

# The *Arabidopsis* NAC Transcription Factor VNI2 Integrates Abscisic Acid Signals into Leaf Senescence via the *COR/RD* Genes <sup>W</sup>

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Leaf aging is a highly regulated developmental process, which is also influenced profoundly by diverse environmental conditions. Accumulating evidence in recent years supports that plant responsiveness to abiotic stress is intimately related with leaf longevity. However, molecular mechanisms underlying the signaling crosstalks and regulatory schemes are yet unknown. In this work, we demonstrate that an abscisic acid (ABA)-responsive NAC transcription factor VND-INTERACTING2 (VNI2) integrates ABA-mediated abiotic stress signals into leaf aging by regulating a subset of *COLD-REGULATED (COR)* and *RESPONSIVE TO DEHYDRATION (RD)* genes. The *VNI2* gene was induced by high salinity in an ABA-dependent manner. In addition, spatial and temporal expression patterns of the *VNI2* gene are correlated with leaf aging and senescence. Accordingly, leaf aging was delayed in transgenic plants overexpressing the *VNI2* gene but significantly accelerated in a *VNI2*-deficient mutant. The *VNI2* transcription factor regulates the *COR* and *RD* genes by binding directly to their promoters. Notably, transgenic plants overexpressing the *COR* or *RD* genes exhibited prolonged leaf longevity. These observations indicate that the *VNI2* transcription factor serves as a molecular link that integrates plant responses to environmental stresses into modulation of leaf longevity.

## INTRODUCTION

Leaf aging, culminating in senescence, or complete breakdown of leaf structures, is a crucial component of later stages in plant development. It is not a passive process but a developmentally programmed and orderly regulated process that is modulated by more than 800 genes (Buchanan-Wollaston et al., 2005). The aging process is initiated by chloroplast degeneration (Gepstein, 2004), which is followed by catabolism of macromolecules, such as nucleic acids, proteins, and lipids (Ulker et al., 2007), and degeneration of mitochondria and nuclei (Buchanan-Wollaston et al., 2003, 2005; Lim et al., 2003, 2007). This orderly process facilitates hydrolysis and recycling of nutrients into the development of reproductive structures to increase reproductive success (Buchanan-Wollaston et al., 2003, 2005; Lim et al., 2007).

Leaf aging is under nuclear control. Inhibition of gene transcription and translation profoundly affects aging progression in leaves (Buchanan-Wollaston et al., 2003; Lim and Nam, 2005). Indeed, leaf aging is accompanied by enormous changes in gene expression. Transcriptome analysis reveals that ~10% of the genes in the *Arabidopsis thaliana* genome are differentially expressed during leaf aging (Guo et al., 2004). Thousands of

potential *SENESCENCE-ASSOCIATED GENES (SAGs)*, which are upregulated during leaf senescence, have been identified through similar approaches, indicating that changes in gene expression are critical in modulating leaf aging (Buchanan-Wollaston et al., 2005).

The leaf aging process is also influenced by environmental factors, such as osmotic stress, temperature extremes, nutrient availability, pathogen attacks, and light quality and intensity (Buchanan-Wollaston et al., 2005). Leaf aging is prematurely induced by abiotic stresses (Buchanan-Wollaston et al., 2005). Salt stress triggers chloroplast degeneration and membrane disintegration and induces natural senescence (Dhindsa et al., 1981). Consistently, abscisic acid (ABA) plays a key role in environmental stress responses and, thus, leaf longevity (Lim et al., 2007). ABA is thought to promote aging and abscission of leaves, and levels of the phytohormone increase dramatically in the leaves that are undergoing senescence (Lim et al., 2007). Leaf aging under stress conditions is also accompanied by differential gene expression. For example, numerous SAGs are influenced by diverse abiotic and biotic stresses (Weaver et al., 1998; Quirino et al., 1999). In addition, genes regulating stress responses also affect leaf aging (Binyamin et al., 2001; Yoon et al., 2008). In this respect, transcriptional control of leaf senescence is believed to be an important mechanism under environmental stress conditions.

It has been estimated that ~100 transcription factors belonging to the APETALA2, basic-leucine zipper, MYB, NAC (NAM/ATAF1, 2/CUC2), WRKY, zinc finger, and GRAS families are differentially regulated during leaf senescence (Buchanan-Wollaston et al., 2005). It is notable that many of the transcription factors

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<sup>W</sup>Online version contains Web-only data.

www.plantcell.org/cgi/doi/10.1105/tpc.111.084913

involved in the developmental processes also play diverse roles in hormone signaling, stress responses, and metabolism, supporting the idea that extensive signaling crosstalk exists between intrinsic aging programs and environmental stress signals (Lim and Nam, 2005; Lim et al., 2007).

The NAC transcription factors constitute one of the largest transcription factor families in plants. The *Arabidopsis* genome contains ~110 NAC members (Riechmann et al., 2000). They are involved in diverse developmental processes and biotic and abiotic stress responses (Olsen et al., 2005b). The NAC transcription factors also constitute a large fraction of senescence-regulated genes in many plant species (Guo et al., 2004; Buchanan-Wollaston et al., 2005; Yoon et al., 2008; Balazadeh et al., 2010a). The *Arabidopsis* *NAP* (*NAC-LIKE, ACTIVATED BY AP3/PI*) gene is transcriptionally regulated during senescing process in *Arabidopsis* leaves (Guo and Gan, 2006). Whereas the *NAP*-deficient mutant exhibits delayed leaf senescence, premature senescence is induced in the transgenic plants overexpressing *NAP*. Notably, *NAP* homologs from rice (*Oryza sativa*) and kidney bean (*Phaseolus vulgaris*), *Os NAP* and *Pv NAP*, restore the delayed leaf senescence in the *At NAP*-deficient mutant (Guo and Gan, 2006), showing that the *NAP* genes are conserved in diverse plant species.

Roles of the *ANAC092/NAC2/ORE1* gene have been recently demonstrated in regulation of leaf longevity (Kim et al., 2009). The *ORESARA1* (*ORE1*) gene is posttranscriptionally regulated by microRNA164 (miR164). miR164 is repressed in an age-dependent manner, and *ORE1* gene expression gradually increases as plants grow (Oh et al., 1997). The *ore1* mutant exhibits delayed leaf senescence, as observed in miR164 overproducing transgenic plants (Kim et al., 2009). It has been found that these regulatory components are further regulated by the *ETHYLENE INSENSITIVE2* gene (Kim et al., 2009), providing an insight into the roles of ethylene signaling in leaf aging and senescence. Notably, the *ANAC092/NAC2/ORE1* gene is also induced by salt stress, and the NAC transcription factor regulates salt-promoted senescing process (He et al., 2005; Balazadeh et al., 2010a, 2010b). According to the EST database, ~20 NAC transcription factors are differentially regulated during senescence (Guo et al., 2004), supporting the roles of NAC transcription factors in leaf aging. Although many transcriptional regulators and senescence-regulated genes have been identified, molecular mechanisms underlying leaf aging process and interactions between environmental signals and intrinsic programs governing leaf aging are largely unknown.

In this study, we identified a NAC transcription factor VND-INTERACTING2 (*VNI2*) that mediates signaling crosstalk between salt stress response and leaf aging process. The *VNI2* transcription factor regulates the *COLD-REGULATED* (*COR*) and *RESPONSIVE TO DEHYDRATION* (*RD*) genes by binding directly to their promoters. Overexpression of *COR* or *RD* led to prolonged leaf longevity, as observed in the *VNI2*-overexpressing transgenic plants, which also exhibit enhanced resistance to salt stress. The *VNI2*-deficient *vni2-1* mutant was susceptible to salt stress and showed reduced leaf longevity, supporting the idea that *VNI2*-mediated salt stress signaling is related to leaf longevity. Our data also indicate that the *COR/RD* genes play an important role in signaling crosstalk.

## RESULTS

### The *VNI2* Transcriptional Repressor Has an Activation Domain

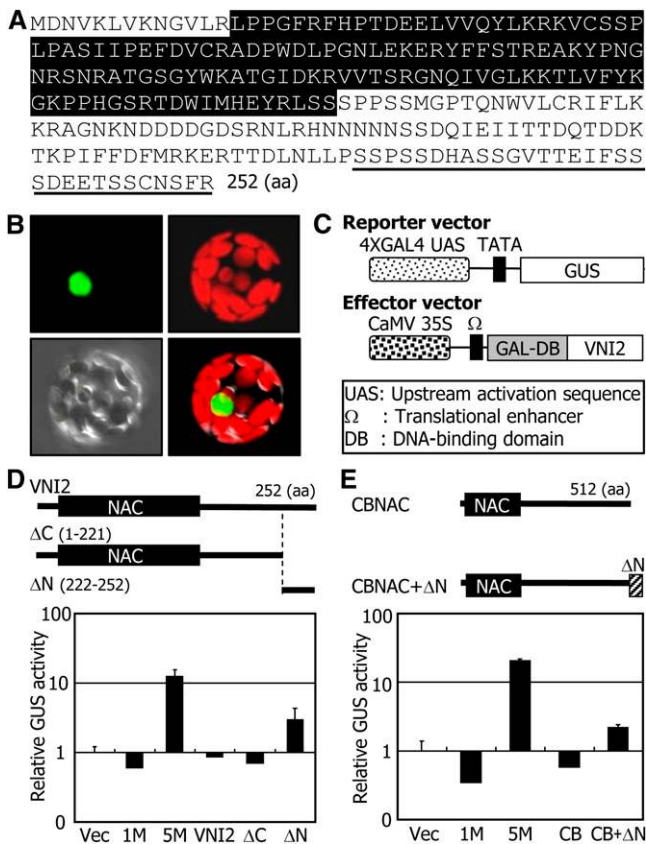
Among the NAC transcription factor genes identified in *Arabidopsis*, *VNI2* (AT5G13180) is of particular interest in that it is influenced by both developmental and environmental cues. Gene expression analysis using the GENEVESTIGATOR database (<https://www.genevestigator.com/gv/index.jsp>) revealed that *VNI2*, which is highly expressed in senescing plant tissues, is also induced by environmental stress conditions, suggesting that it may be involved in modulation of senescence in response to environmental stresses. We therefore decided to examine the potential role of *VNI2* in the functional relationship between leaf aging and environmental stress responses.

The *VNI2* protein consists of 252 amino acid residues with a conserved NAC DNA binding domain in the N-terminal region (Figure 1A) like other nuclear NAC transcription factors. Subcellular localization assays in *Arabidopsis* protoplasts, using a *VNI2-green fluorescent protein* (*GFP*) gene fusion, in which a *GFP*-coding sequence was fused in frame to the 3' end of the *VNI2* gene, showed that the *VNI2* protein was localized exclusively in the nucleus (Figure 1B).

We next examined whether the *VNI2* protein is a transcriptional regulator using a *GAL4* transient expression system in *Arabidopsis* protoplasts (Miura et al., 2007). A full-length *VNI2* sequence was fused in-frame to the 3' end of the *GAL4* DNA binding domain (*GAL4-DB*)-coding sequence in the effector vector (Figure 1C). The effector vector, together with a construct containing the  $\beta$ -glucuronidase (*GUS*) reporter gene and a vector containing the *Renilla* luciferase gene, was cotransformed into *Arabidopsis* protoplasts. Expression of the *VNI2* sequence repressed *GUS* activity by ~20%, compared with expression of vector control (Figure 1D), demonstrating that the *VNI2* protein is a transcriptional repressor.

To map the transcriptional regulation domains, additional constructs expressing a couple of truncated *VNI2* sequences were also assayed (Figure 1D, top panel). Expression of the  $\Delta C$  sequence consisting of residues 1 to 221 but lacking the C-terminal region repressed *GUS* activity by more than 30%, indicating that the *VNI2* protein is a transcriptional repressor. Interestingly, a strong transcriptional activation activity was detected in the C-terminal region consisting of residues 222 to 252 ( $\Delta N$ ). Expression of the  $\Delta N$  construct elevated *GUS* activity more than threefold, showing that the *VNI2* protein possesses both transcriptional activation and repression domains.

To further examine the transcriptional activation activity of the  $\Delta N$  construct, the  $\Delta N$  sequence was fused to the 3' end of the calmodulin binding NAC (*CBNAC*) transcriptional repressor gene (Kim et al., 2007), and the *CBNAC*+ $\Delta N$  sequence was fused in frame to the 3' end of the *GAL4-DB*-coding sequence in the effector vector (Figure 1E, top panel). Whereas the *CBNAC* protein repressed *GUS* activity by ~30% as has been reported (Kim et al., 2007), the *CBNAC*+ $\Delta N$  fusion elevated *GUS* activity more than twofold (Figure 1E), confirming that the  $\Delta N$  region possesses transcriptional activation activity.



**Figure 1.** The VNI2 Transcriptional Repressor Has an Activation Domain.

**(A)** Amino acid (aa) sequence of VNI2 protein. Residues constituting the NAC domain are marked in black. The transcriptional activation domain defined in **(D)** is underlined.

**(B)** Subcellular localization of VNI2 protein. A VNI2-GFP gene fusion, in which the GFP-coding sequence was fused in frame to the 3' end of the VNI2 gene, was transiently expressed in *Arabidopsis* protoplasts and visualized by differential interference contrast microscopy. Top left, VNI2-GFP; top right, autofluorescence; bottom left, differential interference contrast; bottom right, merged.

**(C)** Schematic diagrams of reporter and effector constructs used in transient expression assays using *Arabidopsis* protoplasts. In the effector vectors, the VNI2 coding sequence was fused in frame to the 3' end of the GAL-DB-coding sequence.

**(D)** Relative GUS activities of the effector constructs containing a full-size VNI2, ΔC, and ΔN proteins. The VNI2 constructs assayed are shown (top panel), with the NAC domain indicated. GAL4 transient expression assays were performed as described previously (Miura et al., 2007). Five measurements were averaged (bottom panel). Bars indicate SE. ARF5M (5M) is a transcriptional activator control, and ARF1M (1M) is a transcriptional repressor control (Tiwari et al., 2003). The y axis is presented on a logarithmic scale for better comparison of fold changes. Vec, vector control.

**(E)** Relative GUS activities of the effector constructs containing the CBNAC (CB) and CBNAC+ΔN (CB+ΔN) proteins. In the CB+ΔN fusion (top panel), the ΔN sequence was fused to the C-terminal end of a transcriptional repressor CBNAC (Kim et al., 2007). Measurements of GUS activities and data processing (bottom panel) were performed as described in **(D)**.

## The VNI2 Gene Is Highly Expressed in Senescing Plant Tissues

To obtain clues as to the role played by the VNI2 gene, we examined temporal and spatial expression patterns of the VNI2 gene by quantitative real-time RT-PCR (qRT-PCR). qRT-PCR assays using total RNA samples from different plant organs revealed that VNI2 was expressed to a high level in leaves but to a relatively lower level in shoot apical meristem and reproductive tissues, such as flowers and siliques (Figure 2A). In addition, VNI2 was highly expressed in late stages of plant development (Figure 2B), which is similar to the expression patterns in the public database GENEVESTIGATOR (<https://www.genevestigator.com/gv/index.jsp>), suggesting that VNI2 is involved in leaf senescence.

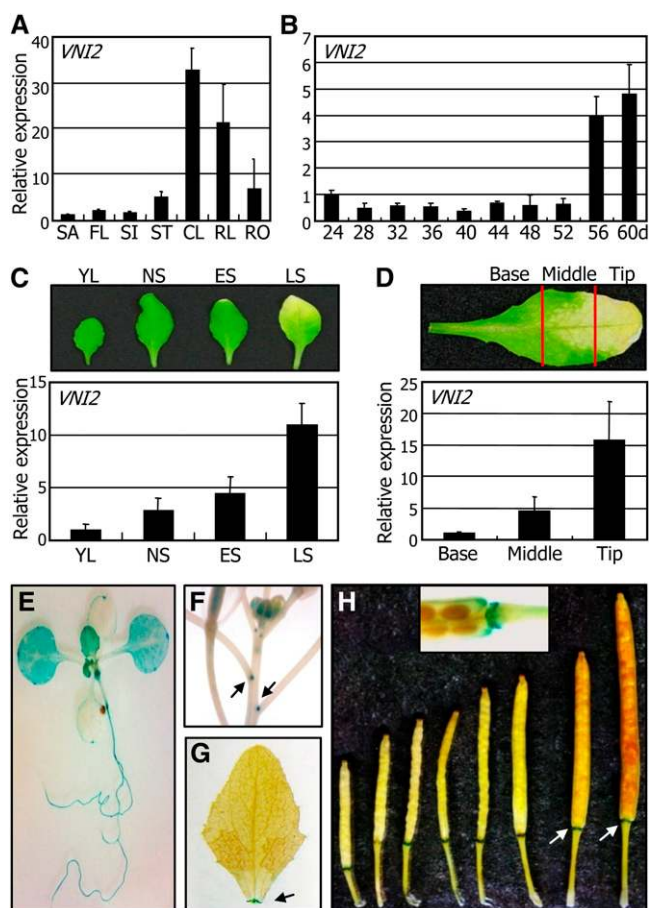
We therefore examined VNI2 expression in leaves at different developmental stages. The levels of the VNI2 transcripts were relatively lower in young leaves, but the transcript level gradually increased as leaves began senescing (Figure 2C). In *Arabidopsis*, leaf development proceeds from the tip toward the base (Lim et al., 2007). The 8th rosette leaves exhibiting ~50% senescence were dissected into basal, middle, and tip parts along the leaf axis (Figure 2D, top panel), and the VNI2 transcript levels were compared by qRT-PCR in individual leaf parts. The results showed that whereas the VNI2 gene was expressed to a relatively low level in the basal leaf, it was highly expressed in the leaf tip (Figure 2D). These observations suggest that VNI2 is closely associated with leaf aging or leaf longevity.

To further examine the expression patterns of VNI2 in different plant tissues, a VNI2 promoter sequence covering 2992 bp upstream of the transcription start site was transcriptionally fused to a GUS-coding sequence, and the pVNI2-GUS construct was transformed into Columbia-0 (Col-0) plants. In young plants, GUS activity was detected mainly in roots and leaves (Figure 2E). Notably, GUS activity was detected to a higher level in senescent leaves and abscission and dehiscence tissues, such as axils of bracts and abscission zones in cauline leaves and siliques (Figures 2F to 2H; see Supplemental Figure 1 online), supporting the idea that the VNI2 gene is involved in late stages of plant development, particularly in leaf aging and abscission.

## Leaf Aging Is Accelerated in vni2-1 Mutants

To investigate the potential role of VNI2 in leaf aging and longevity, we produced transgenic plants overexpressing the VNI2 gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter (35S:VNI2). A T-DNA insertional VNI2-deficient knockout mutant (*vni2-1*) was also obtained from the ABRC (Figure 3A). Overexpression of the VNI2 gene in the 35S:VNI2 transgenic plants and lack of gene expression in the *vni2-1* mutant were verified by qRT-PCR assays before further analysis (Figure 3B).

Analysis of leaf aging revealed that whereas the 35S:VNI2 transgenic plants exhibited prolonged leaf longevity in comparison to Col-0 plants and vector control plants (pB2GW7), leaf aging and senescence were accelerated significantly in the *vni2-1* mutant (Figures 3C and 3D; see Supplemental Figure 2 online). A similar pattern was also observed with measurements of chlorophyll contents in the leaves (Figure 3E; see Supplemental



**Figure 2.** Temporal and Spatial Expression Patterns of the *VNI2* Gene.

In (A) to (D), transcript levels were determined by qRT-PCR. The *eIF4A* gene was included in the assays as an internal control for normalizing the variations in cDNA amounts used. Biological triplicates were averaged. Bars indicate SE.

(A) Tissue-specific expression. Total RNAs were extracted from different plant tissues. SA, shoot apical meristem; FL, flowers; SI, siliques; ST, stems; CL, cauline leaves; RL, rosette leaves; RO, roots.

(B) Growth stage-dependent expression. Total RNAs were extracted from shoots harvested at the indicated time points. d, days after cold imbibition.

(C) Transcript levels in the leaves at different developmental stages. YL, young leaves; NS, no senescence; ES, early senescing step; LS, late senescing step. Leaves of indicated stages are shown above.

(D) Localized expression in a senescing leaf. A rosette leaf showing ~50% senescence was dissected into three parts as indicated.

(E) to (H) Distribution of GUS activities in plant tissues. The *pVNI2-GUS* fusion construct, in which the GUS-coding sequence was transcriptionally fused to the *VNI2* gene promoter sequences consisting of ~3 kb upstream of the transcription start site, was transformed into Col-0 plants. GUS activities were detected in the leaves and roots of young seedlings (E). Higher activities were detected in the axils of bracts (F) and in the abscission zones of cauline leaves (G) and siliques (H), as indicated by arrows.

Figure 2C online) and expression patterns of the cysteine protease-encoding *SAG12* senescence marker gene (Figure 3F; see Supplemental Figure 2D online). *SAG12* exhibited delayed induction in the 35S:*VNI2* transgenic leaves but an accelerated induction in the *vni2-1* mutant (Figure 3F). Because Col-0 and vector control plants were phenotypically indistinguishable in overall plant morphology and senescence (see Supplemental Figure 3 online), Col-0 plants were used as controls in subsequent assays.

The *vni2-1* mutant was phenotypically and developmentally indistinguishable from Col-0 plants. By contrast, the 35S:*VNI2* transgenic plants exhibited somewhat retarded growth in young seedling stages (see below), although the overall size and morphology of full-grown plants are quite similar to that of Col-0 plants (Figure 3C). To eliminate potential indirect effects of growth retardation and differential leaf emergence on leaf senescence, the 4th rosette leaves of plants that exhibit similar patterns of leaf aging were assayed. Whereas the 35S:*VNI2* transgenic leaves exhibited prolonged longevity, leaf senescence was accelerated considerably in the *vni2-1* mutant leaves (see Supplemental Figure 4 online), further supporting that *VNI2* regulates leaf longevity. By contrast, flowering times were essentially unchanged in the transgenic and mutant plants (see Supplemental Figure 5 online), indicating that *VNI2* is involved specifically in leaf aging.

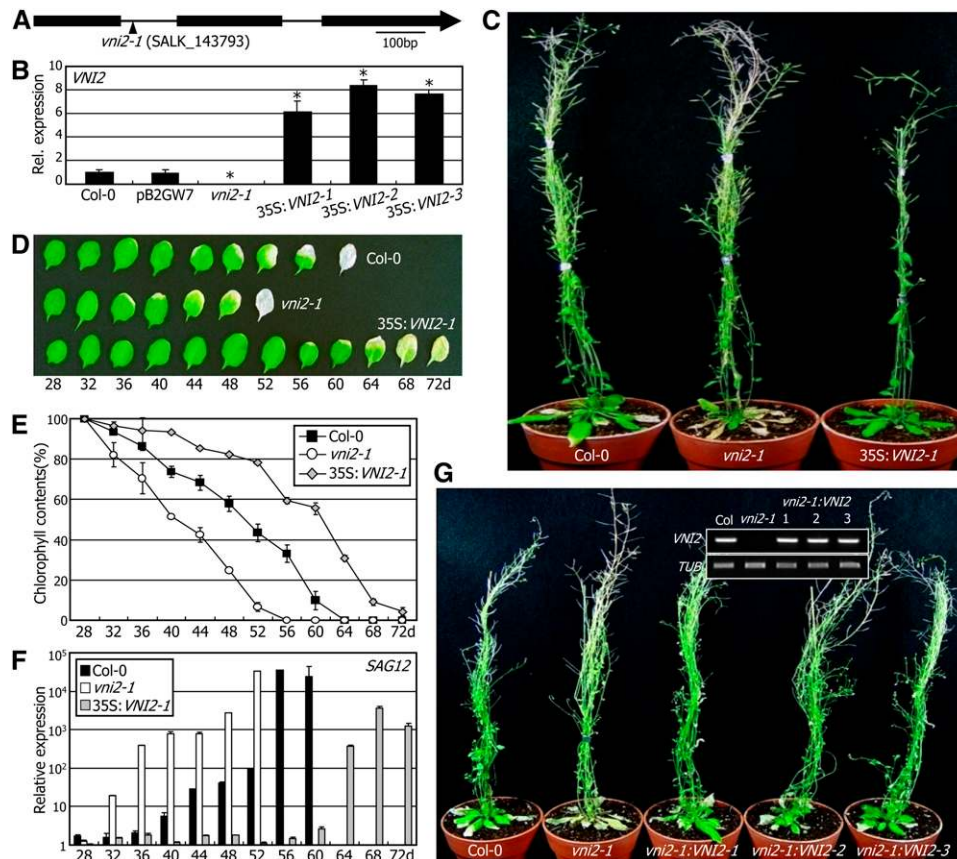
To confirm the role of *VNI2* in leaf longevity, the *vni2-1* mutant was transformed with the *VNI2* gene driven by its own promoter. Leaf aging in the resultant transgenic plants (*vni2-1:VNI2*) was indistinguishable from that in Col-0 plants (Figure 3G), verifying that the differential phenotypes of leaf aging observed in the 35S:*VNI2* transgenic plants and *vni2-1* mutant is linked with the *VNI2* gene.

Darkness is considered to be a strong inducer of leaf senescence (Weaver and Amasino, 2001), and responses to natural leaf aging and dark-induced leaf senescence overlap considerably in signaling mediators and cellular processes, although with some degree of difference (Lin and Wu, 2004). We observed that dark-induced leaf yellowing was accelerated in the *vni2-1* mutants (see Supplemental Figure 6A online). Consequently, chlorophyll degradation was accelerated and *SAG12* was induced more rapidly in the *vni2-1* leaves after exposure to darkness (see Supplemental Figures 6B and 6C online). Expression of *VNI2* was also correlated with the duration of darkness (see Supplemental Figure 6D online). Furthermore, the premature leaf senescence of the *vni2-1* mutant was rescued in the *vni2-1:VNI2* transgenic plants under dark conditions (see Supplemental Figure 7 online), further supporting the role of *VNI2* in modulating leaf longevity.

### The *VNI2* Gene Is Regulated by Salt Stress Partially via ABA

To determine the nature of environmental signals affecting *VNI2*, we investigated the effects of various growth hormones and environmental stress conditions on *VNI2* expression.

*VNI2* expression was induced considerably by ABA but unresponsive to indole-3-acetic acid (IAA), gibberellic acid (GA), 1-aminocyclopropane-1-carboxylic acid, salicylic acid, and methyl jasmonic acid (Figure 4A; see Supplemental Figure 8 online). High salinity also induced the *VNI2* gene approximately twofold, but cold and drought stresses did not affect *VNI2* expression (Figure 4B).



**Figure 3.** Leaf Aging Processes in 35S:VNI2 Transgenic and *vni2-1* Mutant Plants.

(A) Mapping of T-DNA insertion site in *vni2-1* mutant.

(B) Transcript levels of the *VNI2* gene. Transcript levels were examined by qRT-PCR using RNA samples extracted from 2-week-old whole plants grown on MS-agar plates. Biological triplicates were averaged. Bars indicate SE (*t* test, \**P* < 0.01).

(C) Phenotypes of adult plants. Ten-week-old plants grown in soil under LDs were photographed.

(D) Comparison of leaf aging. Fourth leaves from plants grown in soil under LDs for up to 72 d were photographed. d, days after cold imbibition.

(E) Measurements of chlorophyll contents. Fourth leaves of ~30 plants grown in soil were harvested at the indicated time points. Five measurements were averaged. Bars indicate SE. d, days after cold imbibition.

(F) Expression of the *SAG12* gene. Fourth leaves of ~30 plants grown in soil and harvested at the indicated time points were used for total RNA extraction. Transcript levels were determined by qRT-PCR. The *eIF4A* gene was included in the assays as an internal control for normalizing the variations in cDNA amounts used. Biological triplicates were averaged. Bars indicate SE. d, days after cold imbibition.

(G) Complementation of the *vni2-1* mutant. The *VNI2* gene with its own promoter was transformed into the *vni2-1* mutant. Ten-week-old plants grown in soil under LDs were photographed. Transcript levels of *VNI2* were examined by RT-PCR (inset).

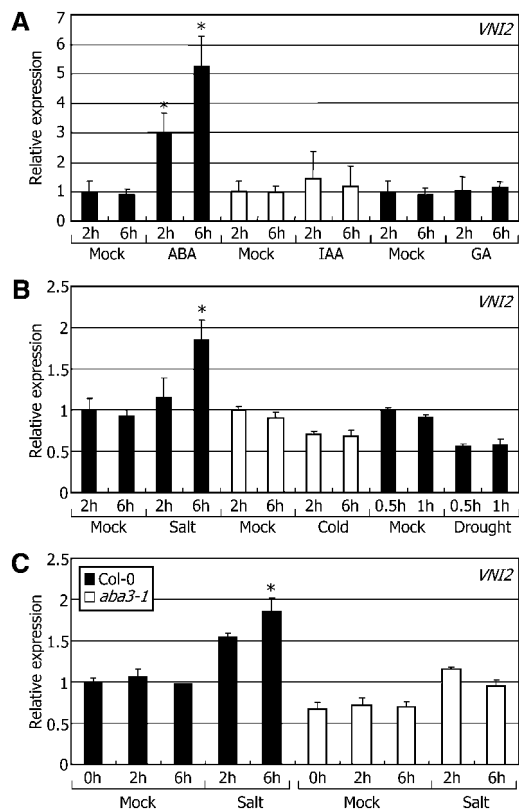
We examined whether the salt induction of *VNI2* depends on ABA or not. Effects of high salt on *VNI2* expression were reduced significantly in the ABA-deficient *aba3-1* mutant (Schwartz et al., 1997; Figure 4C), indicating that the *VNI2* gene is regulated in part by high salinity in an ABA-dependent manner.

The *VNI2* transcription repressor possesses an activation domain in its C-terminal region (Figures 1D and 1E). We examined whether the transcriptional regulation activity of *VNI2* is altered in the presence of high salt. GAL4 transient expression assays in *Arabidopsis* protoplasts were performed in the presence of 50 mM NaCl. Unexpectedly, the *VNI2* transcriptional repressor behaved as a transcriptional activator under high salinity (see Supplemental Figure 9 online). The transcriptional activation

activity of the  $\Delta N$  construct was further elevated under the identical conditions. These observations indicated that the activities of the *VNI2* transcription factor were modulated at a posttranslational level by high salinity, which may be related with the role of the *VNI2* transcription factor in salt stress responses (see below).

### The *VNI2* Transcription Factor Regulates the *COR* and *RD* Genes

It was suspected that the *VNI2* transcription factor plays a role in salt stress regulation of leaf longevity. To look into the molecular mechanisms underlying *VNI2* function in leaf aging under high salt conditions, we searched for genes whose expression levels



**Figure 4.** Effects of High Salinity and ABA on *VNI2* Gene Expression.

Two-week-old plants grown on MS-agar plates were used for subsequent treatments with salt (200 mM NaCl), cold (4°C), drought, and growth hormones, such as ABA, IAA, and GA. Transcript levels were determined by qRT-PCR and normalized to *eIF4A*. Biological triplicates were averaged. Statistical significance was determined by a Student's *t* test (\**P* < 0.01). Bars indicate SE.

- (A)** Effects of growth hormones. Growth hormones were used at 20  $\mu$ M.  
**(B)** Effects of abiotic stresses.  
**(C)** Effects of high salinity on *VNI2* gene expression in *aba3-1* mutant.

were altered in the 35S:*VNI2* transgenic and *vni2-1* mutant plants. Among the stress-responsive genes examined, expression of a subset of *COR* and *RD* genes was altered in the 35S:*VNI2* transgenic and *vni2-1* mutant plants (Figure 5A; see Supplemental Figure 10 online). The *COR15A*, *COR15B*, *RD29A*, and *RD29B* genes were upregulated in the 35S:*VNI2* transgenic plants (Figure 5A). Their expression was unchanged in the *vni2-1* mutant under normal growth conditions. However, effects of ABA and high salinity on gene expression were reduced significantly in the *vni2-1* mutant (Figure 5B; see Supplemental Figure 11 online), indicating that *VNI2* is required for proper induction of the *COR* and *RD* genes under high salt conditions (see Discussion).

The *VNI2* protein is a potential transcriptional activator under high salt conditions (see Supplemental Figure 9 online). We therefore asked whether it directly regulates the *COR* and *RD* genes. Nucleotide sequence analysis revealed that the pro-

motors of the *COR* and *RD* genes contained conserved sequence motifs (Figure 5C, top panel), which are analogous to the NAC binding consensus sequences (CATGT) (Olsen et al., 2005a). Chromatin immunoprecipitation (ChIP) assays were employed to examine whether the *VNI2* protein binds to the gene promoters using 35S:*VNI2*-MYC transgenic plants, in which a MYC-coding sequence was fused in frame to the 3' end of the *VNI2* gene. Quantitative real-time ChIP-PCR assays using an anti-MYC antibody showed that *VNI2* binds to the conserved sequence motifs in the gene promoters (Figure 5C, bottom panel).

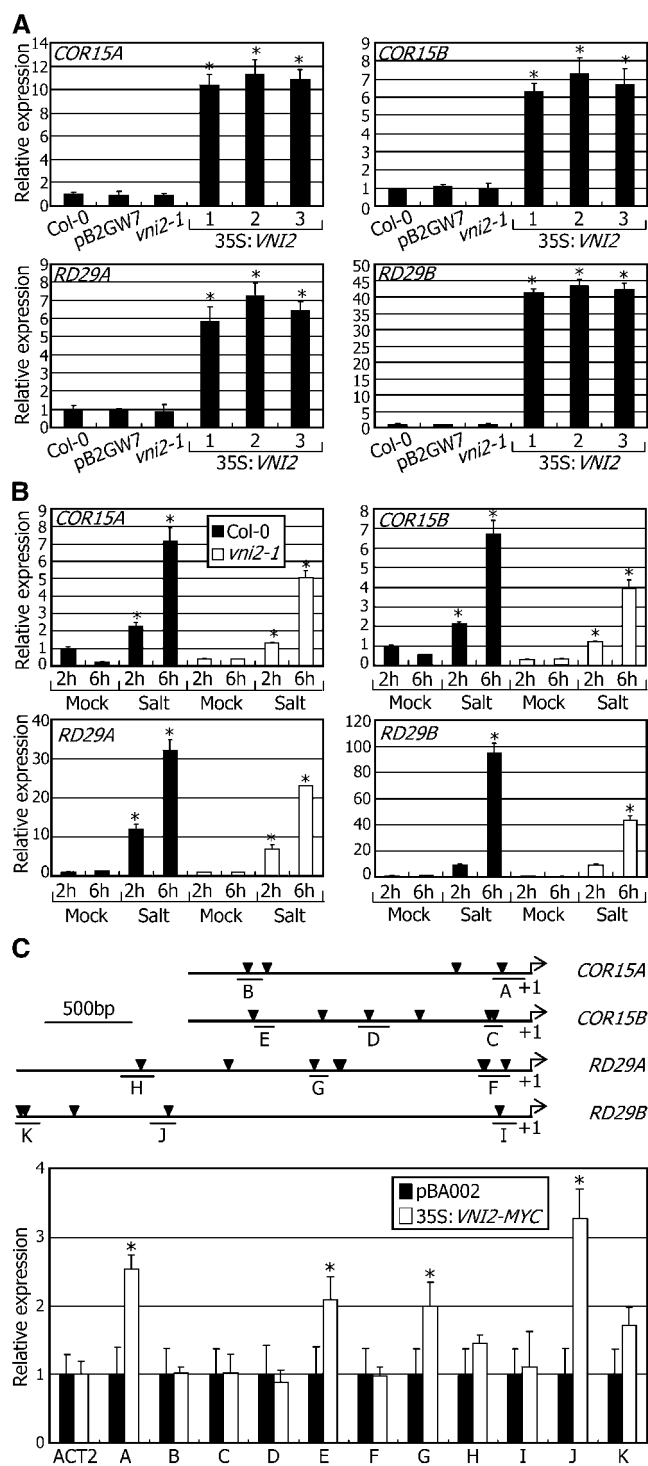
Potential core binding sequences of the *VNI2* transcription factor were predicted by sequence analysis of the *COR* and *RD* gene promoters in conjunction with the ChIP data (see Supplemental Figure 12A online). Electrophoretic mobility shift assays using a recombinant maltose binding protein (MBP)-*VNI2* fusion protein produced in *Escherichia coli* cells showed that the *VNI2* protein bound specifically to the core binding sequences (BSs) of the *COR15A*, *COR15B*, *RD29A*, and *RD29B* gene promoters (see Supplemental Figure 12B online). The MBP protein alone did not bind to the sequences. In addition, *VNI2* binding was significantly reduced in the presence of excess unlabeled BS fragments and was reduced to a lesser degree by mutated DNA fragments (mBSs), supporting the specific binding of *VNI2* to the BSs (see Supplemental Figure 12C online).

We next performed transient GUS expression assays in *Arabidopsis* protoplasts to further investigate the *VNI2* regulation of *COR* and *RD* genes. The *COR*/*RD*-BS and -mBS DNA fragments were transcriptionally fused to the CaMV 35S minimal promoter, resulting in pCOR/*RD*-P or pCOR/*RD*-mP (Figure 6A). The reporter plasmids and an effector plasmid p35S-*VNI2* were cotransformed into *Arabidopsis* protoplasts. A vector construct containing the luciferase gene was included to monitor transformation efficiencies (Miura et al., 2007). Cotransformation of p35S-*VNI2* with pCOR/*RD*-P did not affect reporter gene expression under normal assay conditions (Figure 6B). Under high salinity, reporter gene expression was elevated by four- to sixfold under high salinity. By contrast, cotransformation with pCOR/*RD*-mP did not affect reporter gene expression regardless of assay conditions. These observations indicate that the *VNI2* transcription factor regulates the *COR* and *RD* genes by directly binding to the gene promoters and transcriptional activation activity of *VNI2* is elevated under high salinity.

We also examined whether DNA binding affinity and protein stability of *VNI2* is altered under high salinity. ChIP assays on salt-treated 35S:*VNI2*-MYC transgenic plants revealed that DNA binding affinity of *VNI2* was unaffected by high salt (see Supplemental Figure 13 online). Protein stability was also uninfluenced by high salt (see Supplemental Figure 14 online). It is therefore evident that positive regulation of *COR*/*RD* genes by *VNI2* under high salinity is mediated mainly by increased transcriptional activation activity.

### The *COR* and *RD* Genes Play a Role in Leaf Longevity

Our data indicated that the *VNI2* transcription factor modulates leaf longevity by regulating stress-responsive genes. Therefore, we asked whether *COR15A*, *COR15B*, *RD29A*, and *RD29B* are



**Figure 5.** Binding of VNI2 to Consensus Motifs in the *COR* and *RD* Gene Promoters.

In **(A)** and **(B)**, transcript levels were determined by qRT-PCR and normalized to *eIF4A*. Biological triplicates were averaged. Bars indicate SE ( $t$  test,  $*P < 0.01$ ).

**(A)** Transcript levels of *COR* and *RD* genes. Two-week-old whole plants grown on MS agar plants were used for total RNA extraction. Vector

intimately associated with leaf aging. To answer this question, we analyzed expression of the *COR* and *RD* genes in senescing leaves. They were expressed to a relatively high level in leaf tip parts, similar to *VNI2* expression (Figure 7A). Temporal expression patterns of the *COR* and *RD* genes were also similar to *VNI2* (Figure 7B). *COR* and *RD* transcript levels gradually increased during leaf senescence, but the age-dependent elevation of transcript levels was reduced significantly in the *vni2-1* mutant, suggesting that the *COR* and *RD* genes do play a role in the *VNI2*-mediated modulation of senescence via ABA-mediated salt stress signaling.

To examine more directly the role of the *COR* and *RD* genes in leaf longevity, we produced transgenic plants overexpressing the *COR* and *RD* genes (see Supplemental Figure 15 online). As expected, all the transgenic plants exhibited prolonged leaf longevity (Figures 7C and 7D; see Supplemental Figure 16 online), as observed in the 35S:*VNI2* transgenic plants, indicating that the *COR* and *RD* genes also function in leaf aging in addition to their roles in abiotic stress responses (Yamaguchi-Shinozaki and Shinozaki, 1993; Steponkus et al., 1998; Nakayama et al., 2007, 2008).

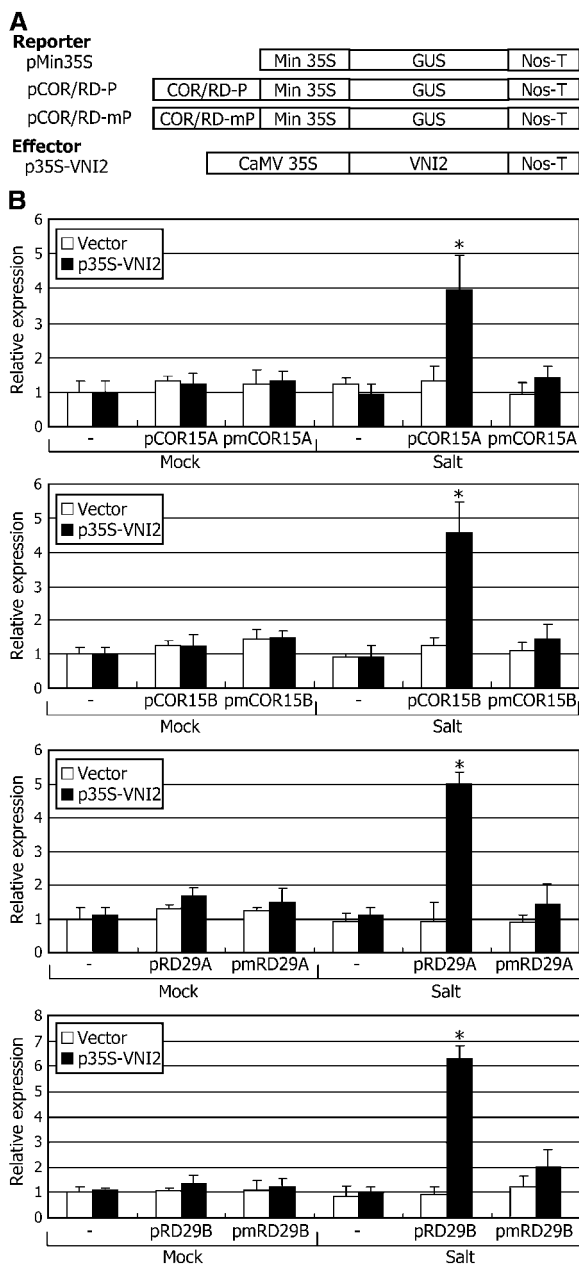
Extensive expression studies on genes involved in the salt stress response and developmental leaf aging revealed that expression of additional genes is also altered in *vni2-1* mutants. Notably, several genes that are responsive to high salt in a *VNI2*-dependent manner, such as *ABA-INSENSITIVE1* (*ABI1*), *ABI2*, *ABI3*, *GH3-3*, *GH3-5*, *GH3-6*, and *RD26*, were also regulated developmentally during leaf senescence (see Supplemental Figure 17 online).

Taken together, our observations demonstrate that the *VNI2* transcription factor serves as a molecular link that integrates developmental and environmental signals in modulating leaf longevity under high salt conditions (Figure 8). Consistent with the view that leaf longevity is closely related with plant responses to high salinity, whereas seedling growth of the 35S:*VNI2* transgenic plants is resistant to high salinity, that of the *vni2-1* mutant was influenced more severely by high salinity (see Supplemental Figure 18 online). In addition, salt-induced leaf senescence was accelerated in the *vni2-1* mutant (see Supplemental Figures 19 to 21 online). Our data provide a molecular mechanism regulating the functional linkage between leaf aging process and environmental stress resistance (Figure 8).

control plants (pB2GW7) and multiple transgenic lines were included in the assays.

**(B)** Effect of high salinity on *COR* and *RD* gene expression in *vni2-1* mutant. Two-week-old plants grown on MS-agar plates were subsequently incubated for 2 or 6 h in MS liquid cultures supplemented with 200 mM NaCl before harvesting plant materials.

**(C)** ChIP assays. In the *COR* and *RD* gene promoters, the sequence regions used for ChIP assays are marked (A to K, top panel). NAC binding motifs are indicated as arrowheads. For ChIP assays, 35S:*VNI2-MYC* transgenic plants grown on MS-agar plates for 3 weeks were used (bottom panel). Three measurements were averaged for individual assays. Bars indicate SE ( $t$  test,  $*P < 0.01$ ). The values in Col-0 plants were set to 1 after normalization against *ACT2* for qPCR analysis.



**Figure 6.** Elevation of Transcriptional Activation Activity of VNI2 under High Salinity.

**(A)** Expression constructs used. The promoter sequence elements of the *COR/RD* genes listed in Supplemental Figure 12A were fused to a minimal 35S promoter (Min 35S) and used as reporters. In the effector construct, the *VNI2* gene was transcriptionally fused to the CaMV 35S promoter. Nos-T, Nos terminator.

**(B)** Transcriptional activation activity assays in *Arabidopsis* protoplasts. The expression constructs were expressed transiently in *Arabidopsis* protoplasts, and GUS activities were determined fluorimetrically. The protoplasts were treated with 50 mM NaCl before use. Luciferase gene expression was used to normalize the GUS activities. The normalized values in control protoplasts were set to 1 and represented as relative activation. Three measurements were averaged. Statistical significance was determined by a Student's *t* test (\* $P < 0.01$ ). Bars indicate se.

## DISCUSSION

### Environmental Stress Regulation of Leaf Longevity

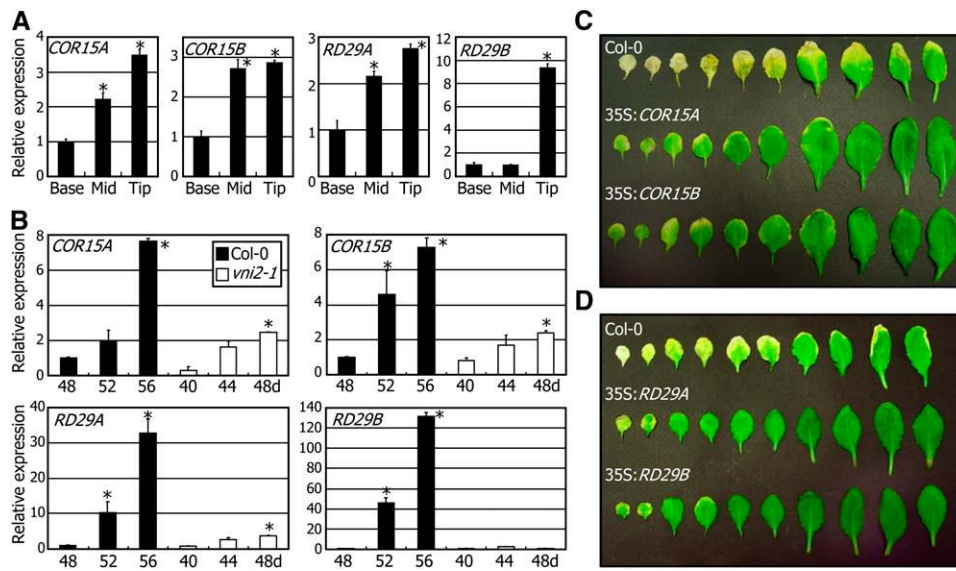
Intrinsic developmental programs are intimately associated with environmental signals in virtually all aspects of plant growth and development and responses to biotic and abiotic stresses. One intriguing example is the close relationship between stress responses and aging and senescence. Indeed, enhanced stress tolerance accompanies increased life span in all living organisms tested (Martin et al., 1996; Finkel and Holbrook, 2000; Johnson et al., 2001). In *Caenorhabditis elegans*, most life extension mutants exhibit enhanced resistance to environmental stresses (Johnson et al., 1996). It has been shown that overexpression of the putative *tyrosine kinase receptor-1* gene increases longevity and improves resistance to heat and UV irradiation in *C. elegans* (Murakami and Johnson, 1998). Association of life span with stress resistance has also been demonstrated in *Drosophila melanogaster*, *Saccharomyces cerevisiae*, and mice, indicating that the underlying mechanisms are widely conserved in living organisms (Longo, 1999; Finkel and Holbrook, 2000; Fabrizio et al., 2001).

In plants, leaf longevity is also intimately linked with stress resistance. Abiotic stresses and leaf aging commonly induce programmed cell death by altering plasma membrane integrity (Rivero et al., 2007; Seo et al., 2011). Therefore, it has been perceived that some plasma membrane proteins integrate incoming developmental and environmental signals into a unified cellular regulatory mechanism that determines leaf longevity under stressful conditions. It has been recently shown that the plasma membrane-localized, senescence-associated protein SAG29 regulates cell viability under high salinity (Seo et al., 2011). SAG29 negatively regulates membrane integrity during leaf aging and stress responses, further supporting the linkages of abiotic stress responses with leaf aging. A similar role has been proposed for the *isopentenyltransferase (IPT)* gene involved in cytokinin biosynthesis, which is closely related with leaf longevity (Smart et al., 1991). Transgenic plants expressing *IPT* under the control of a drought stress-inducible promoter leads to drought tolerance, indicating that suppression of stress-induced senescence confers tolerance to drought stress (Rivero et al., 2007; Zhang et al., 2010). Leaf aging is also regulated by C-repeat responsive element binding factor (CBF) transcriptional activators, which are involved in freezing tolerance (Gilmour et al., 1998, 2004). Transgenic plants overexpressing either *CBF2* or *CBF3* exhibit prolonged leaf longevity (Sharabi-Schwager et al., 2010).

The *VNI2* gene is regulated developmentally by leaf aging processes and by high salinity. Seedling growth of the 35S:*VNI2* transgenic plants, which show prolonged leaf longevity, is less susceptible to high salinity. By contrast, leaf aging is accelerated in the *vni2-1* mutant, and growth of mutant seedlings is severely affected by high salinity. We therefore conclude that plant resistance to salt stress are linked with leaf aging via the *VNI2* transcription factor in modulating leaf longevity under salt stress conditions.

One issue to be considered carefully is the possible indirect effects of growth retardation on delayed leaf senescence in the 35S:*VNI2* transgenic plants. We observed that the *vni2-1* mutant,





**Figure 7.** Roles of *COR* and *RD* Genes in Modulation of Leaf Longevity.

In (A) and (B), transcript levels were determined by qRT-PCR and normalized to *eIF4A*. Biological triplicates were averaged. Statistical significance was determined by a Student's *t* test (\* $P < 0.01$ ). Bars indicate SE.

(A) Localized expression of *COR* and *RD* genes in a senescing leaf. The leaves were dissected into three parts as described in Figure 2D.

(B) Expression of *COR* and *RD* genes in *vni2-1* mutants during leaf aging. Col-0 leaves at 48, 52, and 56 d are developmentally equivalent to *vni2-1* leaves at 40, 44, and 48 d, respectively, as displayed in Figure 3D. d, days after cold imbibition.

(C) and (D) Prolonged leaf longevity in 35S:*COR15A/B* (C) and 35S:*RD29A/B* (D) transgenic plants. Rosette leaves of 9-week-old plants grown in soil were photographed.

in which leaf senescence is significantly accelerated, was morphologically and developmentally identical to Col-0 plants. Seedling growth was delayed to some degree in the 35S:*VNI2* transgenic plants. However, the overall morphology and size of the full-grown 35S:*VNI2* transgenic plants was similar to Col-0 plants. In addition, flowering initiation of the 35S:*VNI2* transgenic plants was induced at the same time as Col-0 and *vni2-1* mutant plants (see Supplemental Figure 5 online). Furthermore, analysis of leaf senescence in rosette leaves at the same developmental stage showed that whereas leaf longevity was prolonged in the 35S:*VNI2* transgenic plants, it was reduced in the *vni2-1* mutant. We therefore conclude that differential leaf longevity is not related with growth retardation in the 35S:*VNI2* transgenic plants, further supporting the role of *VNI2* in leaf longevity.

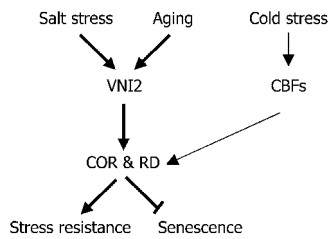
### Regulation of Leaf Longevity by *CORs* and *RDs*

The *COR* and *RD* genes are regulated by both ABA-dependent and -independent stress signals. Promoters of these genes contain conserved sequence motifs, such as CRT/DRE (TACC-GACAT) and ABA-responsive element (PyACGTGGC) (Yamaguchi-Shinozaki and Shinozaki, 2006). The *cis*-acting elements serve as nodes in molecular webs that integrate incoming signals. As inferred from the presence of a series of distinct *cis*-acting elements in the gene promoters, the *COR* and *RD* genes are influenced by various developmental and environmental cues, such as ABA, dehydration, and cold, supporting the idea that the *cis*-acting elements are one of major sites for signaling crosstalk

(Zhu, 2002; Yamaguchi-Shinozaki and Shinozaki, 2006). Despite their prominent roles in plant responses to environmental stresses (Gilmour et al., 1998, 2004), its biological functions in plant development and phase transition are merely reported.

*COR15A* or its processed form Cor15am is a small hydrophilic protein that is located in chloroplast stroma (Nakayama et al., 2007). Overexpression of *COR15A* leads to enhanced freezing tolerance of chloroplasts and leaf protoplasts, suggesting a role for *COR15A* in protecting plants from environmental damage (Nakayama et al., 2007). Indeed, the Cor15am peptide protects chloroplast enzymes, such as L-lactate dehydrogenase, from freeze-induced inactivation *in vitro* (Nakayama et al., 2008). Association of Cor15am with its substrates in chloroplasts may be an important part of protective mechanisms at freezing temperatures. In addition, the *COR15A*-mediated protection of enzyme activities also occurs during dehydration and heat stress responses, supporting a broad spectrum of *COR15* functions (Nakayama et al., 2008).

A small group of hydrophilic polypeptides, such as late embryogenesis abundant proteins, are also associated with environmental stress responses. It has been suggested that late embryogenesis abundant proteins protect enzymes and biological membranes from environmental damage (Danyluk et al., 1998; Goyal et al., 2005; Reyes et al., 2005; Nakayama et al., 2008). The *RD29A* and *RD29B* genes encode desiccation-induced hydrophilic proteins. Although their biological roles and biochemical activities are currently unknown, they may play a role in protecting cells and proteins from stress damages.



**Figure 8.** Model of VNI2 in Modulation of Leaf Longevity under Abiotic Stress Conditions.

VNI2 expression is regulated by both high salinity and aging processes. The VNI2-mediated COR/RD regulation contributes to stress resistance responses and prolonged leaf longevity. The CBF pathway also plays a role in this signaling but acts independently of VNI2-mediated signaling (Sharabi-Schwager et al., 2010).

Our data demonstrate that the *COR* and *RD* genes also play a role in leaf aging. It has been reported that leaf aging and osmotic stresses induce damage in cellular organelles, such as chloroplasts, and cellular proteins. In this regard, involvement of the *COR* and *RD* proteins in the VNI2-mediated integration of developmental and stress signals is significant during leaf aging under salt stress conditions.

### VNI2 Is a Bifunctional Transcription Factor

A number of transcription factors act either as transcriptional repressors or activators, depending on cellular environments (Ikeda et al., 2009; Hao et al., 2010), indicating that one transcription factor can play multiple roles. The *Arabidopsis* WUSCHEL transcription factor possesses both transcriptional activation and repression domains. As a result, it acts either as a repressor of the response regulator genes during stem cell proliferation or as an activator of *AGAMOUS* in floral patterning (Lohmann et al., 2001; Leibfried et al., 2005; Ikeda et al., 2009). It is envisioned that transcriptional regulation activities are also influenced by environmental signals.

Notably, several NAC transcription factors also contain both transcriptional activation and repression domains (Hao et al., 2010). Most of the NAC transcription factors characterized so far are transcriptional activators. However, a few NAC transcription factors contain NAC repression domains consisting of ~35 residues within the NAC domains. Consequently, combinatorial effects of both transcriptional regulation activities determine downstream events (Hao et al., 2010).

VNI2 has been recently reported as a transcriptional repressor functioning in xylem vessel formation (Yamaguchi et al., 2010). The VNI2 protein also possesses a transcriptional repressor activity in our assay system. Interestingly, we found that VNI2 has a transcriptional activation domain consisting of 30 residues in the far C-terminal region. Furthermore, VNI2 exhibited a transcriptional activator activity when 50 mM NaCl was included in the assay system. More careful examination revealed that in the presence of 50 mM NaCl, the transcriptional repressor activity was largely unaffected, but the transcriptional activation activity was greatly elevated. It is possible that high salinity may induce

changes in the structure and/or activities of the VNI2 protein. Based on the previous report (Yamaguchi et al., 2010) and our own data, we propose that the VNI2 protein is a bifunctional transcription factor, which acts as a transcription repressor in xylem vessel development and as a transcriptional activator in stress-mediated regulation of leaf longevity.

VNI2 transcriptional regulation would also be influenced by *cis*-acting elements to which it binds. Although VNI2 acts as a transcriptional repressor under normal growth conditions (Yamaguchi et al., 2010; this work), expression of *COR* and *RD* genes is induced in the 35S:VNI2 transgenic plants. In addition, transient GUS expression assays in *Arabidopsis* protoplasts, using the VNI2 binding *cis*-acting elements of the *COR* and *RD* genes, also showed that VNI2 does not repress expression of the GUS reporter under normal conditions. It seems that the transcriptional regulation activity of VNI2 is modulated by both intrinsic and environmental factors through diverse molecular interactions, elaborating regulation specificity and accuracy.

The relationship between salt stress adaptation and xylem development has been documented extensively in poplar. Environmental stresses influence tracheary element density, cell wall structures, and lumen area (Kozłowski et al., 1997). Especially, differentiation of xylogenic cambium is a flexible developmental process that is also influenced by environmental factors (Junghans et al., 2006). Indeed, xylem differentiation zone and lumina are reduced under high salt conditions (Escalante-Pérez et al., 2009). It is therefore likely that VNI2 transcriptional repressor activity during xylem vessel development would be compromised by the transcription activation domain that is active under high salt conditions, contributing to plant fitness for enhanced stress tolerance and leaf longevity.

## METHODS

### Plant Materials and Growth Conditions

All *Arabidopsis thaliana* lines used were in the Col-0 background. Plants were grown in a controlled culture room at 22°C with a relative humidity of 55% under long-day (LD) conditions (16-h light/8-h dark) with white light illumination (120  $\mu\text{mol photons/m}^2\text{s}$ ) provided by fluorescent FLR40D/A tubes (Osram). The *vni2-1* mutant (SALK-143793) was isolated from a T-DNA insertional mutant pool deposited into the ABRC. Homozygous lines were obtained by herbicide selection for three or more generations and analysis of segregation ratios. Absence of gene expression in the mutant was verified by RT-PCR before use.

To produce transgenic plants overexpressing the VNI2, *COR15A*, *COR15B*, *RD29A*, and *RD29B* genes, full-length cDNAs were subcloned into the binary pB2GW7 vector under the control of the CaMV 35S promoter (Invitrogen) (see Supplemental Table 1 online). *Agrobacterium tumefaciens*-mediated *Arabidopsis* transformation was performed according to a modified floral dip method (Clough and Bent, 1998). T3 transgenic plants having single T-DNA insertional events were used in whole assays.

For the VNI2 complementation test, a genomic clone was amplified using two primers, forward (5'-AAAAGCAGGCTCACACTGTCATCTT-CACGGACC-3') and reverse (5'-AGAAAGCTGGGTTTTGACAGCCTCT-CCGTGCT-3'). The genomic clone containing the VNI2 gene with its own promoter (~3 kb) was subcloned into the promoterless pKGWFS7 Gateway vector (Invitrogen).

### Expression Analysis

qRT-PCR was employed for measuring transcript levels. Preparation of total RNA samples, reverse transcription, and qPCR were performed based on the rules that have been proposed recently to ensure reproducible and accurate measurements (Udvardi et al., 2008). Extraction of total RNA samples from appropriate plant materials and RT-PCR conditions have been described previously (Kim et al., 2006). Total RNA samples were pretreated extensively with an RNase-free DNase to eliminate any contaminating genomic DNA before use.

qRT-PCR was performed in 96-well blocks with an Applied Biosystems 7500 Real-Time PCR system using the SYBR Green I master mix in a volume of 25  $\mu$ L. The PCR primers were designed using the Primer Express Software (Applied Biosystems) and are listed in Supplemental Table 1 online. The two-step thermal cycling profile used was 15 s at 94°C and 1 min at 68°C. An *eIF4A* gene (At3g13920) was included in the assays as an internal control for normalizing the variations in cDNA amounts used (Gutierrez et al., 2008). The qRT-PCR reactions were performed in biological triplicates using total RNA samples extracted from three independent plant materials grown under identical growth conditions. The comparative  $\Delta\Delta$  threshold cycle method was used to evaluate the relative quantities of each amplified product in the samples. The threshold cycle was automatically determined for each reaction by the system set with default parameters. The specificity of the PCR was determined by melt curve analysis of the amplified products using the standard method installed in the System.

### Histological Assays

The primers used for subcloning of the *VNI2* gene promoter were  $P_{VNI2}$ : GUS-F (5'-AAAAGCAGGCTTAGTTCGTTGAATATATTGGAGG-3') and  $P_{VNI2}$ : GUS-R (5'-AGAAAGCTGGGTGGTGGTTCCAAACAAAGAGAG-3'). The PCR product was subcloned into the pHGWFS7 vector (Invitrogen), and the expression construct was transformed into *Arabidopsis* plants.

For histochemical analysis of GUS activities, plant materials were incubated in 90% acetone for 20 min on ice, washed twice with rinsing solution [50 mM sodium phosphate, pH 7.2, 0.5 mM  $K_3Fe(CN)_6$ , and 0.5 mM  $K_4Fe(CN)_6$ ], and subsequently incubated at 37°C for 18 to 24 h in rinsing solution containing 2 mM 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronide (Duchefa). They were then incubated in a series of ethanol solutions ranging from 15 to 80% in order to remove chlorophylls from plant tissues. The plant samples were mounted on microscope slides and visualized using a Nikon SMZ 800 microscope.

### Treatments with Growth Hormones and Abiotic Stresses

Two-week-old plants grown on half-strength Murashige and Skoog (MS)-agar plates (hereafter referred to as MS-agar plates) were transferred to MS liquid cultures supplemented with various growth hormones. ABA, IAA, GA, 1-aminocyclopropane-1-carboxylic acid, and methyl jasmonic acid were used at a final concentration of 20  $\mu$ M. Salicylic acid was used at a final concentration of 100  $\mu$ M.

For the assays on the effects of drought on gene expression, 2-week-old plants grown on MS-agar plates were put on a dry 3MM paper at room temperature for the indicated time periods. To examine the effects of high salinity on gene expression, 2-week-old plants grown on MS-agar plates were soaked in MS liquid cultures containing 200 mM NaCl and incubated under constant light for the indicated time periods. For cold treatments, 2-week-old plants grown on MS-agar plates were transferred to a cold chamber set at 4°C and incubated for the indicated time periods before harvesting plant materials. Whole plants were used for RNA extraction, unless otherwise specified.

### Measurements of Chlorophyll Contents

Measurements of chlorophyll contents were performed as described previously (Oh et al., 1997). Chlorophylls were extracted with *N,N*-dimethylformamide, and the extracted solution was incubated at 4°C for 2 h in complete darkness. Chlorophyll contents were assayed by measuring absorbance at 652, 665, and 750 nm using a diode array spectrophotometer (WPA Biowave).

### Dark-Induced Senescence

Plants were grown on MS-agar plates under LDs for 12 d, transferred to complete darkness, and incubated for appropriate time periods up to 6 d. Representative plants were photographed.

### Protoplast Transfection Assays

For transient expression assays in *Arabidopsis* protoplasts, several reporter and effector plasmids were constructed. The reporter plasmid contains four copies of the GAL4 upstream activation sequence and the GUS gene. To construct the p35S-VNI2 effector plasmid, the *VNI2* cDNA was fused in frame to the GAL4 DNA binding domain-coding sequence and inserted into an expression vector containing the CaMV 35S promoter. The reporter and effector plasmids were cotransformed into *Arabidopsis* protoplasts by polyethylene glycol-mediated transformation (Yoo et al., 2007). The GUS activities were measured by a fluorometric method as described previously (Jefferson et al., 1987). A CaMV 35S promoter-luciferase construct was also cotransformed as an internal control. The luciferase assay was performed using the Luciferase Assay System kit (Promega).

### Subcellular Localization Assays

For detection by fluorescence microscopy, a GFP-coding sequence was fused in frame to the 3' end of the *VNI2* gene sequence (see Supplemental Table 1 online), and the gene fusion was subcloned into the p2FGW7 vector (Invitrogen). The expression construct was transformed into *Arabidopsis* protoplasts by a polyethylene glycol-mediated transformation method (Yoo et al., 2007). The subcellular distribution of the VNI2 protein was visualized by differential interference contrast microscopy and fluorescence microscopy.

### ChIP Assays

A MYC-coding sequence was fused in frame to the 3' end of the *VNI2* gene, and the gene fusion was subcloned under the CaMV 35S promoter in the modified pBA002 vector (Kim et al., 2006). The expression construct was transformed into Col-0 plants. Two-week-old 35S:*VNI2*-MYC transgenic plants grown on MS-agar plates were used for preparation of total protein extracts. Processing of plant materials and qRT-PCR were performed as described previously (Lawrence et al., 2004). The qRT-PCR primers used are listed in Supplemental Table 2 online.

### Electrophoretic Mobility Shift Assays

The *VNI2* cDNA was subcloned into the pMAL-c2X *E. coli* expression vector (NEB) having an MBP-coding sequence. The MBP-VNI2 fusion protein was purified according to the manufacturer's procedure using the pMAL protein fusion and purification system (#E8000S). The DNA fragments were end labeled with [ $\gamma$ - $^{32}P$ ]dATP using T4 polynucleotide kinase. Labeled probes were incubated with  $\sim$ 0.5  $\mu$ g of the purified MBP-VNI2 protein for 30 min at 25°C in a binding buffer (10 mM Tris-HCl, pH 7.6, 50 mM NaCl, 1 mM EDTA, 5 mM DTT, and 5% glycerol) with or without competitor DNA fragments. The reaction mixtures were analyzed on 6%

native PAGE gels. The gels were dried on Whatman 3MM paper and exposed to x-ray film.

### Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative database under the following accession numbers: *VNI2* (At5g13180), *COR15A* (At2g42540), *COR15B* (At2g42530), *RD29A* (At5g52310), and *RD29B* (At5g52300).

### Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Expression of *VNI2* Gene in Young and Senescing Leaves.

**Supplemental Figure 2.** Leaf Aging Process in 35S:*VNI2* Transgenic Plants.

**Supplemental Figure 3.** Comparison of Leaf Aging in Col-0 and Vector Control Plants.

**Supplemental Figure 4.** Comparison of Leaf Longevities in *vni2-1* and 35S:*VNI2* Leaves.

**Supplemental Figure 5.** Flowering Phenotypes of *vni2-1* and 35S:*VNI2* Plants.

**Supplemental Figure 6.** Dark-Induced Senescence in *vni2-1* Mutant.

**Supplemental Figure 7.** Dark-Induced Senescence in *vni2-1*:*VNI2* Plants.

**Supplemental Figure 8.** Effects of Growth Hormones on *VNI2* Gene Expression.

**Supplemental Figure 9.** Salt Effects on Transcriptional Activation Activities of *VNI2* Proteins.

**Supplemental Figure 10.** Expression of Senescence-Related Genes in *vni2-1* and 35S:*VNI2* Plants.

**Supplemental Figure 11.** Effects of ABA on *COR* and *RD* Gene Expression.

**Supplemental Figure 12.** Binding of *VNI2* to Consensus Sequences in the Promoters of *COR* and *RD* Genes in Vitro.

**Supplemental Figure 13.** Chromatin Immunoprecipitation Assays Using Plants with or without Salt Treatments.

**Supplemental Figure 14.** Salt Effects on Protein Stability of *VNI2*.

**Supplemental Figure 15.** Transcript Levels of Transgenes in Transgenic Plants.

**Supplemental Figure 16.** Senescing Phenotypes of Transgenic Plants Overexpressing *COR* or *RD* Genes.

**Supplemental Figure 17.** Expression of Stress-Responsive and Senescence-Associated Genes in the *vni2-1* Mutant.

**Supplemental Figure 18.** Salt Effects on Seedling Growth of *vni2-1* and 35S:*VNI2* Plants.

**Supplemental Figure 19.** Acceleration of Salt-Induced Leaf Senescence in *vni2-1* Mutant.

**Supplemental Figure 20.** Expression of *SAG12* Gene in Salt-Treated *vni2-1* Leaves.

**Supplemental Figure 21.** Salt-Induced Leaf Senescence in 35S:*VNI2* Transgenic Plant.

**Supplemental Table 1.** Primers Used in This Study.

**Supplemental Table 2.** Primers Used in Chromatin Immunoprecipitation Assays.

### ACKNOWLEDGMENTS

This work was supported by the Leaping Research Program (20100014373) from the National Research Foundation of Korea and by grants from the Plant Signaling Network Research Center (20100001457), the National Research Foundation of Korea (20100028147), and the Agricultural R&D Promotion Center (309017-5), Korea Ministry for Food, Agriculture, Forestry, and Fisheries.

### AUTHOR CONTRIBUTIONS

C.-M.P. conceived and designed the research. C.-M.P. wrote the article with the help of P.J.S. S.-D.Y., P.J.S., and H.-K.Y. conducted the experiments and contributed to the study design.

Received March 4, 2011; revised April 27, 2011; accepted June 5, 2011; published June 14, 2011.

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**The *Arabidopsis* NAC Transcription Factor VNI2 Integrates Abscisic Acid Signals into Leaf Senescence via the *COR/RD* Genes**

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*Plant Cell* 2011;23;2155-2168; originally published online June 14, 2011;  
DOI 10.1105/tpc.111.084913

This information is current as of January 15, 2021

<b>Supplemental Data</b>	<a href="/content/suppl/2011/06/06/tpc.111.084913.DC1.html">/content/suppl/2011/06/06/tpc.111.084913.DC1.html</a>
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