Mol. Cells *28*, 375-382, October 31, 2009
DOI/10.1007/s10059-009-0132-x

Molecules and Cells

RNAi Suppression of *RPN12a* Decreases the Expression of Type-A *ARRs*, Negative Regulators of Cytokinin Signaling Pathway, in *Arabidopsis*

Moon Young Ryu, Seok Keun Cho, and Woo Taek Kim*

The 26S proteasome is a 2-MDa complex with a central role in protein turn over. The 26S proteasome is comprised of one 20S core particle and two 19S regulatory particles (RPs). The RPN12a protein, a non-ATPase subunit of the 19S RP, was previously shown to be involved in cytokinin signaling in *Arabidopsis*. To further investigate cellular roles of RPN12a, RNAi transgenic plants of *RPN12a* were constructed. As expected, the *35S:RNAi-RPN12a* plants showed cytokinin signaling defective phenotypes, including abnormal formation of leaves and inflorescences. Furthermore, RNAi knock-down transgenic plants exhibited additional unique phenotypes, including concave and heart-shape cotyledons, triple cotyledons, irregular and clustered guard cells, and defects in phyllotaxy, all of which are typical for defective cytokinin signaling. We next examined the mRNA level of cytokinin signaling components, including type-A *ARRs*, type-B *ARRs*, and *CRFs*. The expression of type-A *ARRs*, encoding negative regulators of cytokinin signaling, was markedly reduced in *35S:RNAi-RPN12a* transgenic plants relative to that in wild type plants, while type-B *ARRs* and *CRFs* were unaffected. Our results also indicate that in vivo stability of the ARR5 protein, a negative regulator of cytokinin signaling, is mediated by the 26S proteasome complex. These results suggest that RPN12a participates in feedback inhibitory mechanism of cytokinin signaling through modulation of the abundance of ARR5 protein in *Arabidopsis*.

INTRODUCTION

The ubiquitination system participates in the control of diverse cellular processes, including cell cycle, regulation of transcription, signal transduction, stress responses, and differentiation (Glickman and Ciechanover, 2002). In the ubiquitination pathway, ubiquitin, a 76-amino acid protein, becomes conjugated to target proteins by specific enzymatic cascades involving three enzymes, E1 (a ubiquitin activating enzyme), E2 (a ubiquitin conjugating enzyme), and E3 (a ubiquitin ligase) (Kraft et al., 2005; Stone et al., 2005; Vierstra, 2003). Poly-ubiquitinated target proteins are then rapidly degraded by the 26S proteasome complex. Mono- or multi-ubiquitination of target proteins often alters their activity, lipidation, cellular localization, and interactions with other proteins (Ichimura et al., 2000; Mukhopadhyay and Riezman, 2007; Pickart, 2001; Pickart and Eddins, 2004; Wojcik, 2001).

The 26S proteasome consists of two complexes, the 20S core particle (CP) and the 19S regulatory particle (RP). The 20S CP is composed of seven α and β subunits in an $\alpha_{1\text{-}7}/\beta_{1\text{-}7}/\beta_{1\text{-}7}/\alpha_{1\text{-}7}$ configuration (Fu et al., 2001). The 19S RP consists of two subcomplexes, the lid and base (Glickman and Ciechanover, 2002; Voges et al., 1999). The lid complex contains nine non-ATPase RPN subunits (RPN3, RPN5 - RPN9, and RPN11 - RPN13), while the base complex has six ATPase subunits, RPT1 - RPT6, and three non-ATPase subunits, RPN1, RPN2, and RPN10 (Vierstra, 2003). Recent studies indicate that the 19S RP functions in important cellular processes, such as transcription elongation (Ferdous et al., 2001; Lee et al., 2005). In addition, some individual subunits of the 19S complex play critical roles in growth and development of higher plants. For example, homozygous embryos of an *rpn1a* mutant were arrested at the globular stage, indicating that RPN1a activity is essential during embryogenesis (Brukhin et al., 2005). The *rpt5b* mutant plant was insensitive to high-glucose conditions similar to a *gin2* mutant. RPT5b was shown to repress the glucose signaling pathway through interaction with HXK-1 in the nucleus (Cho et al., 2006a). The *rpn8a* mutant exhibited the abaxialized leaf phenotype (Huang et al., 2006). The transcriptional repression of *RPN9* promotes xylem formation, a process of programmed cell death, and induces senescence of leaf veins. Thus, the RPN9 subunit was suggested to be essential for the regulation of the plant vascular development and leaf vein formation (Jin et al., 2006). *rpn12a* displayed abnormal growth responses to exogenous cytokinin and auxin, suggesting that this mutant has decreased sensitivity to these plant hormones (Smalle et al., 2002). Finally, the *rpn10* and *rpn12a* mutations altered the sensitivity of plants to heat shock and oxidative stress (Kurepa et al., 2008).

Cytokinin affects many aspects of growth and development, including seed germination, vascular development, cell proliferation, apical dominance, chloroplast development, and leaf senescence (Choi and Hwang, 2007). In *Arabidopsis*, cyto-

Department of Biology, College of Life Science and Biotechnology, Yonsei University, Seoul 120-749, Korea
*Correspondence: wtkim@yonsei.ac.kr

Received July 2, 2009; revised August 5, 2009; accepted August 6, 2009; published online September 30, 2009

Keywords: 26S proteasome complex, *Arabidopsis*, cytokinin signaling, RNAi suppression, RPN12a

kinins are recognized by at least three receptors (CRE1, AHK2, and AHK3), which are all related to bacterial two-component sensor histidine kinases (Sheen, 2002; Urao et al., 2001). After binding of cytokinin to receptors, the phosphate is then transferred from the receptors to a His phosphotransfer protein (AHP) and finally to response regulators (ARRs). There are two groups of ARRs. Type-A ARRs are made up of only a receiver domain, while type-B ARRs contain a receiver domain and an output domain that acts as a transcription factor. The type-A ARRs, such as ARR5, ARR6, and ARR7, are known to be negative regulators of cytokinin signaling and their expression is induced by cytokinin via a feedback inhibitory loop (Brenner et al., 2005; Ferreira and Kieber, 2005; Lee et al., 2007a; Sheen, 2002).

In a previous study, Smalle et al. (2002) showed that a T-DNA insertion knock-out mutant of the *RPN12a* gene, encoding a non-ATPase subunit of the 19S RP, exhibited a reduced rate of leaf formation, decreased root elongation, and delayed skotomorphogenesis in *Arabidopsis*. In addition, the mutant displayed abnormal growth responses to exogenously applied cytokinin, indicating that the *rpn12a* mutant plant had a reduced sensitivity to cytokinin. In the present study, to investigate more detailed function of RPN12a, we generated transgenic *Arabidopsis* plants in which *RPN12a* expression is knocked down by RNAi. The independent RNAi knock-down transgenic plants (*35S:RNAi-RPN12a*) showed pleiotropic phenotypes related to cytokinin signaling, including abnormal leaf formations and inflorescences. Furthermore, *35S:RNAi-RPN12a* plants exhibited additional unique phenotypes, including concave and heart-shape cotyledons, triple cotyledons, irregular and clustered guard cells, and defects in phyllotaxy and organ positioning. Intriguingly, the *35S:RNAi-RPN12a* transgenic plants contained a decreased level of mRNAs for the type-A *ARRs*, including *ARR4*, *ARR5*, *ARR6*, *ARR7*, *ARR8*, *ARR15*, and *ARR16*, which all encode negative regulators of cytokinin signaling. We also provide the first evidence that the ARR5 protein is regulated by the 26S proteasome *in vivo*.

MATERIALS AND METHODS

Plant materials and growth condition

Arabidopsis thaliana ecotype Columbia was used throughout this study. *Arabidopsis* plants were grown, transformed, and treated as described previously (Cho et al., 2008).

Genomic DNA preparation

Isolation of genomic DNA from rosette leaves of 2-week-old, light-grown *Arabidopsis* plants was performed as described previously (Cho et al., 2006b) with modifications. Leaves were frozen in liquid nitrogen, ground in a 1.5-ml microcentrifuge tube with 700 μl of CTAB buffer containing 20 μg/ml RNase A, and incubated for 15 min at 55°C. Samples were mixed with 200 μl chloroform, and the aqueous phase was separated by centrifugation. The genomic DNA was precipitated from the aqueous phase with 0.7 volumes of isopropanol and pelleted by centrifugation. The DNA pellet was washed with 1 ml of 70% ethanol and resuspended in a final volume of 50 μl TE buffer (10 mM Tris-HCl pH 8.0, and 1 mM EDTA).

Southern hybridization

Genomic DNA (10 μg) was digested with *Eco*RI, separated by agarose gel electrophoresis, and blotted onto a nylon membrane (Amersham). The membrane was hybridized with radiolabeled *NPTII* gene probe at 65°C under high stringency conditions as described previously (Cho et al., 2008). The blot was visualized by autoradiography at -80°C using Kodak XAR-5 film and an intensifying screen.

Vector constructions

RPN12a cDNA encompassing 237-bp of the C-terminus was PCR amplified with the forward primers (5′-CCGCTCGAGACTCTAAGCTACGCCAGAGAGCTG-3′ and 5′-GCTCTAGAACTCTAAGCTACGCCAGAGAGCTG-3′) and the reverse primers (5′-GGAATTCACGAGTGAACACTAAACAATAGAATATAACG-3′ and 5′-CGGGATCCACGAGTGAACACTAAACAATAGAATATAACG-3′). The PCR product was ligated into the pKANNIBAL vector in the *Xho*I/*Eco*RI sites and the *Xba*I/*Bam*HI sites, respectively. The vector cassette (*Not*I-*35S:RPN12a-PDK intron-RPN12a*:*OCS-Not*I) was moved from the pKANNIBAL vector into the binary pART27 vector (http://www.pi.csiro.au/RNAi/vectors.htm). The full length *ARR5* cDNA was isolated from the cytokinin treated seedlings, and amplified by PCR (forward primer 5′-CTGCAGTCATGGCTGAGGTTTTGCG-3′ and reverse primer 5′-CTGCAGTCAGATCTTTGCGCGTTT-3′). The *ARR5* cDNA PCR product was then inserted into the pGEM T easy vector (Promega) and confirmed by DNA sequencing. *ARR5* insert digested with *Eco*RI was ligated into the N-terminal 6x Myc tagged pBI221 transient expression vector (Clontech).

Construction of *35S:RNAi-RPN12a* knock-down transgenic *Arabidopsis* plants

RPN12a RNAi construct was introduced into *Agrobacterium tumefaciens* strain GV3101 by electrophoration (Joo et al., 2006). Transformed *Agrobacterium* cells were spread onto an LB agar plate (1.5% agar) containing 25 μg/ml kanamycin. Positive transformants were analyzed by colony PCR with *NPTII* gene specific primers (forward primer 5′-TGTGCTCGACGTTGTCACTGAA-3′ and reverse primer 5′-CACCATGATATTCGGCAAGCAG-3′). *Arabidopsis* plants (Col-0) were transformed by the floral-dip method (Seo et al., 2008) and T_1 seeds were collected from regenerated T_0 plants. T_1 seeds were germinated on half-strength of Murashige and Skoog (MS) medium containing 25 μg/ml kanamycin. At least 10 survived T_1 seedlings from the selective antibiotic MS media were transferred to soil under long day conditions and allowed to self-pollinate. T_2 *RPN12a* RNAi transgenic plants were confirmed by PCR and genomic Southern-blot analysis with *NPTII*. T_4 lines fully resistant to kanamycin, obtained from T_3 lines through self-fertilization, were used in this study.

Scanning microscopy

Scanning electron microscopy was performed as described by Bae et al. (2009) with slight modifications. Seven-day-old wild type and *35S:RNAi-RPN12a* transgenic *Arabidopsis* seedlings were collected and fixed in 4% paraformaldehyde solution under vacuum infiltration. After fixation, specimens were rinsed in sodium phosphate buffer (pH 7.4) and dehydrated with a graded ethanol series. They were then critical point-dried in liquid CO_2. The dried samples were mounted and coated with platinum-palladium in a sputter-coater and subjected to scanning electron microscopy (model S-800, FESEM, Hitachi).

RT-PCR

Total RNAs of wild type and *35S:RNAi-RPN12a* transgenic seedlings were obtained by the Easy-blue trizol method (Intron) as described previously (Oh et al., 2007). To examine the expression level of genes related to cytokinin signaling, such as type-A *ARRs*, type-B *ARRs*, and *CRFs*, first strand cDNA was synthesized from total RNAs (2 μg) and RT-PCR was per-

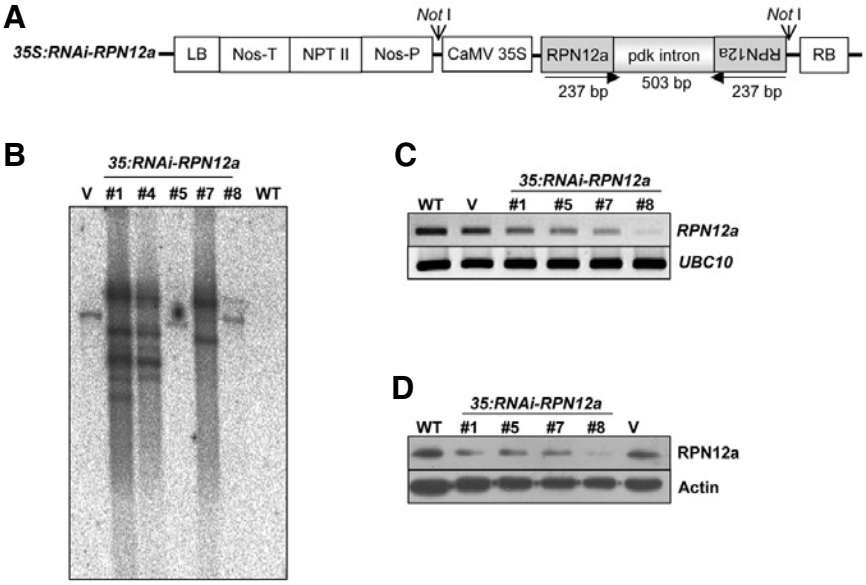

Fig. 1. Construction and analysis of *35S:RNAi-RPN12a* transgenic *Arabidopsis* plants. (A) Schematic representation of *RPN12a* RNAi binary vector construct. The *35S:RNAi-RPN12a* vector carries the 237-bp inverted-repeat sequence of *RPN12a*. (B) Southern blot analysis of wild-type (WT), vector control (V), and five *RPN12a* RNAi lines. Leaf genomic DNAs were restricted with *Eco*RI enzyme and hybridized with ^{32}P-labeled *NPTII* cDNA as a probe under high stringency conditions. Transgenic lines #1, #5, #7, and #8 appeared to be independent lines. (C) Suppression of *RPN12a* expression in *35S:RNAi-RPN12a* transgenic plants. Total RNAs were obtained from leaf tissue of wild type (WT), vector control (V), and *35S:RNAi-RPN12a* transgenic plants, respectively, and analyzed by RT-PCR using a gene-specific primer set. The level of actin mRNA was shown as a loading control. (D) Immunoblot analysis of RPN12a in the wild type (WT), vector control (V), and *RPN12a* RNAi transgenic plants. Total leaf protein (10 μg) was detected using anti-RPN12a antibody or anti-actin antibody. Actin was used as a loading control.

formed with gene specific primers using Ex-Tag polymerase (Takara) as described previously (Lee et al., 2007b. The gene-specific primer sequences are as follows: *RPN12a*, 5′-AAGTTTCACAGCAGTTCAGA-3′ and 5′-CTCTTATGGTCTTTGCCAAGAG-3′, *ARR4*, 5′-GATGAGCGTCGGTGGTATC-3′ and 5′-CGGAACTAGAATCTTCCGGA-3′, *ARR5*, 5′-ATCTCTAACGACACTTCTTCATTAGC-3′ and 5′-GTGCATAATATTCTTAAAAGCTCTTTC-3′, *ARR6*, 5′-CGAGCGTTTGCTCAGAGTA-3′ and 5′-GCTGGCGAGAATCATCAGT-3′, *ARR7*, 5′-TGGAACTAGGGCTTTGCAG-3′ and 5′-TTGCTAAGGTCTTGGCCTC-3′, *ARR8*, 5′-ATGGTAATGGAAACAGAGTCAAA-3′ and 5′-AATCTCTTCTATGGCAACTGGT-3′, *ARR15*, 5′-GGCTCTCAGAGATTTATCTTCTTCT-3′ and 5′-GAATCAATGTCGTTTTGTAGGA-3′, *ARR16*, 5′-ATGAACAGTTCAGGAGGTTCTTG-3′ and 5′-TTAGCTTCTGCAGTTCATGAGA-3′, *ARR1*, 5′-AAGAGGACTCGGATCGGC-3′ and 5′-CAGAATGTTCCGGTACACTCC-3′, *ARR10*, 5′-ATTCATAATCTTTGACGGCG-3′ and 5′-CTTAAGTTTGCTCTTTCTCACAAC-3′, *CRF2* 5′-AGCGACGGAGAAGAAAG-3′ and 5′-CCTCCATGTGCCAGCTGG-3′, *CRF5*, 5′-ACGACTGAGGTGTTACCGG-3′ and 5′-TGAGCTGAAATCTCCGATCACT-3′, *CRF6*, 5′-TCGATCTCCAAACGGTTTC-3′ and 5′-TGGAGAAGACTCGAAATCATCA-3′, and *UBC10*, 5′-CACCATGGCGTCGAAGCGGATC-3′ and 5′-TTAGCCCATGGCATACTTCTG-3′.

Protein stability analysis

The 6x Myc tagged *ARR5* construct (*35S:Myc-ARR5*) was transformed into the protoplasts prepared from wild-type *Arabidopsis* seedlings by polyethylene glycol treatment (Lee et al., 2009) with modifications (Seo et al., 2009). After 16 h, transformants were treated with 100 μM cycloheximide in the presence or absence of 10 μM MG132, respectively. Samples were harvested at an appropriate time, and homogenized with protein extraction buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, and complete protease inhibitor cocktail). Total proteins were separated by 12% SDS-PAGE and then analyzed by immunoblotting using anti-Myc antibody or anti-Actin antibody as described previously (Lee et al., 2006a).

RESULTS

Construction and analysis of *35S:RNAi-RPN12a* knock-down transgenic *Arabidopsis* plants

It was previously suggested that RPN12a, one of the non-ATPase 19S RP subunits, is involved in cytokinin growth responses in *Arabidopsis* (Smalle et al., 2002). To explore more detailed *in vivo* functions of RPN12a, in this study we employed the RNAi knock-down transgenic approach. We first generated transgenic *Arabidopsis* plants (*35S:RNAi-RPN12a*) in which a hairpin-forming DNA fragment of the partial *RPN12a* cDNA was constitutively expressed under the control of the cauliflower mosaic virus 35S promoter (Fig. 1A). Several independent primary transformants were obtained based on the resistance to kanamycin, and T_4 transgenic plants were used for further studies. The presence of transgene was examined by genomic Southern blot analysis. Genomic DNA was prepared from 2-week-old rosette leaves of T_2 *35S:RNAi-RPN12a* transgenic lines, restricted with *Eco*RI enzyme, and hybridized with radiolabeled *NPTII* gene probe under high stringency conditions. This hybridization detected several distinct bands in the RNAi transgenic plants, indicating that several of the knock-down transgenic plants were independent lines (#1, #5, #7, and #8) (Fig. 1B).

To examine whether the *RPN12a* gene was suppressed in these RNAi lines, the levels of *RPN12a* mRNA and its protein were monitored by RT-PCR and immunoblotting, respectively. Total RNAs were isolated from the seedlings of light-grown 10-day-old wild type and *35S:RNAi-RPN12a* plants and PCR amplified using gene-specific primers. The results show that the amount of *RPN12a* transcript was markedly reduced in RNAi transgenic line #8 and significantly decreased in lines #1, #5, and #7, indicating that the *RPN12a* gene was effectively repressed in these RNAi transgenic plants (Fig. 2C). Total proteins were prepared from wild type and RNAi transgenic seedlings and immunologically analyzed using anti-RPN12 antibody. As shown in Fig. 2D, the level of RPN12a protein was clearly decreased in *35S:RNAi-RPN12a* transgenic plants, with the amount of the protein being lowest in RNAi line #8. Overall,

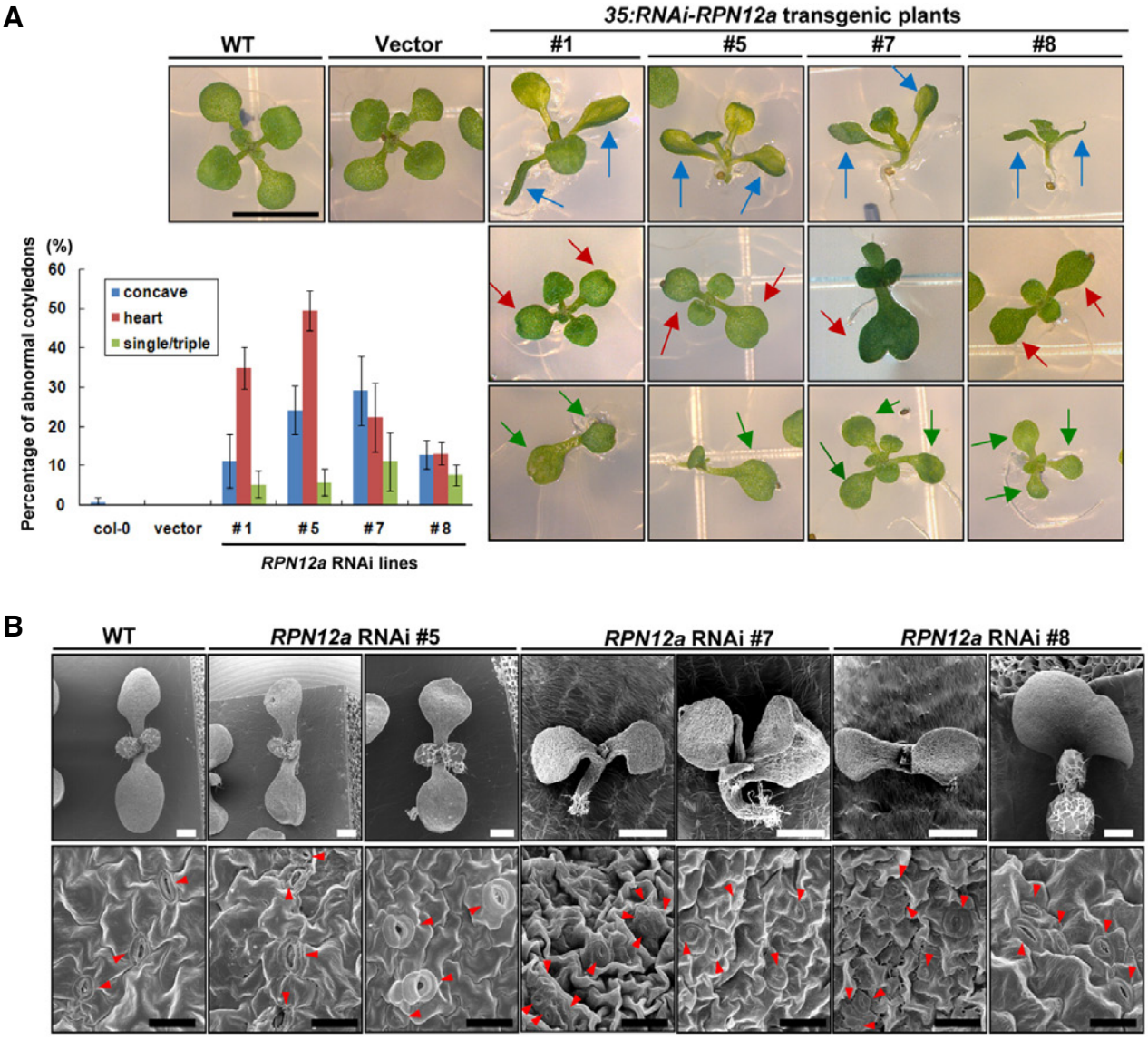

Fig. 2. Phenotypic analysis of *35S:RNAi-RPN12a* transgenic seedlings. (A) Wild type (WT), vector control (V), and *35S:RNAi-RPN12a* transgenic seedlings were grown on MS-agar plates for 14 d under light-grown conditions. The *RPN12a* RNAi transgenic seedlings displayed abnormal concave (blue arrows) and heart-shaped (red arrows) cotyledons and also single or triple cotyledons (green arrows). Scale bar, 5 mm. (B) Scanning electron micrographs of wild type and *35S:RNAi-RPN12a* transgenic cotyledons. Adaxial epidermal pavement cell layers of cotyledons in 7-day-old wild type and *RPN12a* RNAi seedlings was visualized by scanning electron microscopy. Red arrowheads indicate guard cells. White scale bars, 600 μm; black scale bars, 30 μm.

these results indicate that expression of the *RPN12a* gene was effectively suppressed in *35S:RNAi-RPN12a* transgenic plants.

35S:RNAi-RPN12a transgenic seedlings exhibit abnormal development of cotyledons and guard cells

The *rpn12a-1* T-DNA insertion knock-out mutant seedlings exhibited pleiotropic abnormal phenotypes, including increased accumulation of anthocyanin in hypocotyls and petioles, delayed leaf emergence, and reduced root elongation (Smalle et al., 2002). In addition, some of the *rpn12a-1* mutants contained a single cotyledon. While *35S:RNAi-RPN12a* transgenic seedlings showed similar phenotypes to the *rpn12a-1* mutant plant, including anthocyanin accumulation and delayed leaf and root growth, independent RNAi knock-down transgenic seedlings displayed additional aberrant phenotypes. For example, the shape of cotyledons of *35S:RNAi-RPN12a* plants appeared to be abnormal. As shown in Fig. 2A, 27% to 71% of the RNAi transgenic seedlings had unusual concave and/or heart-shape cotyledons depending on the independent lines, which were not observed in knock-out mutant seedlings. Furthermore, 6% to 12% of *35S:RNAi-RPN12a* seedlings contained an abnormal number of cotyledons. As found in *rpn12a-1* knock-out mutant plants, some RNAi transgenic seedlings contain a single cotyledon. However, we often observed *35S:RNAi-RPN12a* seedlings with triple cotyledons (Fig. 2A). These abnormal triple cotyledons were not detected in the knock-out mutant plant. *RPN12a* RNAi transgenic seedlings also exhibited arrested leaf

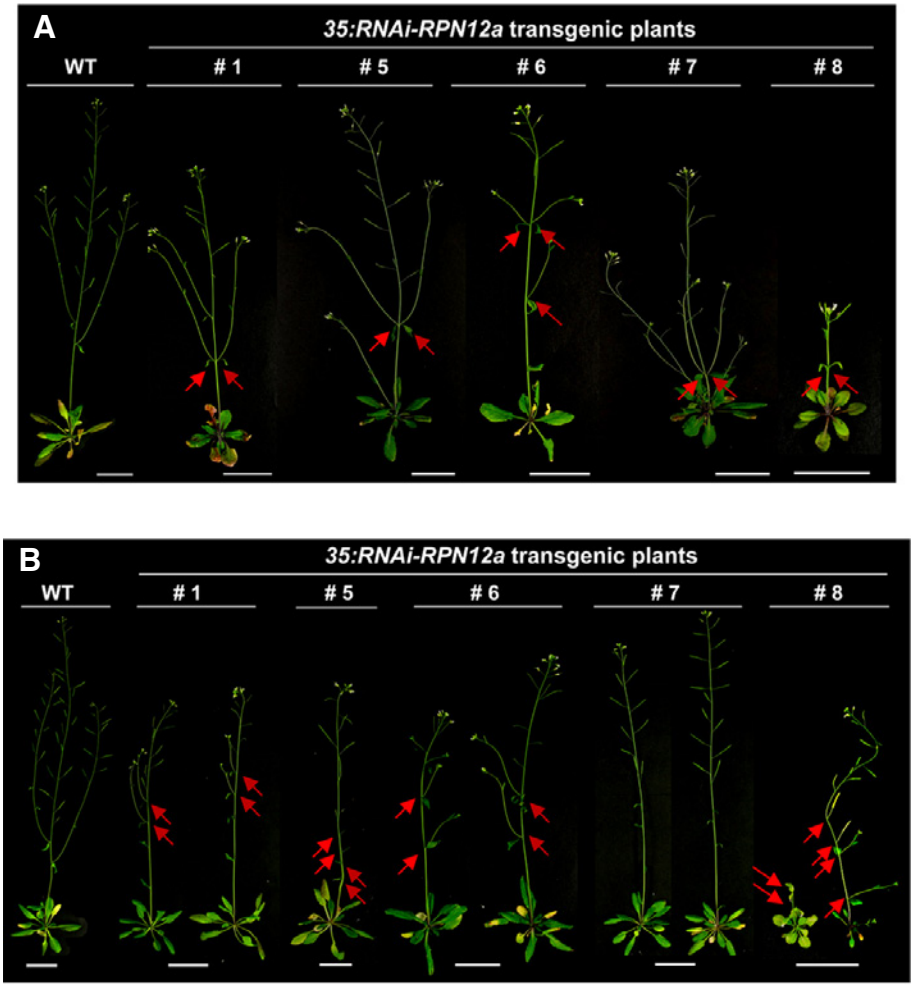

Fig. 3. Abnormal phyllotaxy in *RPN12a* RNAi transgenic plants. Light-grown, 4-week-old *RPN12a* RNAi transgenic plants show irregular organ positioning (A) and irregular side-shoot positions and abbreviated shoot development (B). Red arrows indicate abnormal phyllotaxy. Scale bars, 3.5 cm.

and root development after cotyledons opened.

The more detailed phenotypes of RNAi transgenic cotyledons were investigated by microscopic analysis. We examined the cellular patterns in the epidermal cell layers in cotyledons of 7-day-old *35S:RNAi-RPN12a* transgenic and wild type seedlings. Normal stomata are typically separated from each other because guard cells synthesize inhibitors that prevent the formation of other guard cells in adjacent cells and, thus, stomata are spaced apart from each other (Bergmann and Sack, 2007; Bergmann et al., 2004). However, as illustrated in Fig. 2B, the adaxial surface of the *RPN12a* RNAi cotyledons displayed irregular guard cell development. In *35S:RNAi-RPN12a* transgenic cotyledons, development of guard cells often occurred close to each other, resulting in several stomata being clustered in epidermal cells. However, the leaf cell number and size in *RPN12a* RNAi transgenic cotyledons was highly similar to those in the wild type *Arabidopsis* (Fig. 2B). Therefore, although *35S:RNAi-RPN12a* knock-down transgenic seedlings share similar abnormal phenotype with *rpn12a-1* knock-out seedlings, the RNAi transgenic seedlings exhibited additional distinct phenotypes, including concave and heart-shape cotyledons, abnormal triple cotyledons, and clustered guard cells. Collectively, these results indicate that RPN12a plays an important role not only in the development of leaves and roots (Smalle et al., 2002) but also in normal formation of cotyledons and guard cells (Fig. 2).

35S:RNAi-RPN12a transgenic plants exhibit abnormal phyllotaxy

We next investigated the phenotype of 4-week-old, light-grown *35S:RNAi-RPN12a* plants. As found in seedlings, the mature knock-down transgenic plants also displayed abnormal phenotype. The most specific and distinct phenotype of *35S:RNAi-RPN12a* transgenic plants is an irregular organ positioning. In normal growth conditions, wild type *Arabidopsis* plants have lateral organs that are positioned regularly, and this arrangement of leaves and flowers around the stem is referred to phyllotaxy (Jean, 1994; Kuhlemeier and Reinhardt, 2001). Monocot or dicot mutants with defects in cytokinin signaling, such as *abph1* and *arr*, are known to display defective phyllotaxis (Giulini et al., 2004; Leibfried et al., 2005). Figures 3A and 3B show that, while wild type of *Arabidopsis* shoots and leaves are arranged in radial phyllotaxy, *35S:RNAi-RPN12a* transgenic plants have defects in phyllotaxy and organ initiation. Also, *RPN12a* RNAi transgenic plants have multiple or irregular positioning of inflorescence stems (Fig. 3B). The severity of aberrant phyllotaxy and growth retardation appeared to be linked to the levels of endogenous *RPN12a* mRNA and its protein. RNAi line #8, which showed strong suppression (Figs. 1C and 1D), exhibited severely aberrant phyllotaxy and growth retardation, whereas lines #1, #5, #6, and #7, in which the mRNA and protein levels were moderately decreased, displayed a less serious phenotype (Figs. 3A and 3B). These phenotypes are reminiscent of *arr* mutants, in which type-

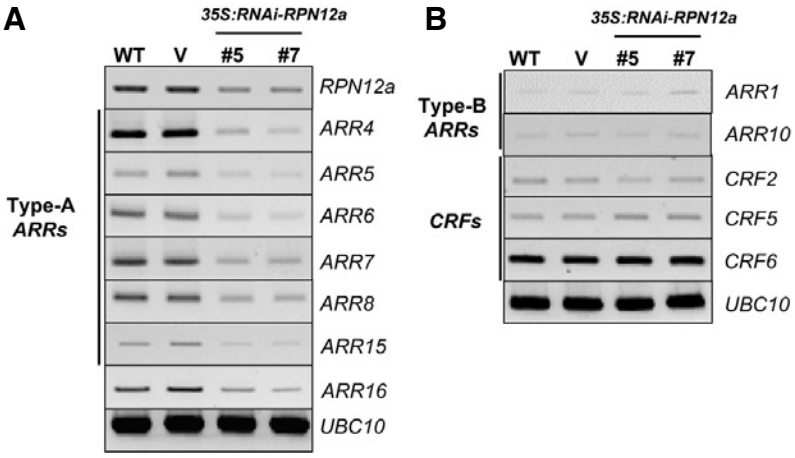

Fig. 4. Expression of type-A *ARRs* is down-regulated in *35S:RNAi-RPN12a* transgenic plants. (A) RT-PCR analysis of Type-A *ARRs*, including *ARR4*, *ARR5*, *ARR6*, *ARR7*, *ARR8*, *ARR15*, and *ARR16*. Total RNAs (2 μg) were obtained from leaf tissue of wild type (WT), vector control (V), and *35S:RNAi-RPN12a* transgenic plants (lines #5 and #8), respectively, and analyzed by RT-PCR using gene-specific primer sets as indicated in "Materials and Methods" section. The level of *UBC10* (ubiquitin conjugating enzyme) mRNA was used as a loading control. (B) RT-PCR analysis of Type-B *ARRs* (*ARR1* and *ARR10*) and *CRFs* (*CRF2*, *CRF5*, and *CRF6*) genes. RT-PCR was performed as described above.

A *ARR* genes, encoding negative regulators of cytokinin signaling, are silenced (Leibfried et al., 2005). Thus, these results are consistent with the notion that suppression of the *RPN12a* gene is intimately tied to the cytokinin signaling defective phenotype.

RNAi suppression of *RPN12a* results in decreased expression of type-A *ARRs*, encoding negative regulators of cytokinin signaling pathway

To provide a molecular link between the abnormal phenotypes of *35S:RNAi-RPN12a* transgenic plants and the cytokinin signaling pathway, we monitored the level of mRNAs for the cytokinin signaling components, including type-A *ARRs*, type-B *ARRs* and *CRFs*. In general, the type-A *ARR* genes, *ARR4*, *ARR5*, *ARR6*, *ARR7*, *ARR8*, *ARR15*, and *ARR16*, encode negative regulators of cytokinin signaling, while type-B *ARRs*, such as *ARR1* and *ARR10*, encode positive regulators (Choi and Hwang, 2007; To and Kieber, 2008). The *CRF* genes are for cytokinin response factors, which belong to the AP2 type transcription factor family (Rashotte et al., 2006). As illustrated in Fig. 4A, all of the type-A *ARRs* genes examined are down-regulated in *RPN12a* RNAi transgenic plants relative to the wild type plant. On the other hand, expression of type-B *ARRs* and *CRF* genes are unchanged in *35S:RNAi-RPN12a* transgenic plants (Fig. 4B). These results indicate that decreased expression of *RPN12a* is accompanied by decreased expression of type-A *ARR* genes.

In vivo* stability of ARR5 protein, a negative regulator of cytokinin signaling, is mediated by the 26S proteasome complex in *Arabidopsis

ARR5, a negative regulator of cytokinin signaling, was previously reported to be rapidly degraded in *Arabidopsis* in the presence of cycloheximide (CHX), an inhibitor of de novo protein synthesis, but stabilized by treatment with cytokinin (To et al., 2007). These results raise the possibility that the 26S proteasome complex affects the stability of ARR5 protein. To test this possibility, we examined the stability of ARR5 using a transient expression system in *Arabidopsis* protoplasts. An N-terminal Myc tagged ARR5 construct (*35S:Myc-ARR5*) (Fig. 5A) was transformed into protoplasts by the polyethylene glycol method. After 16 h, protoplasts were treated with 100 μM CHX in the presence or absence of the proteasome inhibitor MG132. Total proteins were prepared from protoplasts, separated by SDS-PAGE, and subjected to immunoblot analysis using anti-Myc antibody or anti-actin antibody. Figure 5B reveals that

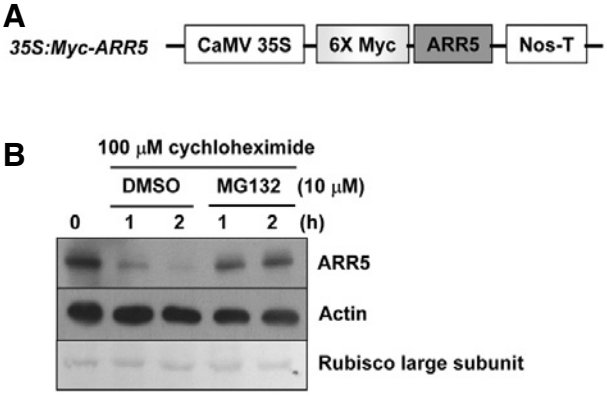

Fig. 5. *In vivo* stability of ARR5 protein is mediated by 26S proteasome complex in *Arabidopsis* protoplasts. (A) Structure of plasmid (*35S:myc-ARR5*) used in a transient assay of *Arabidopsis* protoplasts. The 6x Myc tagged *ARR5* cDNA was inserted between CaMV 35S promoter and Nos-Terminator. (B) *In vivo* stability of ARR5 protein. *Arabidopsis* leaf protoplasts were transformed with *35S:myc-ARR5* and then incubated with 100 μM cycloheximide in the presence or absence of 10 μM MG132. Total proteins were prepared from treated protoplast samples and subjected to immuno-blot analysis with anti-Myc or anti-actin antibody. The amount of actin and Rubisco large subunit proteins is shown as a loading control.

rapid degradation of ARR5 in the presence of CHX is markedly inhibited by MG132. This suggests that ARR5 protein level is regulated by the 26S proteasome complex in *Arabidopsis*.

DISCUSSION

Diverse environmental and developmental responses in higher plants are regulated by the 26S proteasome complex (Dreher and Callis, 2007; Moon et al., 2004; Smalle and Vierstra, 2004). It is well documented that the 26S proteasome plays a critical role in degradation of ubiquitinated proteins (Kraft et al., 2005; Stone et al., 2005; Vierstra, 2003; Yee and Goring, 2009). The 26S proteasome complex is comprised of one 20S core particle and two 19S regulatory subunits. The 19S RP consists of at least 18 polypeptides and can be dissociated into two subcomplexes, the lid and base. The lid complex contains nine non-ATPase RPN subunits, some of which are important for

19S RP assembly (Fu et al., 2001; Vierstra, 2003; Voges et al., 1999). The functions of a number of *Arabidopsis* RPNs have been determined from genetic studies: RPN1 is essential for embryogenesis (Brukhin et al., 2005); RPN8 is required for specifying leaf adaxial identity (Huang et al., 2006); RPN9 regulates vascular formation by targeting a subset of regulatory proteins for degradation (Jin et al., 2006); RPN10 is involved in ABA signaling (Smalle et al., 2003); and RPN12a controls the cytokinin signaling (Smalle et al., 2002). *rpn10* and *rpn12a* mutants were recently reported to be hypersensitive to heat shock and to have increased tolerance to oxidative stress (Kurepa et al., 2008). In hot pepper plant, CaRPN7 was suggested to be involved in programmed cell death in response to the infection of tobacco mosaic virus (Lee et al., 2006b).

We previously reported that RPN12a interacts with AtPUB22 and AtPUB23, the U-box containing E3 Ub ligases, in *Arabidopsis* (Cho et al., 2008). RPN12a is ubiquitinated by AtPUB22 and AtPUB23 *in planta*, and ubiquitination of RPN12a, in turn, may function as a signal for negative regulation of the drought stress signaling pathway in *Arabidopsis* (Cho et al., 2008). Recently, cytokinin signaling pathway and its components were proposed to provide negative feedback regulation of osmotic stress signaling (Tran et al., 2007, Wohlbach et al., 2008). Thus, in this study, to further understand *in vivo* functions of RPN12a, we examined the effects of suppressing the *RPN12a* gene on *Arabidopsis* morphology and development. To this end, we first generated RNAi transgenic plants of *RPN12a*, in which the expression of *RPN12a* is markedly reduced (Fig. 1). The *35S:RNAi-RPN12a* plants exhibited similar phenotypes to the *rpn12a-1* mutant plant, including anthocyanin accumulation, delayed leaf and root growth, and retarded growth. Furthermore, the knock-down transgenic plants displayed additional unique phenotypes, including concave and heart-shape cotyledons (Fig. 2A), triple cotyledons (Fig. 2A), irregular and clustered guard cells (Fig. 2B), and defects in phyllotaxy and organ positioning (Fig. 3), all of which are typical phenotypes of cytokinin signaling defective mutants (Leibfried et al., 2005). Thus, our RNAi knock-down results, along with previous knock-out results (Smalle et al., 2002), are consistent with the view that RPN12a, a non-ATPase subunit of 19S RP, is critically involved in the cytokinin responses in *Arabidopsis*.

Our results reveal that expression of type-A *ARRs* was significantly down-regulated in *35S:RNAi-RPN12a* transgenic plants, but the level of mRNAs for the type-B *ARRs* and *CRFs* was not affected by *RPN12a* RNAi suppression (Fig. 4). In general, type-A ARRs are transcriptional repressors and act as negative regulators in the cytokinin primary responses (Choi and Hwang, 2007; To and Kieber, 2008). Overexpression of the type-A *ARRs* in *Arabidopsis* protoplasts represses *ARR6* promoter activity in the presence of cytokinin (Hwang and Sheen, 2001). Thus, suppression of *RPN12a* is associated with the decrease in the expression of type-A *ARRs* in *Arabidopsis*. More intriguingly, *in vivo* stability of ARR5 protein appears to be modulated by the action of 26S proteasome complex (Fig. 5). It was recently reported that proteasome is possibly involved in A-type ARR7 protein stability (Lee et al., 2008). These results raise the tantalizing possibility that transcriptional repression of *RPN12a* results in an accumulation of type-A ARR proteins through a decrease in the 26S proteasome activity, and increased level of type-A ARR protein, in turn, causes a negative regulation of cytokinin signaling pathway and the aberrant phenotypes of *35S:RNAi-RPN12a* plants. By this negative feedback loop, all of the type-A *ARRs* genes examined were down-regulated in *35S:RNAi-RPN12a* transgenic plants (Fig. 4A).

Taken together, our current results provide the first evidence that *in vivo* stability of ARR5 protein, a negative regulator of cytokinin signaling pathway, is mediated by the 26S proteasome complex. In addition, the data suggest that RPN12a may control the feedback inhibitory mechanisms of cytokinin signaling, possibly through the modulation of ARR5 protein abundance. Further functional studies of RPN12a will be required to understand the physiological relationship between cytokinin and abiotic stress responses in relation with E3 ubiquitin ligases AtPUB22 and AtPUB23 in *Arabidopsis*.

ACKNOWLEDGMENTS

This work was supported by grants from the National Research Foundation (Project No. 2009-0078317 funded by the Ministry of Education, Science, and Technology, Republic of Korea) and from the Plant Diversity Research Center (21st Century Frontier Research Program funded by the Ministry of Education, Science, and Technology) to W.T.K. M.Y.R was the recipient of a Brain Korea 21 graduate student scholarship.

REFERENCES

Bae, H., Choi, S.M., Yang, S.W., Pai, H.-S., and Kim, W.T. (2009). Suppression of the ER-localized AAA ATPase NgCDC48 inhibits tobacco growth and development. Mol. Cells *28*, 57-65.

Bergmann, D.C., and Sack, F.D. (2007). Stomatal development. Annu. Rev. Plant Biol. *58*, 163-181.

Bergmann, D.C., Lukowitz, W., and Somerville, C.R. (2004). Stomatal development and pattern controlled by a MAPKK kinase. Science *304*, 1494-1497.

Brenner, W.G., Romanov, G.A., Köllmer, I., Bürkle, L., and Schmülling, T. (2005). Immediate-early and delayed cytokinin response genes of *Arabidopsis thaliana* identified by genome-wide expression profiling reveal novel cytokinin-sensitive processes and suggest cytokinin action through transcriptional cascades. Plant J. *44*, 314-333.

Brukhin, V., Gheyselinck, J., Gagliardini, V., Genschik, G., and Grossniklausa, U. (2005). The RPN1 subunit of the 26S proteasome in *Arabidopsis* is essential for embryogenesis. Plant Cell *17*, 2723-2737.

Cho, Y-.H., Yoo, S-.D., and Sheen, J. (2006a). Regulatory functions of nuclear hexokinase1 complexin glucose signaling. Cell *127*, 579-589.

Cho, S.K., Chung, H.S., Ryu, M.Y., Park, M.J., Lee, M.M., Bahk, Y.-Y., Kim, J., Pai, H.S., and Kim, W.T. (2006b). Heterologous expression and molecular and cellular characterization of CaPUB1 encoding a hot pepper U-box E3 ubiquitin ligase homolog. Plant Physiol. *142*, 1664-1682.

Cho, S.K., Ryu, M.Y., Song, C., Kwak, J.M., and Kim, W.T. (2008). *Arabidopsis* PUB22 and PUB23 are homologous U-Box E3 ubiquitin ligases that play combinatory roles in response to drought stress. Plant Cell *20*, 1899-1914.

Choi, J., and Hwang, I. (2007). Cytokinin: Perception, signal transduction, and role in plant growth and development. J. Plant Biol. *50*, 98-108.

Dreher, K., and Callis, J. (2007). Ubiquitin, hormones and biotic stress in plants. Ann. Bot. *99*, 787-822.

Ferdous, A., Gonzalez, F., Sun, L., Kodadek, T., and Johnston, S.A. (2001). The 19S regulatory particle of the proteasome is required for efficient transcription elongation by RNA polymerase II. Mol. Cell *7*, 981-991.

Ferreira, F.J., and Kieber, J.J. (2005). Cytokinin signaling. Curr. Opin. Plant Biol. *8*, 518-525.

Fu, H., Reis, N., Lee, Y., Glickman, M.H., and Vierstra, R.D. (2001). Subunit interaction maps for the regulatory particle of the 26S proteasome and the COP9 signalosome. EMBO J. *20*, 7096-7107.

Giulini, A., Wang, J., and Jackson, D. (2004). Control of phyllotaxy by the cytokinin-inducible response regulator homologue ABPHYL1. Nature *430*, 1031-1034.

Glickman, M.H., and Ciechanover, A. (2002). The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol. Rev. *82*, 373-428.

Huang, W., Pi, L., Liang, W., Xu, B., Wang, H., Dai, R., and Huang,

H. (2006). The proteolytic function of the *Arabidopsis* 26S proteasome is required for specifying leaf adaxial identity. Plant Cell *18*, 2479-2492.
Hwang, I., and Sheen, J. (2001). Two-component circuitry in *Arabidopsis* cytokinin signal transduction. Nature *413*, 383-389.
Ichimura, Y., Kirisako, T., Takao, T., Satomi, Y., Shimonishi, Y., and Ishihara, N. (2000). A ubiquitin-like system mediates protein lipidation. Nature *408*, 488-492.
Jean, R. (1994). Phyllotaxis: a systemic study in plant morphogenesis. (Cambridge Univ. Press).
Jin, H., Li, S., and Villegas, A. Jr. (2006). Down-regulation of the 26S proteasome subunit RPN9 inhibits viral systemic transport and alters plant vascular development. Plant Physiol. *142*, 651-661.
Joo, S., Seo, Y.S., Kim, S.M., Hong, D.K., Park, K.Y., and Kim, W.T. (2006). Brassinosteroid-induction of *AtACS4* encoding an auxin-responsive 1-aminocyclopropane-1-carboxylate synthase 4 in *Arabidopsis* seedlings. Physiol. Plant. *126*, 592-604.
Kraft, E., Stone, S.L., Ma, L., Su, N., Gao, Y., Lau, O.-S., Deng, X.-W., and Callis, J. (2005). Genome analysis and functional characterization of the E2 and RING-type E3 ligase ubiquitination enzymes of Arabidopsis. Plant Physiol. *139*, 1597-1611.
Kuhlemeier, C., and Reinhardt, D. (2001). Auxin and phyllotaxis. Trends Plant Sci. *6*, 187-189.
Kurepa, J., Toh-E, A., and Smalle, J.A. (2008). 26S proteasome regulatory particle mutants have increased oxidative stress tolerance. Plant J. *53*, 102-114.
Lee, D., Ezhkova, E., Li, B., Pattenden, S.G., Tansey, W.P., and Workman, J.L. (2005). The proteasome regulatory particle alters the SAGA coactivator to enhance its interactions with transcriptional activators. Cell *123*, 423-436.
Lee, J.-H., Deng, X.W., and Kim, W.T. (2006a). Possible role of light in the maintenance of EIN3/EIL1 stability in *Arabidopsis* seedlings. Biochem. Biophys. Res. Commun. *350*, 484-491.
Lee, B.-J., Kwon, S.J., Kim, S.-K., Kim, K.-J., Park, C.-J., Kim, Y.-J., Park, O.K., and Peak, K.-H. (2006b). Functional study of hot pepper 26S proteasome subunit RPN7 induced by Tobacco mosaic virus from nuclear proteome analysis. Biochem. Biophys. Res. Commun. *351*, 405-411.
Lee, D.J., Park, J.Y., Ku, S.J., Ha, Y.M., Kim, S., Kim, M.D., Oh, M.H., and Kim, J. (2007a) Genome-wide expression profiling of *ARABIDOPSIS* RESPONSE REGULATOR 7 (ARR7) overexpression in cytokinin response. Mol. Genet. Genomics *277*, 115-137.
Lee, M.O., Hwang, J.H., Lee, D.H., and Hong, C.B. (2007b). Gene expression profile for *Nicotiana tabacum* in the early phase of flooding stress. J. Plant Biol. *50*, 496-503.
Lee, D.J., Kim, S., Ha, Y.-M., and Kim, J. (2008). Phosphorylation of *Arabidopsis* response regulator 7 (ARR7) at the putative phosphor-accepting site is required for ARR7 to act as a negative regulator of cytokinin signaling. Planta *227*, 577-587.
Lee, H.K., Cho, S.K., Son, O., Xu, Z., Hwang, I., and Kim, W.T. (2009). Drought stress-induced Rma1H1, a RING membrane-anchor E3 ubiquitin ligase homolog, regulates aquaporin levels via ubiquitination in transgenic *Arabidopsis* plants. Plant Cell *21*, 622-641.
Leibfried, A., To, J.P.C., Busch, W., Stehling, S., Kehle, A., Demar, M., Kieber, J.J., and Lohmann, J.U. (2005). WUSCHEL controls meristem function by direct regulation of cytokinin-inducible response regulators. Nature *438*, 1172-1175.
Moon, J., Parry, G., and Estelle, M. (2004). The Ubiquitin-proteasome pathway and plant development. Plant Cell *16*, 3181-3195.
Mukhopadhyay, D., and Riezman, H. (2007). Proteasome-independent functions of ubiquitin in endocytosis and signaling. Science *315*, 201-205
Oh, M., Lee, H., Kim, Y.-K., Nam, J.-W., Rhee, J.-K., Zhang, B.-T., Kim, V.N., and Lee, I. (2007). Identification and characterization of small RNAs from vernalized *Arabidopsis thaliana*. J. Plant Biol. *50*, 562-572.
Pickart, C.M. (2001). Ubiquitin enters the new millennium. Mol. Cell *8*, 499-504.
Pickart, C.M., and Eddins, M.J. (2004). Ubiquitin: structures, functions, mechanisms. Biochim. Biophys. Acta *1695*, 55-72.
Rashotte, A.M., Mason, M.G., Hutchison, C.E., Ferreira, F.J., Schaller, G.E., and Kieber, J.J. (2006). A subset of *Arabidopsis* AP2 transcription factors mediates cytokinin responses in concert with a two-component pathway. Proc. Natl. Acad. Sci. USA *103*, 11081-11085.
Seo, Y.S., Kim, E.Y., Mang, H.G., and Kim, W.T. (2008). Heterologous expression and biochemical and cellular characterization of *CaPLA1* encoding a hot pepper phospholipase A1 homolog. Plant J. *53*, 895-908.
Seo, Y.S., Kim, E.Y., Kim, J.H., and Kim, W.T. (2009). Enzymatic characterization of class I DAD1-like acylhydrolase members targeted to chloroplast in Arabidopsis. FEBS Lett. *583*, 2301-2307.
Sheen, J. (2002). Phosphorelay and transcription control in cytokinin signal transduction. Science *296*, 1650-1652.
Smalle, J., and Vierstra, R.D. (2004) The ubiquitin 26S proteasome proteolytic pathway. Annu. Rev. Plant Biol. *55*, 555-590.
Smalle, J., Kurepa, J., Yang, P., Babiychuk, E., Kushnir, S., Durski, A., and Vierstra, R.D. (2002). Cytokinin growth responses in *Arabidopsis* involve the 26S proteasome subunit RPN12. Plant Cell *14*, 17-32.
Smalle, J., Kurepa, J., Yang, P., Emborg, T.J., Babiychuk, E., Kushnir, S., Vierstra, R.D. (2003). The pleiotropic role of the 26S proteasome subunit RPN10 in *Arabidopsis* growth and development supports a substrate-specific function in abscisic acid signaling. Plant Cell *15*, 965-980.
Stone, S.L., Hauksdottir, H., Troy, A., Herschleb, J., Kraft, E., and Callis, J. (2005) Functional analysis of the RING-type ubiquitin ligase family of Arabidopsis. Plant Physiol. *137*, 13-30.
To, J.P.C., and Kieber, J.J. (2008). Cytokinin signaling: two-components and more. Trends Plant Sci. *13*, 85-92.
To, J.P.C., Deruère, J., Maxwell, B.B., Morris, V.F., Hutchison, C.E., Ferreira, F.J, Schaller, G.E., and Kieber, J.J. (2007). Cytokinin regulates type-A *Arabidopsis* Response Regulator activity and protein stability via two-component phosphorelay. Plant Cell *19*, 3901-3914.
Tran, L.-S.P., Urao, T., Qin, F., Maruyama, K., Kakimoto, T., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2007). Functional analysis of AHK1/ATHK1 and cytokinin receptor histidine kinases in response to abscisic acid, drought, and salt stress in *Arabidopsis*. Proc. Natl. Acad. Sci. USA *104*, 20623-20628.
Urao, T., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2001). Plant histidine kinases: An emerging picture of two-component signal transduction in hormone and environmental responses. Sci. STKE. *2001*, re18.
Vierstra, R.D. (2003). The ubiquitin/26S proteasome pathway, the complex last chapter in the life of many plant proteins. Trends Plant Sci. *8*, 135-142.
Voges, D., Zwickl, P., and Baumeister, W. (1999). The 26S proteasome: a molecular machine designed for controlled proteolysis. Annu. Rev. Biochem. *68*, 1015-1068.
Wohlbach, D.J., Quirino, B.F., and Sussman, M.R. (2008). Analysis of the *Arabidopsis* histidine kinase ATHK1 reveals a connection between vegetative osmotic stress sensing and seed maturation. Plant Cell *20*, 1101-1117.
Wojcik, C. (2001). Ubiquitin-more than just a signal for protein degradation. Trends Cell Biol. *11*, 397-399.
Yee, D., and Goring, D.R. (2009). The diversity of plant U-box E3 ubiquitin ligases: from upstream activators to downstream target substrates. J. Exp. Bot. *60*, 1109-1121.