Arabidopsis type B cytokinin response regulators ARR1, ARR10, and ARR12 negatively regulate plant responses to drought

Kien Huu Nguyen[a,1], Chien Van Ha[b,1], Rie Nishiyama[a,1], Yasuko Watanabe[a], Marco Antonio Leyva-González[c], Yasunari Fujita[d], Uven Thi Tran[a], Weiqiang Li[a], Maho Tanaka[e], Motoaki Seki[e,f], G. Eric Schaller[g], Luis Herrera-Estrella[h,2], and Lam-Son Phan Tran[a,2]

[a]Signaling Pathway Research Unit, RIKEN Center for Sustainable Resource Science, Yokohama 230-0045, Japan; [b]National Key Laboratory of Plant Cell Biotechnology, Agricultural Genetics Institute, Vietnamese Academy of Agricultural Science, Hanoi 100000, Vietnam; [c]Department of Applied Biotechnology, StelaGenomics México, Irapuato, Guanajuato 36821, Mexico; [d]Biological Resources and Post-Harvest Division, Japan International Research Center for Agricultural Sciences, Tsukuba, Ibaraki 305-8686, Japan; [e]Plant Genomic Network Research Team, RIKEN Center for Sustainable Resource Science, Yokohama 230-0045, Japan; [f]Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology (JST), Kawaguchi, Saitama 332-0012, Japan; [g]Department of Biological Sciences, Dartmouth College, Hanover, NH 03755; and [h]Laboratorio Nacional de Genómica para la Biodiversidad (Langebio)/Unidad de Genómica Avanzada, Centro de Investigación y Estudios Avanzados del Instituto Politécnico Nacional, Irapuato, Guanajuato 36500, Mexico

Contributed by Luis Herrera-Estrella, January 13, 2016 (sent for review December 6, 2015; reviewed by Michael Hasegawa and Yan Wu)

In this study, we used a loss-of-function approach to elucidate the functions of three *Arabidopsis* type B response regulators (ARRs)—namely ARR1, ARR10, and ARR12—in regulating the *Arabidopsis* plant responses to drought. The *arr1,10,12* triple mutant showed a significant increase in drought tolerance versus WT plants, as indicated by its higher relative water content and survival rate on drying soil. This enhanced drought tolerance of *arr1,10,12* plants can be attributed to enhanced cell membrane integrity, increased anthocyanin biosynthesis, abscisic acid (ABA) hypersensitivity, and reduced stomatal aperture, but not to altered stomatal density. Further drought-tolerance tests of lower-order double and single mutants indicated that ARR1, ARR10, and ARR12 negatively and redundantly control plant responses to drought, with ARR1 appearing to bear the most critical function among the three proteins. In agreement with these findings, a comparative genome-wide analysis of the leaves of *arr1,10,12* and WT plants under both normal and dehydration conditions suggested a cytokinin (CK) signaling-mediated network controlling plant adaptation to drought via many dehydration/drought- and/or ABA-responsive genes that can provide osmotic adjustment and protection to cellular and membrane structures. Expression of all three *ARR* genes was repressed by dehydration and ABA treatments, inferring that plants down-regulate these genes as an adaptive mechanism to survive drought. Collectively, our results demonstrate that repression of CK response, and thus CK signaling, is one of the strategies plants use to cope with water deficit, providing novel insight for the design of drought-tolerant plants by genetic engineering.

cytokinin signaling | drought adaption | comparative transcriptome analysis | type B response regulators

Water deficit is one of the most detrimental environmental stresses commonly encountered by plants, resulting in a significant loss of crop yield worldwide. To deal with drought, plants have evolved various adaptive strategies during the process of evolution that help them survive. Various phytohormones, including abscisic acid (ABA) and cytokinin (CK), have been known to regulate the protective responses in plants to drought (1, 2). During water deficit, ABA accumulates to help plants survive by different mechanisms, such as promotion of stomatal closure, acceleration of leaf senescence, and induction of the biosynthesis of various protective metabolites. In contrast, CK is known to delay both stomatal closure and leaf senescence, and thus, to overcome drought, CK biosynthesis has been reported to be repressed at some critical stages in plants with the involvement of ABA (2–5). These findings suggest the existence of an interaction between ABA and CK, and perhaps other hormones, and that regulation of ABA and CK homeostases is critical for plant adaptation to drought (1, 6, 7).

Recently, genetic studies in *Arabidopsis* have provided evidence that CK acts as negative regulator of plant adaptation to drought through the CK phosphorelay system (4, 6, 7), which consists of three CK receptor *Arabidopsis* histidine kinases (AHKs), five authentic His-containing phosphotransfer proteins (AHPs), and 21 typical *Arabidopsis* response regulators (ARRs) (8). The ARRs are further classified into type A and type B ARRs, of which the 10 type A *ARR*s are CK-inducible, and the 11 type B *ARR*s are not CK-inducible. Type A ARRs, which act as negative feedback regulators of CK signaling, have only a receiver domain, whereas type B ARRs, which mediate the CK-regulated gene expression as myeloblastosis (MYB)-like transcription factors (TFs), have both receiver and output domains. Like many gene families in *Arabidopsis*, significant functional overlap has been observed within the AHK, AHP, type A ARR,

Significance

Cytokinin regulates plant drought adaptation via a multistep component system consisting of histidine kinases, histidine phosphotransfer proteins, and type A and B response regulators (RRs). The functional dissection of individual members of cytokinin signaling and identification of their downstream targets in drought responses are of high importance to provide a complete picture of how cytokinin controls plant drought adaptation. Previous studies have identified functions of several histidine kinases, histidine phosphotransfer proteins, and type A RRs in drought responses of *Arabidopsis*; however, the roles of type B RRs remain elusive. This comprehensive functional analysis of three type B RRs provides further insight into how cytokinin signaling regulates plant drought adaptation through the proposed yin-yang strategy, enabling efficient application of cytokinin biology in stress tolerance-oriented plant biotechnology.

Author contributions: L.H.-E. and L.-S.P.T. designed research; K.H.N., C.V.H., R.N., Y.W., Y.F., U.T.T., and M.T. performed research; M.S. and G.E.S. contributed new reagents/analytic tools; K.H.N., C.V.H., R.N., Y.W., M.A.L.-G., W.L., L.H.-E., and L.-S.P.T. analyzed data; and L.H.-E. and L.-S.P.T. wrote the paper.

Reviewers: M.H., Purdue University; and Y.W., Wuhan University.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE74880).

[1]K.H.N., C.V.H., and R.N. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: lherrera@langebio.cinvestav.mx or son.tran@riken.jp.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1600399113/-/DCSupplemental.

and type B ARR families, as evidenced by results of comparative analyses of single and multiple mutants in CK responses (8).

Among the CK signaling elements, a number of studies have indicated that the AHK2 and AHK3, as well as the AHP2, AHP3, and AHP5, play a negative and redundant role in the adaptation of plants to drought in both ABA-dependent and -independent manners (9–11). However, only scant genetic evidence is available with regard to the functions of the ARRs in drought responses, perhaps because of the significantly higher number of *ARR* genes and the complexity resulted from their cross-talk (8, 12). Only a preliminary phenotyping study of three higher-order type A *arr3,4,5,6*, *arr5,6,8,9*, and *arr3,4,5,6,8,9* mutants is available in the literature, which suggests that type A ARR3 and -4 might act as positive regulators, and ARR8 and -9 as negative regulators in plant responses to water stress stimulated by sorbitol (13). On the other hand, no reports are yet available with regard to the function of type B ARRs in plant responses to drought.

Given that dissection of the role of each component of the CK phosphorelay system in drought responses is an important and challenging task, in this study we used a loss-of-function approach to characterize detailed functions of three type B ARR members—namely ARR1, ARR10, and ARR12—in regulating drought responses. Various combinations of *arr* single, double, and triple mutants were explored to elucidate the functions of these three ARRs, as well as the mechanisms by which they modulate *Arabidopsis* plant adaptation to drought. Our results demonstrate that ARR1, ARR10, and ARR12 redundantly act as negative regulators of drought responses in both ABA-dependent and -independent pathways. We also identify an enhanced ABA response in the *arr1,10,12* triple mutant, which is an evidence for cross-talk between ABA and CK signalings, and may be critical for its higher drought tolerance relative to WT. Furthermore, retention of leaf water content, maintenance of cell membrane stability, and enhancement of anthocyanin biosynthesis were found to contribute to the enhanced drought tolerance of the *arr1,10,12* mutant. Comparative genome-wide analyses of the *arr1,10,12* triple mutant and WT reveal several potential pathways, including the anthocyanin biosynthesis pathway, that are activated by mutations of *ARR1*, *ARR10*, and *ARR12* under both normal and dehydration conditions, further strengthening our findings.

Results

arr1,10,12 Triple Mutant Displays Enhanced Tolerance to Drought. Previous reports studying the functions of AHKs and AHPs in drought responses demonstrated that among the various combinations of *ahk* and *ahp* mutations, the *ahk2,3* double and *ahp2,3,5* triple mutants showed the strongest tolerance to drought (9–11). Mutants harboring mutated alleles of these two *ahk* or three *ahp* genes displayed a noticeable shoot-growth retardation compared with other combinations that do not possess these combined *ahk* or *ahp* mutations, suggesting that reduced shoot stature might be, at least in part, an adaptive trait of CK signaling mutants to drought. Thus, to study the functions of other downstream components of the CK signaling, namely the type B ARRs, in drought responses, we screened for type B *ARR* genes whose mutation resulted in dwarfism. Among the 11 type B *ARR* genes, mutant combinations of *arr1*, *arr10*, and *arr12* genes (*arr1–3*, *10–5*, *12–1* alleles, referred to as *arr1*, *arr10*, and *arr12* hereafter) exhibited similar shoot-growth retardation as *ahk2,3* and *ahp2,3,5* mutant plants (Fig. S1) (12). We therefore focused on elucidating the functions of the *ARR1*, *ARR10*, and *ARR12* genes in plant adaptation to drought using a loss-of-function approach.

To examine the function of the *ARR1*, *ARR10*, and *ARR12* genes in drought responses, first the drought tolerance of 3-wk-old *arr1,10,12* mutant and WT plants were compared on drying soil in a long-term drought treatment using the same-tray method developed for comparing genotypes with different growth rates (Fig. 1 *A–C*) (4). A significantly high number of *arr1,10,12* mutant plants survived the water deficit, whereas most of the WT plants died following their drought exposure (Fig. 1 *A* and *B*). Although the same-tray method applied ensures a valid comparison of genotypes with different growth rates (14), a more stringent method was also used to compare the drought tolerance of WT and *arr1,10,12* plants. In this method, the two genotypes were grown in an alternate order, as shown in Fig. S2*A*. Results also indicated that *arr1,10,12* showed higher drought tolerance than the WT (Fig. S2 *B* and *C*). For comparison, both the *arr1,10,12* and WT plants grew healthily under well-watered conditions (Fig. 1*C* and Fig. S2*D*). Additionally, we found that *arr1,10,12* plants retained higher relative water content (RWC) than WT at similar soil moisture content in the soil-drying experiment (Fig. 1 *D* and *E*). These results demonstrate that loss-of-function of *ARR1*, *ARR10*, and *ARR12* genes enhances drought tolerance, suggesting that these three type B ARR proteins mediate *Arabidopsis* responses to drought as negative regulators.

Enhanced Cell Membrane Integrity of _arr1,10,12_ Under Drought Stress. An enhancement in cell membrane integrity and stability might contribute to the differential RWC observed between *arr1,10,12* and WT (Fig. 1*D*) (14). Thus, to elucidate the mechanisms by which ARR1, ARR10, and ARR12 mediate drought responses, first we compared the degree of cell-membrane injury induced by drought in *arr1,10,12* and WT plants by measuring electrolyte leakage from both genotypes at various time points in a soil-drying experiment. Data indicated that loss-of-function mutations in all three *ARR* genes resulted in a remarkably lower electrolyte leakage in the *arr1,10,12* mutant in comparison with WT, especially on the days where significant differences in RWC of the two genotypes were recorded (Fig. 1 *D–F*). Even at the time point when the RWC of the two genotypes was almost equally reduced to below 60%, *arr1,10,12* plants showed significantly lower level of ion leakage than did the WT plants (Fig. 1*G*). These findings suggest that loss-of-function of these three type B *ARR* genes enhances cell membrane integrity and stability as an adaptive mechanism, contributing to the improved drought tolerance of *arr1,10,12* plants by retaining higher water content in the plants under drought.

Comparative Analyses of ABA Responsiveness and Stomatal Regulation in _arr1,10,12_ and WT Plants. Another trait that might result in difference in RWC of the *arr1,10,12* and WT is that related to stomatal regulation and ABA, namely ABA-mediated stomatal closure and/or stomatal density and/or ABA response (3). As shown in Fig. 2 *A* and *B*, the stomatal aperture in the *arr1,10,12* mutant was significantly narrower than that in WT with or without ABA. However, both genotypes exhibited comparable stomata closing rates (after aperture normalization) in response to ABA (Fig. 2*C*). Furthermore, *arr1,10,12* plants were observed to have higher—but not lower—stomatal density than WT plants (Fig. 2*D*). These results indicate that the ABA-independent impairment of stomatal opening by loss-of-function of the three *ARR* genes may reduce the water loss in the mutant, thereby contributing to enhanced drought tolerance of *arr1,10,12* rather than alteration in ABA-mediated stomatal closure or stomatal density.

Although the *arr1,10,12* and WT plants responded similarly to ABA-mediated inhibition of stomatal opening, the *arr1,10,12* mutant exhibited higher ABA responsiveness than WT in a germination assay (Fig. 2*E*). This result suggests that loss-of-function of these three *ARR* genes may also enhance the general ABA response, which induces expression of genes that function in cellular dehydration tolerance, thereby enhancing the survival of *arr1,10,12* plants under drought.

Enhanced Anthocyanin Biosynthesis in _arr1,10,12_ Plants Under Drought. We observed that *arr1,10,12* developed a darker leaf coloration than WT in our drought-tolerance assays (Fig. 1*A* and Fig. S2). Thus, we suspected that *arr1,10,12* might have higher anthocyanin content than WT, which could also improve the performance of *arr1,10,12* under drought because anthocyanins protect plants against various environmental stresses, including drought (15). To test this hypothesis, we measured anthocyanin contents in the aerial parts of *arr1,10,12* and WT plants at various time points after exposing plants to a drought treatment on soil. As shown in Fig. S3, the *arr1,10,12* plants possessed significantly higher

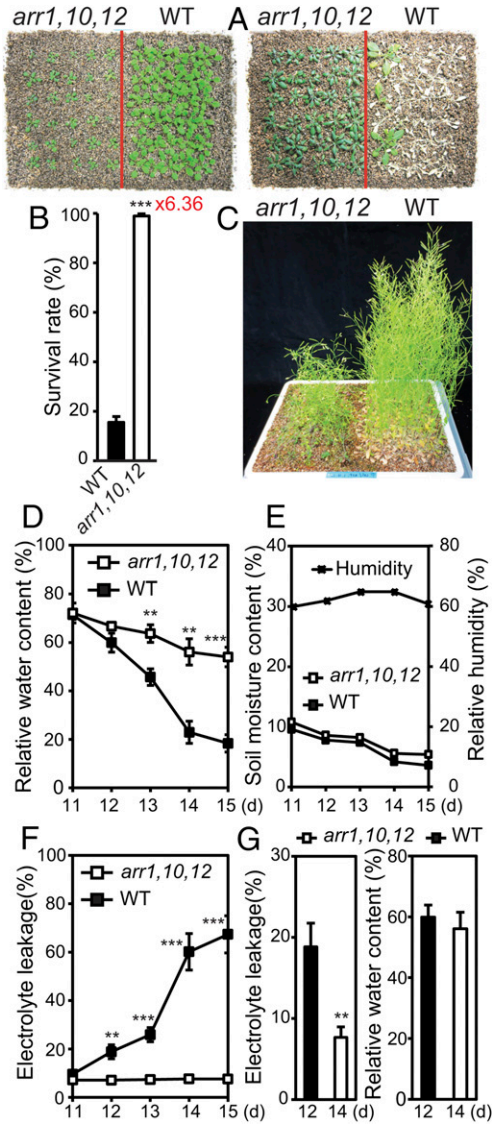

Fig. 1. Loss-of-function of *ARR1*, *ARR10*, and *ARR12* results in enhanced drought tolerance. (*A*) Two-week-old *arr1,10,12* and WT plants were transferred from GM plates to trays containing soil and grown for an additional week (*Left*). Three-week-old plants were exposed to a drought treatment by water withholding for 15 d and then rewatered for 3 d. Photographs were taken subsequent to rewatering and after the removal of inflorescences (*Right*). (*B*) Survival rates and SEs (error bars) were calculated from the results of three independent experiments (n = 30 plants per genotype). Red number next to the asterisks represents the fold-change over the WT. (*C*) For control, 2-wk-old *arr1,10,12* and WT plants were transferred from GM plates to trays and grown under well-watered conditions in parallel with the drought tolerance assay. (*D*) RWC of the *arr1,10,12* and WT plants grown and exposed to drought treatment as described in *A*. Error bars represent SEs (n = 5). (*E*) Soil relative moisture contents (n = 5) and relative humidity were monitored from day 11 until the end of drought tolerance test. (*F*) Electrolyte leakage of the *arr1,10,12* and WT plants grown and exposed to drought treatment as described in *A*. Error bars represent SEs (n = 5). (*G*) Differential electrolyte leakage (*Left*) between *arr1,10,12* and WT plants collected at a similar RWC (*Right*) during drought treatment. Error bars represent SEs (n = 5). Asterisks indicate significant differences as determined by a Student's t test analysis (**P < 0.01; ***P < 0.001).

levels of anthocyanins than WT under both drought and well-watered control conditions. These data support the notion that enhancement of anthocyanin production in the *arr1,10,12* mutant could be interpreted as an adaptive mechanism that contributes to its improved drought tolerance.

ARR1, _ARR10_, and _ARR12_ Are Redundant Regulators of Drought Response, with _ARR1_ Being the Most Critical Gene. We next examined the drought tolerance of lower-order mutant combinations to rank *ARR1*, *ARR10*, and *ARR12* in terms of their importance in controlling drought response. We first subjected all three *arr1,10*, *arr1,12*, and *arr10,12* double-mutant plants to a drought-tolerance assay for comparison with WT. As shown in Fig. S4, all three double mutants showed improved tolerance to drought at different levels compared with WT, demonstrating the redundant and negative regulatory functions of the *ARR1*, *ARR10*, and *ARR12* in drought responses. Furthermore, among the three double mutants, *arr1,12* exhibited the highest drought tolerance, followed by *arr1,10* and *arr10,12*. The data also suggested that *ARR1* plays the most prominent role in regulation of plant responses to drought, followed by *ARR12* and *ARR10*. Additionally, although the single mutant *arr1* exhibited higher drought tolerance relative to WT, both the *arr1,10* and *arr1,12* double mutants still displayed higher drought tolerance than the *arr1* single mutant (Fig. S4), further strengthening that *ARR1*, *ARR10*, and *ARR12* function in combination to effectively regulate drought responses in plants.

Down-Regulation of _ARR1_, _ARR10_, and _ARR12_ by Dehydration and ABA Treatment. Based on their role in mediating drought responses, we investigated the expression patterns of the three *ARR* genes in stress-exposed plants. We treated *Arabidopsis* WT plants with dehydration or ABA over a time-course and analyzed the expression patterns of the three *ARR* genes. Quantitative RT-PCR (RT-qPCR) data indicated that expression of the three *ARR* genes was affected by both dehydration and ABA treatments. At the whole-plant level, all three *ARR* genes were repressed by dehydration and ABA treatments (Fig. 3*A*). Furthermore, in a tissue-specific expression assay, all three *ARR* genes were substantially down-regulated in shoots (>twofold), whereas only *ARR10* showed a comparable decrease in transcript level (>twofold) in roots (Fig. 3*B*). *ARR1* was determined as the most rapidly down-regulated in shoots, followed by *ARR12* and *ARR10* (Fig. 3*B*). The differential expression of *ARR1*, *ARR10*, and *ARR12* under dehydration and ABA treatments corroborates their involvement in regulating plant adaptation to drought, and indicates that these genes may also act in an ABA-dependent manner.

Differential Gene Expression in Shoots of _arr1,10,12_ and WT Plants Under Normal and Dehydration Conditions. A comparative transcriptome analysis of leaves of *arr1,10,12* and WT plants was performed under both normal and dehydration conditions to identify effector genes regulated by the three *ARR* genes. Our experimental design is illustrated in Fig. S5 *A–C*, and results of the microarray analysis are summarized in Dataset S1. A search for differentially expressed genes (DEGs) identified 1,414 up-regulated and 817 down-regulated genes in *arr1,10,12* vs. WT under unstressed conditions (M-C/W-C) (Fig. S5*D* and Dataset S2 *A* and *E*). With regard to the DEGs derived from comparative analysis of *arr1,10,12* and WT under dehydration (M-D/W-D), a total of 676 induced genes were identified, of which a higher number of genes were found to be up-regulated by the long-term 4-h (M-D4/W-D4) dehydration than the short-term 2-h (M-D2/W-D2) dehydration treatment (Fig. S5*D* and Dataset S2 *B–D*). As for the down-regulated DEGs obtained from *arr1,10,12* vs. WT under dehydration, a total of 766 down-regulated genes were noted in M-D/W-D comparison. In a similar manner, more down-regulated genes were identified in the prolonged dehydration rather than in the short-term dehydration (Fig. S5*D* and Dataset S2 *F–H*).

Venn diagram and MapMan analyses indicated that a significant number of the up-regulated genes identified in M-C/W-C and M-D/W-D comparisons are dehydration-inducible (Figs. S5 *E*, *i* and *ii*, and S6*A*, and Datasets S3–S5), whereas a substantial portion of down-regulated genes noted in the same comparisons are dehydration-repressible (Figs. S5 *E*, *iv* and *v*, and S6*A*, and Datasets S3, S4, and S6). A number of overlapped genes were also identified between the up-regulated DEGs (Fig. S5 *E*, *iii* and Dataset S5*H*), as well as between the down-regulated DEGs

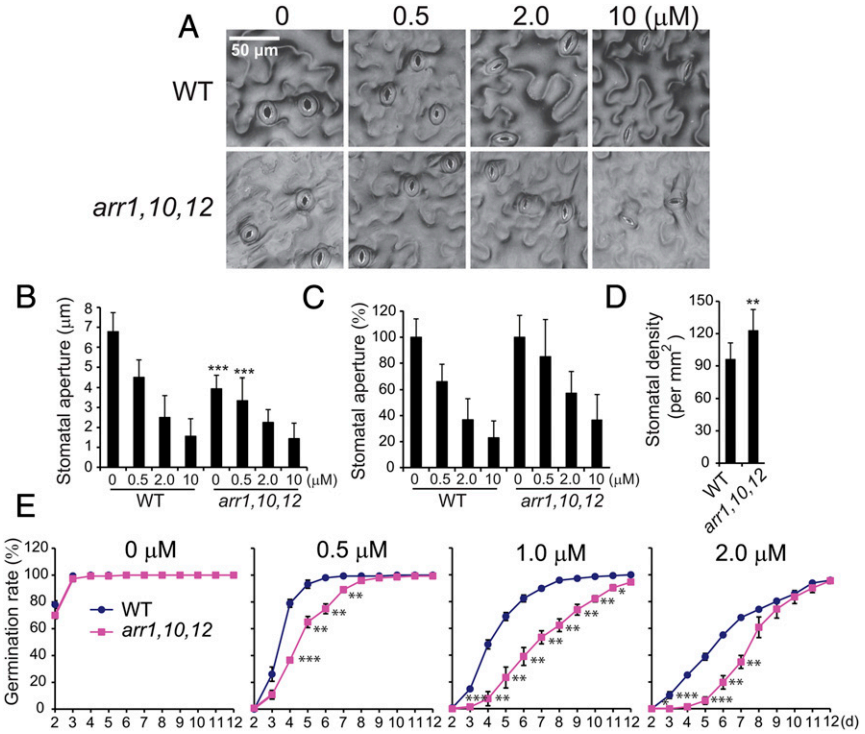

Fig. 2. Comparison of stomatal aperture, stomatal density, and ABA responsiveness of *arr1,10,12* and WT plants. (*A*) Representative guard cells of rosette leaves from 4-wk-old *arr1,10,12* and WT plants in the presence or absence of ABA for 2 h. (*B* and *C*) Average stomatal aperture of rosette leaves from 4-wk-old *arr1,10,12* and WT plants in the absence or presence of indicated ABA concentrations. Aperture data are expressed in micrometers (*B*) or in percentage of the aperture data obtained without ABA (*C*). Error bars represent SD values ($n = 35$). (*D*) Average stomatal density of rosette leaves (abaxial side) from 4-wk-old *arr1,10,12* and WT plants. Error bars represent SDs ($n = 8$). (*E*) Percent germination of *arr1,10,12* and WT seeds treated with different levels of exogenous ABA. Data represent the means and SEs of data pooled from three independent experiments ($n = 3$; 50 seeds were used per genotype per experiment). Asterisks indicate significant differences as determined by a Student's *t* test analysis (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

(Fig. S5 *E*, *vi* and Dataset S6*H*) obtained from comparisons M-C/W-C and M-D/W-D. These transcriptomic changes might have an impact on the improvement of drought tolerance of *arr1,10,12* plants. MapMan was then used to classify the up-regulated genes obtained from M-C/W-C and M-D/W-D comparisons into functional categories, which indicated that "S-assimilation" was the most highly enriched category (Fig. S6*B*), suggesting the importance of sulfur assimilation in enhancement of drought tolerance of *arr1,10,12*. Increasing evidence has indicated that sulfur has a high demand during drought because it plays important role in drought responses by acting in ABA signaling or serving as a precursor for synthesis of several osmoprotectants and antioxidants, such as choline-*O*-sulfate, polyamines, and glutathione (16). Several DEGs were then selected for RT-qPCR to verify the microarray data (Fig. S7).

A closer look of the up-regulated genes obtained from M-C/W-C and M-D/W-D comparisons identified many genes that may contribute to enhanced drought tolerance of *arr1,10,12* (Dataset S2 *A* and *D*). Notably, almost all of the major nuclear factor Y subunit A (*NF-YA*) genes, members of the *NF-Y*-type TF family, which are well-known to confer drought tolerance when being overexpressed in *Arabidopsis* transgenic plants (17, 18), were up-regulated in *arr1,10,12* under well-watered and/or dehydration conditions (Fig. S7 and Dataset S7*A*). Up-regulation of *SHINE1* (*SHN1*) and *SHN3*, two members of the AP2/EREBP-type TF family that activate epidermal wax biosynthesis (19), was also observed in stressed *arr1,10,12* plants (Fig. S7 and Dataset S7*A*). An enhanced wax synthesis might contribute to protect the plants from cellular dehydration during drought (20). Additionally, a subset of the up-regulated genes identified in the *arr1,10,12* mutant with respect to the WT under unstressed and/or stressed conditions were found to be involved in anthocyanin/flavonoid biosynthesis, including *CHS*, *F3H*, *FLS1-5*, *PAP1*/*MYB75*, *PAP2*/*MYB90*, and *MYB112* genes (Figs. S7–S9 and Dataset S7*B*) (15, 21, 22), supporting the results of the anthocyanin assay (Fig. S3). Furthermore, an increase in transcript levels of late embryogenesis abundant (*LEA*) genes, such as responsive to dehydration 29B (*RD29B*), responsive to ABA 18 (*RAB18*), and ABA-inducible LEA class gene 1 (*AIL1*) (23, 24), and osmolyte synthesis-related δ1-pyrroline-5-carboxylate synthase 1 (*P5CS1*) and sucrose synthase 1 (*SUS1*) genes in the *arr1,10,12* mutant relative to WT might also contribute to the better survival of *arr1,10,12* plants under drought through protection of membrane structure and osmotic adjustment (Fig. S7 and Dataset S7*C*) (13, 14). Most of these genes are induced by dehydration, some of which are also induced by ABA (Dataset S7), providing evidence that ARR1, ARR10, and ARR12 regulate drought responses through both ABA-dependent and ABA-independent stress-responsive pathways. Regarding the down-regulated DEGs identified in M-C/W-C and M-D/W-D comparisons, it is worth mentioning that 8 of 10 type A *ARR* genes (except *ARR3* and *ARR8*) are down-regulated in M-C/W-C and/or M-D/W-D comparisons (Fig. S7 and Dataset S7*D*), providing a hint for their potential function in drought responses.

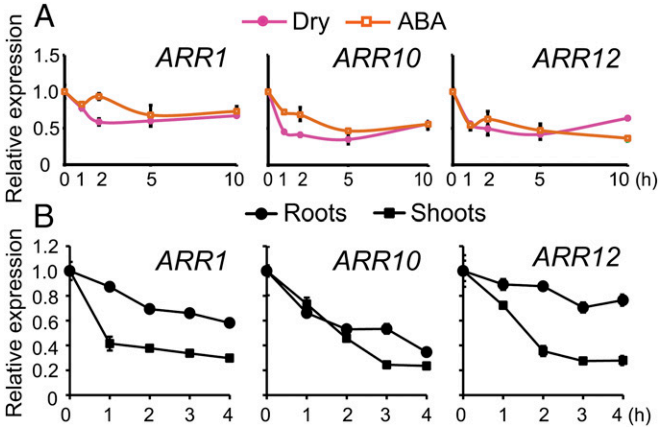

Fig. 3. Expression of *ARR1*, *ARR10*, and *ARR12* genes under stress conditions. (*A*) Expression of *ARR1*, *ARR10*, and *ARR12* genes in 14-d-old WT plants exposed to dehydration and ABA treatments for indicated time points. (*B*) Expression of *ARR1*, *ARR10*, and *ARR12* genes in roots and shoots of 17-d-old WT plants exposed to a dehydration treatment for indicated time points. Relative expression levels were normalized to a value of 1 in the respective control plants. Data represent the means and SEs of three independent biological replicates ($n = 3$). *UBQ10* was used as reference gene.

Discussion

In this study, we explored the function of the type B *ARR1*, *ARR10*, and *ARR12* in drought responses by investigating the consequences of their loss-of-function on the adaptation of *Arabidopsis* plants to drought. Our data demonstrate that ARR1, ARR10, and ARR12 redundantly act as negative regulators of plant responses to drought, with ARR1 appearing to be the most critical protein (Fig. 1 and Figs. S2 and S4). This study has therefore taken one more step into fulfilling the challenging task of dissecting the roles of different components of the CK signaling pathway in drought responses. Previously, upstream components AHK2 and AHK3, as well as AHP2, AHP3, and AHP5 were identified as negative regulators in drought tolerance (9–11), which together with the results of this study demonstrate that repression of the CK response, and thus CK signaling, is one of the strategies plants use to cope with water deficit.

In accordance with its enhanced drought tolerance (Fig. 1 *A*–*C* and Fig. S2), the *arr1,10,12* mutant maintained higher RWC than WT (Fig. 1*D*), which can be attributed to its enhanced ability to protect the membrane structure (Fig. 1 *F* and *G*), reduced stomatal opening (Fig. 2*B*) and enhanced sensitivity to ABA (Fig. 2*E*), rather than an alteration in stomatal density (Fig. 2*D*). In agreement with this finding, the repression of CK response through down-regulation of CK signaling-related genes or reduction of endogenous CK also improved drought tolerance of *Arabidopsis* mutant plants by protecting membrane structures and enhancing ABA sensitivity, independently from the status of stomatal density (4, 11). An enhanced ABA response may lead to up-regulation of downstream ABA-responsive genes, such as *LEA* and osmolyte biosynthesis-related genes, to activate protective mechanisms, including damage repair, protection of membrane structure, and maintenance of osmotic homeostasis in plants during water deficit, which in turn improves the overall performance of plants (14, 23, 24). For example, constitutive overexpression of the active form of *AREB1* gene (*AREB1ΔQT*) and the *AHK1* gene improved drought tolerance in *Arabidopsis*, which might be ascribed to the enhanced cellular dehydration tolerance through up-regulation of ABA-responsive *LEA* class genes and osmolyte synthesis-related *P5CS1* and *SUS1* genes, respectively (13, 23). Concurrently, a higher expression of *LEA* class genes, including *RD29B*, *RAB18*, and *AIL1*, as well as *P5CS1* and *SUS1*, was found in *arr1,10,12* relative to WT under normal or dehydration conditions (Fig. S7 and Dataset S7*C*), which might partially explain the enhanced drought tolerance of the *arr1,10,12* mutant. Furthermore, up-regulation of *SHN1* and *SHN3* genes detected in the *arr1,10,12* mutant (Fig. S7) might result in a reduction in electrolyte leakage noted in the *arr1,10,12* mutant (Fig. 1 *F* and *G*), through enhancement of the membrane permeability barrier by activating cuticular wax biosynthesis, thereby enhancing drought tolerance (19, 20). Additionally, transcriptome analysis also identified a number of anthocyanin biosynthesis-related genes up-regulated in the *arr1,10,12* mutant under both unstressed and dehydration conditions (Fig. S7 and Dataset S7*B*), which is in agreement with anthocyanin accumulation in the *arr1,10,12* mutant under the same conditions (Fig. S3). A previous study also detected many anthocyanin/flavonoid biosynthesis-related genes induced in the drought-tolerant *ahp2,3,5* mutant in comparison with WT under well-watered and/or drought conditions (Dataset S7*B*) (11). Taken together, these results strengthen that repression of CK signaling leads to increased anthocyanin biosynthesis, contributing to plant adaptation to drought, as anthocyanins can protect cells from various stresses by acting as reactive oxygen species-scavenging antioxidants and absorbing UV and high light irradiation (15, 22).

The impairment of stomatal opening observed in the *arr1,10,12* mutant might help the plant avoid dehydration (Fig. 2 *A* and *B*) (17); however, it may cause aberrant photosynthesis as a trade-off, resulting in its dwarf phenotype (Fig. S1) (12). This finding also implies that CK signaling positively regulates light-stimulated stomatal opening. In support of our finding, a recent study reported that the *ahk2,3* double mutant also showed a significantly narrower stomatal aperture than WT (25), which correlates with its enhanced drought tolerance and dwarf-shoot phenotype (Fig. S1) (9, 10). Taken together, these data provide genetic evidence that CK and CK signaling regulate plant drought adaptation through controlling stomatal closure. In line with this finding, the up-regulation of 7 of 10 *NF-YA* genes (except *NF-YA1*, *-4*, and *-9*) in *arr1,10,12* under normal and/or dehydration conditions deserves mention. Several reports studying *NF-YA2*, *-3*, *-5*, *-7*, and *-10* showed that overexpression of these *NF-YA* genes remarkably enhanced drought tolerance in *Arabidopsis* transgenic plants (17, 18). Moreover, detailed analysis of *NF-YA5* overexpressor plants indicated a link between enhanced drought tolerance and impairment in stomatal opening (17), as observed with the *arr1,10,12* mutant (Fig. 2 *A* and *B*), further supporting that CK signaling is involved in regulation of *NF-YA* genes and stomatal closure in plant acclimation to drought. Some of the overexpressor plants, such as those overexpressing *NF-YA2*, *-7*, and *-10* (18), also exhibited growth retardation, which together with the down-regulation of the *CYCD3;1* gene, encoding a D-type cyclin implicated in regulating CK effects on cell division (12), may explain the reduced plant size of the *arr1,10,12* mutant as well. It was also interesting to discover that the majority of type A *ARR* genes were down-regulated in the *arr1,10,12* mutant under both normal and dehydration conditions (Fig. S7 and Dataset S7*D*). Several lines of evidence indicate, although indirectly, that drought tolerance correlates with down-regulation of type A *ARR* genes. For example, it was reported that *arr4* and *arr7* mutants exhibited smaller stomatal aperture than WT (26), which also suggests that impairment in stomatal opening of *arr1,10,12* may be attributed to the down-regulation of type A *ARR* genes. More evidence is that CK-deficient mutant and type C *ARR22* overexpressor plants exhibited drought tolerance, which coincided with down-regulation of type A *ARR5* and *ARR7*, and *ARR4*, *ARR5*, *ARR6*, *ARR7*, *ARR9*, and *ARR16* genes, respectively (4, 27). The biological functions of the type A ARRs under drought remain to be determined to directly provide further mechanistic explanation.

In agreement with the negative regulatory functions of *ARR1*, *ARR10*, and *ARR12* in drought responses, the expression of all three genes was found to be repressed by dehydration and ABA treatments (Fig. 3). This finding suggests that during drought exposure, plants may down-regulate *ARR1*, *ARR10*, and *ARR12* genes, and thereby the CK response to enhance survival. The differential down-regulation of the three *ARR* genes in shoots and roots points to a particular role for drought in reducing the CK sensitivity in shoots (Fig. 3*B*), which is consistent with the results of our physiological analyses of shoot-related phenotypes. Plants with reduced endogenous CK levels (e.g., *CKX* overexpressor plants) or CK sensitivity (e.g., *ahk2,3* mutant) display retarded shoot growth but enhanced root growth, which correlates with their enhanced drought tolerance (4, 6, 9, 10). Restraint of shoot growth is known as an adaptive strategy of plant responses to water deficit, through which plants can reallocate limited energy resources from high energy-demanding processes, such as photosynthesis, for stimulating defense pathways to deal with adverse environmental conditions (4, 6, 7). Moreover, enhanced root growth may help plants respond to drought by obtaining water from deeper soil layers or foraging subsoil surface moisture (28). Mutations in various type B *ARR* genes—including *ARR1*, *ARR10*, and *ARR12*—result in shoot retardation or root enhancement (12, 29), strengthening the importance of morphological adjustment through CK signaling in drought responses. The finding that CK levels are reduced by drought at some periods during long-term stress (2, 4) implies that plants may trigger mechanisms to suppress the negative effect of CK signaling to survive drought by either reducing endogenous CK content or directly downregulating CK-signaling genes, including type B *ARR* genes. This stress-induced suppression of CK signaling appears to be a yin-yang strategy facilitating environmental acclimation/adaptation of plants. Detailed functional analysis of all of the components of CK signaling in plant responses to drought, as well as other stresses, remains an important future task to elucidate this complex network underlying plant adaptation to stress.

Materials and Methods

Plant Materials, Dehydration, and Treatments. The *arr1* single, *arr1,10*, *arr1,12*, *arr10,12* double, and *arr1,10,12* triple mutants used in this study are different combinations of *arr1-3*, *arr10-5*, and *arr12-1* mutants. All of the *arr* mutants are in Columbia genetic background and were obtained from Argyros et al. (12). For dehydration and ABA treatments, WT plants were grown on germination medium (GM) agar plates for 14 or 17 d (22 °C, 16-h light/8-h dark cycle, 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photon flux density) and treated with dehydration, water (hydroponic control), or 100 μM ABA for the indicated time periods. Samples were collected in whole plants or separately in shoot and root fractions in at least three biological replicates, and immediately frozen in liquid nitrogen for further analyses.

Assessment of Drought Tolerance. The same-tray method that ensures a valid comparison of genotypes with different growth rates was used to evaluate drought tolerance, as previously described (4). Rewatering was performed when the greatest difference in appearance was observed between the mutant and control plants. Three days after rewatering, trays were photographed after removal of inflorescences from the survived plants.

Measurements of RWC and Electrolyte Leakage from Drought-Stressed Plants. RWC and electrolyte leakage of the detached aerial parts of plants during exposure to soil drying treatment were measured according to the previously described methods (4).

Stomatal Movement Assays. Assay for ABA-mediated stomatal closure and measurement of stomatal density were conducted as previously described (4).

Assay for Sensitivity to ABA. Germination and growth inhibition assays were performed on GM medium containing 1% sucrose and various concentrations of ABA, as described in Nishiyama et al. (4).

Determination of Anthocyanin Content. Plants were subjected to a drought tolerance treatment on the same tray as described above. At indicated time points, aerial parts (without inflorescence) of well-watered control and stressed plants were separately collected in two sets for measurement of fresh weight, dry weight, and RWC (4), and anthocyanin content (30), respectively. For comparison, anthocyanin contents per plant dry weight were estimated using the ratios of fresh and dry weights of collected plant parts.

Expression Analyses. Total RNA was extracted using RNeasy Plant Mini Kit (Qiagen). Methods described in ref. 31 were used for cDNA synthesis and RT-qPCR. *UBQ10* was used as a reference gene in analysis of RT-qPCR data. Gene-specific primers used for RT-qPCR are listed in Dataset S8.

Dehydration Treatment and Transcriptome Analysis. Fourteen-day-old *arr1,10,12* and WT plants germinated on GM agar plates were transferred to soil (30 plants/each in the same tray) and grown under well-watered conditions for an additional 10 d. Aerial portions of 24-d-old plants were then detached and subjected to dehydration by placing them on paper towels on a laboratory bench. During dehydration, RWC of treated samples was determined at the indicated time points ($n = 5$) (23). Rosette leaves collected in three biological repeats from *arr1,10,12* and WT plants treated by dehydration for 0, 2, and 4 h were used for microarray and expression analyses. Transcriptome analysis using the *Arabidopsis* Oligo 44K DNA microarray (v4.0, Agilent Technology) was performed as described in ref. 32. The criteria of fold-change ≥ 2 and a false-discovery rate-corrected *P* value (q-value) < 0.05 were used in microarray analysis to search for DEGs. The detailed protocol and raw microarray data were deposited in the Gene Expression Omnibus database (GSE74880). MapMan (mapman.gabipd.org/web/guest;jsessionid=B426E14286E9DFBEF97E1474C00C2D95.ajp13_mapman_gabipd_org), Genevestigator (https://www.genevestigator.com), and *Arabidopsis* eFP browser (bar.utoronto.ca/efp_arabidopsis/cgi-bin/efpWeb.cgi) were used to analyze the data.

ACKNOWLEDGMENTS. We thank M. Toyoshima (Japan International Research Center for Agricultural Sciences) for excellent technical support. This project was supported in part by the Core Research for Evolutional Science and Technology, Japan Science and Technology (M.S.); and National Science Foundation Grant IOS-1022053 (to G.E.S.). The PhD study of K.H.N. and C.V.H. was supported by the International Program Associate of RIKEN.

1. Peleg Z, Blumwald E (2011) Hormone balance and abiotic stress tolerance in crop plants. *Curr Opin Plant Biol* 14(3):290–295.
2. Zwack PJ, Rashotte AM (2015) Interactions between cytokinin signalling and abiotic stress responses. *J Exp Bot* 66(16):4863–4871.
3. Fujita Y, Fujita M, Shinozaki K, Yamaguchi-Shinozaki K (2011) ABA-mediated transcriptional regulation in response to osmotic stress in plants. *J Plant Res* 124(4):509–525.
4. Nishiyama R, et al. (2011) Analysis of cytokinin mutants and regulation of cytokinin metabolic genes reveals important regulatory roles of cytokinins in drought, salt and abscisic acid responses, and abscisic acid biosynthesis. *Plant Cell* 23(6):2169–2183.
5. Hwang I, Sheen J, Müller B (2012) Cytokinin signaling networks. *Annu Rev Plant Biol* 63:353–380.
6. Werner T, et al. (2010) Root-specific reduction of cytokinin causes enhanced root growth, drought tolerance, and leaf mineral enrichment in *Arabidopsis* and tobacco. *Plant Cell* 22(12):3905–3920.
7. Ha S, Vankova R, Yamaguchi-Shinozaki K, Shinozaki K, Tran LS (2012) Cytokinins: Metabolism and function in plant adaptation to environmental stresses. *Trends Plant Sci* 17(3):172–179.
8. Kieber JJ, Schaller GE (2014) Cytokinins. *Arabidopsis Book* 12:e0168.
9. Tran LS, et al. (2007) Functional analysis of AHK1/ATHK1 and cytokinin receptor histidine kinases in response to abscisic acid, drought, and salt stress in *Arabidopsis*. *Proc Natl Acad Sci USA* 104(51):20623–20628.
10. Kang NY, Cho C, Kim NY, Kim J (2012) Cytokinin receptor-dependent and receptor-independent pathways in the dehydration response of *Arabidopsis thaliana*. *J Plant Physiol* 169(14):1382–1391.
11. Nishiyama R, et al. (2013) *Arabidopsis* AHP2, AHP3, and AHP5 histidine phosphotransfer proteins function as redundant negative regulators of drought stress response. *Proc Natl Acad Sci USA* 110(12):4840–4845.
12. Argyros RD, et al. (2008) Type B response regulators of *Arabidopsis* play key roles in cytokinin signaling and plant development. *Plant Cell* 20(8):2102–2116.
13. Wohlbach DJ, Quirino BF, Sussman MR (2008) Analysis of the *Arabidopsis* histidine kinase ATHK1 reveals a connection between vegetative osmotic stress sensing and seed maturation. *Plant Cell* 20(4):1101–1117.
14. Verslues PE, Agarwal M, Katiyar-Agarwal S, Zhu J, Zhu JK (2006) Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. *Plant J* 45(4):523–539.
15. Nakabayashi R, et al. (2014) Enhancement of oxidative and drought tolerance in *Arabidopsis* by overaccumulation of antioxidant flavonoids. *Plant J* 77(3):367–379.
16. Chan KX, Wirtz M, Phua SY, Estavillo GM, Pogson BJ (2013) Balancing metabolites in drought: The sulfur assimilation conundrum. *Trends Plant Sci* 18(1):18–29.
17. Li WX, et al. (2008) The *Arabidopsis* NFYA5 transcription factor is regulated transcriptionally and posttranscriptionally to promote drought resistance. *Plant Cell* 20(8):2238–2251.
18. Leyva-González MA, Ibarra-Laclette E, Cruz-Ramírez A, Herrera-Estrella L (2012) Functional and transcriptome analysis reveals an acclimatization strategy for abiotic stress tolerance mediated by *Arabidopsis* NF-YA family members. *PLoS One* 7(10):e48138.
19. Aharoni A, et al. (2004) The SHINE clade of AP2 domain transcription factors activates wax biosynthesis, alters cuticle properties, and confers drought tolerance when overexpressed in *Arabidopsis*. *Plant Cell* 16(9):2463–2480.
20. Zhou X, et al. (2015) OsGL1-3 is involved in cuticular wax biosynthesis and tolerance to water deficit in rice. *PLoS One* 10(1):e116676.
21. Saito K, et al. (2013) The flavonoid biosynthetic pathway in *Arabidopsis*: Structural and genetic diversity. *Plant Physiol Biochem* 72:21–34.
22. Lotkowska ME, et al. (2015) The *Arabidopsis* transcription factor MYB112 promotes anthocyanin formation during salinity and under high light stress. *Plant Physiol* 169(3):1862–1880.
23. Fujita Y, et al. (2005) AREB1 is a transcription activator of novel ABRE-dependent ABA signaling that enhances drought stress tolerance in *Arabidopsis*. *Plant Cell* 17(12):3470–3488.
24. Jin X, et al. (2013) Transcription factor *OsAP21* gene increases salt/drought tolerance in transgenic *Arabidopsis thaliana*. *Mol Biol Rep* 40(2):1743–1752.
25. Marchadier E, Hetherington AM (2014) Involvement of two-component signalling systems in the regulation of stomatal aperture by light in *Arabidopsis thaliana*. *New Phytol* 203(2):462–468.
26. Mira-Rodado V, et al. (2012) Identification of two-component system elements downstream of AHK5 in the stomatal closure response of *Arabidopsis thaliana*. *Plant Signal Behav* 7(11):1467–1476.
27. Kang NY, Cho C, Kim J (2013) Inducible expression of *Arabidopsis response regulator 22* (*ARR22*), a type-C ARR, in transgenic *Arabidopsis* enhances drought and freezing tolerance. *PLoS One* 8(11):e79248.
28. Manavalan LP, Guttikonda SK, Tran LS, Nguyen HT (2009) Physiological and molecular approaches to improve drought resistance in soybean. *Plant Cell Physiol* 50(7):1260–1276.
29. Mason MG, et al. (2005) Multiple type-B response regulators mediate cytokinin signal transduction in *Arabidopsis*. *Plant Cell* 17(11):3007–3018.
30. Ito S, et al. (2015) Strigolactone regulates anthocyanin accumulation, acid phosphatases production and plant growth under low phosphate condition in *Arabidopsis*. *PLoS One* 10(3):e0119724.
31. Le DT, et al. (2011) Genome-wide expression profiling of soybean two-component system genes in soybean root and shoot tissues under dehydration stress. *DNA Res* 18(1):17–29.
32. Nishiyama R, et al. (2012) Transcriptome analyses of a salt-tolerant cytokinin-deficient mutant reveal differential regulation of salt stress response by cytokinin deficiency. *PLoS One* 7(2):e32124.