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DEG9, a serine protease, modulates cytokinin and light signaling by regulating the level of *ARABIDOPSIS* RESPONSE REGULATOR 4

Wei Chi[a,1], Jing Li[a,1], Baoye He[a], Xin Chai[a,b], Xiumei Xu[a], Xuwu Sun[a], Jingjing Jiang[a,b], Peiqiang Feng[a,b], Jianru Zuo[c,d], Rongcheng Lin[a], Jean-David Rochaix[e,f], and Lixin Zhang[a,2]

[a]Photosynthesis Research Center, Key Laboratory of Photobiology, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China; [b]University of Chinese Academy of Sciences, Beijing 100049, China; [c]State Key Laboratory of Plant Genomics, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China; [d]National Plant Gene Research Center, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China; [e]Department of Molecular Biology, University of Geneva, 1211 Geneva, Switzerland; and [f]Department of Plant Biology, University of Geneva, 1211 Geneva, Switzerland

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Cytokinin is an essential phytohormone that controls various biological processes in plants. A number of response regulators are known to be important for cytokinin signal transduction. *ARABIDOPSIS* RESPONSE REGULATOR 4 (ARR4) mediates the cross-talk between light and cytokinin signaling through modulation of the activity of phytochrome B. However, the mechanism that regulates the activity and stability of ARR4 is unknown. Here we identify an ATP-independent serine protease, degradation of periplasmic proteins 9 (DEG9), which localizes to the nucleus and regulates the stability of ARR4. Biochemical evidence shows that DEG9 interacts with ARR4, thereby targeting ARR4 for degradation, which suggests that DEG9 regulates the stability of ARR4. Moreover, genetic evidence shows that DEG9 acts upstream of ARR4 and regulates the activity of ARR4 in cytokinin and light-signaling pathways. This study thus identifies a role for a ubiquitin-independent selective protein proteolysis in the regulation of the stability of plant signaling components.

protease | cytokinins | light | DEG | *ARABIDOPSIS* RESPONSE REGULATOR 4

Cytokinins are essential plant hormones that are involved in the regulation of cell division and metabolism, chloroplast development, shoot and root development, delay of leaf senescence, and stress responses (1–3). Cytokinin signals are transmitted through a multistep histidine-to-aspartate phosphorelay system that is evolutionarily related to the two-component signaling systems of prokaryotes (4–6). In *Arabidopsis thaliana*, the final targets of this phosphorelay system are two functionally antagonistic classes of *Arabidopsis* response regulators (ARRs) that contain a receiver domain with a conserved Asp phosphorylation site. *Arabidopsis* contains 24 ARRs, which are subdivided into types A and B. Type A ARRs include 10 typical members (ARR3–9 and 15–17) and 2 atypical members (ARR22 and 24, also called type C ARRs), whereas type B ARRs include 12 members (ARR1, 2, 10–14, 18–21, and 23) (7–10). Type B ARRs function as transcription factors for a subset of cytokinin-regulated targets, including the type A ARRs that act as negative regulators of the cytokinin signal transduction pathway. The induction of type A ARR genes creates a negative-feedback loop that regulates the strength and duration of the cytokinin response (11–14). ARRs also play an important role in the interactions of cytokinin with Auxin and other signal transduction pathways (3, 14–16).

In addition to the transcriptional regulation of ARRs by type B ARRs, posttranslational modification is important in the two-component signaling pathway (17–21). One transcription-independent cytokinin response occurs through the regulation of ARR stability (19, 22–27). In plants, the best-characterized route for selective protein proteolysis is the ubiquitin-proteasome system, which contributes to the regulation of a wide range of growth and developmental processes (28). Nevertheless, the ubiquitin-proteasome system does not account for the degradation of all cytokinin signaling components, as several ARR proteins do not depend on the ubiquitin-proteasome system for their degradation (19, 23). However, alternative ubiquitin-independent protein degradation mechanisms that may regulate the degradation of ARR proteins have not yet been identified.

Aside from the ubiquitin-proteasome system, plants contain hundreds of proteases, which have been subdivided into families and clans based on evolutionary relationships (29, 30). Many of these proteases are highly conserved and widely distributed in eukaryotes and prokaryotes. Recent studies have shown that distinct proteases are expressed at specific times and locations and accumulate in different subcellular compartments. These observations suggest diverse roles for plant proteases (29, 30). Degradation of periplasmic proteins (DEG), also called high-temperature requirement A proteins, are ATP-independent serine proteases that are found in almost all organisms, including bacteria, protozoa, fungi, plants, and mammals (31). DEG proteases, with the exception of some plant and mammalian family members, contain a chymotrypsin-type serine protease domain and one or two C-terminal PDZ domains (domain present in PSD-95/SAP90, disc-large and ZO-1 proteins), which mediate protein–protein interactions and are necessary for the formation of functional oligomeric complexes (32, 33).

Significance

Selective protein proteolysis is essential for many plant signal transduction pathways and regulates developmental stages of a plant. In addition to the well-characterized ubiquitin-proteasome system, other factors appear to be involved in the degradation of plant signaling components. Here we describe the function of the serine protease degradation of periplasmic protein 9 (DEG9) in plant signaling. We found that DEG9 mediates the degradation of *ARABIDOPSIS* RESPONSE REGULATOR 4, which is critical for regulating the cross-talk between cytokinin and light-signaling pathways. This study adds to our knowledge about the function of DEG proteases, which are common in the plant kingdom, and emphasizes their importance in plant development.

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[1]W.C. and J.L. contributed equally to this work.

[2]To whom correspondence should be addressed. Email: zhanglixin@ibcas.ac.cn.

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Although DEG proteases have been intensively studied in prokaryotes, the underlying molecular mechanisms and functional significance of DEG proteases in eukaryotes are poorly understood.

A striking feature of DEGs in plants is their relative abundance and their diversity in plants (34, 35). Most prokaryotes contain three DEG proteases, whereas fungi have only one and mammals have five. However, the genomes of *A. thaliana*, *Oryza sativa*, and *Populus trichocarpa* contain 16, 15, and 20 DEG genes, respectively (34). The high number of DEG proteases in plants may be due to gene duplications that are unique to the respective species (34, 35). Most proteases in plants are predicted to be located in organelles. Six DEG proteases from *Arabidopsis* localize to chloroplasts and are involved in the degradation of damaged photosynthetic proteins, specifically photosystem II reaction center D1 protein, under excess light conditions (36–39). However, little is known about the function of plant DEGs that are targeted to other compartments, with the exception of a peroxisomal DEG protease involved in the processing of the N-terminal peroxisomal targeting signal 2 (40).

In this study, we show that DEG9, a nuclear-localized DEG protease, regulates the stability of ARR4. The results provide evidence that the proteolysis of ARR4 mediated by the DEG9 protease contributes to the interaction between the cytokinin and phytochrome signaling pathways.

Results

Inactivation of DEG9 Affects the Cytokinin Response. Sixteen genes encoding proteins related to DEG from *Escherichia coli* are present in the *Arabidopsis* genome (*DEG1–16*) (34). The function of DEG9 in cytokinin signaling is implied by the high accumulation of *DEG9* mRNA induced by cytokinin (*t*-zeatin), as analyzed by the *Arabidopsis* electronic Fluorescent Pictograph (eFP) browser (41) (Fig. S1). To confirm the induction of DEG9 in response to cytokinin, we investigated the accumulation of DEG9 transcript and protein in wild-type (WT) plants after *t*-zeatin treatment (Fig. 1 *A* and *B*). The results confirmed that exogenous cytokinin induced the accumulation of DEG9. The amount of DEG9 protein increased approximately eightfold after 12 h of cytokinin treatment compared with untreated plants (Fig. 1*B*). By contrast, treatment with 1-aminocyclopropane-1-carboxylic acid, gibberellins, or brassinolide did not induce DEG9 (Fig. 1 *A* and *B*). These results show that DEG9 specifically responds to a cytokinin signal and suggest that DEG9 is involved in the cytokinin signaling pathway.

To investigate the role of DEG9 in cytokinin signaling, we isolated a null mutant allele of *DEG9* and generated transgenic lines that overexpress *DEG9* under the constitutive 35S cauliflower mosaic virus promoter (*DEG9*-OX) (Fig. S2). The expression of the cytokinin primary response genes *ARR4*, *ARR5*, *ARR7*, and *ARR15* after cytokinin treatment was examined by quantitative (q)RT-PCR in WT, *deg9*, and *DEG9*-OX seedlings (Fig. 1*C*). In WT plants, *ARR4*, *ARR5*, *ARR7*, and *ARR15* were rapidly induced and increased 3- to 10-fold after treatment with 100 nM *t*-zeatin for 1 h. In the *deg9* mutant, cytokinin-dependent induction of *ARR4*, *ARR5*, *ARR7*, and *AAR15* expression was inhibited compared with WT plants. The expression of these genes in response to cytokinin was not altered in *DEG9*-OX plants (Fig. 1*C*). The

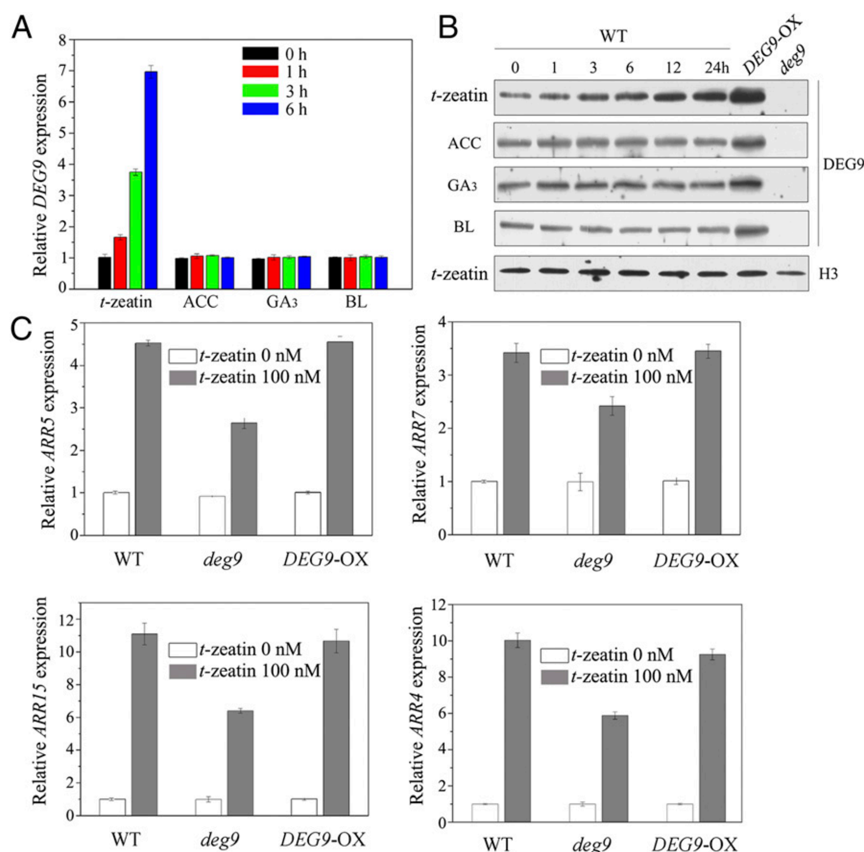

Fig. 1. Involvement of DEG9 in cytokinin signaling. (*A*) Expression of *DEG9* assayed by qRT-PCR. Ten-day-old WT seedlings were treated with 20 μM *t*-zeatin, aminocyclopropane-1-carboxylic acid (ACC), gibberellin 3 (GA_3), or brassinolide (BL) for 0–6 h and the accumulation of *DEG9* transcripts was analyzed by qRT-PCR at different times. Means ± SD ($n = 6$) are shown. (*B*) Accumulation of DEG9 protein under different phytohormone treatments. The histone H3 protein was used as a control. The treatment was as in *A*. (*C*) Accumulations of *ARR4*, *ARR5*, *ARR7*, and *ARR15* mRNAs were analyzed by qRT-PCR in WT, *deg9*, and *DEG9*-OX plants treated with 100 nM *t*-zeatin for 1 h. Data are means ± SD ($n = 10$).

inhibited induction of cytokinin primary response genes in *deg9* indicates that DEG9 positively regulates the primary cytokinin signal transduction pathway.

***DEG9* Genetically Interacts with *ARR4*.** Because the accumulation of *DEG9* transcripts was induced by exogenous cytokinin, we analyzed the promoter sequences of *DEG9* through the *Arabidopsis cis*-regulatory element database (AtcisDB; arabidopsis.med.ohio-state.edu/AtcisDB/) to obtain information about possible gene regulatory elements. Two phytochrome-responsive elements, GT-1 and SORLIP2 (42–44), were identified five times in the *DEG9* promoter. The abundant representation of these two elements suggested a potential relationship between DEG9 and ARR4 because ARR4 is known to interact with PhyB to mediate red-light signaling (18). We found that the levels of ARR4 induced by cytokinin in *deg9* were higher than those in WT plants (Fig. S3), which suggests a functional association between DEG9 and ARR4. In addition, the transcript profiles of *deg9* and *ARR4*-OX in response to cytokinin overlapped considerably when we compared the global gene expression responses using RNA-sequencing analysis (Fig. S4*A*). Clustering analysis of genes that were differentially expressed compared with WT in response to cytokinin showed a similar pattern in *deg9* and *ARR4*-OX plants (Fig. S4*B*). These results strongly suggest shared features between ARR4 and DEG9 function.

To further explore the functional relationship between DEG9 and ARR4 in cytokinin signaling, we assessed the physiological response to exogenous cytokinin in plant lines with altered levels of these two proteins. Growth of the primary root is sensitive to cytokinin and its elongation is inhibited by exogenous cytokinin, as shown in WT plants (Fig. 2*A*). *DEG9*-OX, *arr4*, and *arr4deg9* plants were indistinguishable from the WT plants, whereas the primary root growth of *deg9* mutant and *ARR4*-OX plants was less sensitive to cytokinin compared with WT plants. In addition, when *ARR4* was overexpressed in a *deg9* background (*ARR4*-OX/*deg9* plants), the sensitivity to cytokinin was significantly lower than in the single mutant (Fig. 2*A*). *ARR4*-OX/*DEG9*-OX, *deg9*, and *ARR4*-OX plants were equally sensitive to cytokinin. The levels of ARR4 were increased in the *deg9* background (Fig. S5), and these increased ARR4 levels negatively correlated with the inhibition of root elongation by cytokinin in these mutant lines (Fig. 2*A* and Fig. S5). These results suggest that DEG9 might be involved in cytokinin signaling by negatively regulating the accumulation of ARR4.

arr4 plants were indistinguishable from WT plants in the cytokinin response, which may be due to the genetic redundancy of

Fig. 2. Genetic interaction between *DEG9* and *ARR4*. (*A* and *B*) Effects of cytokinin on root growth in WT and mutant plants with distinct genetic backgrounds. WT and mutant seedlings were grown vertically on plates supplemented with dimethyl sulfoxide (DMSO) or 50 nM *t*-zeatin under constant light at 22 °C. After growth for 10 d, the primary root length was measured. The relative inhibition of primary roots is shown below. (*C* and *D*) Effects of red light on hypocotyls of WT and mutant seedlings. After seedlings were grown on 1/2 MS medium under red light for 4 d, hypocotyl length was measured. Mutant lines were analyzed for significant differences in their responsiveness to cytokinin or red light based on ANOVA ($P < 0.01$). Data are means ± SD ($n > 30$). Lines indicated with the same letter exhibited no significant difference.

type A ARRs (19). To further address the genetic relationship between *DEG9* and *ARR4*, we introduced the *deg9* mutation into double and quadruple mutants (*arr3,4* and *arr3,4,5,6*) and analyzed their cytokinin response. The double and quadruple mutants were more sensitive to cytokinin compared with WT plants (Fig. 2*B*), which is in agreement with a previous report (19). However, neither the *deg9* mutation nor *DEG9* overexpression in the *arr3,4* and *arr3,4,5,6* backgrounds caused a change in cytokinin response from that observed in *arr3,4* and *arr3,4,5,6* (Fig. 2*B*). By contrast, *DEG9* overexpression in the *arr3,5,6* mutant significantly enhanced cytokinin sensitivity compared with *arr3,5,6* plants (Fig. S6). Taken together, these results suggest that ARR4 and DEG9 act in the same pathway mediating cytokinin signaling.

Because of the involvement of ARR4 in phytochrome-mediated light signaling, we examined the light response of the mutant plant lines. *deg9*, *ARR4*-OX/*DEG9*-OX, *ARR4*-OX, and *ARR4*-OX/*deg9* plants showed shortened hypocotyls compared with WT plants under red light, suggesting a red-light hypersensitivity of these plants (Fig. 2*C*). By contrast, *arr4*, *arr4deg9*, and *DEG9*-OX plants showed an inhibited response to red light compared with WT plants. Similar to observations after cytokinin treatment, ARR4 levels negatively correlated with hypocotyl elongation under red light in these lines (Fig. 2*C* and Fig. S5). Hypocotyl elongation in *arr4deg9* was similar to that in *arr4* but not to that in *deg9* (Fig. 2*C*). Moreover, mutation or overexpression of *DEG9* in *arr3,4* and *arr3,4,5,6* had no effect on red-light sensitivity (Fig. 2*D*), similar to the case for cytokinin sensitivity. The *deg9* mutant did not show hypersensitivity in the hypocotyl growth response to far-red light, blue light, or darkness (Fig. S7*A*). A fluence-rate/response analysis of hypocotyl elongation revealed that hypersensitivity of the *deg9* mutant to red light increased over a broad range of light intensities (Fig. S7*B*). These results indicate that DEG9 is specifically involved in red-light signaling through regulating ARR4 activity.

Subcellular Localization and Expression Pattern of DEG9. Previous proteomic data indicated the presence of DEG9 in the nucleus of *Arabidopsis* (45). However, DEG9 is predicted to reside in chloroplasts as well as in the nucleus by the TargetP program (www.cbs.dtu.dk/services/TargetP/). To determine the subcellular location of DEG9, GFP fusion constructs for full-length *DEG9* under the control of the 35S cauliflower mosaic virus promoter were constructed and expressed in *Arabidopsis* protoplasts. The DEG9–GFP fusion protein localized to the nucleus, similar to the nucleolar fibrillarin that was used as a control for nuclear localization (46), and was not detected within chloroplasts (Fig. 3*A*). Two typical nuclear localization signals (amino acids 6–12 and 53–64) are predicted within the N-terminal sequence of DEG9. In agreement with this prediction, the N-terminal sequence (amino acids 1–128) of DEG9 was sufficient to target GFP to the nucleus (Fig. 3*A*). Immunoblot analysis of protein extracts from the nucleus and chloroplasts confirmed the nuclear localization of DEG9 (Fig. 3*B*).

Histochemical analysis of stable *Arabidopsis* lines expressing β-glucuronidase (GUS) under the control of the *DEG9* promoter revealed *DEG9* promoter activity in all vegetative and reproductive tissues, including roots, cotyledons, rosette leaves, siliques, and flowers (Fig. 3*C*), indicating that *DEG9* is ubiquitously expressed within the plant.

DEG9 Protein Physically Interacts with ARR4. Protein stability of ARRs is critical for cytokinin signaling (19, 23). To explore the mechanism of regulation of ARR4 stability, we treated seedlings overexpressing MYC-tagged ARR4 (ARR4-MYC) with various protease inhibitors in the presence of the protein biosynthesis inhibitor cycloheximide (CHX) and then investigated ARR4 accumulation (Fig. S8). CHX treatment alone reduced ARR4 accumulation significantly in comparison with the control (DMSO treatment alone). However, in the presence of the serine protease inhibitors phenylmethylsulfonyl fluoride, *N*-tosylphenylalanine

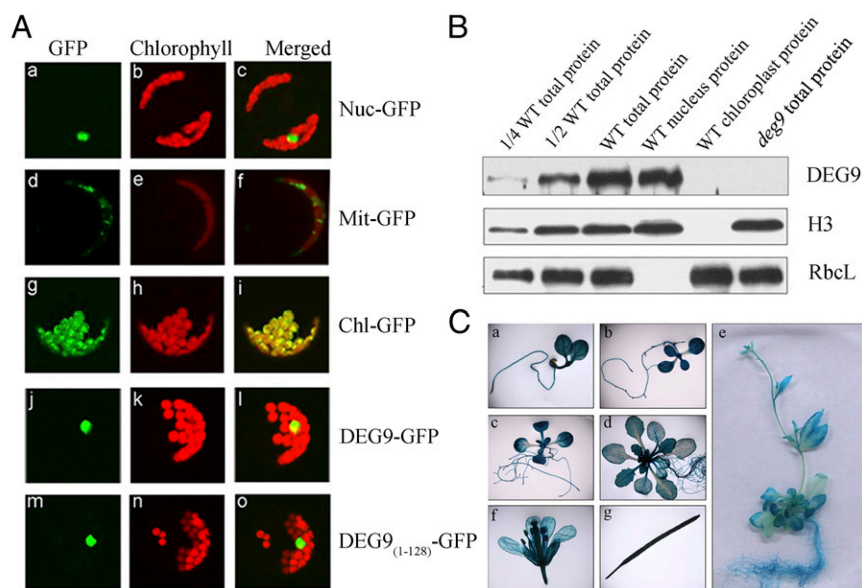

Fig. 3. Subcellular localization and expression patterns of DEG9. (*A*) Subcellular localization of DEG9 protein visualized by GFP analysis. The GFP signal was obtained by confocal microscopy (*a*, *d*, *g*, *j*, and *m*). Chloroplasts were visualized by chlorophyll autofluorescence (*b*, *e*, *h*, *k*, and *n*). The colocalization of GFP and chloroplasts is shown in merged images (*c*, *f*, *i*, *l*, and *o*). The constructs used for transformation are indicated (*Right*): Nuc-GFP, control showing the nuclear localization signal of fibrillarin; Mit-GFP, control showing the mitochondrial localization signal of FROSTBITE1 (FRO1); Chl-GFP, control showing the transit peptide of the ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit; DEG9–GFP, signals from the DEG9–GFP fusion protein; and $DEG9_{(1–128)}$-GFP, signals from the N-terminal 128 amino acid–GFP fusion protein. (*B*) Immunoblot analysis of DEG9 subcellular localization. Total protein, chloroplast protein, and nucleoprotein preparations from WT and *deg9* plants were analyzed using immunoblot analysis with specific antisera against DEG9, H3, and ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (RbcL). Total protein (100 μg), nucleoprotein (10 μg), and chloroplast protein (10 μg) extracts were loaded into the indicated lanes. (*C*) Expression analysis of the *DEG9* promoter. *DEG9* promoter-driven GUS constructs were generated and introduced into WT plants. The GUS activities of 10 transformed lines were examined at different growth stages and one representative line was photographed.

chloromethyl ketone, and aprotinin supplemented with CHX, ARR4 accumulation was restored to control levels. By contrast, other protease inhibitors or the proteasome inhibitor MG132 were not able to recover ARR4 control levels under the same conditions (Fig. S8). These findings suggested that the stability of ARR4 is controlled by serine proteases.

The subcellular colocalization of the serine protease DEG9 and ARR4, together with the genetic interaction data between DEG9 and ARR4 in the light and cytokinin signaling pathways, raised the possibility that DEG9 might target ARR4 for degradation. If so, direct physical interaction between ARR4 and DEG9 could occur in the nucleus. We performed pull-down assays to test the potential direct interaction between DEG9 and ARR4 proteins. His-tagged ARR4 was precipitated with MBP-tagged DEG9 but not with MBP, whereas His-tagged ARR3 was precipitated with neither MBP-tagged DEG9 nor MBP, suggesting a specific interaction between ARR4 and DEG9 in vitro (Fig. 4*A*). To confirm this interaction in vivo, bimolecular fluorescence complementation (BiFC) analysis was performed in protoplasts of *Arabidopsis* mesophyll cells. A fluorescent signal was detected in the nucleus of protoplasts transfected with DEG9 and ARR4 constructs but not with those for other type A ARRs (Fig. 4*B*). This result confirms that DEG9 interacts with ARR4 in vivo. Interaction in vivo between ARR4 and DEG9 was further confirmed by coimmunoprecipitation experiments using *Arabidopsis* transgenic seedlings expressing ARR4-MYC. The DEG9 protein could be precipitated by ARR4-MYC and vice versa (Fig. 4*C*).

The type A ARRs of *Arabidopsis* contain a conserved receiver domain at the N terminus and a short variable extension with unknown function at the C-terminal end (Fig. S9). The receiver domain of ARR4 does not appear to be responsible for its interaction

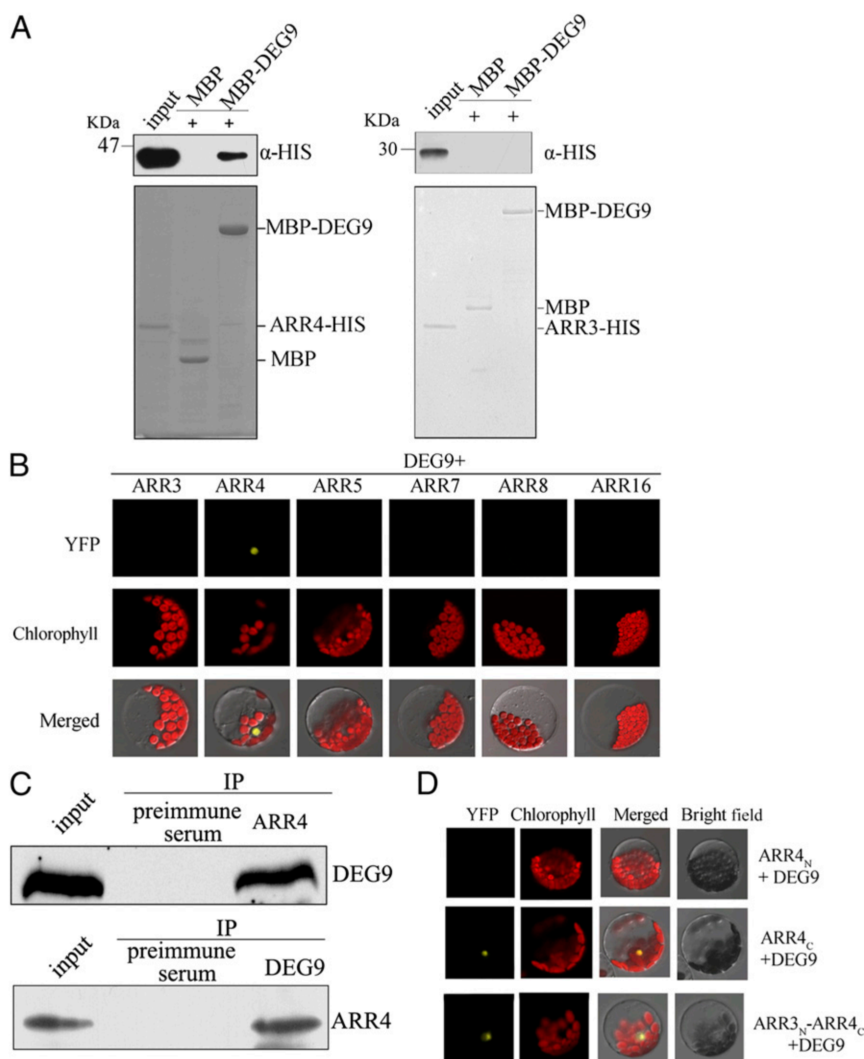

Fig. 4. DEG9 physically interacts with ARR4. (*A*) Pull-down assay. Full-length MBP-tagged DEG9 was used as bait, MBP was used as vehicle control, and full-length His-tagged ARR4/ARR3 was used as prey. The purified recombinant proteins were bound to amylose resin, and the bound proteins were eluted and analyzed by immunoblotting or staining. Input, 4% of the prey protein. (*B*) BiFC analysis of interactions between DEG9 and type A ARRs. Plasmids encoding fusion constructs with the N- or C-terminal part of YFP were transiently expressed in *Arabidopsis* protoplasts. Yellow fluorescence indicates YFP fluorescence; red fluorescence shows chloroplast autofluorescence. (*C*) Coimmunoprecipitation of ARR4 and DEG9 in transgenic *Arabidopsis* seedlings expressing ARR4-MYC. Total protein extracts (input) and immunoprecipitated (IP) fractions using anti-DEG9 (*Upper*) or anti-MYC (*Lower*) antibody were analyzed by immunoblotting. The IP fractions using preimmune serum were used as controls. (*D*) The C terminus of ARR4 interacts with DEG9. The interaction between DEG9 with the N and C termini of ARR4 as shown in Fig. S9 was investigated using BiFC (*Top* and *Middle*). The C terminus of ARR3 was replaced with the C terminus of ARR4, and its interaction with DEG9 was assayed as well (*Bottom*).

with DEG9, because ARR3, which displays high sequence identity with ARR4 within the receiver domain, did not interact with DEG9. In fact, we found that the short C terminus, rather than the receiver domain of ARR4, interacted with DEG9, although both the C terminus and N terminus of ARR4 could be properly expressed in the nucleus of transfected protoplasts, respectively (Fig. 4*D* and Fig. S10). In addition, when the C terminus of ARR4 was fused to the N terminus of ARR3, the interaction with DEG9 remained intact (Fig. 4*D*). These results indicate that the short variable extension of ARR4 is responsible for the protein–protein interaction.

DEG9 Participates in the Degradation of ARR4. To test whether DEG9 is capable of degrading ARR4, we first investigated the proteolytic activity of DEG9. To this aim, DEG9 was incubated with β-casein, which is the preferred substrate for assaying bacterial DEGP activity in vitro (39). β-Casein was efficiently degraded by DEG9 at a rate of ∼50% within 2 h (Fig. 5*A*), showing that recombinant DEG9 was proteolytically active. When we used recombinant ARR4 as the substrate, more than 90% of recombinant ARR4 was degraded by DEG9 within 2 h (Fig. 5*A*). Recombinant ARR3, used as a control, was not degraded by DEG9 under the same conditions (Fig. 5*A*). Therefore, DEG9 is proteolytically active toward ARR4. We subsequently investigated the degradation of recombinant ARR4 proteins purified from *E. coli* in cell-free extracts from WT, *deg9*, and *DEG9*-OX plants. The degradation of ARR4-His was almost completely blocked in *deg9* extracts, but the degradation of ARR4 was accelerated significantly in *DEG9*-OX extracts compared with WT extracts (Fig. 5*B*). These results suggest that DEG9 can facilitate degradation of recombinant ARR4 in vitro.

We next investigated whether DEG9 is able to degrade ARR4 in vivo by crossing the *ARR4-MYC*-OX transgenic line with the *deg9* and *DEG9-HIS*-OX lines, respectively. The degradation of ARR4-MYC protein in these lines was compared by immunoblotting with an antibody against MYC (Fig. 5*C*). The ARR4-MYC protein was gradually degraded under the inhibition of de novo protein biosynthesis by CHX in *ARR4-MYC*-OX, and ARR4 levels were reduced by ∼90% after 3.5 h. However, the degradation of ARR4-MYC protein was significantly delayed in the *deg9* mutant background and ARR4 levels decreased only by ∼30% after 3.5 h. The ARR4-MYC protein in *DEG9-HIS*-OX plants was degraded rapidly, and only trace amounts of protein were observed after 2.5 h (Fig. 5*C*). This result is consistent with the degradation kinetics of ARR4-MYC in cell-free extracts. Using a similar approach, we investigated whether DEG9 is required for

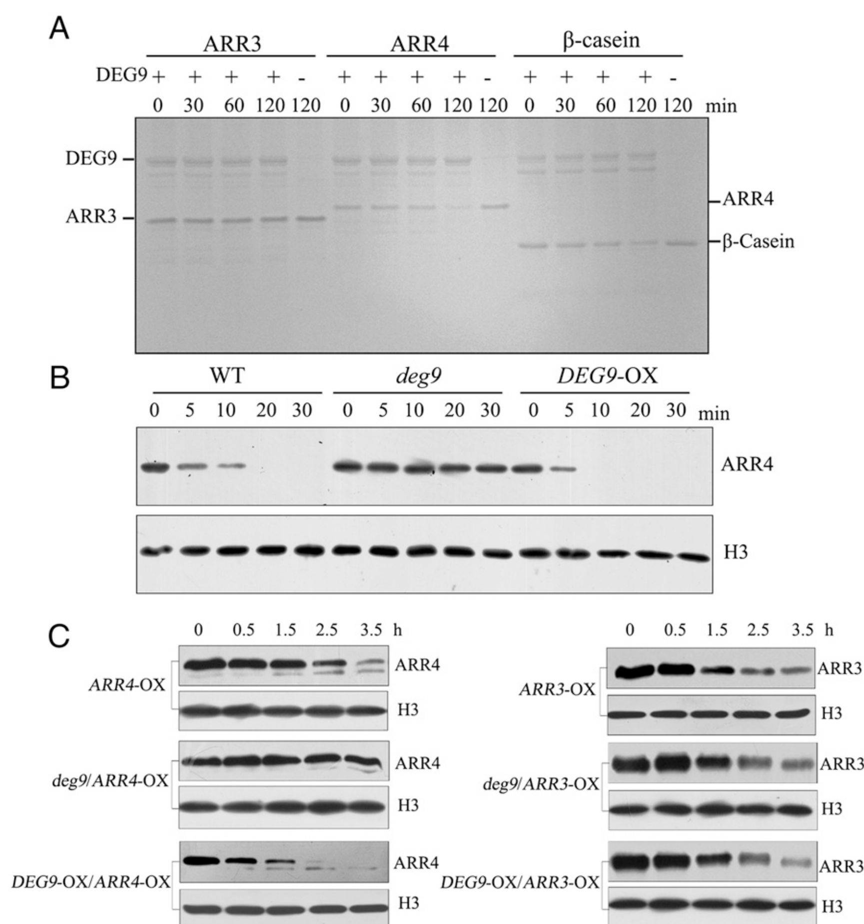

Fig. 5. DEG9 targets ARR4 for degradation. (*A*) Proteolytic activities of DEG9. DEG9 (0.5 μg) proteins were incubated with a mixture of β-casein (15 μg), ARR3, or ARR4 for 30, 60, or 120 min at 37 °C. A mixture without DEG9 was used as a control. After terminating the reaction, the reaction mixtures were subjected to SDS/PAGE using 12% (wt/vol) acrylamide gels. The locations of DEG9, β-forms of casein, ARR3, and ARR4 in the gel are indicated. Similar results were obtained from three independent experiments; results from a representative experiment are shown. (*B*) Cell-free degradation of His-tagged ARR4 proteins. His-ARR4 was expressed and purified from *E. coli* and then added to extracts from WT, *DEG9*-OX, and *deg9* plants. H3 was used as a loading control. (*C*) Immunoblot detection of ARR4/3-MYC degradation after CHX treatment in 7-d-old *ARR4/3*-OX, *deg9/ARR4/3*-OX, and *DEG9*-OX/*ARR4/3*-OX plants. H3 was used as a loading control.

the degradation of AAR3. Our results indicated that the degradation of AAR3 is not DEG9-dependent (Fig. 5C). Taken together, we conclude that DEG9 is critical for the control of ARR4 levels, probably through the direct degradation of ARR4.

Discussion

Although the role of regulated protein turnover is well-documented in many plant hormone signaling pathways, including those for auxin, ethylene, gibberellins, and jasmonic acid, the identification of regulatory components for the cytokinin signaling pathway has emerged only recently (47). A family of F-box proteins, designated the KMD (KISS ME DEADLY) family, targets type B ARRs for degradation through the formation of an S-PHASE KINASE-ASSOCIATED PROTEIN 1 (SKP1)–Cullin F-box (SCF) E3 ubiquitin ligase complex (25). AXR1, a subunit of the E1 enzyme in the RUB (RELATED TO UBIQUITIN) modification pathway, was found to mediate the *Arabidopsis* response to cytokinin by facilitating ARR5 degradation (27). Here we report that a prokaryote-derived protease is involved in cytokinin signal transduction through regulation of the degradation of ARR4. Evidence is accumulating that plant proteases are key regulators of a large variety of biological processes (29, 30). However, for most of these proteases, the substrates and activation mechanisms remain elusive (29). With the identification of the role of DEG9 in the cytokinin response, the importance of DEG proteases extends beyond their role in organelle biogenesis and maintenance.

The fact that a prokaryote-derived protease was recruited to degrade signaling proteins of the cytokinin signaling pathway is not entirely surprising. Cytokinins are evolutionarily ancient and highly conserved small molecules that are present in almost all known organisms, and they evolved into an important group of hormones in plants (48). Homologs of components of the cytokinin signaling pathway are found in bacteria, where they form the archetype of two-component signaling systems. In these bacterial signaling systems, the degradation of response regulators similarly serves as a pivotal mechanism for transcriptional regulation (49, 50). Despite these commonalities between plant and prokaryote systems, it does seem surprising that the DEG protease has evolved to degrade ARR4 in plants, as no DEG participating in the degradation of response regulators has been described in prokaryotes. For example, the response regulators DegU and DegP of *Bacillus subtilis* are degraded by ClpCP and not by DEG proteases (50). Nevertheless, similar to the conservation of the cytokinin signaling pathway, orthologs of DEG9 exist in the monocotyledon rice, as well as in the bryophyte *Physcomitrella patens* and the lycophyte *Selaginella moellendorffii*, suggesting that the use of DEG proteases for the cytokinin signaling pathway is universal in plants.

Previous studies showed that SCF^{KMD} targets at least two members of type B ARRs (ARR1 and ARR12) for degradation in *Arabidopsis* (25). However, this is not the case for DEG9, based on our analysis. Two lines of evidence support the notion that DEG9 targets ARR4 specifically for degradation, although the possibility that DEG9 targets other ARR proteins for degradation cannot be excluded. First, the interaction between DEG9 and ARR4 was specific in our BiFC assay and no interaction between DEG9 and other A-type ARRs was found (Fig. 4). Second, the cytokinin response of the *DEG9*-overexpression line was not affected in our primary root growth assay (Fig. 2*A*). If DEG9 targeted multiple A-type ARRs for degradation, the overexpression line of *DEG9* would be expected to show an altered cytokinin response similar to that of the overexpression line of *KMD* (25). Considering the redundant role of type A ARRs, the purpose of the specific degradation of ARR4 by DEG9 could be questioned, because the loss of ARR4 could be compensated for by other ARR members. However, ARR4 plays multiple roles in plant growth and development, and not only acts as a negative regulator of cytokinin signaling but is also involved in the regulation of light signaling through modulation of PhyB activity (18). The involvement of ARR4 in light signaling might be specific, as several other ARRs do not interact with PhyB (18). In fact, we found that the light response was affected in both *deg9* and *DEG9*-OX plants (Fig. 2*C*). The ARR4 degradation that is mediated by DEG9 might be used to fine-tune the cross-talk of cytokinin signaling with other signaling pathways, which is critical for plants to adjust light responsiveness to endogenous requirements for growth and development (51). The *deg9* mutant has a more pronounced phenotype in hypocotyl elongation under red light but a less severe root phenotype after treatment with cytokinin (Fig. 2). This result indicates that the DEG9-ARR4 pathway likely plays a more dominant role in red-light signaling.

Cytokinin influences several light-regulated processes. It can partially induce photomorphogenesis in etiolated seedlings (52, 53), suggesting a functional cross-talk between cytokinin signaling and light-signal transduction pathways. It is conceivable that ARR4 mediates the output of an independent two-component signaling system that acts on PhyB activity and therefore red-light photomorphogenesis. In addition, ARR4 signals, together with those of ARR3, mediate the phase of the circadian clock through regulation of PhyB (54). Therefore, ARR4 appears to play a central role in the interaction between cytokinin signaling and light signal transduction. Similar to other ARRs, ARR4 activity is thought to be regulated by a phosphorelay mechanism that depends on the AHK family of cytokinin receptors. Indeed, changing the phosphorylatable aspartate to asparagine within the receiver domain creates a version of ARR4 that negatively affects photomorphogenesis (51). In this study, we have shown that ARR4 activity is controlled by another process that is associated with protease-mediated degradation. With the identification of a role for DEG9 in ARR4 degradation, it becomes increasingly clear that targeting and degradation of key elements of two-component signaling systems function in the modulation of cytokinin perception and in light signaling.

Materials and Methods

Plant Material and Growth Conditions. *A. thaliana* ecotype Columbia was used for all WT and mutant plants. Seeds of the T-DNA insertion line *deg9* (*SALK_125251C*), *arr4*, *arr3,4*, *arr3,4,5,6*, and *phyB* mutants were obtained from the *Arabidopsis* Biological Resource Center. The *deg9* homozygous plants were identified by a standard procedure based on PCR analysis. To obtain *DEG9*-OX plants, a fragment containing the full-length *DEG9* coding sequence and His tag was cloned into pSN1301 under the control of the cauliflower mosaic virus 35S promoter. For the generation of *DEG9 promoter-GUS* transgenic lines, a 1.0-kb DNA fragment upstream of the start codon of *DEG9* was PCR-amplified and cloned into the pCAMBIA1305 vector. All of the constructed plasmids were transformed into an *Agrobacterium tumefaciens* strain using electroporation and subsequently introduced into WT or mutant plants by the floral-dip method. When not specified, the *Arabidopsis* plants were grown under short-day conditions (10-h-light/14-h-dark cycles) with a photon flux density of 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at a temperature of 22 °C.

Analysis of Light and Cytokinin Responses. Treatment of seedlings with red light was performed as previously described with minor modifications (52). Mutant and WT seeds were sown on 1/2 Murashige and Skoog (MS) medium containing 1% sucrose and 0.8% agar and incubated at 4 °C in darkness for 3 d, followed by various light treatments. Light-emitting diode light sources were used for far-red (726 nm, 12 $\mu mol \cdot m^{-2} \cdot s^{-1}$), red (667 nm, 10 $\mu mol \cdot m^{-2} \cdot s^{-1}$), and blue (425 nm, 14 $\mu mol \cdot m^{-2} \cdot s^{-1}$) light treatments. Light intensities that deviated from these treatments are specifically indicated. After a 4-d light exposure, the seedlings were scanned and the hypocotyls were measured using ImageJ software (NIH). The assay to analyze the inhibition of root elongation was carried out according to a method previously described (23). The seedlings were grown vertically on 1/2 MS agar supplemented with the appropriate concentrations of cytokinin. The plates were photographed after 10 d, and root length was measured using ImageJ software.

In Vivo Degradation Assays. Seedlings of WT and mutant plants were infiltrated in liquid 1/2 MS medium supplemented with 200 μM CHX. After the indicated time, the samples were immediately extracted in 125 mM Tris·HCl (pH 8.8), 1%

(wt/vol) SDS, 10% (vol/vol) glycerol, 50 mM $Na_2S_2O_5$, and microcentrifuged for 10 min. The supernatants were mixed with one-tenth volume of loading buffer [125 mM Tris·HCl, pH 6.8, 12% (wt/vol) SDS, 10% (vol/vol) glycerol, 22% (vol/vol) β-mercaptoethanol, 0.001% (wt/vol) bromophenol blue]. The samples were separated by 12% (wt/vol) SDS/PAGE and subjected to immunoblot analysis.

Quantitative PCR. Total RNA was extracted from 10-d-old seedlings with the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. The first-strand cDNA was generated using the SuperScript III First-Strand Synthesis System (Invitrogen). Quantitative PCR analysis was performed using SYBR Green Master Mix with Chromo4 as described in the manufacturer's protocol (Bio-Rad). RNA levels of genes in each sample were normalized to those of *ACTIN*, and the measurements were performed using three biological replicates. The comparative C_T method means and SDs were used to calculate and analyze the results (55).

Immunoblot Analysis. The nucleotide sequence encoding the 98 amino acids of DEG9 (amino acids 1–98) was amplified by PCR and inserted into the expression vector pET28a, and this fusion protein construct was transformed into *E. coli* BL21(DE3) for expression. The recombinant protein was purified using a Ni-NTA agarose resin matrix (Novagen) according to the manufacturer's instructions. Polyclonal antibodies were raised in rabbits against the purified antigens. The antibodies against the His, H3, and MYC tags were obtained from Sigma-Aldrich. Total plant proteins and intact chloroplasts were extracted as previously described (56). Nuclear protein extracts were isolated using the CelLytic PN Isolation/Extraction Kit (Sigma-Aldrich) according to the manufacturer's instructions. Protein concentrations were determined using the Bio-Rad DC protein assay. For immunoblot analysis, proteins were separated by SDS/PAGE and transferred to nitrocellulose membranes. Membranes were incubated with specific primary antibodies, and signals from secondary conjugated antibodies were detected by enhanced chemiluminescence.

GFP and BiFC Assays. For the subcellular localization assay of DEG9–GFP, the full-length or the fragment encoding the N-terminal 128 amino acids of *DEG9* was amplified by RT-PCR and then subcloned into SalI and NcoI of pUC18-35s-SGFP to create fusion proteins with GFP fused at the C terminus. The control plasmids were constructed as described previously (57). BiFC analysis was performed with the pSATN series of vectors as described previously (58). The coding sequence of *DEG9* was cloned into the SalI and BamHI sites of pSAT1-cEYFP-N1 to generate a fusion construct with the C-terminal fragment of YFP. The coding sequences of *ARR* genes were individually cloned into the SalI and BamHI sites of pSAT1-nEYFP-N1 to create fusion constructs with the N-terminal fragment of YFP. The resulting constructs were transfected into *Arabidopsis* mesophyll protoplasts according to the method described previously (59). Fluorescence analysis was performed on an LSM 510 META confocal laser-scanning system (Zeiss).

Pull-Down Assay. The DEG9–MBP fusion protein was coupled to amylose resin (New England Biolabs) according to the manufacturer's instructions. The purified ARR4-His proteins were incubated with DEG9-MBP–amylose resin for 2 h, and subsequently the resin was washed five times with washing buffer containing 50 mM Tris·HCl (pH 7.5), 150 mM NaCl, and 0.5% Nonidet P-40. The bound proteins were eluted with SDS/PAGE sample buffer and resolved by SDS/PAGE followed by immunoblot analysis.

GUS Activity Assay. To detect GUS activity, seedlings were vacuum-infiltrated at 130 mbar for 10 min in X-Gluc (5-bromo-4-chloro-3-indolyl β-d-glucuronide) buffer (100 mM sodium phosphate, pH 7.0, 0.5% Triton X-100, 100 μM X-Gluc). The color reaction was performed at 37 °C overnight. Chlorophyll was extracted three times with 100% ethanol, and the seedlings were examined under a dissecting microscope.

RNA-Sequencing Analysis. RNA-sequencing and data analysis was performed by BGI Tech Solutions. *Arabidopsis* seedlings were treated with 2 μM *t*-zeatin for 30 min as described previously (60) and total RNA was extracted from *Arabidopsis* seedlings using TRIzol reagent (Invitrogen) and treated with RNase-free DNaseI. Poly(A) mRNA was isolated using oligo(dT) beads. The first-strand cDNA was subsequently generated using random hexamer-primed reverse transcription, followed by synthesis of the second-strand cDNA using RNaseH and DNA polymerase I. Then, single-end and paired-end RNA-sequencing libraries were prepared following Illumina's protocols and sequenced using the Illumina GA II platform.

Gene expression profiling analysis was based on the number of tags matching exon regions, and RPKMs (reads per kilobase of exon model per million mapped reads) were used to evaluate the expressed value and quantify transcript levels (61). Audic and Claverie's method was used to analyze differential expression (62). The RNA-sequencing datasets were deposited in the ArrayExpress database (accession no. E-MTAB-4603).

Proteolytic Degradation Assays. The proteolytic activity of DEG9 was assayed in a reaction buffer (250 mM Na_2HPO_4, 70 mM sodium citrate, pH 6.0) including 0.2 mg purified DEG9 and 0.2 mg β-casein (Sigma-Aldrich) or purified ARR4 or ARR3 in a total volume of 200 μL. The mixtures were incubated for 0, 30, 60, and 120 min at 37 °C and subjected to SDS/PAGE. Subsequently, the gels were stained with Coomassie Brilliant Blue G-250.

Cell-Free Degradation Assays. The cell-free degradation assay was performed as described previously (63). Total protein was extracted from 2-wk-old *Arabidopsis* seedlings with degradation buffer containing 25 mM Tris·HCl (pH 7.5), 10 mM NaCl, and 10 mM $MgCl_2$. After two 10-min centrifugations at 17,000 × *g* at 4 °C, the supernatant was collected and the protein concentration was determined using the Bio-Rad Protein Assay Kit. Total protein extracts prepared from WT, *deg9*, and *DEG9*-OX were then adjusted to 2 mg/mL in degradation buffer for each assay. Each cell-free degradation assay was performed in 250 μL degradation buffer including 500 μg total proteins of WT, *deg9*, and *DEG9*-OX separately, and 100 ng purified ARR4-His was added to the reaction buffer. The mixtures were incubated for 0, 5, 10, 20, and 30 min at room temperature and subjected to SDS/PAGE followed by immunoblot analysis.

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1. Haberer G, Kieber JJ (2002) Cytokinins. New insights into a classic phytohormone. *Plant Physiol* 128(2):354–362.
2. Werner T, Schmülling T (2009) Cytokinin action in plant development. *Curr Opin Plant Biol* 12(5):527–538.
3. Hwang I, Sheen J, Müller B (2012) Cytokinin signaling networks. *Annu Rev Plant Biol* 63:353–380.
4. To JP, Kieber JJ (2008) Cytokinin signaling: Two-components and more. *Trends Plant Sci* 13(2):85–92.
5. Perilli S, Moubayidin L, Sabatini S (2010) The molecular basis of cytokinin function. *Curr Opin Plant Biol* 13(1):21–26.
6. Schaller GE, Shiu SH, Armitage JP (2011) Two-component systems and their co-option for eukaryotic signal transduction. *Curr Biol* 21(9):R320–R330.
7. To JP, et al. (2004) Type-A *Arabidopsis* response regulators are partially redundant negative regulators of cytokinin signaling. *Plant Cell* 16(3):658–671.
8. 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.
9. D'Agostino IB, Deruère J, Kieber JJ (2000) Characterization of the response of the *Arabidopsis* response regulator gene family to cytokinin. *Plant Physiol* 124(4): 1706–1717.
10. Hill K, et al. (2013) Functional characterization of type-B response regulators in the *Arabidopsis* cytokinin response. *Plant Physiol* 162(1):212–224.
11. Hwang I, Sheen J (2001) Two-component circuitry in *Arabidopsis* cytokinin signal transduction. *Nature* 413(6854):383–389.
12. Mason MG, et al. (2005) Multiple type-B response regulators mediate cytokinin signal transduction in *Arabidopsis*. *Plant Cell* 17(11):3007–3018.
13. Hutchison CE, et al. (2006) The *Arabidopsis* histidine phosphotransfer proteins are redundant positive regulators of cytokinin signaling. *Plant Cell* 18(11):3073–3087.
14. El-Showk S, Ruonala R, Helariutta Y (2013) Crossing paths: Cytokinin signalling and crosstalk. *Development* 140(7):1373–1383.
15. Feng J, et al. (2013) *S*-nitrosylation of phosphotransfer proteins represses cytokinin signaling. *Nat Commun* 4:1529.
16. Guan C, et al. (2014) Cytokinin antagonizes abscisic acid-mediated inhibition of cotyledon greening by promoting the degradation of abscisic acid insensitive5 protein in *Arabidopsis*. *Plant Physiol* 164(3):1515–1526.
17. Suzuki T, Sakurai K, Ueguchi C, Mizuno T (2001) Two types of putative nuclear factors that physically interact with histidine-containing phosphotransfer (Hpt) domains, signaling mediators in His-to-Asp phosphorelay, in *Arabidopsis thaliana*. *Plant Cell Physiol* 42(1):37–45.
18. Sweere U, et al. (2001) Interaction of the response regulator ARR4 with phytochrome B in modulating red light signaling. *Science* 294(5544):1108–1111.
19. To JP, et al. (2007) Cytokinin regulates type-A *Arabidopsis* response regulator activity and protein stability via two-component phosphorelay. *Plant Cell* 19(12):3901–3914.
20. Marhavý P, et al. (2011) Cytokinin modulates endocytic trafficking of PIN1 auxin efflux carrier to control plant organogenesis. *Dev Cell* 21(4):796–804.
21. Zhang W, To JP, Cheng CY, Schaller GE, Kieber JJ (2011) Type-A response regulators are required for proper root apical meristem function through post-transcriptional regulation of PIN auxin efflux carriers. *Plant J* 68(1):1–10.

22. Smalle J, et al. (2002) Cytokinin growth responses in *Arabidopsis* involve the 26S proteasome subunit RPN12. *Plant Cell* 14(1):17–32.
23. Ren B, et al. (2009) Genome-wide comparative analysis of type-A *Arabidopsis* response regulator genes by overexpression studies reveals their diverse roles and regulatory mechanisms in cytokinin signaling. *Cell Res* 19(10):1178–1190.
24. Kim K, et al. (2012) Cytokinin-facilitated proteolysis of *ARABIDOPSIS* RESPONSE REGULATOR 2 attenuates signaling output in two-component circuitry. *Plant J* 69(6):934–945.
25. Kim HJ, Chiang YH, Kieber JJ, Schaller GE (2013) SCF(KMD) controls cytokinin signaling by regulating the degradation of type-B response regulators. *Proc Natl Acad Sci USA* 110(24):10028–10033.
26. Zheng X, et al. (2011) AUXIN UP-REGULATED F-BOX PROTEIN1 regulates the cross talk between auxin transport and cytokinin signaling during plant root growth. *Plant Physiol* 156(4):1878–1893.
27. Li Y, Kurepa J, Smalle J (2013) AXR1 promotes the *Arabidopsis* cytokinin response by facilitating ARR5 proteolysis. *Plant J* 74(1):13–24.
28. Sadanandom A, Bailey M, Ewan R, Lee J, Nelis S (2012) The ubiquitin-proteasome system: Central modifier of plant signalling. *New Phytol* 196(1):13–28.
29. van der Hoorn RA (2008) Plant proteases: From phenotypes to molecular mechanisms. *Annu Rev Plant Biol* 59:191–223.
30. Tsiatsiani L, Gevaert K, Van Breusegem F (2012) Natural substrates of plant proteases: How can protease degradomics extend our knowledge? *Physiol Plant* 145(1):28–40.
31. Clausen T, Kaiser M, Huber R, Ehrmann M (2011) HTRA proteases: Regulated proteolysis in protein quality control. *Nat Rev Mol Cell Biol* 12(3):152–162.
32. Krojer T, et al. (2008) Interplay of PDZ and protease domain of DegP ensures efficient elimination of misfolded proteins. *Proc Natl Acad Sci USA* 105(22):7702–7707.
33. Kim S, Grant RA, Sauer RT (2011) Covalent linkage of distinct substrate degrons controls assembly and disassembly of DegP proteolytic cages. *Cell* 145(1):67–78.
34. Schuhmann H, Huesgen PF, Adamska I (2012) The family of Deg/HtrA proteases in plants. *BMC Plant Biol* 12:52.
35. Schuhmann H, Adamska I (2012) Deg proteases and their role in protein quality control and processing in different subcellular compartments of the plant cell. *Physiol Plant* 145(1):224–234.
36. Haussühl K, Andersson B, Adamska I (2001) A chloroplast DegP2 protease performs the primary cleavage of the photodamaged D1 protein in plant photosystem II. *EMBO J* 20(4):713–722.
37. Kapri-Pardes E, Naveh L, Adam Z (2007) The thylakoid lumen protease Deg1 is involved in the repair of photosystem II from photoinhibition in *Arabidopsis*. *Plant Cell* 19(3):1039–1047.
38. Sun X, et al. (2007) Formation of DEG5 and DEG8 complexes and their involvement in the degradation of photodamaged photosystem II reaction center D1 protein in *Arabidopsis*. *Plant Cell* 19(4):1347–1361.
39. Sun X, et al. (2010) The thylakoid protease Deg1 is involved in photosystem-II assembly in *Arabidopsis thaliana*. *Plant J* 62(2):240–249.
40. Schuhmann H, Huesgen PF, Gietl C, Adamska I (2008) The DEG15 serine protease cleaves peroxisomal targeting signal 2-containing proteins in *Arabidopsis*. *Plant Physiol* 148(4):1847–1856.
41. Winter D, et al. (2007) An "electronic Fluorescent Pictograph" browser for exploring and analyzing large-scale biological data sets. *PLoS One* 2(8):e718.
42. Lam E, Chua NH (1990) GT-1 binding site confers light responsive expression in transgenic tobacco. *Science* 248(4954):471–474.
43. Gilmartin PM, Chua NH (1990) Localization of a phytochrome-responsive element within the upstream region of pea *rbcS-3A*. *Mol Cell Biol* 10(10):5565–5568.
44. Hudson ME, Quail PH (2003) Identification of promoter motifs involved in the network of phytochrome A-regulated gene expression by combined analysis of genomic sequence and microarray data. *Plant Physiol* 133(4):1605–1616.
45. Pendle AF, et al. (2005) Proteomic analysis of the *Arabidopsis* nucleolus suggests novel nucleolar functions. *Mol Biol Cell* 16(1):260–269.
46. Barneche F, Steinmetz F, Echeverría M (2000) Fibrillarin genes encode both a conserved nucleolar protein and a novel small nucleolar RNA involved in ribosomal RNA methylation in *Arabidopsis thaliana*. *J Biol Chem* 275(35):27212–27220.
47. Santner A, Estelle M (2009) Recent advances and emerging trends in plant hormone signalling. *Nature* 459(7250):1071–1078.
48. Pils B, Heyl A (2009) Unraveling the evolution of cytokinin signaling. *Plant Physiol* 151(2):782–791.
49. Li L, Kehoe DM (2008) Abundance changes of the response regulator RcaC require specific aspartate and histidine residues and are necessary for normal light color responsiveness. *J Bacteriol* 190(21):7241–7250.
50. Ogura M, Tsukahara K (2010) Autoregulation of the *Bacillus subtilis* response regulator gene degU is coupled with the proteolysis of DegU-P by ClpCP. *Mol Microbiol* 75(5):1244–1259.
51. Mira-Rodado V, et al. (2007) Functional cross-talk between two-component and phytochrome B signal transduction in *Arabidopsis*. *J Exp Bot* 58(10):2595–2607.
52. Chory J, Reinecke D, Sim S, Washburn T, Brenner M (1994) A role for cytokinins in de-etiolation in *Arabidopsis*. *Plant Physiol* 104(2):339–347.
53. Lochmanová G, et al. (2008) Cytokinin-induced photomorphogenesis in dark-grown *Arabidopsis*: A proteomic analysis. *J Exp Bot* 59(13):3705–3719.
54. Salomé PA, To JP, Kieber JJ, McClung CR (2006) *Arabidopsis* response regulators ARR3 and ARR4 play cytokinin-independent roles in the control of circadian period. *Plant Cell* 18(1):55–69.
55. Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C_T method. *Nat Protoc* 3(6):1101–1108.
56. Ouyang M, et al. (2011) LTD is a protein required for sorting light-harvesting chlorophyll-binding proteins to the chloroplast SRP pathway. *Nat Commun* 2:277.
57. Cai W, et al. (2009) LPA66 is required for editing psbF chloroplast transcripts in *Arabidopsis*. *Plant Physiol* 150(3):1260–1271.
58. Citovsky V, et al. (2006) Subcellular localization of interacting proteins by bimolecular fluorescence complementation in planta. *J Mol Biol* 362(5):1120–1131.
59. Kovtun Y, Chiu WL, Tena G, Sheen J (2000) Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. *Proc Natl Acad Sci USA* 97(6):2940–2945.
60. Goda H, et al. (2008) The AtGenExpress hormone and chemical treatment data set: Experimental design, data evaluation, model data analysis and data access. *Plant J* 55(3): 526–542.
61. Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B (2008) Mapping and quantifying mammalian transcriptomes by RNA-seq. *Nat Methods* 5(7):621–628.
62. Audic S, Claverie JM (1997) The significance of digital gene expression profiles. *Genome Res* 7(10):986–995.
63. Wang F, et al. (2009) Biochemical insights on degradation of *Arabidopsis* DELLA proteins gained from a cell-free assay system. *Plant Cell* 21(8):2378–2390.