constitute a His to Asp phosphorelay pathway, we examined protein–protein interactions between them using a pairwise yeast two-hybrid analysis, as an initial step. We detected a specific interaction between ATHK1 and ATHP1. We further examined protein–protein interactions between ATHP1–3 and other histidine kinases. We detected interactions between ETR1 and all ATHPs, and between CKI1 and ATHP1 or ATHP2. Interestingly, ERS1 could not interact with any ATHPs. We also examined protein–protein interactions between ATHP1-3 and ATRR1-4. The results indicated that ATHP2 could interact with ATRR4, and that ATHP3 could interact with ATRR1 or ATRR4. However, ATHP1 could not interact with any ATRRs. On the basis of these results, we discuss the possible phosphorelay networks in an *Arabidopsis* two-component system.

Key words: Histidine kinase; Histidine-containing phosphotransfer domain; Protein–protein interaction; Response regulator; Two-component system; Yeast two-hybrid analysis; *Arabidopsis thaliana*

1. Introduction

Protein phosphorylation is a key mechanism for intracellular signal transduction in both eukaryotic and prokaryotic cells. This process is catalyzed by protein kinases, which are classified into three major groups based on their substrate specificities: serine/threonine kinases, tyrosine kinases, and histidine kinases. Histidine kinases in prokaryotes play key roles in sensing and transducing extracellular signals, including chemotactic factors, changes in osmolarity, and nutrient deficiency. This signal transduction system is mediated by phosphotransfer from a histidine residue to an aspartate residue between two types of signal transducers, histidine kinase and response regulator, and is therefore referred to as 'two-component system' or 'His to Asp phosphorelay' [1–6].

Since 1993, when bacterial-type histidine kinases were first discovered in yeast and *Arabidopsis* [7,8], a large number of histidine kinases have been described in plants (for review, see [9–17]). Genetic and biochemical analyses indicate that ETR1 and its homologs function as ethylene receptors. Two other histidine kinases, CKI1 and ATHK1, are suggested to be involved in cytokinin signaling and osmosensing processes, respectively [18,19]. Moreover, many homologous genes encoding phosphorelay intermediates and response regulators have been cloned and characterized [20–27]. It was thus apparent that the two-component system is not confined to prokaryotes.

An increase in the number of the two-component regulators has raised the question of which molecules constitute a His to Asp phosphorelay pathway. To address this issue, several studies have been conducted as an initial characterization using two strategies, yeast two-hybrid interaction and in vitro phosphotransfer analysis. First, ETR1 and ERS1 have been shown to interact directly with CTR1 by both yeast two-hybrid analysis and in vitro interaction assay [28]. Second, two closely related proteins, ATDBP1 and ATDBP2, have been identified as proteins interacting with response regulator ARR4 by yeast two-hybrid screening [29]. ATDBP1 has a sequence similarity with remorin from potato, which is a membrane-associated and uranide-binding phosphoprotein. Third, in vitro phosphotransfer analysis has indicated that ARR3 and ARR4 can accept a phosphoryl group from the phosphorylated Histidine-containing phosphotransfer (HPt) domain of AHP1 (or ATHP3) and AHP2 (or ATHP1), but that ARR10 is incapable of exhibiting phosphoacceptor activity toward any AHPs tested [25]. These results provide some preliminary insight into the presumed phosphorelay network in plants. However, there have been no reports of a comprehensive demonstration of direct protein–protein interactions between two-component regulators in plants.

In this study, we examined protein–protein interactions between histidine kinases (ATHK1, ETR1, ERS1, and CKI1) and phosphorelay intermediates (ATHP1–3), and between phosphorelay intermediates and response regulators (ATRR1–4), using a pairwise yeast two-hybrid analysis. Here, we discuss possible His to Asp phosphorelay pathways in an *Arabidopsis* two-component system based on pairwise yeast two-hybrid analysis.

2. Materials and methods

2.1. Yeast strains, media, and transformation

Yeast strain L40 was used as a host cell for a yeast two-hybrid assay [30]. Transformation to yeast cells was performed by using a lithium-acetate method [31]. The transformants were cultured on SD

*Corresponding author. Fax: (81)-298-38 6643.
E-mail: kazukoys@jircas.affrc.go.jp

0014-5793/00/$20.00
PII: S0014-5793(00)01860-3

medium (2% glucose, 0.7% yeast nitrogen base without amino acids), supplemented with appropriate amino acids for marker selection.

2.2. Plasmid construction

Expression plasmids used in this study were constructed with pBTM116 (a fusion to a LexA DNA binding protein) [30] and pVP16 (a fusion to a VP16 activation domain) [30]. To construct expression plasmids ATHK1-RD and ATHK1-RD (D1074E), the receiver domain of ATHK1 (amino acids 961–1207) was amplified by PCR with oligonucleotide primers containing a *Bam*HI or *Eco*RI site at the 5′ ends. Site-directed mutagenesis for a nucleotide substitution on Asp-1074 within the ATHK1 receiver domain was performed as previously described [19]. The PCR-amplified fragments were digested with *Bam*HI and *Eco*RI and then purified on an agarose gel. Resulting fragments were fused in-frame downstream of the coding region of the LexA DNA binding domain in pBTM116, and of the VP16 activation domain in pVP16. Expression plasmids for the cytoplasmic region of ATHK1 (amino acids 477–1207), ETR1 (amino acids 321–721), ERS1 (amino acids 321–613), CKI1 (amino acids 373–1122), and SLN1 (amino acids 547–1220) were generated by PCR-based construction, as described above. Expression plasmids for the full-length coding regions of ATHP1–3 and ATRR1–4 were also constructed by PCR, as described above.

2.3. Yeast two-hybrid assay

The transformants were cultured on His-lacking medium containing 3-aminotriazole (3AT) for 3–7 days at 30°C, and their ability to activate the expression of a *HIS3* reporter gene was tested. Subsequently, the colonies were transferred to nylon filters and the activity of a β-galactosidase reporter gene was made visible by a 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) filter assay. For the β-galactosidase assay, the colonies on the filter were cracked open by being frozen in liquid nitrogen, and then the thawed filters were placed on Whatman 3MM paper soaked in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0) containing 0.27% β-mercaptoethanol and 0.5 mg/ml X-gal. The filters were incubated at 30°C in the dark for several hours, and the appearance of blue color was monitored. In all experiments, reciprocal combinations of the pLexA and pVP16 plasmids were tested to confirm the positive interactions.

3. Results

3.1. Analysis of protein–protein interactions between ATHK1 and phosphorelay intermediates ATHP1–3

We have so far cloned a cDNA encoding a hybrid-type histidine kinase, ATHK1 [19], three cDNAs encoding phosphorelay intermediates ATHP1–3 [26], and four cDNAs encoding response regulators ATRR1–4 [22] from *Arabidopsis*. We have also shown that ATHK1 can act as an osmosensor by analyzing both sensing (input) and catalytic (output) activities with yeast osmosensing-defective mutants [19]. To analyze the protein–protein interactions between them, we examined whether ATHK1 physically interacts with ATHP1–3 by using a pairwise yeast two-hybrid analysis. It had already been shown that the physical interaction between the yeast histidine kinase SLN1 and the phosphorelay intermediate YPD1 requires the receiver domain of SLN1 [32]. Therefore, the receiver domain of ATHK1 (ATHK1-RD) and the full-length coding regions of ATHP1–3 were expressed as fusion proteins to a LexA DNA-binding domain (pLexA) and to a VP16 activation domain (pVP16), and were tested for both growth on His-lacking medium and β-galactosidase activity in yeast. Reciprocal combinations of the pLexA and pVP16 plasmids were also tested to confirm the positive interactions. ATHK1 interacted with ATHP1 (Fig. 1), but not with ATHP2 or ATHP3 (data not shown). To examine whether the phosphorylation state of ATHK1 affects the interaction between ATHK1 and ATHP1, we expressed a mutated receiver domain of ATHK1 in which a putative phosphorylation site, Asp-1074, had been substituted with Glu (ATHK1-RD(D1074E)) as a fusion protein to a LexA DNA-binding domain and to a VP16 activation domain. The mutated ATHK1-RD(D1074E) had already failed to complement an

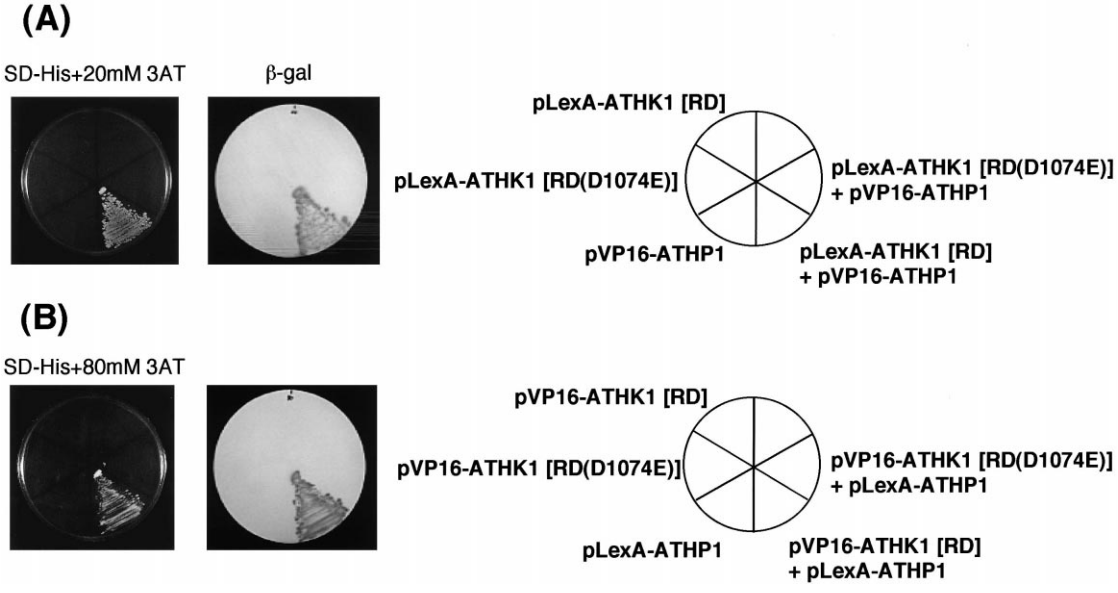

Fig. 1. Two-hybrid interactions between ATHK1 and ATHP1. A: Growth on SD medium lacking histidine supplemented with 20 mM 3AT (left). Their β-galactosidase activities in the filter assay (middle). The yeast strains transformed with indicated plasmids (right). B: Growth on SD medium lacking histidine supplemented with 80 mM 3AT (left). Their β-galactosidase activities in the filter assay (middle). The yeast strains transformed with indicated plasmids (right).

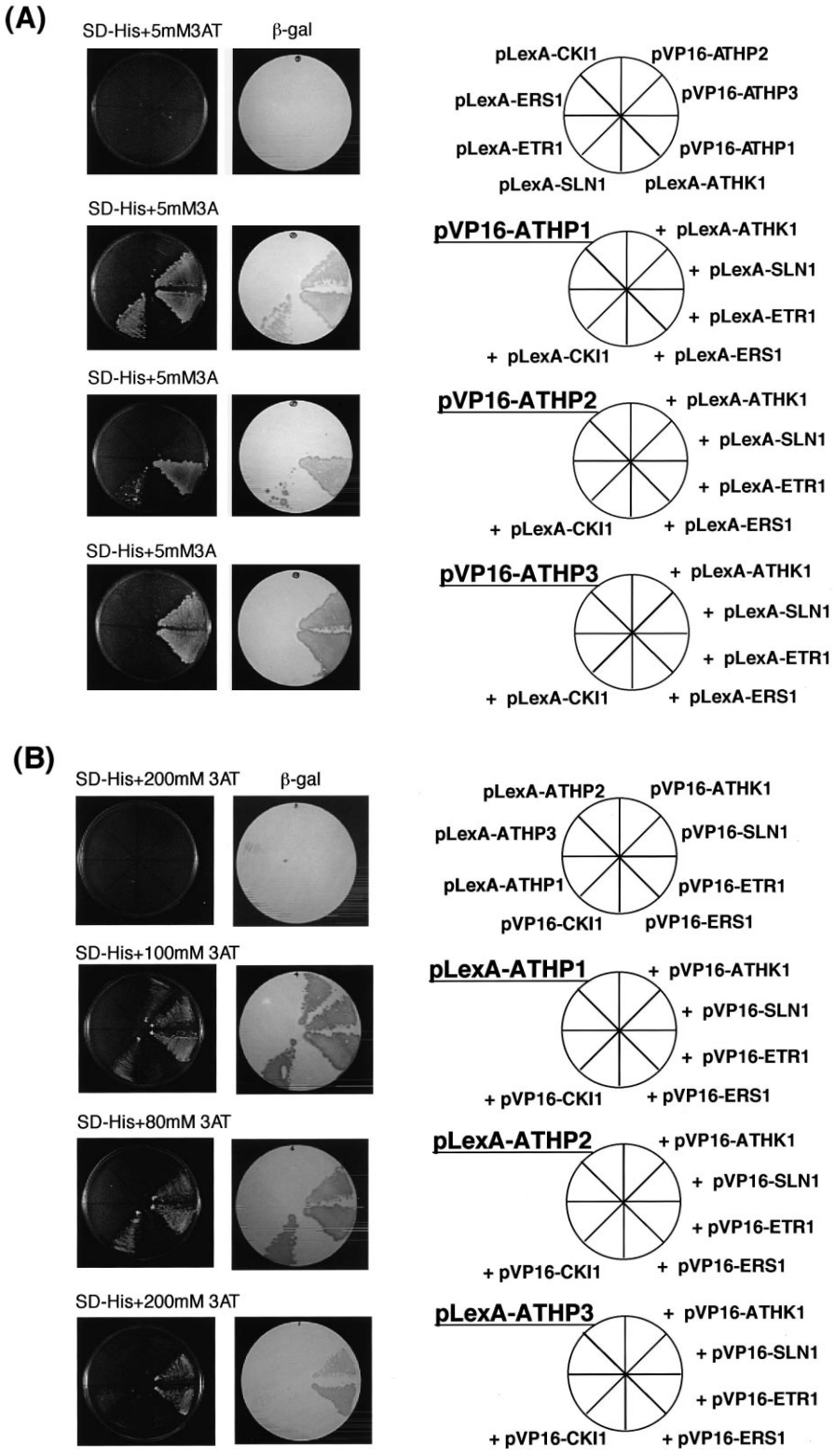

Fig. 2. Two-hybrid interactions between other histidine kinases and ATHP1-3. A: Growth on SD medium lacking histidine supplemented with 5 mM 3AT (left). Their β-galactosidase activities in the filter assay (middle). The yeast strains transformed with indicated plasmids (right). B: Growth on SD medium lacking histidine supplemented with indicated concentrations of 3AT (left). Their β-galactosidase activities in the filter assay (middle). The yeast strains transformed with indicated plasmids (right).

sln1-ts mutant [19]. As shown in Fig. 1, ATHP1 could interact with ATHK1-RD, but not with ATHK1-RD(D1074E). Each plasmid itself used as a negative control had no binding activity (Fig. 1). This interaction was confirmed by reciprocal combination of the pLexA and pVP16 plasmids (Fig. 1). These results suggest that the interaction between ATHK1 and ATHP1 depends on the phosphorylation state of the receiver domain of ATHK1.

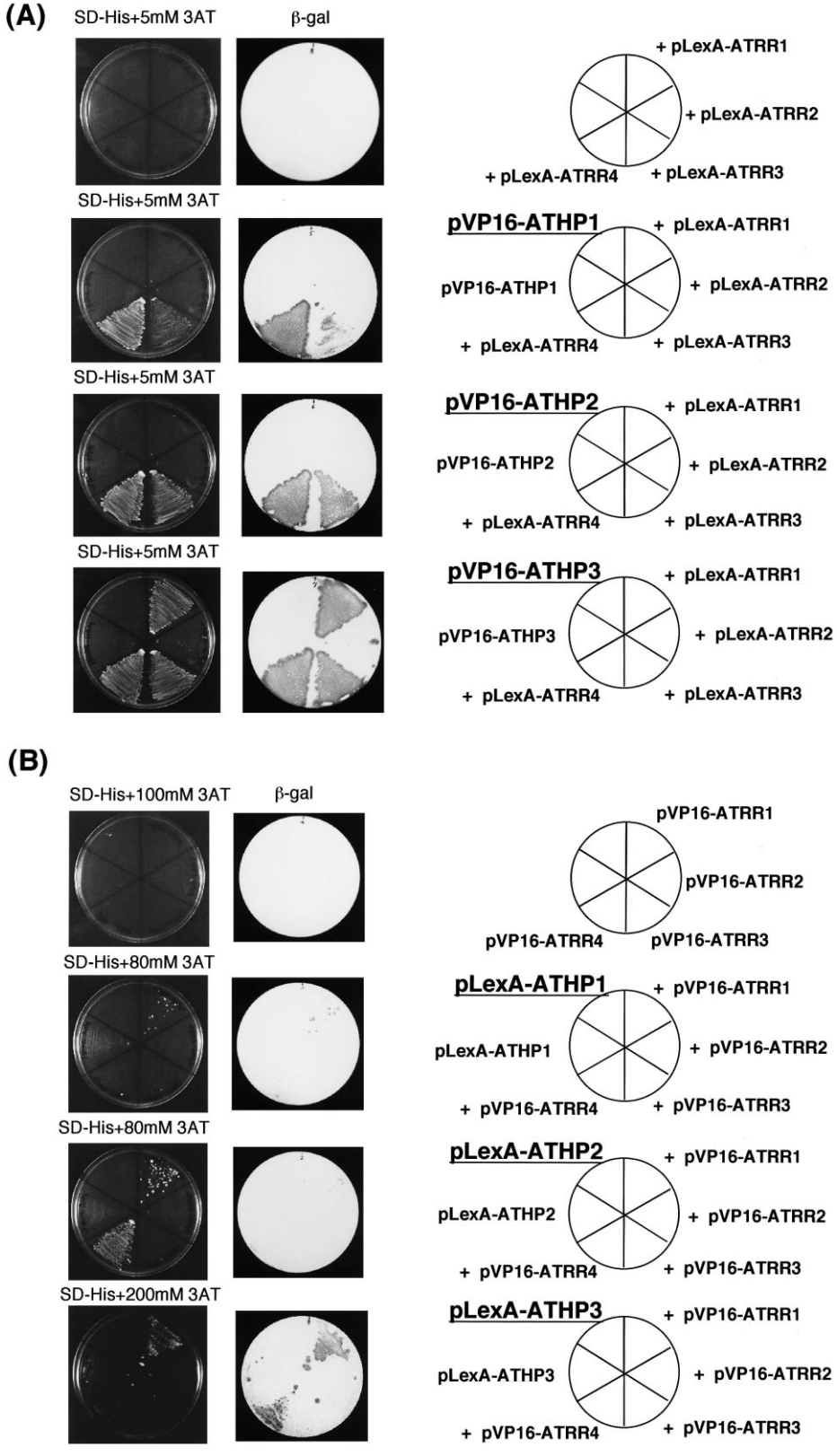

Fig. 3. Two-hybrid interactions between ATHP1-3 and ATRR1-4. A: Growth on SD medium lacking histidine supplemented with 5 mM 3AT (left). Their β-galactosidase activities in the filter assay (middle). The yeast strains transformed with indicated plasmids (right). B: Growth on SD medium lacking histidine supplemented with indicated concentrations of 3AT (left). Their β-galactosidase activities in the filter assay (middle). The yeast strains transformed with indicated plasmids (right).

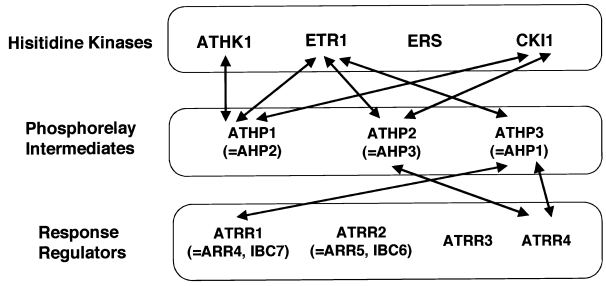

Fig. 4. Schematic presentation of possible His to Asp phosphorelays in the *Arabidopsis* two-component system. Lines indicate the direct protein–protein interactions detected in this study.

3.2. Analysis of protein–protein interactions between other histidine kinases and phosphorelay intermediates

HPt domain is implicated in His to Asp phosphorelay signaling. Indeed, functional analysis using a yeast *ypd1* mutant showed that all ATHPs act as phosphorelay intermediates between SLN1 and SSK1, a response regulator, in yeast [26]. In addition, the recombinant AHP1 (or ATHP3) protein has been shown to be phosphorylated by an uncertain histidine kinase in *Escherichia coli* membrane fractions [27]. Subsequent analysis has revealed that the phosphoryl group on the histidine residue of AHP1 (or ATHP3) and AHP2 (or ATHP1) is transiently transferred to the receiver domain of ARR3 and ARR4 (or ATRR1, IBC7) in vitro [25,27]. These results suggest that ATHPs might function as phosphorelay mediators between histidine kinases and response regulators in *Arabidopsis*.

Therefore, we examined whether ATHP1-3 can interact with other histidine kinases. To this end, we expressed the cytoplasmic regions of *Arabidopsis* ATHK1, ETR1, ERS1, and CKI1 and yeast SLN1 as fusion proteins to a LexA DNA- binding domain and to a VP16 activation domain, and tested them for direct interactions with ATHPs in both combinations of the pLexA and pVP16 plasmids. As shown in Fig. 2, we detected direct interactions between ETR1 and all ATHPs, and between CKI1 and ATHP1 or ATHP2. We also detected interactions between yeast SLN1 and ATHP1 or ATHP3 (Fig. 2). However, ERS1 could not interact with any ATHPs tested (Fig. 2). Each plasmid alone showed no activity, and we observed the specific interactions were only when both the pLexA and pVP16 plasmids were coexpressed (Fig. 2).

3.3. Analysis of protein–protein interactions between phosphorelay intermediates and response regulators

Recent study has shown that genes for response regulators, *ARR3*, *ARR4* (or *ATRR1*, *IBC7*), *ARR5* (or *ATRR2*, *IBC6*), *ARR6*, and *ARR7*, are induced by exogenous cytokinins, but not by any other plant hormones [23,33]. Moreover, reapplication of nitrate to N-starved plants also resulted in the accumulation of the transcripts, as previously observed in maize *ZmRR1* [20]. These results led to a hypothesis that these cytokinin-responsive ARRs function with CKI1 in the nitrate signal transduction mediated by cytokinin in *Arabidopsis*. To gain further evidence to support this hypothesis, we examined whether ATHP1–3 can interact with ATRR1–4. To this end, we expressed the full-length coding regions of ATRR1-4 as fusion proteins to the LexA DNA-binding domain and to the VP16 activation domain, and tested the direct interactions with ATHP1–3 in both combinations of the pLexA and pVP16 plasmids. We detected interactions between ATHP2 and ATRR4, and between ATHP3 and ATRR1 or ATRR4 (Fig. 3). No interaction was detected between ATHP1 and any other ATRRs tested (Fig. 3). These interactions were evident only when both the pLexA and pVP16 plasmids were coexpressed, and neither plasmid itself showed any activity (Fig. 3).

4. Discussion

In this study, we comprehensively examined protein–protein interactions between histidine kinases (ATHK1, ETR1, ERS1, and CKI1) and phosphorelay intermediates (ATHP1–3), and between phosphorelay intermediates and response regulators (ATRR1–4), using a pairwise yeast two-hybrid analysis. Fig. 4 summarizes our results.

ATHK1 could interact only with ATHP1, and the receiver domain of ATHK1 was sufficient for the interaction (Fig. 1). We also examined interactions between the cytoplasmic region, including the receiver domain of ATHK1 and ATHP1, as examined for other histidine kinases (Fig. 2). However, no interaction was found in both combinations of the pLexA and pVP16 plasmids. A significant interaction was found only when the cytoplasmic region of ATHK1 was fused to the VP16 activation domain (Fig. 2). The structure of the cytoplasmic region of ATHK1 may have be come abnormal, when it was expressed as a fusion protein to the LexA DNA-binding domain in yeast.

It is noteworthy that ATHP1 could interact with ATHK1-RD, but not with ATHK1-RD(D1074E) (Fig. 1). This result may indicate that the phosphorylation on Asp-1074 is necessary for sufficient binding to ATHP1, although we do not know whether the Asp-1074 of ATHK1 was phosphorylated in yeast cells. A previous study indicated that the receiver domain of SLN1 interacts with YPD1, and that the phosphoryl group on Asp within the receiver domain of SLN1 can be transferred to the His of YPD1 [32]. Therefore, it is possible that only phosphorylated receiver domains can interact with HPt domains, and that unphosphorylated receiver domains cannot. Alternatively, the replacement of Asp by Glu may cause conformational changes in the receiver domain of ATHK1 and eliminate the ability to interact with the HPt domain of ATHP1. In this regard, it is of interest to note that a substitution of Asp with Glu within the receiver domain of SKN7, a yeast response regulator, has been shown to mimic phosphorylation of Asp and to cause constitutive activation of SKN7 in vivo [34]. Therefore, it is also possible that the failure of ATHK1-RD(D1074E) to interact with ATHP1 is due to the mimicking of phosphorylation, rather than dephosphorylation, of Asp-1074 of ATHK1. In any event, the phosphorylation state of Asp-1074 might affect the interaction between ATHK1 and ATHP1.

ETR1 could interact with all ATHPs (Fig. 2). This indicates that all ATHPs are potential downstream targets for ETR1. Recently, ETR1 and ERS1 have been shown to interact directly with CTR1 by both yeast two-hybrid analysis and in vitro binding assay [28]. Epistasis analysis indicated that CTR1 acts downstream of ETR1 [35]. CTR1 is a protein kinase that resembles a Raf protein kinase, a MAP kinase kinase kinase (MAPKKK), which suggests that the ethylene signaling pathway is similar to the yeast osmoregulation path-

way. This interaction requires the N-terminal domain of CTR1 and the kinase domain of ETR1 or ERS1. The N-terminal domain of Raf, which corresponds to the region that is necessary for the interaction with ETR1 and ERS1, has been shown to be associated with a number of signal transducers such as Ras and the 14-3-3 proteins, and therefore is believed to function as a regulatory domain. This observation, together with the direct interaction between ETR1 and CTR1, led to a hypothesis that the activity of CTR1 is regulated by direct interaction with ETR1. As shown in this study, however, ETR1 could interact with all ATHPs (Fig. 2). Therefore, it is likely that other components, such as ATHP1-3, containing an HPt domain, are also involved in the formation of the ETR1–CTR1 complex, and that the activity of CTR1 is regulated by output activity initiated from ETR1-mediated phosphorelay signaling.

Interestingly, ERS1 could not interact with any ATHPs (Fig. 2). Among the histidine kinases examined in this study, only ERS1 does not have a receiver domain. We showed that the receiver domain of ATHK1 could interact with the HPt domain of ATHP1 (Fig. 1). As described previously, the interaction between SLN1 and YPD1 requires the receiver domain of SLN1 [32]. These results imply that HPt domains can mediate phosphotransfer between hybrid-type histidine kinases and response regulators in multi-step phosphorelay. Therefore, the lack of a receiver domain by ERS1 may account for the failure to interact with ATHPs.

CKI1 could interact with ATHP1 or ATHP2 (Fig. 2). ATHP2 could interact with ATRR4, but ATHP1 could not interact with any ATRRs (Fig. 3). It has recently been shown that AHP2 (or ATHP1) can transfer the phosphoryl group to ARR3 and ARR4 (or ATRR1) [25]. These observations suggest that ATHP1 may function as a phosphorelay intermediate between ATHK1 and ATRR1. Therefore, it is possible that CKI1, ATHP1, and ATRR1 function together in a particular two-component system involved in cytokinin signal transduction in *Arabidopsis*. In this regard, it is noteworthy that CKI1 is suggested to function as a cytokinin receptor [18], and ATRR1 is induced by cytokinin [23,33].

In conclusion, we showed several possible signal flows and cross-talks between two-component regulators in this study. Although our data may indicate His to Asp phosphorelay networks in a plant two-component system, this study, using a yeast two-hybrid interaction analysis, is the first comprehensive demonstration of their physical relationships. To verify the possible signaling pathways presented here, further experiments, such as analysis of phospho-transfer activity in vitro or in vivo using yeast mutants, are currently under way.

Acknowledgements: We thank Drs. Tatsuo Kakimoto (Osaka University, Osaka, Japan) and Tatsuya Maeda (Cancer Institute, Tokyo, Japan) for providing us with the CKI1 cDNA and the *SLN1* gene, respectively. This work was supported in part by a grant from the Program for Promotion of Basic Research Activities for Innovative Bioscience to K.Y.-S. It was also supported in part by the Special Coordination Fund of the Science and Technology Agency of the Japanese Government, a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan, and the Human Frontier Science Program to K.S.

References

[1] Parkinson, J.S. and Kofoid, E.C. (1992) Annu. Rev. Genet. 26, 71–112.
[2] Alex, L.A. and Simon, M.I. (1994) Trends Genet. 10, 133–138.
[3] Swanson, R.V., Alex, L.A. and Simon, M.I. (1994) Trends Biochem. Sci. 19, 485–490.
[4] Mizuno, T., Kaneko, T. and Tabata, S. (1996) DNA Res. 3, 401–414.
[5] Wurgler-Murphy, S.M. and Saito, H. (1997) Trends Biochem. Sci. 22, 172–176.
[6] Mizuno, T. (1997) DNA Res. 4, 161–168.
[7] Ota, I.M. and Varshavsky, A. (1993) Science 262, 566–569.
[8] Chang, C., Kwok, S.F., Bleecker, A.B. and Meyerowitz, E.M. (1993) Science 262, 539–544.
[9] Ecker, J.R. (1995) Science 268, 667–675.
[10] Chang, C. and Meyerowitz, E.M. (1995) Proc. Natl. Acad. Sci. USA 92, 4129–4133.
[11] Chang, C. (1996) Trends Biochem. Sci. 21, 129–133.
[12] Bleecker, A.B. and Schaller, G.E. (1996) Plant Physiol. 111, 653–660.
[13] Kieber, J.J. (1997) Annu. Rev. Plant Physiol. Plant Mol. Biol. 48, 277–296.
[14] Chang, C. and Stewart, R. (1998) Plant Physiol. 117, 723–731.
[15] Bleecker, A.B. (1999) Trends Plant Sci. 4, 269–274.
[16] D'Agostino, I.B. and Kieber, J.J. (1999) Trends Biochem. Sci. 24, 452–456.
[17] Urao, T., Shinozaki, K.-Y. and Shinozaki, K. (2000) Trends Plant Sci. 5, 67–74.
[18] Kakimoto, T. (1996) Science 274, 982–985.
[19] Urao, T., Yakubov, B., Satoh, R., Yamaguchi-Shinozaki, K., Seki, M., Hirayama, T. and Shinozaki, K. (1999) Plant Cell 11, 1743–1754.
[20] Sakakibara, H., Suzuki, M., Takei, K., Deji, A., Taniguchi, M. and Sugiyama, T. (1998) Plant J. 14, 337–344.
[21] Imamura, A., Hanaki, N., Umeda, H., Nakamura, A., Suzuki, T., Ueguchi, C. and Mizuno, T. (1998) Proc. Natl. Acad. Sci. USA 95, 2691–2696.
[22] Urao, T., Yakubov, B., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1998) FEBS Lett. 427, 175–178.
[23] Brandstatter, I. and Kieber, J.J. (1998) Plant Cell 10, 1009–1019.
[24] Sakai, H., Aoyama, T., Bono, H. and Oka, A. (1998) Plant Cell Physiol. 39, 1232–1239.
[25] Imamura, A., Hanaki, N., Nakayama, A., Suzuki, T., Taniguchi, M., Kiba, T., Ueguchi, C., Sugiyama, T. and Mizuno, T. (1999) Plant Cell Physiol. 40, 733–742.
[26] Miyata, S., Urao, T., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1998) FEBS Lett. 437, 11–14.
[27] Suzuki, T., Imamura, A., Ueguchi, C. and Mizuno, T. (1998) Plant Cell Physiol. 39, 1258–1268.
[28] Clark, K.L., Larsen, P.B., Wang, X. and Chang, C. (1998) Proc. Natl. Acad. Sci. USA 95, 5401–5406.
[29] Yamada, H., Hanaki, N., Imamura, A., Ueguchi, C. and Mizuno, T. (1998) FEBS Lett. 436, 76–80.
[30] Vojtek, A.B., Hollenberg, S.M. and Cooper, J.A. (1993) Cell 74, 205–214.
[31] Ito, H., Fukuda, Y., Murata, K. and Kimura, A. (1983) J. Bacteriol. 153, 163–168.
[32] Posas, F., Wurgler-Murphy, S.M., Maeda, T., Witten, E.A., Thai, T.C. and Saito, H. (1996) Cell 86, 865–875.
[33] Taniguchi, M., Kiba, T., Sakakibara, H., Ueguchi, C., Mizuno, T. and Sugiyama, T. (1998) FEBS Lett. 429, 259–262.
[34] Brown, J.L., Bussey, H. and Stewart, R.C. (1994) EMBO J. 13, 5186–5194.
[35] Kieber, J.J., Rothenberg, M., Roman, G., Feldmann, K.A. and Ecker, J.R. (1993) Cell 72, 427–441.