Cytokinin-mediated control of leaf longevity by AHK3 through phosphorylation of ARR2 in *Arabidopsis*

Hyo Jung Kim*, Hojin Ryu*, Sung Hyun Hong*, Hye Ryun Woo*†, Pyung Ok Lim‡, In Chul Lee*, Jen Sheen§, Hong Gil Nam*¶‖, and Ildoo Hwang*‖

*Division of Molecular and Life Sciences, ¶National Core Research Center for Systems Bio-Dynamics and the I-BIO Graduate Program, Pohang University of Science and Technology, Pohang 790-784, Korea; ‡Department of Science Education, Cheju National University, Cheju 690-756, Korea; and §Department of Molecular Biology, Massachusetts General Hospital and Department of Genetics, Harvard Medical School, Boston, MA 02114

Edited by Hans Janos Kende, Michigan State University, East Lansing, MI, and approved November 14, 2005 (received for review June 21, 2005)

Cytokinins are plant hormones with profound roles in growth and development, including control of leaf longevity. Although the cytokinin signal is known to be perceived by histidine kinase receptors, the underlying molecular mechanism and specificity of the receptors leading to delayed leaf senescence have not yet been elucidated. Here, we found that AHK3, one of the three cytokinin receptors in *Arabidopsis*, plays a major role in controlling cytokinin-mediated leaf longevity through a specific phosphorylation of a response regulator, ARR2. This result was obtained through identification of a gain-of-function *Arabidopsis* mutant that shows delayed leaf senescence because of a missense mutation in the extracellular domain of AHK3. A loss-of-function mutation in *AHK3*, but not of the other cytokinin receptors, conferred a reduced sensitivity to cytokinin in cytokinin-dependent delay of leaf senescence and abolished cytokinin-dependent phosphorylation of ARR2. Consistently, transgenic overexpression of wild-type, but not an unphosphorylatable mutant ARR2, led to delayed senescence of leaves.

histidine kinase 3 | cytokinin | leaf senescence

Leaf senescence is typically observed in autumn leaves and during the death process of monocarpic plants (1). During senescence, leaf cells experience striking changes in cellular metabolism and structure (2). The first visible change is yellowing of the leaves caused by chlorophyll breakdown during chloroplast disassembly (3), accompanied by hydrolysis of macromolecules such as lipids, proteins, and nucleic acids. The products of this catabolic process are reallocated to developing organs, such as younger leaves and germinating seeds (4). Therefore, senescence is not a simple degenerative process but is considered as a genetically programmed, active process that contributes to the fitness of plants. Although occurring in an age-dependent manner, leaf senescence is greatly affected by various endogenous and environmental signals to attune the life span of leaves to optimized fitness of plants (5, 6). Among the endogenous developmental signals, the plant hormones cytokinins have a particularly profound effect on longevity of plant organs (7–9). For example, it has been shown that the transgenic modification of cytokinin biosynthesis during the senescence phase can significantly delay the senescence of plant organs, including leaves, and improve plant productivity by up to 50% (8, 10).

Besides control of longevity, cytokinins have many critical functions in plants, such as the control of cell proliferation, shoot formation, nutrient relocation, and shoot branching (11, 12). In *Arabidopsis*, cytokinins are known to be perceived by three cytokinin receptors, AHK2, AHK3, and AHK4/CRE1/WOL, which possess the structural features of hybrid histidine kinases (13, 14). In the model proposed for the cytokinin signal-transduction pathway (15–17), a histidine protein kinase initiates the cytokinin signal-transduction pathway through phosphorelay to histidine phosphotransfer (AHP) proteins. AHP proteins are then translocated to the nucleus, and transfer a phosphoryl group to response regulator (ARR) proteins. The phosphotransfer changes the transactivation capacity of ARR proteins, which, in turn, either activates or represses the expression of cytokinin-responsive genes. The specific combination of *Arabidopsis* histidine kinase (AHK), AHP, and ARR proteins in a phosphorelay may determine the specificity of cytokinin responses during plant growth and development. However, the actual molecular mechanisms and the specificity of cytokinin signaling in controlling the various cytokinin-mediated responses are still largely unknown, especially for the control of longevity.

We report here on the genetic, genomic, and molecular analysis of an *Arabidopsis* cytokinin-receptor mutant *ore12-1* that exhibits extended life span of leaves and show that the phosphorylation of ARR2 mediated by AHK3 is essential in controlling their longevity.

Results

The *Arabidopsis ore12-1* Mutant Exhibits Delayed Leaf Senescence. The leaf of *Arabidopsis* is a suitable experimental system, because it shows a reproducible senescence pattern with age and is amenable to genetic analysis. To elucidate the genetic regulatory mechanisms of leaf senescence, we screened for mutants with delayed senescence. One of the mutants, *ore12-1*, was identified from a screen of plants derived from an ethylmethanesulfonate-mutagenized seed pool. The mutant leaves maintained their green pigmentation and architectural integrity for a longer period than did wild-type leaves (Fig. 1*A*). The senescence of leaves was then examined at increasing leaf age. Visual examination of individual leaves throughout their life spans showed that the *ore12-1* mutant had delayed leaf senescence (Fig. 1*B Top*). In addition, the chlorophyll content and the photochemical efficiency of photosystem II (Fv/Fm), two physiological markers of leaf senescence (18), were maintained at higher levels in the *ore12-1* mutant with age of the leaves (Fig. 1*B Middle*). Moreover, in the mutant, a photosynthesis-related chlorophyll a/b-binding protein (*CAB*) gene and a senescence-associated gene *SAG12* were expressed at higher and lower levels, respectively (Fig. 1*B Bottom*). Darkness is one of the most potent external stimuli that accelerate leaf senescence. The *ore12-1* mutation greatly delayed dark-induced leaf senescence (see Fig. 7 *Top* and *Middle*, which is published as supporting information on the PNAS web site). In addition, the induction of *SEN4*, a marker gene for dark-induced and age-dependent leaf senescence, was delayed in the mutant during dark-induced senescence (Fig. 7 *Bottom*). These results show that the *ore12-1*

Conflict of interest statement: No conflicts declared.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: AHK, *Arabidopsis* histidine kinase; ARR, *Arabidopsis* response regulator; HA, hemagglutinin.

†Present address: Department of Biology, Washington University, St. Louis, MO 63130.

‖To whom correspondence may be addressed. E-mail: ihwang@postech.ac.kr or nam@bric.postech.ac.kr.

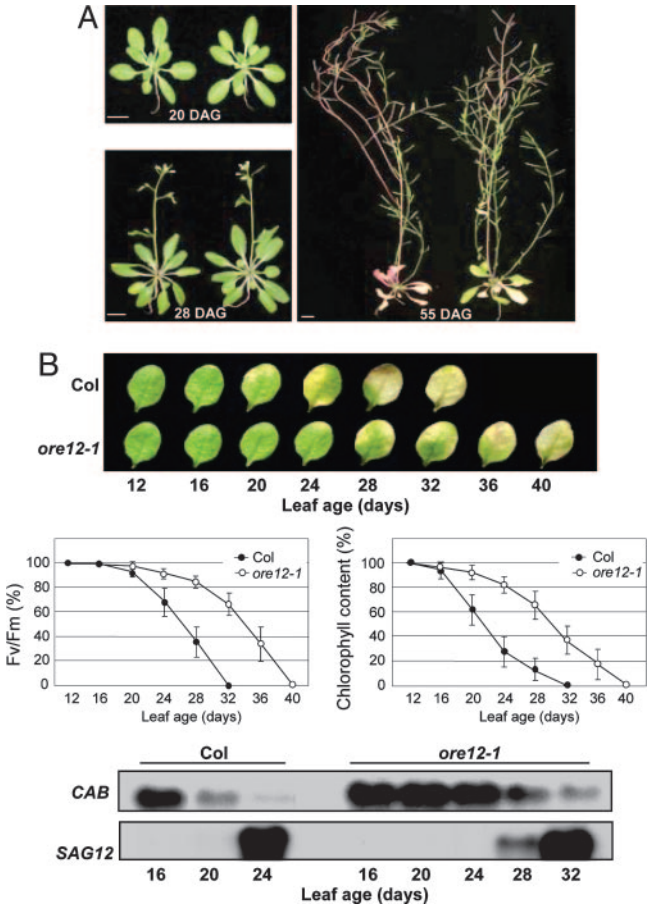

Fig. 1. The *Arabidopsis ore12-1* mutant shows extended leaf longevity. (*A*) Phenotypes of whole wild-type (Col, *Left*) and *ore12-1* (*Right*) plants at different ages. DAG, days after germination. (Scale bar, 1 cm.) (*B*) Extended life span of an *ore12-1* mutant leaf. (*Top*) Age-dependent senescence phenotype of a wild-type and an *ore12-1* leaf. (*Middle*) The chlorophyll content and the photochemical efficiency (Fv/Fm) in wild-type and *ore12-1* leaves were examined at different ages starting at 12 days after leaf emergence when the leaf had just reached its full growth. Error bar indicates standard deviation (n = 30). (*Bottom*) Expression of the molecular-senescence markers *CAB* and *SAG12* in wild-type and the *ore12-1* leaves.

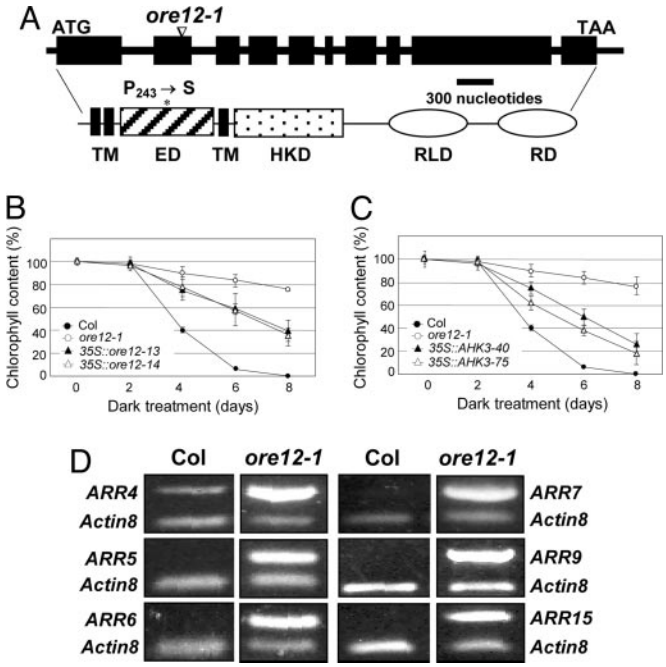

Fig. 2. ORE12 is AHK3, a histidine kinase cytokinin receptor. (*A*) The structure of the *ORE12*/*AHK3* gene and of its protein product. The open triangle and asterisk indicate the position of the *ore12-1* mutation. TM, transmembrane domain; ED, extracellular domain; HKD, histidine kinase domain; RLD, receiver-like domain; RD, receiver domain. (*B* and *C*) Ectopic expression of either *ore12-1* (*B*) or *AHK3* (*C*) recapitulates the *ore12-1* phenotype. The chlorophyll content was examined every 2 days during dark-induced senescence. Error bar indicates standard deviation (n = 12). (*D*) Up-regulation of cytokinin-inducible A-type *ARR* genes in the *ore12-1* mutant. Total RNA extracted from wild-type (Col) and the *ore12-1* leaves was used to examine the expression of the A-type *ARR* genes *ARR4–9* and *15* for RT-PCR.

mutation affects various senescence-associated symptoms, indicating that ORE12 is a key regulator of leaf longevity in *Arabidopsis*.

ORE12 Is Allelic to AHK3, a Histidine Kinase Cytokinin Receptor. The gene responsible for the *ore12-1* mutation was identified by map-based cloning as a missense mutation in the *AHK3* gene, which encodes a histidine kinase cytokinin receptor (see Fig. 8, which is published as supporting information on the PNAS web site) (19, 20). The missense mutation resulted in a substitution of Pro-243 to Ser in the predicted extracellular domain (Fig. 2*A*; and see Fig. 8). The *ore12-1* mutation behaved as a recessive allele in conferring the delayed-leaf-senescence phenotype (data not shown). AHK3-GFP was mainly, if not exclusively, localized to the plasma membrane in *Arabidopsis* protoplasts (see Fig. 9, which is published as supporting information on the PNAS web site), indicating that cytokinin signal through AHK3 is perceived at the plasma membrane. The delayed-leaf-senescence symptoms of the *ore12-1* plants were recapitulated in transgenic plants overexpressing the mutant *ore12-1* gene in wild-type background (Fig. 2*B*; see also Fig. 10, which is published as supporting information on the PNAS web site), indicating that the phenotype is derived from the mutation in the *AHK3* gene. We later found another allele of *AHK3* that conferred delayed leaf senescence because of an amino acid substitution of Asp-448 to Asn (data not shown), further supporting the role of this gene in control of leaf longevity. In addition, the transgenic plants overexpressing *AHK3* (Figs. 2*C* and 10) showed delayed leaf senescence, indicating that AHK3 positively regulates leaf longevity and that the increased leaf life span phenotype observed in the *ore12-1* mutant is due to a gain-of-function mutation.

A genome-wide expression analysis of the *ore12-1* mutant using the *Arabidopsis* GeneChip (Affymetrix, Santa Clara, CA) showed that ≈110 genes were up- or down-regulated >2-fold in the mutant compared with wild type (see Tables 1 and 2, which are published as supporting information on the PNAS web site). Among the 40 up-regulated genes, most notable were many A-type *ARR* genes including *ARR4*, *-5*, *-6*, *-7*, *-9*, and *-15*. The higher expression of A-type *ARR*s in the *ore12-1* mutant was confirmed by RT-PCR (*Supporting Materials and Methods*, and Fig. 2*D*). The A-type *ARR*s are primary cytokinin-responsive genes (15, 21). Other cytokinin-inducible genes, such as those encoding the chalcone synthase, cytokinin oxidase, and dihydroflavonol 4-reductase, were also up-regulated in the mutant. These results show that the mutation in *ore12-1* results in a gain-of-function that leads to constitutive or enhanced expression of cytokinin-responsive genes. Senescence-associated genes encoding nucleases (BFN1), proteases (SAG12), and lipases (acylhydrolase), all of which are hydrolytic enzymes that may be involved in degradation of the respective macromolecules during senescence, were suppressed in the mutant. Of down-regulated genes in the mutant, another major group was genes encoding

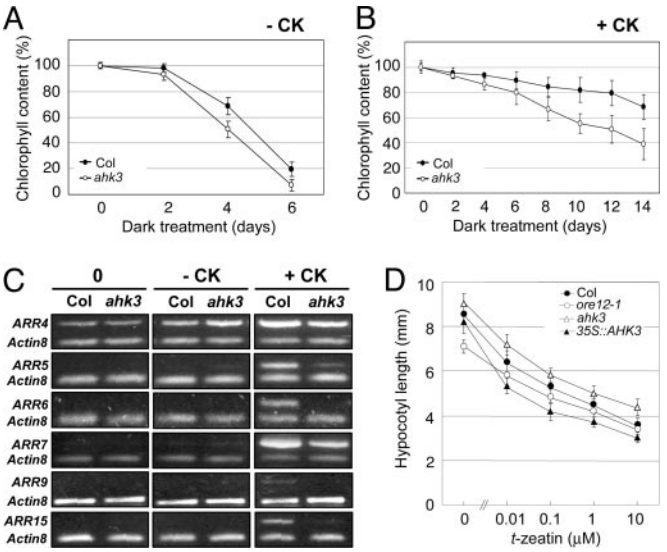

Fig. 3. AHK3 positively transmits cytokinin signals. (*A* and *B*) Senescence response of the loss-of-function *ahk3* mutant. Detached leaves of wild-type and *ahk3* plants were incubated in darkness with or without treatment with BA, a synthetic cytokinin. (*C*) Reduction of cytokinin-induced A-type *ARR* gene expression in the *ahk3* mutant. RT-PCR was performed with total RNA extracted from wild-type or the *ahk3* leaves before (0) and after treatment with (+CK) or without (−CK) *t*-zeatin for 1 h. (*D*) Inhibition of cytokinin-mediated hypocotyl growth in wild-type, *ore12-1*, *ahk3*, and an *AHK3* overexpression *35S::AHK3–40* line. Seedlings were grown in the presence of *t*-zeatin at the indicated concentrations. At least 15 hypocotyls were examined for each treatment.

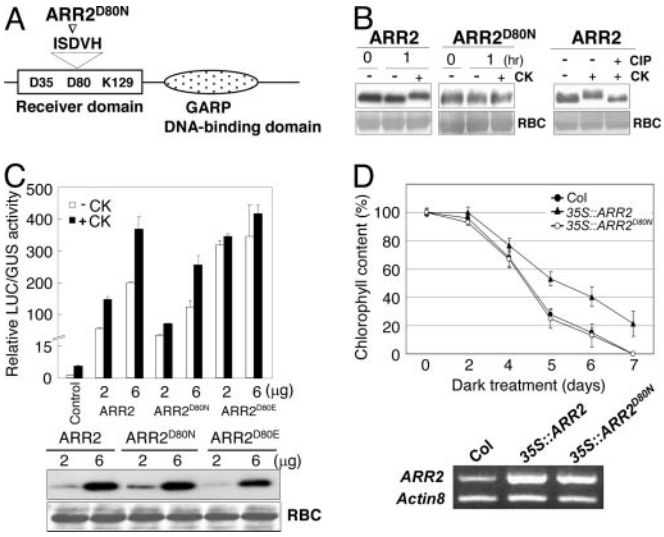

Fig. 4. Cytokinin induces phosphorylation of ARR2 in mature leaf cells, leading to increased leaf longevity. (*A*) Structure of ARR2. The conserved amino acid residues in the receiver domain are noted. D80 is the predicted target residue for phosphorylation and is mutated to Asn in ARR2^{D80N}. (*B*) Cytokinin-induced mobility shift of ARR2. Cells transfected with *ARR2-HA* were preincubated for 4 h (0 h) before treatment for 1 h with (+) or without (−) *t*-zeatin (100 nM). Cytokinin-induced mobility shift is abolished by phosphatase treatment. Cells transfected with *ARR2* were treated with (+CK) or without (−CK) *t*-zeatin for 1 h. Crude extracts of cytokinin-treated cells were incubated with (+CIP) or without (−CIP) calf intestine alkaline phosphatase. The proteins were detected with an anti-HA antibody. The Coomassie-brilliant-blue-stained RbcS protein (RBC) serves as a protein-loading control. (*C*) *ARR2^{D80N}* mutation reduced its capacity to transactivate the *ARR6* promoter (*Upper*). Protoplasts were cotransfected with the *ARR6-LUC* reporter and an effector plasmid expressing ARR2, ARR2^{D80N}, or ARR2^{D80E} mutant proteins. Vector DNA was used as a control. A limited amount (2 μg) of an effector plasmid was transfected to avoid nonspecific activation by protein overexpression. Immunoblot with an anti-HA antibody shows protein level of ARR2, ARR2^{D80N}, and ARR2^{D80E} (*Lower*). (*D*) Ectopic expression of *ARR2* (*^{35}S::ARR2*), but not that of *ARR2^{D80N}* (*^{35}S::ARR2^{D80N}*), resulted in delayed leaf senescence during dark incubation. Error bar indicates standard deviation (n = 30).

nutrient-transporter proteins, including amino acid, sucrose, metal ion, and peptide transporters. This indicates that the *ore12-1* mutation results in reduction of hydrolytic activity and a decreased remobilization and export of metabolites from leaves, which is physiologically consistent with delay of leaf senescence. A few potential regulatory elements of senescence, such as WRKY transcription factors and receptor-like kinase genes, were also down-regulated.

AHK3 Transmits Cytokinin Signals to Positively Control Leaf Longevity. The loss-of-function *ahk3* mutant (see Fig. 11, which is published as supporting information on the PNAS web site) showed an early senescence phenotype during dark-induced senescence (Fig. 3*A*), as evident from its lower chlorophyll content. Furthermore, application of cytokinins to wild-type leaves resulted in markedly reduced senescence symptoms (22), but this response was much reduced in the loss-of-function *ahk3* mutant (Fig. 3*B*). This observation supports the notion that AHK3 plays a positive role in cytokinin-mediated control of leaf longevity.

We examined cytokinin-induced expression of the A-type *ARR* genes in the *ahk3* mutant (Fig. 3*C*). Many of the A-type *ARR* genes were up-regulated by cytokinin treatment in wild-type plants, but cytokinin-dependent induction of *ARR5*,- *6*, *-9*, and *-15* was much suppressed in the loss-of-function mutant. This observation is consistent with the reduced sensitivity of the mutant with respect to cytokinin-mediated longevity control.

A naturally occurring cytokinin *t*-zeatin reduces hypocotyl growth of the wild-type seedlings (23). Compared with the wild-type seedlings, the gain-of-function *ore12-1* lines showed reduced hypocotyl growth, even in the absence of cytokinin (Fig. 3*D*). However, in the presence of 10 μM cytokinin, this difference was eliminated, supporting the argument that the *ore12-1* mutation confers a partially constitutive cytokinin response. The *AHK3*-overexpressing transgenic lines showed an increased sensitivity to the exogenous cytokinin in hypocotyl growth inhibition, whereas the loss-of-function *ahk3* mutant showed a slightly reduced sensitivity to the exogenous cytokinin. The hypocotyl response of these lines to cytokinin is consistent with the senescence response observed above, together supporting the notion that AHK3 positively transmits cytokinin signaling. The data also showed that, in addition to control of leaf longevity, AHK3 is involved in hypocotyl growth control.

Cytokinin Induces Phosphorylation of ARR2, a B-Type Response Regulator. Cytokinin signal perceived by the histidine kinase cytokinin receptors was transduced to the B-type ARRs via a His-to-Asp phosphorelay cascade (16, 17). The *Arabidopsis* B-type ARRs are transcription factors containing an N-terminal receiver domain and a C-terminal GARP DNA-binding domain (16) (Fig. 4*A*). We reported that ectopic expression of *ARR2*, a B-type ARR, delays leaf senescence (15) (Fig. 4*D*). Thus, it is plausible that cytokinin signal is transmitted from AHK3 to ARR2 to control leaf longevity via a phosphorelay cascade. We tested this notion using protoplasts from mature leaf cells and a gel-shift assay. The gel-shift assay showed that the position of ARR2 was shifted by cytokinin treatment from a faster- to a slower-migrating band (Fig. 4*B*). The cytokinin-induced shift of ARR2 was also observed in *ARR2*-overexpressing transgenic plants (data not shown). Phosphatase treatment shifted the slower-migrating ARR2 band back to the faster-migrating one

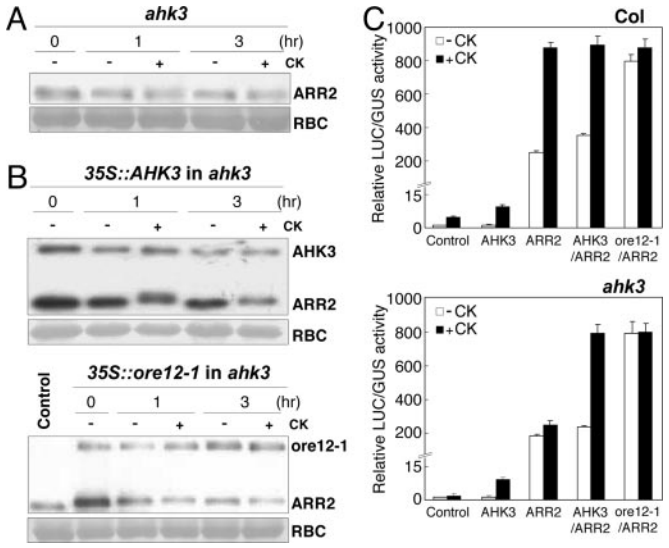

Fig. 5. Cytokinin-induced phosphorylation of ARR2 is mediated by AHK3. (*A*) Impaired phosphorylation of ARR2 in the *ahk3* mutant. (*B*) Restoration of impaired ARR2 phosphorylation in the *ahk3* cells by AHK3 and ore12-1. *ARR2* was transfected into the *ahk3* cells with *AHK3* (*Upper*) or *ore12-1* (*Lower*). In the case of *ore12-1* transfection, protein extract from the *ahk3* cells transfected with *ARR2* was included as a control. The transfected cells were treated with (+CK) or without (−CK) *t*-zeatin for 1 and 3 h. The proteins were detected with an anti-HA antibody. (*C*) AHK3-dependent action of ARR2 in inducing the *ARR6-LUC* expression. Wild-type or *ahk3* mutant cells were transfected with the *ARR6-LUC* reporter alone (Control) or cotransfected with *ARR6-LUC* and the indicated plasmids. Transfected cells were treated with (+CK) or without (−CK) *t*-zeatin. Error bar indicates standard deviation ($n = 3$).

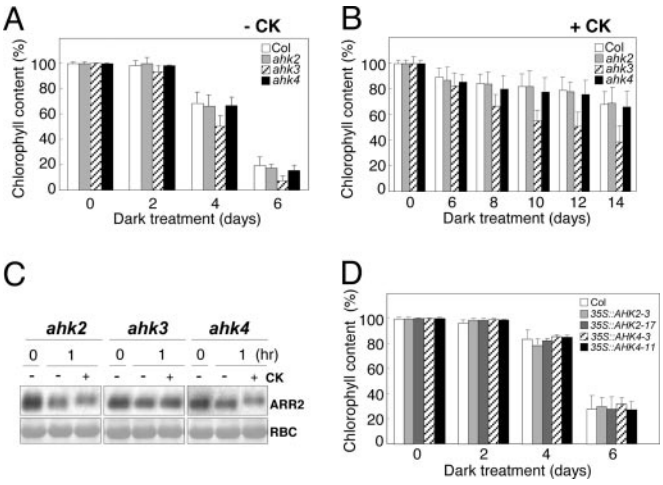

Fig. 6. Specificity of AHK3 in cytokinin-mediated leaf senescence and phosphorylation of ARR2. (*A* and *B*) Senescence response of the loss-of-function *ahk2* and *ahk4* mutants with or without cytokinin. Error bar indicates standard deviation ($n = 36$). (*C*) Impaired ARR2 phosphorylation in the *ahk3* cells but not in the *ahk2* and *ahk4* cells. Note that the mobility shift is observed in the *ahk2* and *ahk4* cells. (*D*) Senescence response of *AHK2-* and *-4*-overexpression transgenic lines. Error bar indicates standard deviation ($n = 24$). The chlorophyll content was examined every 2 days during dark-induced senescence.

(Fig. 4*B*). $ARR2^{D80N}$, in which the phosphotransfer to ARR2 is predicted to be abolished because of the mutation in the conserved Asp-80 residue to Asn in the receiver domain (24) (Fig. 4*A*), did not show a mobility shift to a slower-migrating band upon cytokinin treatment (Fig. 4*B*). This result proved that the cytokinin-dependent mobility shift of ARR2 is due to phosphorylation on the Asp-80 residues of ARR2. The $ARR2^{D80N}$ showed a lower transactivation capacity on *ARR6-LUC* expression than did wild-type ARR2 (Fig. 4*C*). $ARR2^{D80E}$, which mimics phosphorylated ARR2 because of the amino acid substitution of Asp to Glu, constitutively activated the *ARR6* promoter (15, 25) (Fig. 4*C*). This result indicated that the phosphorylation on the Asp-80 residue of ARR2 leads to activation of ARR2 for positive cytokinin response.

We examined the effect of transgenic overexpression of wild-type *ARR2* and $ARR2^{D80N}$ on leaf senescence. The effect of $ARR2^{D80E}$ was not examined, because the transgenic plants overexpressing $ARR2^{D80E}$ showed severe developmental defects, including dwarfism (25). The transgenic plants overexpressing wild-type *ARR2* showed delayed leaf senescence during dark-induced (Fig. 4*D*) and age-dependent senescence (see Fig. 12, which is published as supporting information on the PNAS web site). However, the transgenic plants overexpressing $ARR2^{D80N}$ did not show a noticeable difference in senescence compared with wild-type plants (Figs. 4*D* and 12). Collectively, these data suggest that the cytokinin-induced phosphorylation on the conserved Asp-80 residue of ARR2 plays a positive role in cytokinin-mediated control of leaf longevity.

Cytokinin-Induced Phosphorylation of ARR2 Is Mediated by AHK3. We then tested whether AHK3 mediates phosphorylation of ARR2. When ARR2 protein is expressed in protoplasts from the *ahk3* mutant, cytokinin-dependent mobility shift of ARR2 is abolished (Fig. 5*A*), indicating that AHK3 is required for phosphorylation of ARR2. The requirement of AHK3 for the phosphorylation of ARR2 was further confirmed by the observation that expression of *AHK3* in the *ahk3* cells restored the cytokinin-dependent mobility shift of ARR2 (Fig. 5*B*). Expression of the *ore12-1* mutant gene in the *ahk3* cells caused a mobility shift of ARR2, even in the absence of cytokinin, consistent with the gain-of-function nature of the mutation (Fig. 5*B*). These data suggest that ARR2 is a target for the cytokinin-dependent AHK3-mediated phosphorelay and that control of leaf longevity by cytokinin signals is positively mediated by the phosphorelay pathway from AHK3 to ARR2. We further provided evidence that ARR2 functions downstream of AHK3 in cytokinin signaling by the observation that cytokinin-dependent induction of *ARR6-LUC* through ARR2 is AHK3-dependent (Fig. 5*C*). As reported in ref. 15, overexpression of *ARR2* led to enhanced expression of *ARR6-LUC*, and cytokinin treatment further enhanced expression of *ARR6-LUC* in wild-type cells, showing that the cytokinin signal induces ARR2-dependent expression of *ARR6-LUC*. Cytokinin-induced, ARR2-dependent expression of *ARR6-LUC* was almost completely abolished in the *ahk3* cells but was restored by coexpression of wild-type *AHK3*. Consistent with the gain-of-function nature of the *ore12-1* mutation, overexpression of *ore12-1* together with *ARR2* in wild-type or the *ahk3* cells resulted in cytokinin-independent constitutive expression of *ARR6-LUC*.

AHK3, Among the Three Cytokinin Receptors, Plays a Major Role in Controlling Cytokinin-Mediated Leaf Longevity Through a Specific Phosphorylation of ARR2. *Arabidopsis* has three histidine kinase cytokinin receptors, AHK2, AHK3, and AHK4/CRE1/WOL. We thus asked whether these receptors have overlapping functions in the control of cytokinin-mediated leaf longevity. We first examined the senescence response of the loss-of-function mutants of the three receptors during dark-induced senescence in the absence of exogenous cytokinin. The loss-of-function *ahk3* mutant displayed a noticeable early-senescence phenotype (Fig. 6*A*); however, neither the *ahk2* nor *ahk4* mutant showed any noticeable senescence phenotype. We then examined the senescence response of these mutants to exogenous cytokinin treatment. Whereas the *ahk3* mutant exhibited a clearly reduced

PLANT BIOLOGY

cytokinin response compared with wild type in delaying leaf senescence (Fig. 6*B*), the *ahk2* and *ahk4* mutants responded to cytokinins in a similar manner to wild-type plants. The result showed that AHK2 and AHK4, unlike AHK3, may have no, or only a minor, role in cytokinin-mediated control of senescence. AHK3 was responsible for the phosphorylation of ARR2, which, in turn, caused increased leaf longevity. We thus asked if the lack of senescence phenotype in the *ahk2* and *ahk4* mutants was associated with phosphorylation of ARR2. As shown in Fig. 6*C*, the cytokinin-mediated mobility shift of ARR2 was detected in *ahk2* and *ahk4* mutants.

We further examined the involvement of AHK2 and AHK4 in the control of leaf longevity by analyzing the transgenic plants that ectopically express these genes. Unlike the *AHK3* transgenic plants, the *AHK2* and *AHK4* transgenic lines did not show any noticeable delay of senescence (Fig. 6*D*). Delay of leaf senescence was also not observed in transgenic plants that ectopically express the corresponding *ore12-1* mutant forms of either *AHK2* or *AHK4* (data not shown). These results support the conclusion that AHK3, but not AHK2 or AHK4, plays a major role in control of leaf longevity.

Discussion

Although cytokinins have been known for many decades to be senescence-delaying plant hormones (7), the mechanism underlying cytokinin-mediated senescence control remains largely unknown. Here, we show that a cytokinin receptor, AHK3, is, indeed, involved in the control of leaf longevity. The unique function of AHK3 receptor in cytokinin-mediated senescence control was most clearly revealed by a gain-of-function *ore12-1* mutation. The gain-of-function mutation significantly delayed the senescence symptoms in *Arabidopsis*. The *ore12-1* mutation occurred in the extracellular domain of the receptor, which is predicted to be the cytokinin-binding site. The constitutive cytokinin response observed in this mutation may be due to a constitutively active state of ore12-1 that was structurally altered to resemble a cytokinin-bound form of AHK3. Ectopic expression of *AHK3*, but not the other receptors, displayed delayed senescence, although these lines still showed increased sensitivity to cytokinins in other responses, such as shoot induction. Applied cytokinins markedly increase leaf longevity, and this response is highly attenuated in the absence of AHK3, as shown in the loss-of-function *ahk3* mutant. These observations led to the conclusion that AHK3 is the major cytokinin receptor, among the three *Arabidopsis* cytokinin receptors, to mediate the antisenescing effect of cytokinins, although AHK3 is involved in other cytokinin-mediated responses as well.

Although the loss-of-function *ahk3* plants showed a noticeable early-senescence phenotype during dark-induced senescence (Fig. 3), they did not show a significant early-senescence phenotype during age-dependent senescence (see Fig. 13, which is published as supporting information on the PNAS web site). Because the other two cytokinin receptors, AHK2 and AHK4, did not appear to be involved in the control of senescence, the lack of senescence phenotype in the loss-of-function *ahk3* mutant during age-dependent senescence is not likely because of the redundancy of the *AHK* genes. The deficiency of AHK3 during age-dependent senescence may be compensated by another unknown cytokinin-signaling pathway, as suggested by the moderate phenotype of an *ahk2 ahk3 ahk4* triple mutant in embryogenesis (20). Another possibility is functional compensation by other longevity-controlling pathways, because leaf senescence is the integrated result of the activity of various pathways that incorporate many internal and external signals to finely adjust the life span of a leaf (1).

By analogy with the two-component signaling mechanism in bacteria and yeast, a His-to-Asp phosphorelay cascade has been suggested as a regulatory mechanism in the cytokinin signal-transduction pathway (15, 16, 26). However, no *in vivo* evidence has been provided regarding the existence and the molecular specificity of the phosphorelay cascade in cytokinin signaling, although it was shown that ARRs could function as phosphoacceptors in the *Escherichia coli* His-Asp phosphotransfer system (27). Here, we provide *in vivo* evidence that cytokinins, indeed, induce phosphorylation of ARR2, a B-type ARR, and that this phosphorylation of the conserved Asp residue of ARR2 is essential to exert its action in delaying leaf senescence. Mutational analysis and transgenic phenotype of ARR2 demonstrated that the cytokinin-induced phosphorylation on Asp-80 in the receiver domain of ARR2 is essential for its function on the downstream cytokinin responses. However, $ARR2^{D80N}$ mutant protein was still partially responsive to cytokinin in inducing the *ARR6* promoter in protoplasts (Fig. 4*C*). The mutant protein may still be activated for cytokinin responses by an alternative mechanism that plays a limited role in leaf senescence (Fig. 12).

Whereas transgenic overexpression of *AHK2* or *AHK4* could enhance cytokinin-mediated shoot regeneration and induction of cytokinin-responsive genes (data not shown), only AHK3, but not AHK2 or AHK4, had a major role in delaying leaf senescence. Furthermore, the cytokinin-dependent ARR2 phosphorylation in mature leaf cells was impaired specifically in the *ahk3* mutant but not in the *ahk2* or *ahk4* mutants. These results suggested that the specificity of AHK3 in leaf-senescence control and ARR2 phosphorylation is an intrinsic property of the AHK3 protein. However, transfection of *AHK2* or *AHK4* as well as *AHK3* into the *ahk3* mutant cells restored ARR2 phosphorylation (data not shown), implying that ARR2 phosphorylation can occur by an excess upstream signaling input. Thus, the specificity of AHK3 in the control of leaf longevity and ARR2 phosphorylation may depend not only on the intrinsic property of the cytokinin signaling components but also on their spatially and temporally differential expression.

Whereas the short cytokinin-signaling circuit consisting of two-component elements emerged as a core signaling pathway for various cytokinin responses, there has been no report on the signaling specificity of each cytokinin response. The interactions among two-component elements and other unknown components may be critical to decisively transmit the cytokinin signal to the downstream response, and differential expressions of histidine kinases and response regulators could add some degree of specificity (28, 29). Our data show how a specificity of signaling initiated from a histidine kinase in senescence control is achieved. Based on the data in this report, we propose the following model for cytokinin-mediated control of leaf longevity. The cytokinin signal is perceived by AHK3, and this induces the cytokinin-dependent phosphorelay to ARR2. Although the cytokinin signals may be perceived by the other cytokinin receptors in *Arabidopsis*, ARR2 phosphorylation is specifically mediated by AHK3. Thus, the phosphorelay of AHK3 to ARR2 is a signaling pathway specific to the control of cytokinin-mediated leaf longevity. The phosphorylated ARR2 then induces downstream cytokinin-responsive genes and, directly or indirectly, leads to induction or repression of a set of target genes responsible for regulating and executing leaf senescence, resulting in increased leaf longevity.

The observation that phosphorylation on a specific residue of ARR2 mediated by AHK3 is critical for delaying leaf senescence led us to propose the role of ARR2 in the control of leaf senescence. However, we could not detect an early-senescence symptom in the *arr2* knockout plants (see Fig. 14, which is published as supporting information on the PNAS web site), although the *arr2* knockout plants were less sensitive to cytokinins in inhibition of hypocotyl growth (data not shown). We should note that there are 12 members of B-type *ARR*s in *Arabidopsis*. Thus, it is probable that other B-type ARRs are also involved in cytokinin-mediated control of leaf longevity and

complement the loss of ARR2 activity in the knockout plants. Alternatively, other leaf-longevity-controlling mechanisms may compensate for the loss of ARR2 activity.

Materials and Methods

Plant Materials and Growth Conditions. The *ore12–1* mutant in the Col wild-type background was identified by screening ethylmethanesulfonate-mutagenized M_2 seeds for delayed senescence, as determined by a delayed loss of chlorophyll during the dark-induced senescence of detached leaves (22). For screening of mutants and a dark-induced senescence assay, the 3rd and 4th leaves at 12 days after leaf emergence were detached and floated on 3 mM Mes buffer (pH 5.7) in the dark. The loss-of-function *ahk3* mutant was screened from the Salk T-DNA collection by using a PCR-based method (see *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site). These mutant plants were backcrossed twice with wild-type (Col) plants and used for all analyses. The plants were grown in an environmentally controlled growth room at 22°C with a 16-hr-light/8-hr-dark cycle.

Generation of Transgenic Plants and RT-PCR. Transgenic plants expressing *AHK2*, $AHK2^{P380S}$, *AHK3*, *ore12-1*, *AHK4/CRE1/WOL*, or $AHK4^{P263S}$ under the control of the CaMV ^{35}S promoter were generated by the floral-dip method (30). All mutant clones were produced by QuickChange site-directed mutagenesis (Stratagene). All PCR products and the mutations were confirmed by DNA sequencing. Phenotypic analyses in the transgenic lines were performed in T_3 homozygous plants. For RT-PCR analysis, total RNA was isolated from leaves with the TriReagent kit (Molecular Research Center, Cincinnati, OH). First-strand cDNA was synthesized with 1 μg of RNA with the ImProm-II Reverse Transcription system (Promega). All RT-PCR experiments were repeated at least three times.

Assays of Leaf Senescence and Inhibition of Hypocotyl Elongation. Chlorophyll contents of individual leaves were measured as described in ref. 31. The photochemical efficiency of photosystem II was measured by the Plant Efficiency Analyzer (18) (Hansatech Instruments, Morfolk, U.K.). For measurement of hypocotyls length, *Arabidopsis* seedlings were grown in continuous darkness on the 0.1× Murashige and Skoog (MS) plates, which contained various concentrations of *t*-zeatin, for 4 d. The length of the hypocotyl of individual plants was measured ($n > 15$), as described in ref. 23.

Transient Expression Assay in *Arabidopsis* Protoplasts. *AHK3*, *ore12-1*, and $ARR2^{D80N}$ cDNAs were fused to either the hemagglutinin (HA) epitope or GFP coding sequence in a plant-expression vector containing the *35SC4PPDK* promoter and the *NOS* terminator (32). *Arabidopsis* protoplasts were isolated and transfected as described in ref. 15. For protein phosphorylation assays, protoplasts from wild-type, *ahk2*, *ahk3*, *ahk4*, or *ore12-1* plants were transfected with plasmids expressing *ARR2*, $ARR2^{D80N}$, *ARR2* and *AHK3*, or *ARR2* and *ore12-1*. Transfected protoplasts were incubated for 4 h for protein expression and treated with 100 μM of cycloheximide and 100 nM of *t*-zeatin for indicated times. Total protein (10 μg) was resolved by 7.5% SDS/PAGE and electroblotted to Immobilon-P membrane (Millipore, Bedford, MA). HA-tagged proteins were detected by using a peroxidase-conjugated anti-HA antibody (Roche Applied Science, Indianapolis). For a transient expression assay, the *ARR6-LUC* reporter gene was cotransfected with *ARR2*, $ARR2^{D80N}$, or $ARR2^{D80E}$ into protoplasts isolated from wild-type or *ahk3* plants. Transfected protoplasts were incubated with or without 100 nM *t*-zeatin for 3 h. The *UBQ10-GUS* construct was used as an internal control.

We thank Filip Rolland for critical reading of the manuscript. H.G.N. was supported by the National Research Laboratory Program, the Crop Functional Genomics Research Grant, and the Nanoelectronics Collaborative Research Center Program from the Ministry of Science and Technology of Korea; H.J.K. was supported, in part, by the Program for the Training of Graduate Students from the Ministry of Commerce, Industry and Energy of Korea; and I.H. was supported by the Plant Diversity Research Center of the Ministry of Science and Technology of Korea, Pohang University of Science and Technology research fund and the Plant Signaling Network Research Center of the Korea Science and Engineering Foundation.

1. Noodén, L. D. (1988) in *Senescence and Aging in Plants*, eds. Noodén, L. D. & Leopold, A. C. (Academic, San Diego), pp. 1–50.
2. Nam, H. G. (1997) *Curr. Opin. Biotech.* **8,** 200–207.
3. Gut, H., Ruts, C., Matile, P. & Thomas, H. (1987) *Physiol. Plant.* **70,** 659–663.
4. Himelblau, E. & Amasino, M. (2001) *J. Exp. Bot.* **158,** 1317–1323.
5. Gan, S. & Amasino, R. M. (1997) *Plant Physiol.* **113,** 313–319.
6. Lim, P. O., Woo, H. R. & Nam, H. G. (2003) *Trends Plant Sci.* **8,** 272–278.
7. Richmond, A. E. & Lang, A. (1957) *Science* **125,** 650–651.
8. Gan, S. & Amasino, R. M. (1995) *Science* **270,** 1986–1988.
9. McCabe, M. S., Garratt, L. C., Schepers, F., Jordi, W. J., Stoopen, G. M., Davelaar, E., van Rhijn, J. H., Power, J. B. & Davey, M. R. (2001) *Plant Physiol.* **127,** 505–516.
10. Nelson, C. J. (1988) *Plant Physiol. Biochem.* **26,** 543–554.
11. Mok, D. W. & Mok, M. C. (2001) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **52,** 89–118.
12. Howell, S. H., Lall, S. & Che, P. (2003) *Trends Plant Sci.* **8,** 453–459.
13. Ueguchi, C., Koizumi, H., Suzuki, T. & Mizuno, T. (2001) *Plant Cell. Physiol.* **42,** 231–235.
14. Inoue, T., Higuchi, M., Hashimoto, Y., Seki, M., Kobayashi, M., Kato, T., Tabata, S., Shinozaki, K. & Kakimoto, T. (2001) *Nature* **409,** 1060–1063.
15. Hwang, I. & Sheen, J. (2001) *Nature* **413,** 383–389.
16. Kakimoto, T. (2003) *Annu. Rev. Plant Biol.* **54,** 605–627.
17. Heyl, A. & Schmulling, T. (2003) *Curr. Opin. Plant Biol.* **6,** 480–488.
18. Oh, S. A., Park, J. H., Lee, G. I., Paek, K. H., Park, S. K. & Nam, H. G. (1997) *Plant J.* **12,** 527–535.
19. Nishimura, C., Ohashi, Y., Sato, S., Kato, T., Tabata, S. & Ueguchi, C. (2004) *Plant Cell* **16,** 1365–1377.
20. Higuchi, M., Pischke, M. S., Mahonen, A. P., Miyawaki, K., Hashimoto, Y., Seki, M., Kobayashi, M., Shinozaki, K., Kato, T., Tabata, S., *et al.* (2004) *Proc. Natl. Acad. Sci. USA* **101,** 8821–8826.
21. D'Agostino, I. B., Deruere, J. & Kieber, J. J. (2000) *Plant Physiol.* **124,** 1706–1717.
22. Oh, S. A., Lee, S. Y., Chung, I. K., Lee, C. H. & Nam, H. G. (1996) *Plant Mol. Biol.* **30,** 739–754.
23. Cary, A. J., Liu, W. & Howell, S. H. (1995) *Plant Physiol.* **107,** 1075–1082.
24. Stock, A. M., Robinson, V. L. & Goudreau, P. N. (2000) *Annu. Rev. Biochem.* **69,** 183–215.
25. Hass, C., Lohrmann, J., Albrecht, V., Sweere, U., Hummel, F., Yoo, S. D., Hwang, I., Zhu, T., Schafer, E., Kudla, J. & Harter, K. (2004) *EMBO J.* **23,** 3290–3302.
26. Ferreira, F. J. & Kieber, J. J. (2005) *Curr. Opin. Plant Biol.* **8,** 518–525.
27. Imamura, A., Hanaki, N., Umeda, H., Nakamura, A., Suzuki, T., Ueguchi, C. & Mizuno, T. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 2691–2696.
28. Mason, M. G., Li, J., Mathews, D. E., Kieber, J. J. & Schaller, G. E. (2004) *Plant Physiol.* **135,** 927–937.
29. To, J. P., Haberer, G., Ferreira, F. J., Deruere, J., Mason, M. G., Schaller, G. E., Alonso, J. M., Ecker, J. R. & Kieber, J. J. (2004) *Plant Cell* **16,** 658–671.
30. Clough, S. J. & Bent, A. F. (1998) *Plant J.* **16,** 735–743.
31. Lichtenthaler, H. K. (1987) *Methods Enzymol.* **148,** 350–382.
32. Sheen, J. (1996) *Science* **274,** 1900–1902.