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

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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 (U¨ lker 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...
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 over-expressing 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/OR1 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/OR1 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. 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 (G4DB) cDNA sequence in the effector vector (Figure 1C). The effector vector, together with a construct containing the β-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 ΔC 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 (ΔN). Expression of the ΔN 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 ΔN construct, the ΔN sequence was fused to the 3’-end of the calmodulin binding NAC (CBNAC) transcriptional repressor gene (Kim et al., 2007), and the CBNAC+ΔN sequence was fused in frame to the 3’-end of the GAL4-DB cDNA 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+ΔN fusion elevated GUS activity more than twofold (Figure 1E), confirming that the ΔN region possesses transcriptional activation activity.
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 axes 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 elf4A 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 cauleine leaves (G) and siliques (H), as indicated by arrows.

Figure 2C online) and expression patterns of the cystein 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 un-influenced by indole-3-acetic acid (IAA), gibberellic acid (GA), 1-aminoacyclopropane-1-carboxlyic 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).
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 was 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 ΔN 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
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 promoters 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 uninfuenced 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
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 over expressing 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).

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 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.
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 *isopentenylation transferase* (*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 VNII2 gene is regulated developmentally by leaf aging processes and by high salinity. Seedling growth of the 35S:VNII2 transgenic plants, which show prolonged leaf longevity, is less susceptible to high salinity. By contrast, leaf aging is accelerated in the vnii2-1 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 VNII2 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:VNII2 transgenic plants. We observed that the vnii2-1 mutant,
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:\textit{VNI2} transgenic plants. However, the overall morphology and size of the full-grown 35S:\textit{VNI2} transgenic plants was similar to Col-0 plants. In addition, flowering initiation of the 35S:\textit{VNI2} transgenic plants was induced at the same time as Col-0 and \textit{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:\textit{VNI2} transgenic plants, it was reduced in the \textit{vni2-1} mutant. We therefore conclude that differential leaf longevity is not related with growth retardation in the 35S:\textit{VNI2} transgenic plants, further supporting the role of \textit{VNI2} in leaf longevity.

Regulation of Leaf Longevity by CORs and RDs

The \textit{COR} and \textit{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 \textit{cis}-acting elements serve as nodes in molecular webs that integrate incoming signals. As inferred from the presence of a series of distinct \textit{cis}-acting elements in the gene promoters, the \textit{COR} and \textit{RD} genes are influenced by various developmental and environmental cues, such as ABA, dehydration, and cold, supporting the idea that the \textit{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.

\textit{COR15A} or its processed form Cor15am is a small hydrophilic protein that is located in chloroplast stroma (Nakayama et al., 2007). Overexpression of \textit{COR15A} leads to enhanced freezing tolerance of chloroplasts and leaf protoplasts, suggesting a role for \textit{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 \textit{COR15A}-mediated protection of enzyme activities also occurs during dehydration and heat stress responses, supporting a broad spectrum of \textit{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 \textit{RD29A} and \textit{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.
The VNI2 protein also possesses a transcriptional repressor domain, functioning in xylem vessel formation (Yamaguchi et al., 2010). 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 transcriptional 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 (Kozlowski et al., 1997). Especially, differentiation of xylemogenic 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.

**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 \( \sim 35 \) 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 transcriptional repressor in xylem vessel development and as a transcriptional activator in stress-mediated regulation of 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 μmol photons/m²s) 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 insertion events were used in whole assays.

For the VNI2 complementation test, a genomic clone was amplified using two primers, forward (5'-AAAAACGAGCTCACATTGTCTTTCAGGACC-3') and reverse (5'-AGAAGCTGGTTTGGACGCTCCTCCGTC-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-qPCR conditions have been described previously (Kim et al., 2008). 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 μL. 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 elf4A 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 ΔΔ 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 PVNI2-GUS-F (5′-AAAAAGCAGCTAGTCTGTAATATTGAGG-3′) and PVNI2-GUS-R (5′-AGAAAAGCTGGTGAGTCTCAGAAAGAGAG-3′). The PCR product was subcloned into the pHGWS7 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 K3Fe(CN)6, and 0.5 mM K4Fe(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-β-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 μM. Salicylic acid was used at a final concentration of 100 μM.

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 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 a MBP-coding sequence. The MBP-VNI2 fusion protein was purified according to the manufacturer’s procedure using the pMAL protein fusion and purification system (NEB000S). The DNA fragments were end labeled with [γ-32P]dATP using T4 polynucleotide kinase. Labeled probes were incubated with ~0.5 μg 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 (At5g52309).

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.

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 3SS: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 3SS:VNI2 Leaves.

Supplemental Figure 5. Flowering Phenotypes of vni2-1 and 3SS: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 3SS: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 3SS: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 3SS:VNI2 Transgenic Plant.

Supplemental Table 1. Primers Used in This Study.

Supplemental Table 2. Primers Used in Chromatin Immunoprecipitation Assays.

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