環境変動に応答するシロイヌナズナNACドメイン転写因子VNI2の生物学的役割

Biological roles of an NAC domain transcription factor, VNI2, in response to environmental statuses in Arabidopsis

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by

Isura Sumeda Priyadarshana Nagahage

Approved by

Associate Professor Masatoshi Yamaguchi, Academic supervisor and Committee chair

Professor Maki Kawai-Yamada, Committee member

Professor Toshihisa Kotake, Committee member

Associate Professor Takeshi Fujino, Committee member

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Table of Contents

LIST OF FIGURES

Summary

Plants are sensitive to environmental changes that may lead to making changes in developmentally and physiologically to acclimate it. The mechanism of perceiving environmental changes by plants is well studied so far and that knowledge has been utilized to breed stress tolerant plants, high-quality yield plants, and biofuel production to compensate human needs. There is a tight relationship between environmental changes and plant senescence. Senescence is a highly organized process and essential set of functions to nutrient remobilization between senescence materials to newly generating tissues of the plant. Besides to natural senescence, pre-mature senescence is also induced in response to environmental stress or changes of light-dark diurnal rhythm. Thus, to elucidate the molecular mechanisms of how induction of senescence is taken place, a number of studies have been conducted so far. Indeed, a number of genes including transcriptional factors are isolated as key regulators of promotion or repression of senescence. However, how plants induce senescence in response to environmental statuses is currently still elusive.

NAC domain transcriptional factor family is a plant-specific transcriptional factor family. It has been known to be involved in various developmental and physiological functions such as seed development, environmental stress responses, flowering time management, and embryo development. In addition, Some NAC domain transcription factors such as VNI2, ORE1, ORS1, and ANAC046 are reported to be associated with regulation of senescence.

VNI2 is originally isolated as an interacting factor with another NAC domain transcriptional factor VND7, a master regulator of xylem vessel differentiation. VNI2 inhibits the transcriptional activities of VND7. Additionally, VNI2 is expressed in various types of cells suggesting that VNI2 plays pivotal roles in various biological events as well as xylem vessel formation. Here, to understand the biological roles of VNI2 further, we screened interacting factors, characterize the interacting factors and then try to understand the role of interaction in stress responses.

I focused on two NAC domain transcriptional factors, ATAF2 and ANAC102, isolated as interacting proteins with VNI2 through Yeast two-hybrid screening. To verify their transcriptional activities, I carried out a transactivation assay using protoplast cells. ATAF2

showed transcriptional activation activity, while ANAC102 neither show transcriptional activity nor repression activity. Thus, further studies were carried out by focusing on ATAF2 and VNI2.

To verify the transcriptional activities of ATAF2, the transient assay was performed. Obtained data suggested that ATAF2 could be a bi-functional transcriptional factor. Moreover, I found that the ATAF2 upregulates expression of *ORE1*, a key regulator of senescence. Although ATAF2 is known to be associated with biotic stress responses, it is unclear that ATAF2 is also involved in the promotion of senescence. Thus, I investigated the relationship between ATAF2 and senescence. The transient assay showed that ATAF2 has transcriptional activity on some senescence regulatory transcriptional factors including ORS1 and ANAC046 as well as ORE1. In addition, loss of function mutants (*ataf2*) and overexpression lines (*ATAF2-OX*) showed delaying and accelerating senescence phenotypes, respectively under a dark condition. Consistent the phenotypes of the loss of and the gain of function lines, the expression level of senescence marker genes were elevated in *ATAF2-OX* while low-level expression was observed in *ataf2* under the long day condition, suggesting that ATAF2 promotes senescence in response to the light/dark condition via regulation of these genes.

To unveil the biological roles of VNI2 in response to environmental statuses, I investigated VNI2-ATAF2 protein complex. The transient assay showed that the transcriptional activity of ATAF2 on ORE1 was significantly inhibited by VNI2. Genetic interaction results based on *vni2*, *ataf2*, *vni2ataf2* mutant lines under the long-day condition showed that senescence promotion phenotype of *vni2* can be cancelled under the *ataf2* background suggesting that VNI2 has ATAF2 dependent function in senescence. However, such a phenotype difference was not observed under the continuous light condition. Thus, it is possible that VNI2-ATAF2 interaction leads to senescence in the light-dependent pathway. In addition, ORE1 and ORS1 have been highly induced in 55 days old *vni2* plant lines together with wild-type plants but a significantly low expression of them in *ataf2* plant line. The *vni2ataf2* line showed intermediate expression level that is comparable with chlorophyll content and its phenotype. By contrast, senescence marker genes expression was significantly high in vni2 mutant but low in wild-type, suggesting that there might be an interaction of VNI2 with ORE1 and ORS1, and VNI2 effectively inhibits them to results in lower expression of their downstream target genes.

ORE1 was identified as VNI2 interacting protein in large-scale screening and the molecular mechanism between ORE1 and VNI2 was not characterized so far. Here, I showed that senescence marker genes *SAG12* and *SAG29* can be transcriptionally activated by ORE1 under the transient assay system. Co-transfection of *ORE1* together with *VNI2* showed that ORE1 transcriptional activity on *SAG12* and *SAG29* could be inhibited by VNI2 suggesting that VNI2 regulates ORE1 too.

Besides, I found that the expression level of *VNI2* is strongly activated by ATAF2 and ORE1, suggesting that the existence of a feedback mechanism to fine tune the light-dependent senescence pathway involving ATAF2, ORE1 and VNI2. VNI2 is known to be a very fragile protein and its function as a transcriptional inhibitor at a different level of light-dependent senescence pathway explains that short-term inhibition at partially and temporally makes a significant contribution on natural senescence as well as dark-induced senescence in the process of fine-tuning.

Chapter 1

General introduction

"Stress" in plants can be explained as an external factor that may cause significant changes in the system such as adversely affecting on plant growth, productivity, reproductive capacity or survival depending on intensity and duration (Godbold, 1998; Rhodes and Nodolska-Orczyk, 2001; Hale and Orcutt, 1987). Basically, there are two types of stresses defined as abiotic stress and biotic stress. Abiotic stressors may be of a physical or chemical character such as high temperature, salinity, and drought. (Mandre, 2002; Hale and Orcutt, 1987). Biotic stresses include a wide range of plant pathogens (bacteria, fungai, and viruses) and herbivorous animals that adversely affect on plant growth (Hale and Orcutt, 1987; Atkinson and Urwin, 2012). To overcome these stresses, plants have individual genetic tolerance mechanisms. As a result, energy consumption, growth, development, and productivity can be protected by the regulatory mechanisms.

Plants adopt several strategies to overcome environmental stresses. In drought stress, they make changes from root tips to leaves and flowers to accomplish their single most pressing goal – survival (Sah et al. 2016). In order to conserve water, their leaves close stomata to stop water loss, slow their own growth, and keep seeds in dormant states. Soil salinity makes two kinds of stresses such as osmotic stress and sodium ion toxic stress (Gilroy et al. 2014; Roy et al. 2014). To combat the osmotic stress, stomatal closure and the inhibition of leaf expansion are taken place (Munns and Termaat, 1986) in the initial stage. However, stress prevails over a long period of time, build-up of ions in the shoot to toxic concentrations, particularly in old leaves, causing premature senescence of leaves and ultimately reduced yield or even plant death (Munns and Tester, 2008; Sade et al. 2018a,b). Annual and perennial plant species generally adopt different strategies to ameliorate environmental stresses. Through stress-induced senescence, annual plants escape stress by accelerating their transition to a reproductive stage, and enhance biomass allocation and nutrients for seed production (Albacete et al. 2014; Schippers et al. 2015). Perennial plants avoid stresses by developing larger and deeper root systems, and having different source–sink transitions, allocating biomass and nutrients to vegetative growth (i.e. roots and shoots) as a main strategy (Zwicke et al. 2015). In addition, an important sink organ involved in stress tolerance is the meristematic tissues (meristematic leaves and crowns) that have the capacity to remain alive, ensuring growth once the stress episode has ended (Munné-Bosch, 2008). In annual plants, abiotic stress such as water deficit will induce leaf senescence at

the whole-plant level, starting in older leaves first and progressing later to younger leaves, leading to remobilization processes to seeds and, eventually, to plant death. In contrast, temperate perennial grasses gradually decrease leaf elongation, and induced-senescence processes only occur in the oldest leaves (Volaire and Norton, 2006).

Senescence occurs autonomously in an age-dependent manner and induces by environmental stress. It is known that plant senescence is governed in a well-organized set of cascades involving a number of genes. It is also important to regulate senescence in local tissues or organs during plant life cycle, resulting in nutrient remobilization to newly generating components. Regulation of senescence is mediated through various factors, such as phytohormones, light-dark condition, circadian clock, and microRNAs (miRNAs) (Graff et al. 2006; Zhu et al. 2009; Thatcher et al. 2015; Liebsch and Keech, 2016; Song et al. 2018). It has been known that a number of phytohormones positively or negatively promote senecence. Salicylic acid is a hormone in resopnse to pathogen infection, and also promotes senescence (Morris et al. 2000; Zhao et al. 2016). Ethylene biosynthesis is increased in the later phase of the life cycle, resulting in promotion of senescence. In addition, ethylen gas is easily spreaded out, and affected to other plants (Grbic and Bleecker, 1995, Kim et al. 2015). Abscisic acid (ABA) is a strongly positive senescence regulator in response to environmental stresses. Exogenous application of ABA on healthy tissues induces senescence (Lee et al. 2011; Gao et al. 2016). On the other hand, cytokinins is known to suppress expression of senescence-associated genes (SAG) (Wingler et al. 1998; Zwack and Rashotte, 2013). Brassinosteroids (BRs), which play essential roles in diverse developmental programs, also regulate senescence (Clouse and Sasse, 1998). External application of epibrassinolide induces leaf senescence in mung bean plants (He et al. 1996). In addition, several *Arabidopsis* mutants deficient in brassinosteroid biosynthesis or in the brassinosteroid signal transduction pathway show the delayed leaf senescence phenotype (Clouse and Sasse, 1998).

Numerous studies demonstrated that phytochromes (PHYs), red/far-red light (R/FR) receptors, is involved in senescence in response to light conditions (Biswal and Biswal, 1984; Lers, 2007; Brouwer et al. 2012, 2014; Sakuraba et al. 2014). It was shown that pulses of R can delay senescence, unless they are immediately followed by FR pulses, a typical hallmark of

phytochrome function in the low fluence response (LFR) mode. This behavior is explained by the special characteristics of phytochromes, which are converted to their active Pfr form by absorbing R, but effectively reverted to the inactive Pr form by absorbing FR (Franklin, 2008). Consequently, low R/FR ratio conditions (e.g. as experienced in shade under dense canopies) induce senescence, and R supplementation can delay it (Guiamet et al. 1989; Rousseaux et al. 1996; 2000).

Accumulating evidences display that circadian clock regulate expression of genes involved in various biological events, such as biotic stress response, photosynthesis, and flowering time (Nusinou et al. 2011; Sawa and Kay, 2011; Nakamichi et al. 2012; Lie et al. 2013; 2016; Ezer et al. 2017). For examle, CCA1 is associated with leaf senescence by directly regulating *ORE1* and *GLK2* expression (Song et al. 2018).

Micro RNAs (miRNAs) regulate target gene expressions at the post-transcriptional manner by cleaving the target transcripts (Jones-Rhoades et al. 2006; Brodersen et al. 2008). At the younger stage of plants, miR164 is highly accumulated, and protects progression of senescence by donwnregulatoin of *ORE1* expression (Kim et al. 2009). As the plants grow older, expression of *miR164* is downregulated by ethylene (Kim et al. 2009). Another senescence-associated miRNA, miR319, suppress senescence regulatory transcriptional factors such as TEOSINTE *BRANCHED/CYCLOIDEA/PCF2* (*TCP2*), *TCP3*, *TCP4*, *TCP10*, and *TCP24* (Koyama et al. 2007; Schommer et al. 2008; Qiu et al. 2015). Additionally, osa-miR159, osa-miR160/miR167, osa-miR164, and osa-miR172 are also found to be involved in leaf senescence through phytohormone signalling pathways in rice (Xu et al. 2014).

Transcription factors bind to specific DNA sequences and act as molecular switches. The mode of transcription factors is to recognize and bind to a DNA in a promoter and/or enhancer region called cis-acting element. As a result, they can simultaneously regulate a number of genes by recognizing specific cis-acting element sites. In recent years, increasing evidences have implicated a range of transcription factors directly or indirectly regulating plant stress responses. Transcription factors are grouped into different families on the basis of conserved structural domains that are involved in DNA binding activity. In the model plant *Arabidopsis thaliana*, dozens of transcriptions factors, belonging to APETALA2/ethylene-responsive element binding

factor (AP2/ERF), **my**elo**b**lastosis (MYB), NAC (NAM, ATAF, CUC) domain, basic leucine zipper (bZIP), MYC, WRKY and several classes of zinc finger (ZF) domain families contribute to gain particular stress tolerances (Satoh et al. 2004; Tran et al. 2004; Fujita et al. 2005; Wu et al. 2009; Kim et al. 2011; Mizoi et al. 2011).

NAC domain transcription factors, one of the plant-specific families, have been known to be associated with not only stress and senescence but also physiological development such as cell proliferation, flowering time, secondary cell wall biosynthesis (Souer et al. 1996; Kim et al. 2006; Yamaguchi et al. 2008). Approximately, a hundred members exist in Arabidopsis. Accumulating evidence indicates that a number of NAC domain family proteins, including ATAF1, ATAF2, ANAC102, ANAC019, ANAC055, ANAC072, ANAC096, ORE1, ORS1, AtNAP, ANAC046, and VNI2, play pivotal roles in stress regulation and senescence (Fujita et al. 2004; Tran et al. 2004; Delessert et al. 2005; Guo and Gan, 2006; Christianson et al. 2009; Wang et al. 2009; Nakashima et al. 2012; Yang et al. 2011; Wang et al. 2012; Xu et al. 2013; Kim et al. 2014). Gene expression analysis revealed that seven genes including *ANAC055*, *ANAC019*, *ANAC072/RD26*, *ANAC002/ATAF1*, *ANAC081/ATAF2*, *ANAC102*, and *ANAC032* were induced by long-term treatment with ABA and/or during age-dependent senescence (Takasaki et al. 2015). The septuple mutant, lacking the all seven NAC domain transcription factors, clearly showed retardation of ABA-inducible leaf senescence, suggesting that they play crucial roles in ABA-induced leaf senescence signaling (Takasaki et al. 2015). *ARABIDOPSIS THALIANA ACTIVATION FACTOR 1* (*ATAF1*) gene expression is induced by drought, high salinity, ABA, methyl jasmonate, mechanical wounding, and *Botrytis cinerea* infection (Wu et al, 2009; Jensen et al. 2013). Furthermore, ATAF1 was reported as a positive senescence regulator by its bi-functional transcriptional activities. Namely, ATAF1 directly activates *ORE1* while directly represses *GLK1* expression through the ABA-dependent pathway (Garapati et al. 2015a). ORE1 is a key player in senescence. *ORE1* expression is basically upregulated by ethylene pathway by downregulation of miRNA164 (Kim et al. 2009). Recent studies have shown that ORE1 directly regulates *BFN1* (Matallana-Ramirez et al. 2013), and senescence-associated genes (SAGs) (Balazadeh et al. 2010).

An NAC domain protein, VND-INTERACTING2 (VNI2) is originally isolated as an interacting factor with VND7, a master regulator of xylem vessel differentiation. Further characterization revealed that VNI2 acts as a negative regulator in xylem vessel differentiation, by inhibiting VND7 function (Yamaguchi et al. 2010b). Interestingly, *VNI2* is not only expressed in differentiating cells into xylem vessel cells but also many types of tissues and cells, including phloem, guard cells, endodermis cells (Yamaguchi et al. 2010b). In addition, *VNI2* expression is induced in response to various stress conditions (Yang et al. 2011). Indeed, VNI2 has been reported as a mediator of signaling crosstalk between salt stress response and leaf ageing process (Yang et al. 2011). These data strongly suggested that VNI2 play pivotal roles in various regulations during plant developments.

A previous report demonstrated that VNI2 is able to interact with not only VND7, but also other NAC domain proteins, including VND1-6, NAC1, CUC2 and VNI2 itself (Yamaguchi et al. 2010b). Furthermore, when I screened for interacting factors with VNI2, a number of NAC domain transcription factors were isolated including ATAF2 (Nagahage, Master thesis, 2015). ATAF2 has been known to be involved in various biological events including wounding and biotic stress responses, photomorphogenesis, and brassinosteroid and auxin catabolism (Delessert et al. 2005, Peng et al. 2015). However, molecular function of ATAF2 still remains unknown.

The aim of this thesis

Environmental stress responses are a complex mechanism by which plants process to survive in adverse conditions. As I described above, NAC domain protein, VND-INTERACTING2 (VNI2) has been isolated as an interacting factor with VND7, a master regulator of xylem vessel differentiation (Yamaguchi et al. 2010b). Meanwhile, VNI2 has been reported as a mediator of signaling crosstalk between salt stress response and leaf ageing process (Yang et al. 2011).

I hypothesized that VNI2 interacts with and regulates NAC domain proteins in response to environmental status as in the case of VND7 during vascular development. So my contribution was to understand biological roles of VNI2 in environmental status by interacting with NAC domain proteins. To this end, I tried to isolate and characterize VNI2 interacting proteins.

Particularly, I focused on characterization of ATAF2, and then tried to understand biological roles of the VNI2-ATAF2 complex during plant development. It is expected that the obtaining findings here are important not only for basic sciences in the field of molecular biology but also applications in order to generate stress-tolerant crops, high yield turn-over crops.

Chapter 2

Isolation of NAC domain proteins ANAC102 and ATAF2 as interacting factors of VNI2

2.1 Introduction

NAC domain transcriptional factors play roles in various biological processes, such as seed germination, secondary cell wall formation, senescence, and biotic and abiotic stress responses (Christianson et al. 2009; Nakano et al. 2015; Yamaguchi and Demura 2010). Previous researches demonstrate that NAC domain transcription factor family has a tendency to form protein-protein complexes with other transcription factors. Tran et al. (2007) reported that ANAC019, ANAC055, and ANAC072 interact with a zinc finger homeodomain protein. In addition, ANAC096, involved in the regulation of dehydration and osmotic stress, interacts with ABF2 and ABF4 (Xu et al. 2013). NAC53 and NAC78 also form homo- and heterodimers to obtain tolerance for proteotoxic stress (Gladman et al. 2016). VNI1 and ANAC103 interact with VND family proteins as well as other NAC domain proteins such as NAC1 and CUC2 (Yamaguchi et al. 2015).

A NAC domain transcription factor, VND-INTERACTING2 (VNI2) is originally isolated as an interacting factor with VASCULAR-RELATED NAC-DOMAIN 7 (VND7) (Yamaguchi et al. 2010b), and further studies reported that VNI2 regulates xylem vessel differentiation by inhibiting VND7 functions (Yamaguchi et al. 2010b). The developmental expression profile of *VNI2* showed that *VNI2* is expressed in most parts of the plant including meristematic and procambial regions at the root tip, central cylinder, and vascular cells in aerial parts of the plants (Yamaguchi et al. 2010). Yang et al. (2011), reported that *VNI2* is 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. In addition, *VNI2* was highly expressed in late stages of plant development, suggesting that VNI2 is involved in leaf senescence. Further, VNI2 is reported as a mediator of signalling crosstalk between salt stress response, and longevity through the ABA pathway (Yang et al. 2011). Pull-down assay data suggested that VNI2 strongly binds to VND1, VND2, VND3, VND4, VND5 and weakly binds to VND6, NAC1 and CUC2 (Yamaguchi et al. 2010b). Thus, in my research work, I hypothesised that VNI2 interacts with other NAC domain proteins and regulates various biological events including environmental stress responses.

Yeast two-hybrid analysis is a simple and fast procedure to identify direct protein-protein interactions. The method is based on the reconstruction of yeast GAL4 transcriptional factor

when two protein interact. The genetically modified yeast strain is utilized and the specific phenotypes caused by reporter genes are allowed to select interacting protein combination (Brückner et al. 2009). GAL4 transcriptional factor is split into the GAL4 binding domain (GAL4-BD) and GAL4 transcriptional activation domain (GAL4-AD). One of the interested protein is fused to GAL4-BD and the resulting construction is referred to as bait and another protein fused to GAL4-AD is referred to as prey (Figure 1). A yeast two-hybrid analysis was performed to identify novel NAC domain proteins interacting with VNI2 (Nagahage, Master thesis, 2015). From a cDNA library containing more than 1,600 full-length coding sequences of transcription factors, a number of NAC domain transcription factors were isolated and transcriptional activities of them were investigated. Most of them did not show clear transcriptional activity or repression activity.

ANAC102 and ATAF2, two NAC domain transcription factors which were isolated as interacting factors with VNI2 (Matsuda master thesis, 2012), were further checked by yeast two-hybrid analysis to confirm the interaction (Nagahage, Master thesis, 2015). ANAC102 was reported as a putative transcription factor whose expression was induced early in low-oxygen stress (Klok et al. 2002). In adult plants, *ANAC102* is highly expressed in the lateral root cap and radicle (Christianson et al. 2009) in low oxygen condition. Further, they reported that ANAC102 did not affect on germination in unstressed seeds, nor was any effects on germination observed under salt, osmotic, or ABA stress, but required for tolerance to low oxygen at the seed stage. In addition, *ANAC102* is predicted to be activated by ORE1 during senescence in network modelling analysis (Breeze et al. 2011).

ATAF2 protein has been known to be involved in various biological events including wounding and biotic stress responses, photomorphogenesis, brassinosteroid signaling, and auxin catabolism (Delessert et al. 2005; Huh et al. 2012; Peng et al. 2015; Wang et al. 2009). *ATAF2* shows ubiquitous expression, but also revealed localized high expression in the root vascular cylinder, root cap, hydathodes, cells surrounding the substomatal cavity and guard cells of senescing cotyledons (Delessert et al. 2005). In addition, *OsNAC6*, closest homologue in rice, was also expressed at relatively high levels in all tissues (Kikuchi et al. 2000). *ATAF2* expression is also induced by methyl jasmonate, salicylic acid, auxin, and pathogen infection (Hauck et al. 2003;

Delessert et al. 2004; Huh et al. 2012;) whereas ABA treatment does not have any effect for *ATAF2*. *ATAF2* is induced in wounding, as is its potato homologue *StNAC* (Collinge and Boller, 2001). There could be a relationship between ATAF2 and water potential since *ATAF2* is highly expressed in dehydration and salt stress (Seki et al. 2002; Delessert et al. 2005; Huh et al. 2012). The *ATAF2* expression is negatively influenced by light condition (Delessert et al. 2005; Peng et al. 2012). Furthermore, sucrose is acting as an obstacle to induce *ATAF2* expression in the dark (Delessert et al. 2005).

It is important to confirm the physical interaction of isolated protein in yeast two-hybrid analysis. Pull-down assay is a good method to confirm the protein-protein interaction. The theory of pull-down assay is the immobilization of the tag-fused "bait" protein to the affinity resin (Figure 2). If "prey" proteins bind to the bait protein, the immoblized fractions include the prey protein dependent on co-incubation of the "bait" proteins, and are able to detect by using immuno blot analysis.

In this study, We tried to isolate VNI2 interacting NAC domain proteins to understand VNI2 stress responses by forming a protein complex. VNI2 interacting region which is responsible to form VNI2-ATAF2 protein complex was studied. Further, physical interaction between VNI2 and ATAF2 was investigated through a pull-down assay. In addition, ANAC102 and ATAF2 transcriptional activity were investigated by transient assay system. VNI2-ATAF2 protein interaction was studied in transient assay system to elucidate changes of transcriptional activity of ATAF2 after forming a VNI2-ATAF2 protein complex.

2.2 Materials and methods

Solutions and media

MVD media (1L)

Reagents	Amount (g)
Glucose	10
Yeast nitrogen base (Difco)	20
Agar (Nacalai)	20

Added $Leu^{-}Trp^{-}$ supplement or $Leu^{-}Trp^{-}His^{-}$ supplement according to bottle instruction

Media were autoclaved at 121ºC for 15 min and then transferred to plates. Plates were stored at 4ºC.

LB liquid (1L)

Reagents	Amount (g)
Yeast extract	5
Polypepton	10
NaCl	5

Media were autoclaved at 121ºC for 15 min.

LB media

Reagents	Amount (g)
Yeast extract	5
Polypepton	10
NaCl	5
Agar	15

Media were autoclaved at 121ºC for 15 min and then transferred to plates. Plates were stored at 4ºC.

LB media (1L) antibiotics

Reagents	Amount (mL)
Ampicillin (100 mg/mL)	1
Kanamycin (50 mg/mL)	1
Spectinimycine (50 mg/mL)	1

200 mM MES (pH5.7) (1L)

MES (42.65 g) adjust with KOH (pH 5.7) ** Sterilized with a filter membrane

1 M $CaCl_2$ (300 mL)

$CaCl_2$:$2H_2O$ (44.10 g) is dissolved in 300 mL of water

Digestion Buffer (500 mL)

Reagents	Amount
Mannitol	36.43 g
KCl	0.75 g
1 M $CaCl_2$	5 mL
200 mM MES (pH 5.7)	50 mL

** Sterilize with filter membrane

W5 buffer

Reagents	Amount
5 M NaCl	8.77 g
1 M $CaCl_2$	125 mL
KCl	0.37 g
200 mM MES (pH 5.7)	50 mL

** Sterilize with filter membrane

MMg solution

Reagents	Amount
Mannitol	36.44 g
$MgCl_2$ $6H_2O$	1.55 g
200 mM MES (pH 5.7)	10 mL

** Sterilize with filter membrane

PEG solution

Reagents	Amount
PEG 4000 (Fluka)	16 g
Mannitol	1.44 g
1 M $CaCl_2$	4 mL

Enzyme solution

Reagents	Amount
Cellulase “onozuka” R10 (Yakulto)	16 g
Macerozyme R10 (Yakulto)	0.05 g
β-mercaptoethonol	14 µL

Digestion buffer up to 20 mL

Vector construction

The coding sequences of (CDSs) of NAC domain transcription factors were amplified by polymerase chain reaction (PCR) with the gene specific primer sets (Table S1). The amplified fragments were subcloned into the pENT/D-TOPO vector (Life Technology) to generate the entry clones. The entry clones were integrated into Gateway destination vectors for yeast two-hybrid analysis (pBD-GAL4-GWFC and pAD-GAL4-GWRFC; Yamaguchi et al. 2008). To perform transient assay, ANAC102 and ATAF2 cDNA was subcloned into the pENTR/D-TOPO vector, and then integrated into pA35BDG, a GATEWAY destination vector, which contains the GAL4-BD driven by the cauliflower mosaic virus *CaMV35S* (35S promoter) (Yamaguchi et al. 2015). Entry clone contained *VNI2* cDNA was integrated to p35G, a Gateway destination vector, which contains the *CaMV35S* promoter (Endo et al. 2015). The GATEWAY destination vectors containing the multi-cloning site (MCS) fragment were used as controls (Yamaguchi et al. 2008).

Yeast two-hybrid assay

S. c. EasyCompTM Transformation kit (Invitrogen) was used according to the protocol (https://www.lifetechnologies.com/order/catalog/product/K505001) and pBD-GAL4-GWRFC and/or pAD-GAL4-GWRFC plasmids were introduced into S. cerevisiae strain AH109 by using S.c. solution III. Transformants were incubated at 30 °C on MVD medium lacking with tryptophan and leucine for 3 days. pBD-wt and pAD-wt were used as the positive controllers. Yeast which was successfully grown above was transformed to MVD medium lacking tryptophan, leucine and histidine and incubated at 30 °C for 3 days. pBD-GAL4-MCS and pAD-GAL4-MCS were used as negative controllers. 0.1 mM 3AT was added to minimize the background false positive effect.

Dual luciferase transient expression assay

The effector, reporter, and reference plasmids were used to transfect Arabidopsis protoplasts, which were obtained from 3- to 4-week-old leaves of plants that were grown under long-day conditions, via a PEG transformation method (Sakamoto et al. 2016). Luciferase activities were measured with the Dual-Luciferase Reporter Assay System (Promega,

http://www.promega.com) using a Mithras LB940 Multimode Microplate Reader (Berthold, http://berthold.com).

In vitro pull-down assay

Poly-His tagged VNI2 (His-VNI2) was prepared according to the previous reports (Yamaguchi et al. 2010b). *ATAF2* cDNA was integrated into the pMAL-GWRFC for MBP tag (Yamaguchi et al. 2010b). MBP-ATAF2 was expressed in an *E. coli* strain, BL21 *trxB* (DE3; Cosmo Bio) in the presence of isopropyl ß-D-thiogalactoside (IPTG) and purified with amylose resin (Nes England Biolabs). His-VNI2 and/or MBP-ATAF2 proteins were incubated with the amylose resin for 90 min at 4˚C. The proteins immobilized with the resin were subjected with the immunoblot analysis. His-VNI2 protein was detected with the anti-His antibody (Santa Cruz Biotechnology), and the anti-rabbit IgG antibody (Amersham Biosciences).

2.3 Results

Two NAC domain transcriptional factors, ANAC102 and ATAF2 were isolated as VNI2 interacting proteins by yeast two-hybrid assay.

Two NAC domain transcriptional factors, ATAF2 and ANAC102, are originally isolated as interacting proteins with VNI2 through a yeast two-hybrid screening by using cDNA library obtained from Arabidopsis primary roots (Matsuda, Master thesis, 2012). Since the rescued ATAF2 and ANAC102 cDNAs lacked a part of 5' coding region or contained 5' UTR region, yeast two-hybrid analysis was performed by using full-length coding sequences (Figure 3). VNI2 was fused to GAL4-BD, and ANAC102 and ATAF2 were fused to GAL4-AD. MCS were used as negative controls. Constructed vectors combinations were transformed into budding yeast AH109 strain. I confirmed that all transformants grew on a control medium containing histidine. However, only cells expressing *ANAC102* and *ATAF2* together with *VNI2* were able to grow on a selective medium lacking the histidine with 0.1 mM amino-1,2,4-triazole (3-AT) (Figure 3). Thus, I confirmed that full-length ANAC102 and ATAF2 interact with VNI2 in the yeast cells.

ANAC102 and ATAF2 are closely related to each other and categorized under the stress regulatory transcriptional factors.

In the *Arabidopsis* genome, there are approximately 100 NAC domain transcription factors (Nakashima et al. 2012). Phylogenetic analysis showed that ANAC102 and ATAF2 are the most closely related proteins to each other, and are classified into a clade including ATAF1, ANAC032, ANAC019, ANAC055 and ANAC072. It is known that these members are associated with environmental stress responses (Nakashima et al. 2012; Figure 4). In addition, some of the members were identified as interacting proteins with VNI2 (Nagahage, Master thesis, 2015). It is possible that VNI2 should be involved in environmental stress responses via regulation of ANAC102 and ATAF2 by forming protein complexes.

ATAF2 is a transcriptional activator

NAC domain transcription factors are characterized as transcriptional activators and repressors (Olsen et al. 2005; Yamaguchi et al. 2010b). To investigate the transcriptional activities of ATAF2 and ANAC102, a transient assay system was carried out by using protoplast cells

prepared from *Arabidopsis* mesophyll cells. A reporter construct containing *luc* linked to GAL4 binding sites, and an effector construct containing VND7, VNI2, ANAC102, or ATAF2 fused to GAL4-BD under the control of *35S* promoter was delivered to the *Arabidopsis* protoplasts. GAL4-BD-VND7 and GAL4-BD-VNI2 used as positive and negative controllers, respectively. As reported previously, GAL4-BD-VND7 has a transcriptional activity while GAL4-BD-VNI2 does not show transcriptional activity compared with the control GAL-BD-MCS (Yamaguchi et al. 2010b; Figure 5). In addition, GAL4-BD-ATAF2 showed transcriptional activity while GAL4-BD-ANAC102 showed neither strong transcriptional activity nor repression activity (Figure 5). These data suggested that ATAF2 is a transcriptional activator. Thus, I focused on ATAF2 further.

Whole NAC domain of VNI2 requires to interact with ATAF2

To find the interacting region of VNI2, bait was constructed by using different sizes of truncated VNI2 while GAL4-AD fused full-length ATAF2 was used as a prey. Results showed that full-length VNI2 and truncated VNI2 which contained only NAC domain (coding region between 1 to 170, $VNI2^{1-170}$) were able to grow in the selective medium (Figure 6). However, yeast cells expressing $VNI2^{1-138}$, lacking the 5^{th} subdomain of NAC domain construct, or $VNI2^{147-252}$, containing the 5^{th} subdomain and following C-terminal region were unable to grow (Figure 6). This results suggested that the N - terminal NAC domain of VNI2 is necessary and sufficient to interact with ATAF2.

VNI2 interacts with ATAF2 *in vitro*

To confirm the interaction between VNI2 and ATAF2 furhter, I performed *in vitro* pull-down assay (Figure 7). Maltose binding protein (MBP) tagged full-length ATAF2 (MBP-ATAF2) was expressed in *Escherichia coli* and immobilized on amylose resin. Polyhistidine-tagged full-length VNI2 (His-VNI2) was prepared as a prey protein. Proteins immobilized to the resin were used for immunoblot analysis with an anti-His antibody. Consistent with the results of my yeast two-hybrid assay, VNI2 efficiently bound to the ATAF2 (Figure 7).

VNI2 and *ATAF2* expression patterns are partially overlapped

Basically, *VNI2* and *ATAF2* are expressed at varying levels in all most all parts of the plants. Both of them are highly expressed in cotyledons, stomatal guard cells, vascular cylinder, and root caps (Yamaguchi et al. 2010; Yang et al. 2011; Figure 8). Guard cells regulate the rate of transpiration by opening and closing the stomata. In addition, it controls water loss in drought stress including osmotic stress, salinity stress and dark. These data suggest that *VNI2* and *ATAF2* could be involved in the regulation of stomata in stress condition and light-dark condition. In *Arabidopsis*, two-week-old cotyledons start senescence, and both genes are highly expressed at that stage (Delessert et al. 2005; Yamaguchi et al. 2010b; Yang et al. 2011). In addition, both genes are strongly expressed in the tips of roots and leaves (Figure 8). Usually, senescence is taken place from the tip or border of the leaves. Furthermore, *VNI2* expression is elevated in late stages of plant leaves, and abscission and dehiscence tissues, such as axils of bracts, abscission zones in cauline leaves and siliques (Yang et al. 2011). Thus, there may be a relationship between senescence and VNI2-ATAF2 interaction.

Transcriptional activation activity of GAL4-BD-ATAF2 was inhibited by VNI2

ATAF2 shows transcriptional activation activity in the transient assay system (Figure 9). It is possible that VNI2 regulates transcriptional activation activity of ATAF2 by forming the complex. To verify the possibility, the transient assay was carried out. A reporter construct containing firefly *luc* linked to GAL4 binding sites, and effector construct containing ATAF2 fused to GAL4-BD under the control of *35S* promoter together with effector construct containing VNI2 under the control of *35S* promoter were delivered to *Arabidopsis* protoplasts. Consistent with previous data ATAF2 was shown to possess transcriptional activity (Figure 9). VNI2 itself neither shows any transcriptional activity nor repression activity for the reporter gene. In contrast, VNI2 repressed *luc* expression transactivated by ATAF2. These data suggested that VNI2 is able to inhibit the transcriptional activity of ATAF2 without interfering with the DNA binding ability of ATAF2.

2.4 Discussion

NAC domain transcriptional factors form homo or heterodimers. VNI2 was originally identified as an interacting protein of VND7. VNI2 regulates transcriptional activities of VND7 resulting in repression of xylem vessel differentiation. Later, VNI2 was reported as senescence and salt stress regulator. My yeast two-hybrid data indicates that VNI2 forms protein complexes with ANAC102 and ATAF2 (Figure 3). ANAC102 and ATAF2 are known as stress regulatory NAC domain transcriptional factors, and closely related to each other (Figure 4). There had not been reported that ANAC102 and ATAF2 form protein complexes with other NAC domain proteins.

VND7 has strong transcriptional activation activities while VNI2 has a transcriptional repressing activity (Yamaguchi et al. 2010b). It was easy to see how VNI2 regulates VND7 because VNI2 effectively inhibits VND7 function. Likewise, it is important that ATAF2 and ANAC102 have strong transcriptional activation (or repression) activities to see how VNI2 regulates their functions. Here, because the transient assay showed that ATAF2 but not ANAC102 has transcriptional activation activity, I focused on ATAF2 for further analyses (Figure 5). VNI2 significantly inhibits transcriptional activation activities of GAL4-BD fused ATAF2 (Figure 9), suggesting that VNI2 also inhibits expression of downstream genes of ATAF2. VND7 is known as a key regulator for xylem vessel differentiation, and a number of direct targets of VND7 are identified (Kubo et al. 2005; Yamaguchi et al. 2008; Yamaguchi et al. 2010b). Similarly, a number of downstream targets of ATAF2 have been reported (Delesert et al. 2005; Wang et al. 2009; Wang et al. 2012; Huh et al. 2012; Peng et al. 2015). It is necessary to isolate useful downstream genes effectively upregulated by ATAF2 for investigating how VNI2 regulates ATAF2.

Yeast two-hybrid analysis demonstrated that the whole NAC domain of VNI2 is necessary and sufficient to interact with ATAF2 (Figure 6). Previously, it was reported that NAC subdomain V with the C-terminal transcriptional activation domain of VNI2 was enough to interact with VND7 (Yamaguchi et al. 2010). Thus, although both VND7 and ATAF2 are interacting proteins with VNI2, it seems that they bind to VNI2 in different manners.

It is quite important to understand the biological meanings of the VNI2-ATAF2 complexes. Because ATAF2 and ANAC102, their closely related genes are known to be associated with stress responses (Figure 4), VNI2 might be involved in stress regulation by forming protein complexes with ANAC102 and ATAF2. According to the previous reports, spatial and temporal expression patterns of *VNI2* and *ATAF2* are highly overlapped (Figure 8). Particularly, both genes are strongly expressed in tissues at which senescence is taken place (Figure 8). Senescence is an irreversible process after some stage. Thus, it is important to inhibit senescence when it is not required. VNI2 is known to be a transcriptional repressor (Yamaguchi et al. 2010). Thus, it is possible that VNI2-ATAF2 interaction inhibits senescence in a short period of time. In addition, both genes are highly expressed in dark condition (Delessert et al. 2005; Yang et al. 2011; Peng et al. 2015). These data suggest that VNI2 and ATAF2 fucntions are controled by light/dark condition. In addition, sucrose inhibits *ATAF2* expression even in dark, biotic and abiotic stress conditions (Delessert et al. 2005). Thus, there may be possibile that *ATAF2* expression is controlled in response to energy starvation, rather than environmental statuses. Transcriptional regulation in response to light and dark is very complex, including ABA-mediating pathway (Liebsch and Keech, 2016). It is noteworthy that *ATAF2* is not induced by ABA (Delessert et al. 2005), while *VNI2* expression is induced by ABA (Yang et al. 2011). ATAF1 is the closest homolog to ATAF2, and is involved in energy starvation and senescence as well as ATAF2 (Garapati et al. 2015a; Garapati et al. 2015b). However, in contrast to *ATAF2*, *ATAF1* expression is regulated by ABA. Thus, it is possible that ATAF1 and ATAF2 modulate senescence in ABA-dependent and independent pathway, respectively. VNI2 is quite unstable protein, and C-terminally truncated VNI2 which becomes more stable than full length, severely inhibits VND7 functions (Yamaguchi et al. 2010b), suggesting that VNI2 may regulate interacting proteins for short time. Considered together, my findings suggest that VNI2 forms a protein complex with ATAF2 and inhibits its function in a short time in stress reponseses or progression of senescence.

2.5 Figures

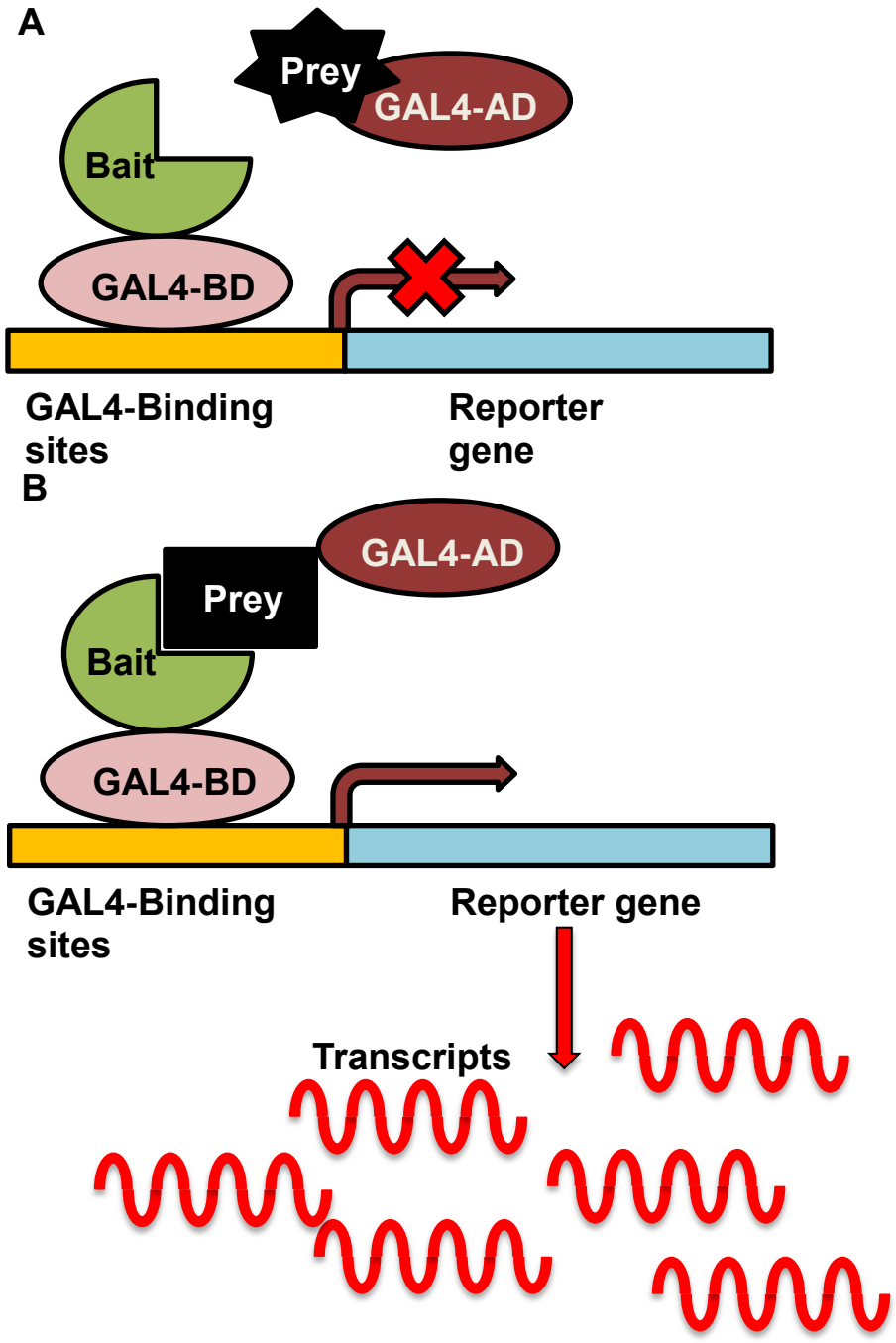


Figure 1. Schematic diagram of yeast two-hybrid analysis. (A) Schematic diagram of the bait and prey constructs. When they do not have binding affinity to each other, GAL4 transcriptional factor cannot be formed and consequently transcription is not taken place. (B) Expression of the reporter gene is induced due to interaction of bait and prey proteins.

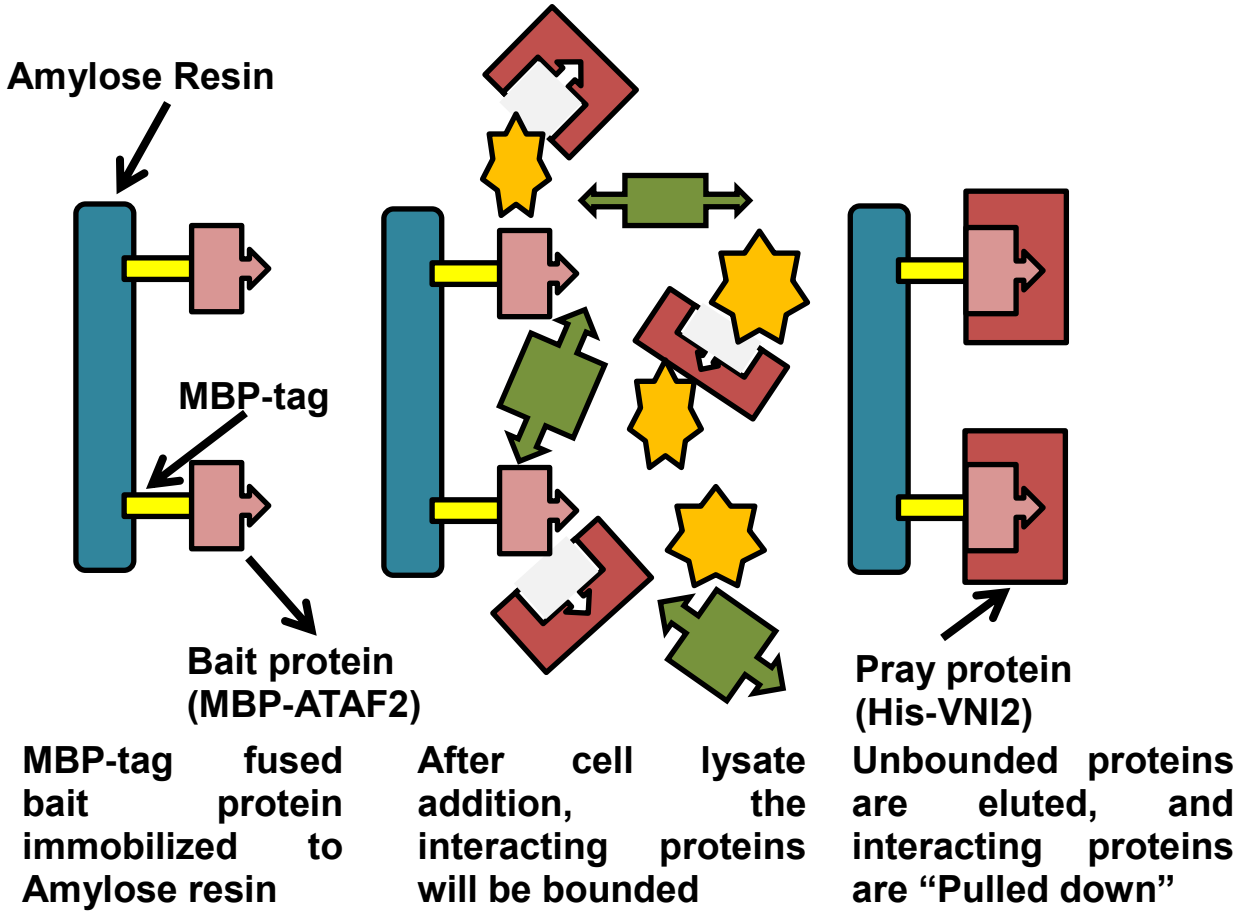


Figure 2. Schematic diagram of pull-down assay. MBP-ATAF2 is called as bait protein and His-VNI2 is called as pray protein.

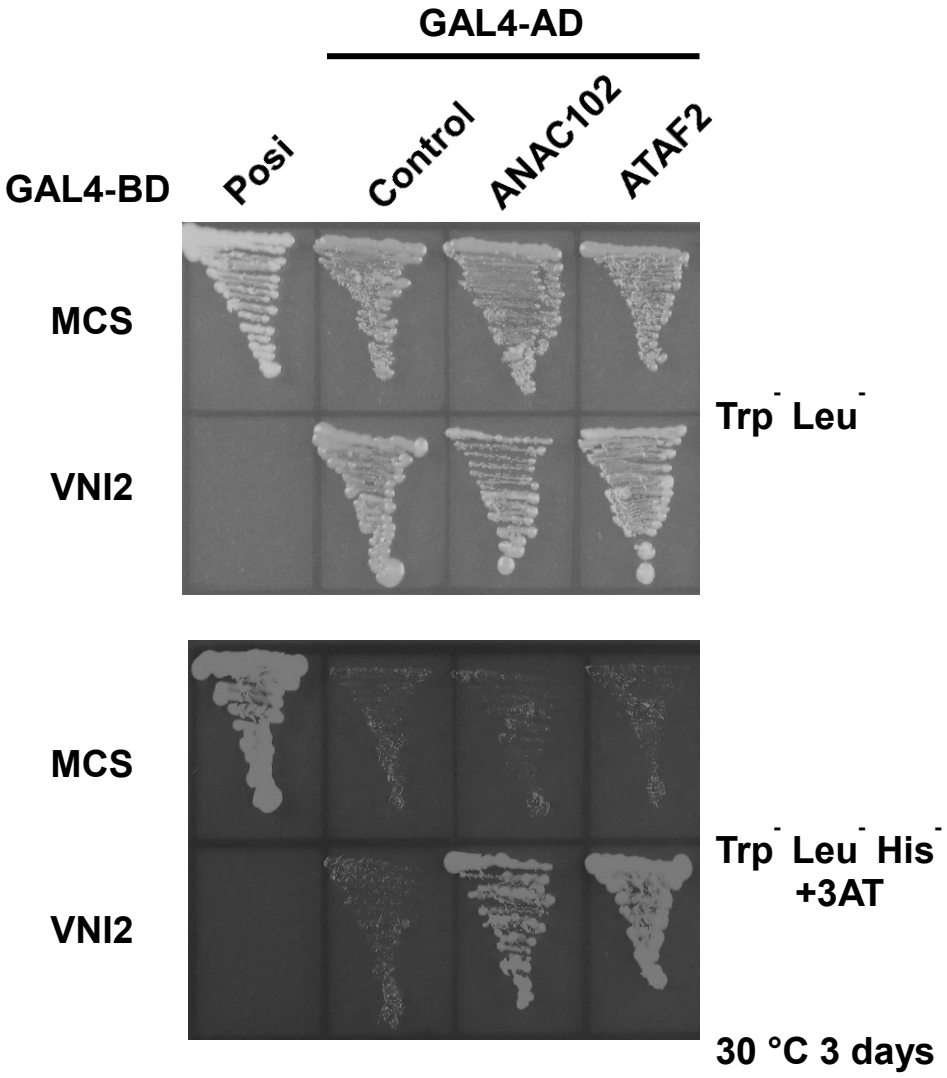
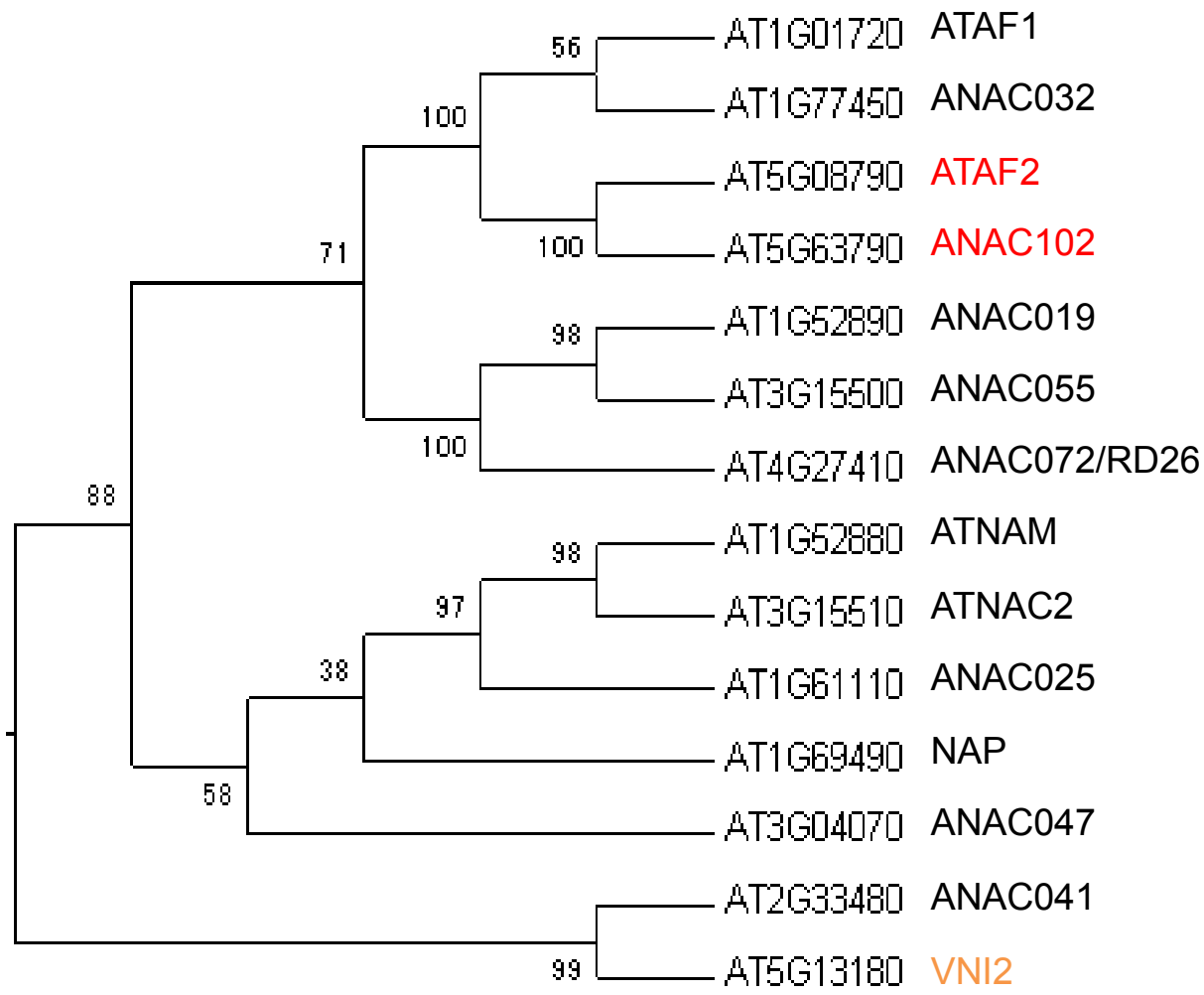


Figure 3. VNI2 interacts with ANAC102 as well as ATAF2.

The multi cloning site (MCS), and VNI2 fused to the GAL4-BD, and ANAC102, ATAF2 fused to GAL4-AD were introduced into AH109 and grown on the control (Trp^-Leu^-) and selective media ($Trp^-Leu^-His^-$). Plasmids containing a MCS fused to GAL-BD or GAL4-AD were used as the negative controls, and pBD-wt and pAD-wt were used as the positive controls.



From FioreDB database (http://www.cres-t.org/cgi-bin2/open_tree.cgi)

Figure 4. Phylogenetic tree of Arabidopsis NAC domain family involved in environmental stress. ANAC102 and ATAF2, isolated as the interacting factors with VNI2, are closely related to each other.

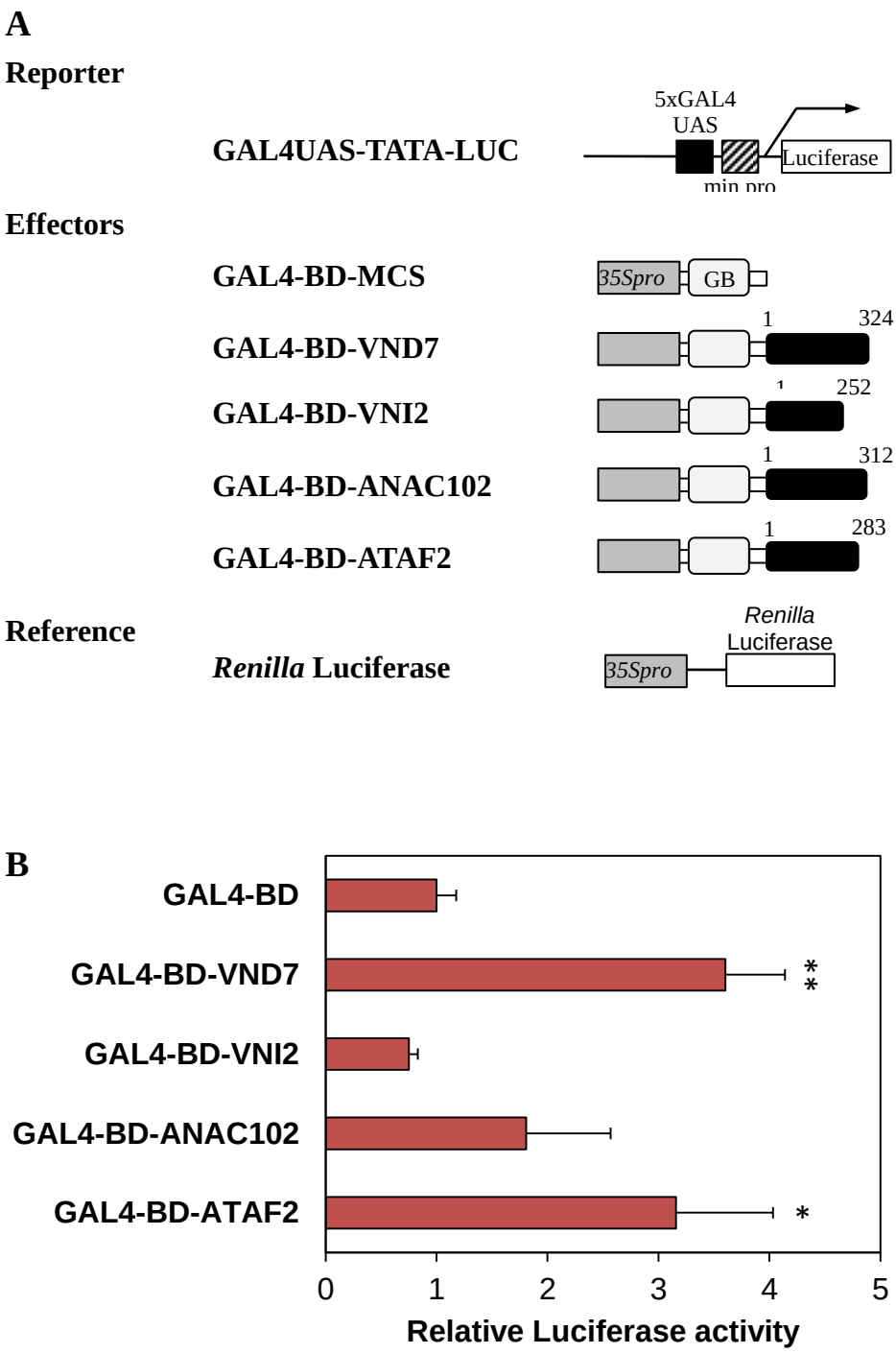


Figure 5. ATAF2 has transcriptional activation activity.

(A) Schematic diagrams of the constructs used in the dual luciferase transient assay. The reporter construct contained the firefly luciferase reporter gene under the control of five repeats of the upstream activation sequence of GAL4 (5x GAL4 UAS) fused to a minimal *CaMV35S* promoter

(min pro). The effector constructs contained GAL4-BD bound to an empty multiple cloning site (GAL4-BD-MCS) or to coding sequences corresponding to full-length *VND7*, *VNI2*, and *ATAF2* driven by the *CaMV35S* promoter (*35S* promoter). (B) Results of the transient transfection assay. Firefly luciferase activities were normalized by *Renilla* luciferase activities. Error bars indicate SD (n = 4). Asterisks indicate statistically significant differences (Students t-test **$p < 0.01$ *$p < 0.05$) compared to the GAL4-BD-MCS vector control. ATAF2 has transcriptional activation activity.

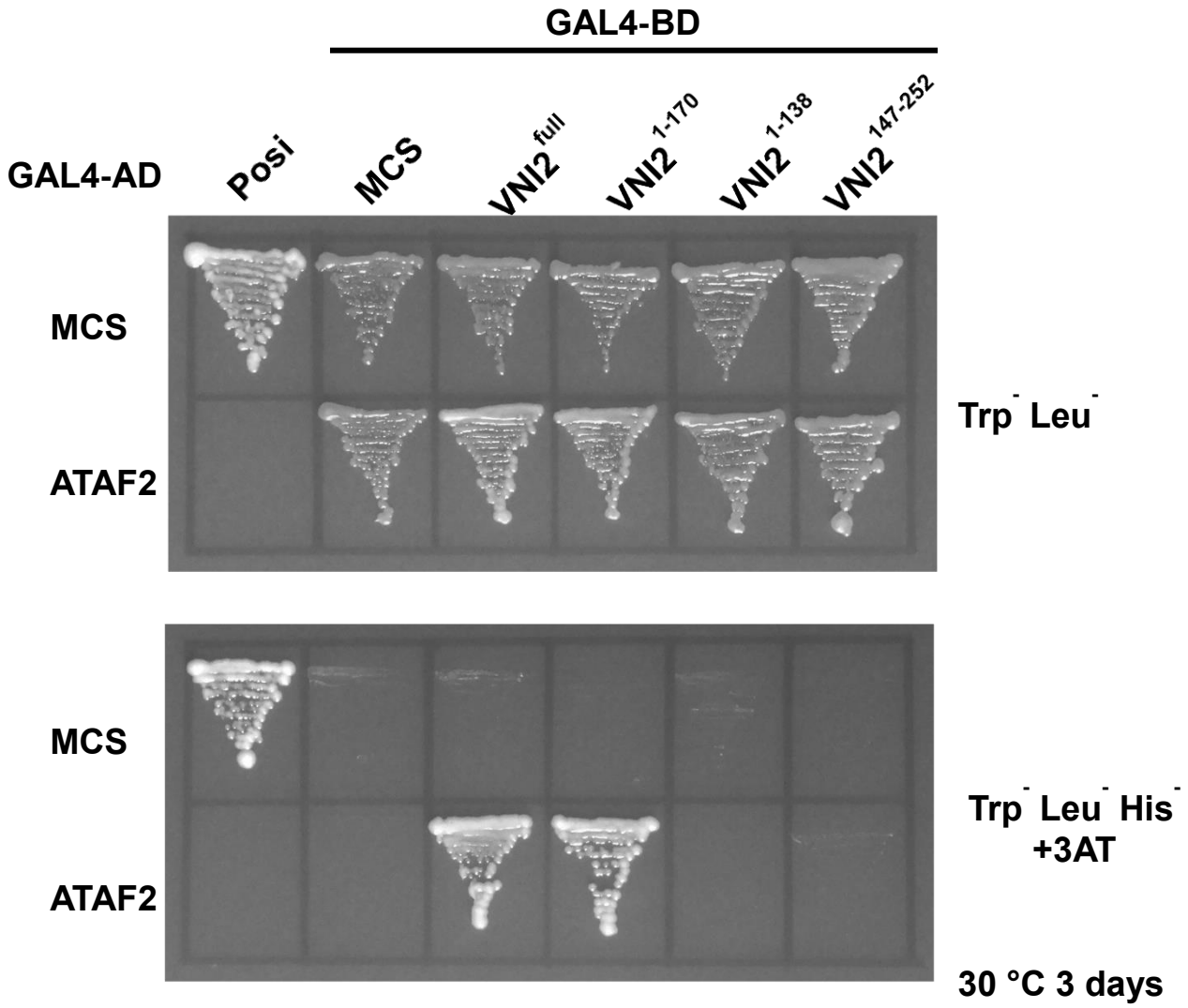


Figure 6. VNI2 requires full NAC domain region to interact with ATAF2.

Full length or truncated VNI2 fused to the GAL4-BD, and full length ATAF2 fused to GAL4-AD were introduced into AH109 and grown on the control (Trp^-Leu^-) and selective media ($Trp^-Leu^-His^-$). Plasmids containing a MCS fused to GAL-BD or GAL4-AD were used as the negative controls, and pBD-wt and pAD-wt were used as the positive controls. $VNI2^{1-170}$ contains the whole NAC domain. $VNI2^{1-138}$ lacks fifth subdomain of NAC domain. $VNI2^{147-252}$ contains the transcriptional repression domain of VNI2 + fifth subdomain.

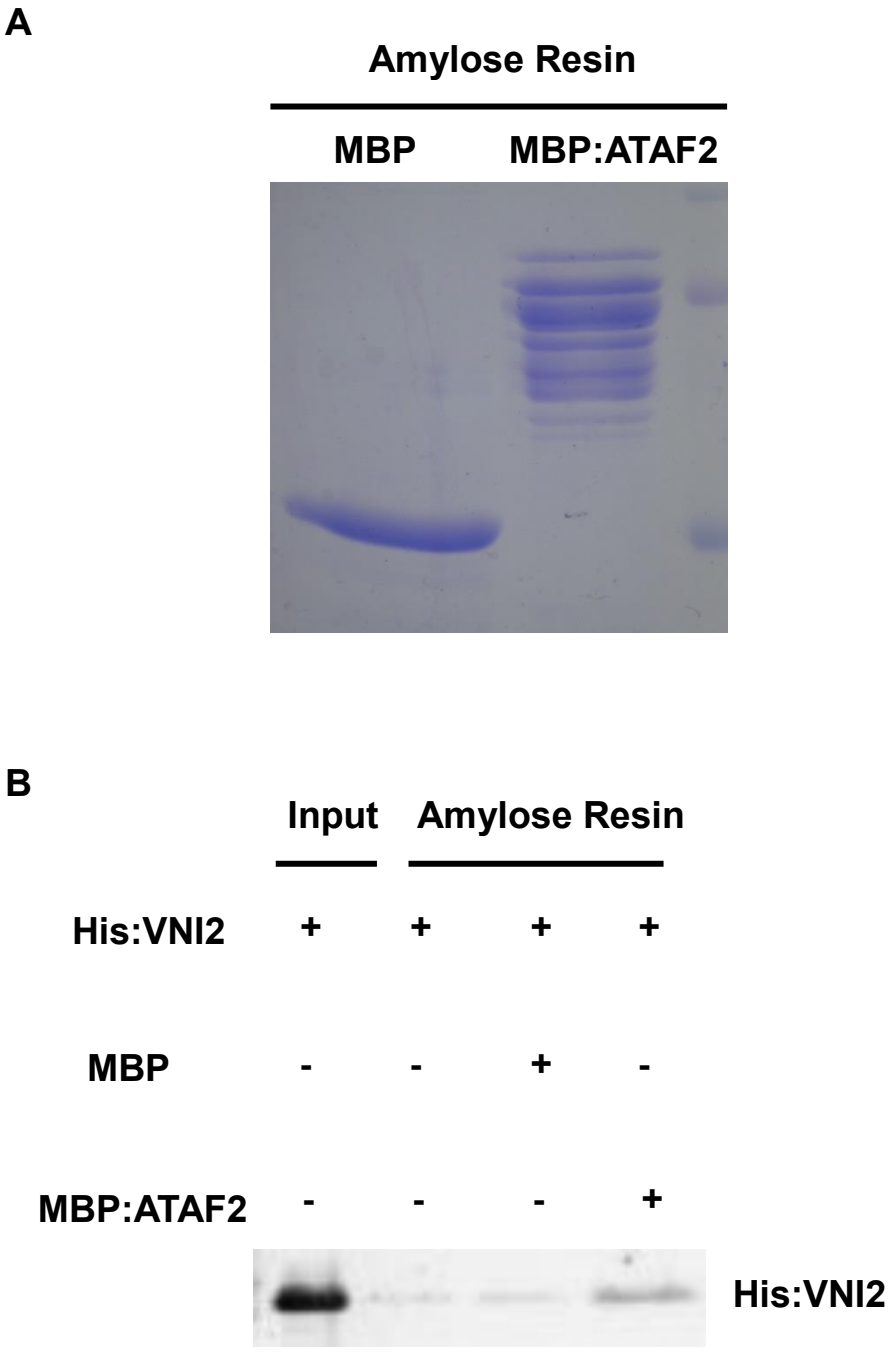


Figure 7. In vitro binding of VNI2 to ATAF2.

(A) CBB staining of MBP-ATAF2. (B) ATAF2 interacts with VNI2. The proteins immobilized with the resin were subjected with the immunoblot analysis. His-VNI2 protein was detected with the anti-His antibody

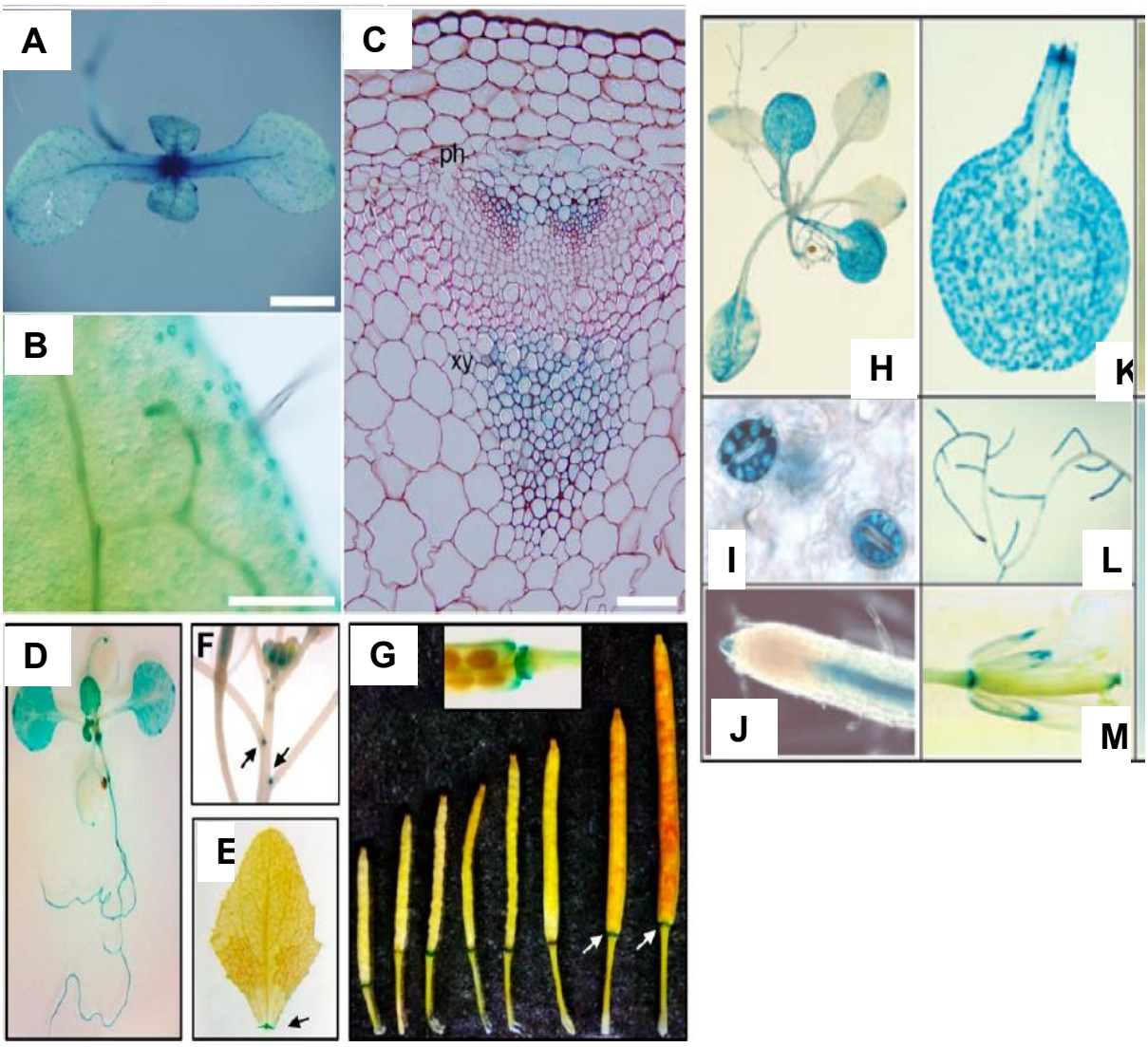


Figure 8. Expression pattern of *VNI2* and *ATAF2* are partially overlapped.

(A) to (C) figures were referred from Yamaguchi et al. (2010). *VNI2* is expressed in (A) cotyledons, (B) guard cells, (C) phloem cells and xylem cells. (D) to (G) figures were referred from Yang et al. (2011). (D) *VNI2* is expressed leaves and roots of young seedlings, (E) abscission zones of cauline leaves (F) axils of bracts and (G) siliques. (H) to (M) figures were referred from Delessert et al. (2005). *ATAF2* is expressed in (H) leaves of 2-weeks plant (I) senescence cotyledon (J) root tips (K) cotyledon (L) roots (M) and, flower of 10-week old plant.

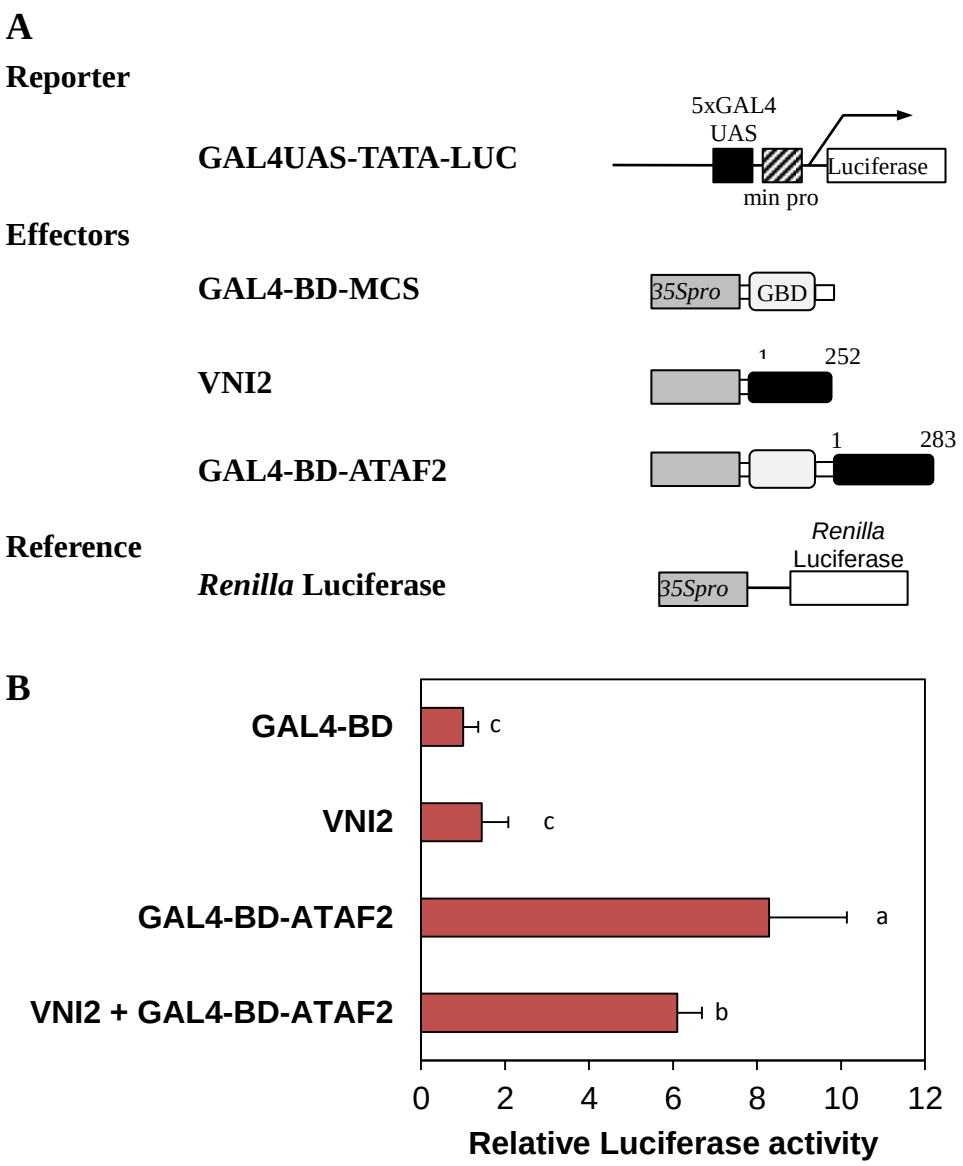


Figure 9. VNI2 interacts with ATAF2 and inhibits ATAF2 transcriptional activity.

(A) Schematic diagram of the effector and reporter construct. The effector constructs contained GAL4-BD bound to (GAL4-BD-MCS, GAL4-BD-ATAF2) coding sequences corresponding to full-length MCS, and *ATAF2* driven by the *35S* promoter or VNI2 coding sequence driven by the *35S* promoter. (B) Results of the transient transfection assay. Firefly luciferase activities were normalized by *Renilla* luciferase activities. Error bars indicate SD (n = 4). $p < 0.05$ (Tukey`s HSD).

Chapter 3

Characterization of ATAF2 biological roles in senescence

3.1 Introduction

NAM/ATAF/CUC (NAC) domain transcription factor family is a plant-specific transcription factor family, and involved in various biological events such as seed germination, secondary cell wall formation, senescence, and biotic-abiotic stress responses (Christiansan et al. 2009, Yamaguchi and Demura, 2010, Nuruzzaman et al. 2013, Shao et al. 2015). Most of NAC domain transcription factors are reported as transcriptional activators. However, some NAC domain transcription factors are known to be act as transcriptional repressors, such as CALMODULIN BINDING NAC PROTEIN (CBNAC) and VND-INTERACTING2 (VNI2) (Kim et al. 2007, Yamaguchi et al. 2010).

ATAF2 is known to be involved in biotic stress responses (Delessert et al. 2005; Wang et al. 2009; Wang et al. 2012). In addition, ATAF2 is also associated with various types of regulations, such as wounding responses, inactivation of brassinosteroid, and auxin biosynthesis (Delessert et al. 2005; Huh et al. 2012; Peng et al. 2015). It has been reported that a number of genes were identified as a direct or indirect target of ATAF2. Delessert et al. (2005) reported that ATAF2 represses a number of pathogenesis-related proteins. Likewise, ATAF2 directly represses the expression of brassinosteroid inactivation enzymes, *BAS1* and *SOB7* (Peng et al. 2015). In contrast to these reports, I showed that ATAF2 acts as a transcriptional activator in chapter 2 (Figure 4). In addition, Wang and Culver (2012) showed that ATAF2 upregulates expression of pathogen-related genes. Moreover, ATAF2 directly induces expression of *NIT2*, encoding an auxin biosynthetic enzyme which converts indole-3-acetonitrile (IAN) to indole-3-acetic acid (IAA) (Huh et al. 2012). Although ATAF2 was studied in several discrete biological functions, still transcriptional regulation of ATAF2 protein is elucidated further.

It has been reported that a number of NAC domain transcription factors regulate senescence (Guo et al. 2006; Balazadeh et al. 2010; Balazadeh et al. 2011; Yang et al. 2011; Garapati et al. 2015). ATAF1, a close homolog of ATAF2, regulates NAC domain transcription factor, *Orresara 1* (*ORE1*). ORE1 is a famous senescence regulatory transcription factor and many senescence-associated genes are controlled by it. Orresara sister1 (ORS1), closely related to ORE1, also is reported as a senescence regulatory transcription factor (Balazadeh et al. 2011). Recently, another NAC domain transcription factor, a close homolog to ORE1 and ORS1,

ANAC046 also regulates senescence (Oda-Yamamizo et al. 2016). In addition, some other NAC domain transcription factors, including ANAC016, AtNAP, JUNGBRUNNEN1 (JUB1), and VNI2 positively and negatively regulate senescence (Guo et al. 2006; Yang et al. 2011; Podzimska-Sroka et al. 2015).

In this chapter, I focus on characterization of ATAF2 to obtain some evidence regarding the biological roles of VNI2 and ATAF2 complex. First, to isolate the promoters effectively regulated by ATAF2. Among 21 promoters were used as reporter constructs, ATAF2 effectively upregulated the reporter genes driven by *ORE1* promoter, a key regulator of senescence (Figure 10). Thus, I investigated further whether ATAF2 regulates senescence by using the loss-of-function and gain-of-function plants.

3.2 Materials and methods

Solutions and media

GM media (1L)

Reagents	Amount
MS plant salt mixture	1 packet
Sucrose	5 g
5% MES-KOH	10 mL
500 X vitamin mix	2 mL
Gellan gum	6 g

Media were autoclaved at 121ºC for 15 min and then transferred to plates. Plates were stored at 4ºC.

5% MES-KOH (1L)

2-(N-Morpholino) ethane sulfonic acid: MES	50 g

Stored at 4ºC

500 X Vitamin mix

Reagents	Amount (g)
myo-Inositol	50
Thiamine hydrochloride	10
Pyridoxine hydrochloride	0.5
Nicotinic Acid	0.5

Stored at -30ºC

PPM solution (100 mL)

Reagents	Amount (mL)
PPM	3
1g/L $MgCl_2$	5
Distilled water	98

1000 X B5 vitamin solution (100 mL)

Reagents	Amount
myo-Inositol	10 g
Thiamine hydrochloride	1 g
Pyridoxine hydrochloride	100 mg
Nicotinic Acid	100 mg

Sterilize with filtration and store at 4ºC

Vector construction

The coding sequences of (CDSs) of NAC domain transcription factors and promoter fragments were amplified by polymerase chain reaction (PCR) with the gene specific primer sets (Table S1). Effector constructs were prepared as described in materials and methods in chapter 2. The amplified fragments were subcloned into the pENT/D-TOPO vector (Life Technology) to generate the entry clones. The promoter fragments contained vectors were integrated into the pAGL Gateway destination vector containing the firefly *luc* (Endo et al. 2015). *ATAF2* cDNA contained pENT/D-TOPO vector was integrated to pH2GW7 to transformation of plants.

Plant materials

The *Arabidopsis thaliana* wild type (Columbia – 0 (Col)) and T-DNA insertion plants were obtained from Arabidopsis biological resource center (SALK_136355C). Plants were grown in Jiffy-7 soil pots (Sakata Seed) under a 16-h-light/8-h-dark cycle.

***ATAF2* overexpression construct**

The resultant plasmids were electroporated into *Agrobacterium tumefaciens* strain *GV3101/pMP90*, which was used to transform *Atabidopdis* Col-0. The T1 generation of transgenic seedlings was selected on GM medium supplemented with 20 µg/mL hygromycin under continuous light 15 days. The plants were transferred to antibiotic-free medium for 7 days and then planted in Jiffy 7 pots under continuous light.

Expression analysis

RNA from 5th or 7th leaves of wild-type plants, transgenic plants or T-DNA insertion lines was extracted using RNeasy mini prep kit (Qiagen). cDNA was synthesized from 400 ng total RNA using SuperScript II reverse transcriptase (Invitrogen). qPCR reactions were performed with 7300 read Time PCR system (Applied Biosystems) and the primer sets shown in Table S1.

Dual luciferase transient expression assay

The effector, reporter, and reference plasmids were used to transfect Arabidopsis protoplasts, which were obtained from 3- to 4-week-old leaves of plants that were grown under long-day conditions, via a PEG transformation method (Sakamoto et al. 2016). Luciferase activities were measured with the Dual-Luciferase Reporter Assay System (Promega, http://www.promega.com) using a Mithras LB940 Multimode Microplate Reader (Berthold, http://berthold.com).

Chlorophyll analysis

Chlorophyll was extracted with *N*,*N*-dimethylformamide from *Arabidopsis* leaves. The extracts were spectrophotometrically measured at wavelengths of 646.8 and 663.8 nm (Porra et al. 1989).

3.3 Results

ATAF2 acts as both transcriptional activator and repressor

As shown in chapter 2, ATAF2 potentially has transcriptional activation activities. To find downstream genes whose expression is upregulated by ATAF2, reporter constructs were prepared containing the candidate promoters connecting to firefly *luc* (Figure 10A). Totally, 21 genes were chosen (Figure 10) as the candidate genes of ATAF2 according to the previous reports (Tran et al. 2004; Delessert et al. 2005; Wang et al. 2009; Yang et al. 2011; Huh et al. 2012; Wang et al. 2012; Jenson et al. 2013; Kim et al. 2014; Peng et al. 2015; Garapati et al. 2015a; Takahasi et al. 2015). Effector plasmids containing *35S* promoter linked to *ATAF2* cDNA was delivered to *Arabidopsis* protoplasts with a reporter plasmid. Results showed that ATAF2 did not upregulate most of the reporter constructs (Figure 10). Rather, some of the reporters, such as under controls of *NIT2* and *ANAC055* promoters, were downregulated by ATAF2 (Figure 10B). By contrast, the only *ORE1* promoter was significantly upregulated by ATAF2 (Figure 10B). ORE1, a NAC domain transcription factor, is known as one of principle senescence regulator (Rauf et al. 2013). It has been reported that *ORE1* expression is directly regulated by ATAF1, closely related to ATAF2 (Garapati et al. 2015a).

I investigated ATAF2 transcriptional activity further by using SRDX fused ATAF2 (Nagahage et al. 2018). The SRDX motif is known as a strong transcriptional repression motif modified from the repression domain of SUPRMAN (SUP; Hiratsu et al. 2003). When this motif is fused to transcriptional activators, the chimeric transcription factors convert into the dominant repressors (Hiratsu et al. 2003). The reporter gene expression driven by *ORE1* promoter was significantly downregulated by ATAF2-SRDX, suggesting that ATAF2 has a direct influence on activating *ORE1* expression.

ATAF2 binds to the promoter region of *NITRILASE2* (*NIT2*) encoding a nitrilase, which converts indole-3-acetonitrile (IAN) to an auxin, indole-3-acetic acid (IAA) (Huh et al. 2012). When *NIT2* promoter was used as a reporter, both ATAF2 and ATAF2-SRDX significantly downregulated the luciferase activity (Nagahage et al. 2018). This data strongly suggested that ATAF2 represses *NIT2* expression.

In addition, Peng et al. (2015) showed that *BAS1* expression is downregulated by ATAF2. However, ATAF2 did not downregulate reporter which contained *BAS1* promoter (Nagahage et al. 2018). In contrast, ATAF2-SRDX significantly upregulated *BAS1* expression suggesting an existence of a transcriptional cascade for *BAS1* expression.

These results suggested that ATAF2 has a very complex molecular function to regulate target genes (Figure 19).

ATAF2 upregulates several transcriptional factors that promote senescence

Since a senescence regulator, *ORE1*, is upregulated by ATAF2, I thought that it was worth to check whether ATAF2 also regulates other senescence regulatory transcriptional factors in the transient assay system. *ORS1* and *ORE1* are phylogenetically closely related proteins (Ooka et al. 2003). Further, Balazadeh et al. (2011) reported that ORS1 as a positive senescence regulator in salt and H_2O_2-dependent signalling pathways. ANAC046 is belonging to the same subgroup of ORE1, and shares some function of ORE1 and ORS1 in senescence (Oda-Yamamizo et al. 2016). In addition, it has a specific function in controlling the expression of chlorophyll catabolic genes, and senescence-associated genes (Oda-Yamamizo et al. 2016).

Reporter constructs containing *ORS1* and *ANAC046* promoters were prepared to check the transcriptional activity of ATAF2 (Figure 11A). As shown in Figure 11B, as well as *ORE1*, *ORS1* and *ANAC046* were upregulated by ATAF2. These results strongly suggested that ATAF2 plays role in the promotion of senescence through regulation of these transcription factors.

Overexpression of *ATAF2* accelerates senescence

To examine whether ATAF2 plays a pivotal role in senescence, I generated *ATAF2* overexpression lines (*ATAF2OX-3-1*, *ATAF2OX-2-1*) which were homozygous single insertion (Figure 12A), and obtained T-DNA inserted loss-of-function line (*ataf2*) to observe the phenotype (Figure 12A). *ATAF2OX-3-1* had the highest *ATAF2* expression level while *ATAF2OX-2-1* had slightly high ATAF2 expression compared with wild-type (Figure 12B). Consistent with previous data (Delessert et al. 2004; Huh et al. 2012), *ATAF2OX-3-1* lines exhibited leaf-yellowing phenotype (Figure 13). ATAF2 loss-of-function line did not show a clear difference compared to wild-type. In order to quantify the senescence phenotype,

chlorophyll content was measured in *ATAF2OX-3-1*, *ATAF2OX-2-1* together with *ataf2* (Figure 14). Consistent with the phenotype, *ATAF2OX-3-1* showed the lowest chlorophyll content while *ataf2* did not show a significant difference compared to wild-type (Figure 14). Likewise, *ATAF2OX-2-1* showed neither clear senescence accelerating phenotype nor lower chlorophyll content compared to the wild-type (Figure 14). It was possible that expression level in *ATAF2OX-2-1* line was not enough to promote senescence (Figure 12A).

Expression levels of genes associated with senescence were altered in *ataf2 and ATAF2OX-3-1* lines

To clarify the senescence phenotype associated with ATAF2 more deeply, expression of *ORE1*, *ORS1*, *ANAC046* and *SAG12* (senescence-associated gene), were measured (Figure 15). In 45 days old plants, expression levels of *ORE1*, *ORS1*, *ANAC046*, and *SAG12* were significantly higher in *ATAF2OX-3-1* line than those in wild-type (Figure 15A). In contrast, the expression levels of aforesaid genes in *ATAF2OX-2-1* were comparable with those of wild-type plants (Figure 15A). It might be possible that the expression level of *ATAF2* in *ATAF2OX-2-1* was just slightly higher than that in wild-type while *ATAF2* transcript was highly accumulated in *ATAF2OX-3-1*(Figure 12A). Besides, *ataf2* did not show a significant difference in expression level of *ORE1*, *ORS1*, and *SAG12* compared to wild-type while the elevated expression level of *ANAC046* was observed in *ataf2*, suggesting that ATAF2 homologs may compensate ATAF2 function at this age.

To investigate the expression levels of the senescence-associated genes at the later stage, I also extracted RNA from 55 day-old-plants of wild-type and *ataf2* (Figure 15B). In wild-type plants, the expression level of the above genes was significantly elevated in 55-day-old plants compared to 44-day-old plants (Figure 15A). By contrast, the elevation of the expression of these genes was significantly reduced in *ataf2* (Figure 15B). These results suggested that ATAF2 promotes natural senescence at a later stage.

***ATAF2* is involved in dark-induced senescence**

It was reported that *ATAF2* is transcriptionally suppressed by light (Peng et al. 2015). Thus, I speculated that dark-induced senescence might involve ATAF2. Fifth leaeves of 4-weeks-old

plants were incubated for 5 days on wet filter papers. Control samples were incubated under the continuous light while treatment samples were incubated under the dark condition. *ataf2* exhibited senescence delaying phenotype while *ATAF2OX-3-1* showed senescence accelerating phenotype compared to wild-type (Figure 16). To quantify the intensity of the senescence, chlorophyll content of the leaves were measured (Figure 17). Consistent of the observation of the leaves, the loss of chlorophyll contents in *ataf2* and in *ATAF2OX-3-1* were milder and greater, respectively, than those in wild-type (Figure 16). *ATAF2OX-2-1* line expression level of ATAF2 is not so high compared to wild-type. It could be the reason that this line phenotype is similar to the wild-type and similar chlorophyll content to the wild-type. These data indicated that ATAF2 promotes senescence in response to the dark condition.

3.4 Discussion

ATAF2 is reported as a transcriptional activator as well as transcriptional repressor (Huh et al. 2012; Peng et al. 2015; Wang et al. 2009), and a number of candidates of the direct target genes of ATAF2 have been identified (Huh et al. 2012; Peng et al. 2015; Wang et al. 2009). The consensus DNA binding sequence for ATAF2 (TCAGAAGAGCAATCAAATTAAAACACATAT) has been proposed by Wang et al. (2012). Delessert et al. (2005) reported that ATAF2 represses expression of a number of pathogenesis-related genes including *PR1*, *PR2* and *PDF1.2* while Wang et al. (2009) showed that ATAF2 activates the *PR1*, *PR2* and *PDF1.2* expression. It was originally shown that ATAF2 was a transcriptional activator for *NIT2* (Huh et al. 2012). However, *NIT2* was repressed by ATAF2 in my study (Figure 18). It might be possible that ATAF2 is able to upregulate and downregulate expression of some target genes including *NIT2* in response to environmental conditions. Peng et al. (2015) showed that *BAS1* expression is downregulated by ATAF2. However, ATAF2 was unable to repress the *BAS1* expression in the transient assay system. Interestingly, ATAF2-SRDX, which was converted into a strong transcriptional repressor, upregulates *BAS1* expression (Nagahage et al. 2018; Figure 18). Thus, this result suggested the existence of a transcriptional cascade for *BAS1* expression (Nagahage et al. 2018; Figure 18). There is a report showing similar case; A member of TEOSINETE BRANCHED1, CYCLOIDEA and PCF (TCP), TCP3 upregulates expression of *miR164* which negatively regulates *CUC* genes (Baker et al. 2005; Laufs et al. 2004; Mallory et al. 2004). Overexpression of *TCP3-SRDX* induces ectopic expression of *CUC* genes (Koyama et al. 2007). As in the case of TCP3, ATAF2 may activate unknown transcriptional factor or microRNA that represses *BAS1* (Nagahage et al. 2018). Although ATAF2 binds to the *BAS1* promoter (Peng et al. 2015), the direct regulation of ATAF2 for *BAS1* expression may not largely contribute to the entire regulation of *BAS1* expression. These data suggested that ATAF2 shows complex transcriptional regulation depend on the condition and promoter context (Figure 19).

ORE1 is known as a key regulator of senescence (Rauf et al. 2013), and has been reported as a direct target of ATAF1, closely related to ATAF2 (Garapati et al. 2015a). The reporter gene expression driven by the *ORE1* promoter was significantly upregulated by ATAF2 (Nagahage et

al. 2018; Figure 11), suggesting that ATAF2 would be associated with senescence. Consistent with this hypothesis, Interestingly, my data shows that *ORS1* and *ANAC046* can be upregulated by ATAF2 (Figure 11). As well as *ORE1*, *ORS1*, and *ANAC046* are known as positive senescence regulators (Balazadeh et al. 2011; Rauf et al. 2013; Oda-Yamamizo et al. 2016). *ORE1* binds to the promoters of a number of senescence-associated genes, such as *SAG29/SWEET15* and *SINA1 in vivo*. *ORS1* also regulates senescence-associated genes but the number of target genes of *ORS1* seems to be less than *ORE1* (Balazadeh et al. 2011). ANAC046 directly binds to the promoter regions of *NYC1*, *SGR1*, *SGR2* and promotes senescence (Oda-Yamamizo et al. 2016). Although *ORS1*, *ORE1*, and *ANAC046* all regulate senescence, how the expression of these transcription factors is regulated has not been understood well. *ORE1* is upregulated by EIN3, and complex regulation was discovered with miRNA164. (Kim et al. 2009; Kim et al. 2014). Further, *ORE1* is directly regulated by ATAF1 under oxidative stress and ABA-mediated pathway (Garapati et al. 2015). Previously, *ORE1*, *ORS1* and *ANAC046* are reported as ABA-dependent senescence mediation. ATAF1 is also reported as an ABA-dependent senescence regulator on *ORE1*. However, ATAF2 is neither upregulated nor downregulated by ABA. Thus, regulation of senescence by ATAF2 should be independent on the ABA-mediated pathway. In addition, these three transcription factors have different functions in senescence (Balazadeh et al. 2011; Rauf et al. 2013; Oda-Yamamizo et al. 2016). Thus, it is possible that ATAF2 regulates them in a different way depending on the condition.

In my data, *ATAF2* overexpression line (*ATAF2OX-3-1*) showed leaf yellowing phenotype compared to wild-type (Figure 13). This phenotype was consistent with previous data which were described independently (Delessert et al. 2005; Huh et al. 2012). The expression level of *ATAF2OX-3-1* was significantly higher compared to wild-type (Figure 12). However, *ATAF2OX-2-1* expression level was not so high compared with wild-type (Figure 12). Thus, it is likely that leaf yellowing phenotype is observed dependent on the expression levels of exogenous *ATAF2* (Figure 13). Previous data showed that overexpression of *ORE1* and *ORS1* also make leaf yellowing phenotype due to senescence acceleration (Rauf et al. 2013; Qiu et al. 2015). In transient assay shows that ATAF2 upregulates *ORE1* and *ORS1*. Thus, my data suggested that leaf yellowing phenotype of *ATAF2* overexpression is associated with senescence promotion. However, Delessert et al. (2005) suggested that leaf yellowing was not due to a

senescence response. The expression profile of the senescence marker gene *SEN1* was not affected, nor could the phenotype be alleviated by adding the cytokinins BAP or zeatin (0.1 or 10 μM, respectively), two potent senescence inhibitors (Singh et al. 1992) to the growth media (Delessert et al. 2005). Furthermore, resluts of microarray analyses did not show that *ATAF2* expression is not changed by ethylene (Delessert et al. 2004). In contrast, this study shows that specific senescence marker gene, *SAG12* (Gan and Amasino, 1995; Noh and Amasino, 1999a,b) expression was elevated in *ATAF2OX-3-1* together with *ORE1, ORS1,* and *ANAC046* (Figure 15). Interestingly, *ATAF2* expression is not responded to common senescence promoting hormone ABA (Delessert et al. 2005). Thus, it is possible that the ATAF2 senescence promotion pathway via *ORE1, ORS1,* and *ANAC046* is ABA and cytokinin-independent.

In my data, *ataf2* single mutant did not show a clear difference compared to wild-type (Figure 13). ANAC102 is the closest homolog to ATAF2 and predicted as a senescence regulator (Kim et al. 2014). ATAF1 is also a close homolog to ATAF2 and known as a senescence regulator (Garapati et al. 2015a). Besides, ANAC032 is also a close homolog to ATAF2 and reported as a regulator of senescence in stress and age-dependent manner. Takasaki et al. (2015) showed that seven NAC domain proteins including ATAF2, ATAF1, ANAC032, and ANAC102 play crucial roles in ABA-induced leaf senescence signalling. Collecting together, above information suggested that there might be a functional redundancy among ATAF2 and close homologs. Thus, it could be possible that *ataf2* single mutant did not show clear phenotype regarding natural senescence.

I further analysed the expression level of *ORE1, ORS1, ANAC046,* and *SAG12* (Figure 15). There was a significantly high expression of the above genes in *ATAF2OX-3-1* in 45 days old plants compared to wild-type. However, the gene expression was not altered in *ataf2* suggesting that there may be a functional redundancy to compensate the *ataf2* loss of function. In contrast, the above genes expression was significantly lower in the *ataf2* plants compared to wild-type in the older plants (Figure 15B). These data suggested that *ataf2* single mutant affects the downstream target genes though it is not enough to delay senescence.

In addition, I observed the effect of dark condition on plants which their *ATAF2* expression is altered (Figure 16). Interestingly, *ataf2* shows that senescence accelerating phenotype while

ATAF2 overexpression gave the opposite phenotype compared to wild-type (Figure 16). It is known that the *ATAF2* expression is modulated by light condition (Delessert et al. 2005; Peng et al. 2015). *ATAF2* expression is elevated under the dark condition and downregulated by application of sucrose (Delessert et al. 2005). Garapati et al. (2015b) highlighted the existence of a positively acting feedforward loop between *ATAF1* expression and carbon utilization of carbon stock. Namely, *ATAF1* is induced by carbon starvation, and ATAF1 regulates the genes involved in the depletion of cellular carbon/energy pools. Chung et al. (1997) suggested that low sugar levels might be natural inducers of senescence. In addition, Thimann et al. (1977) showed that chlorophyll degradation could be prevented by sugar treatment. These data suggested that dark condition reduces the photosynthesis activity resulting in low sugar condition. Furthermore, senescence is known to associate with nutrient recycling process. When leaves reduce the sugar assimilation due to low light condition, senescence is induced accompanied with relocation of nutrients to the sink. *ATAF2* is known to be a cytochrome A responsive gene (Peng et al. 2015). Thus, the low light condition may induce *ATAF2*, and ATAF2 may lead leaf to the senescence through the stress-induced senescence pathway. These data suggested that ATAF2 seems to be involved in light-dependent senescence mediating energy starvation (Figure 20).

3.5 Figures

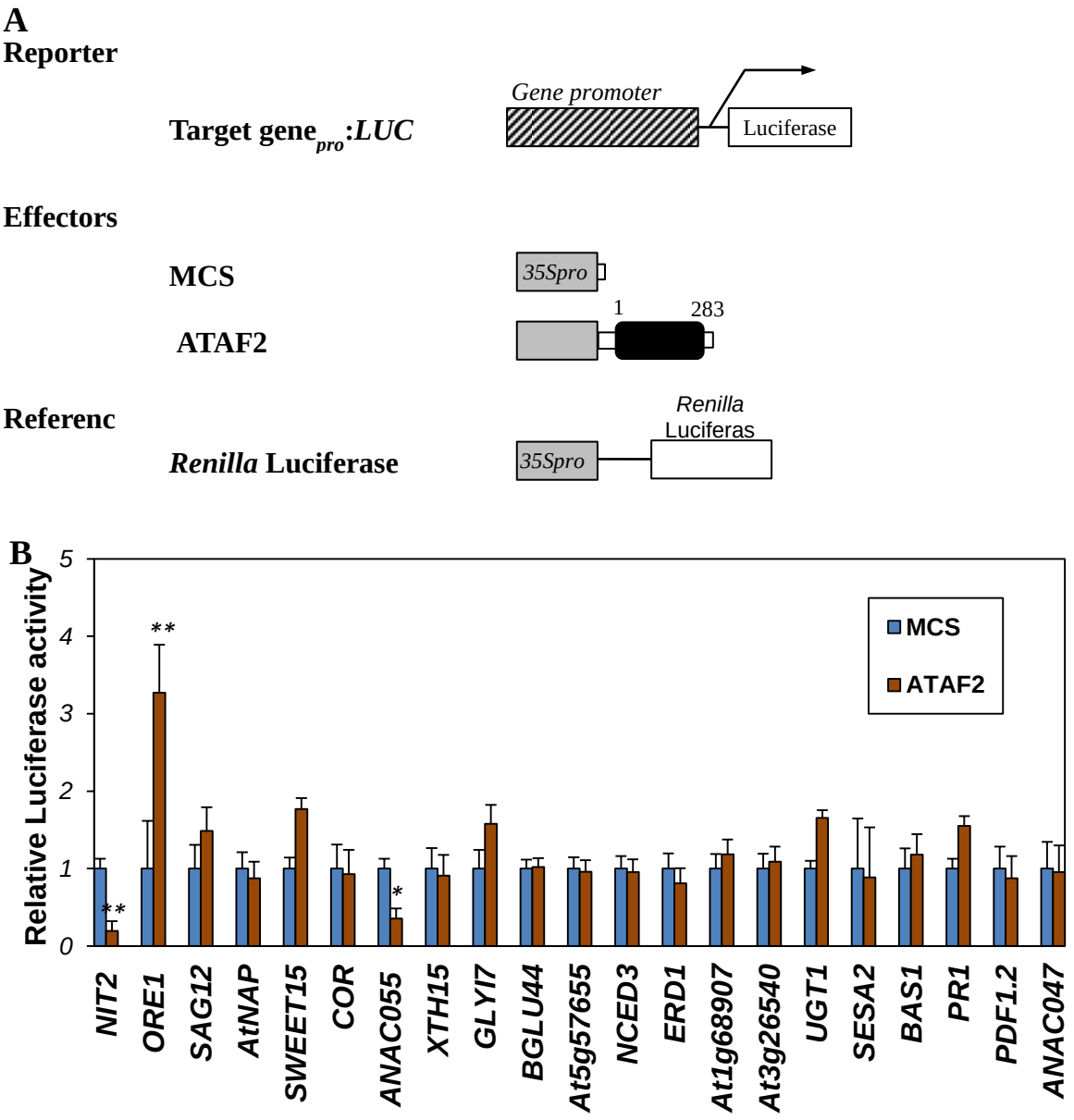


Figure 10. Identification of possible downstream target genes, which can be regulated by ATAF2. (A) Schematic diagram of the effector and reporter constructs. The effector constructs contain the MCS and ATAF2 driven by the *35S* promoter. The reporter constructs contained the promoters of potential direct target genes with a min pro (B) Results of the transient transfection assay. Firefly luciferase activities were normalized by *Renilla* luciferase activities. Error bars indicate SD (n = 4). Asterisks indicate statistically significant differences (Students t-test **p < 0.01 *p < 0.05) compared to the MCS vector control.

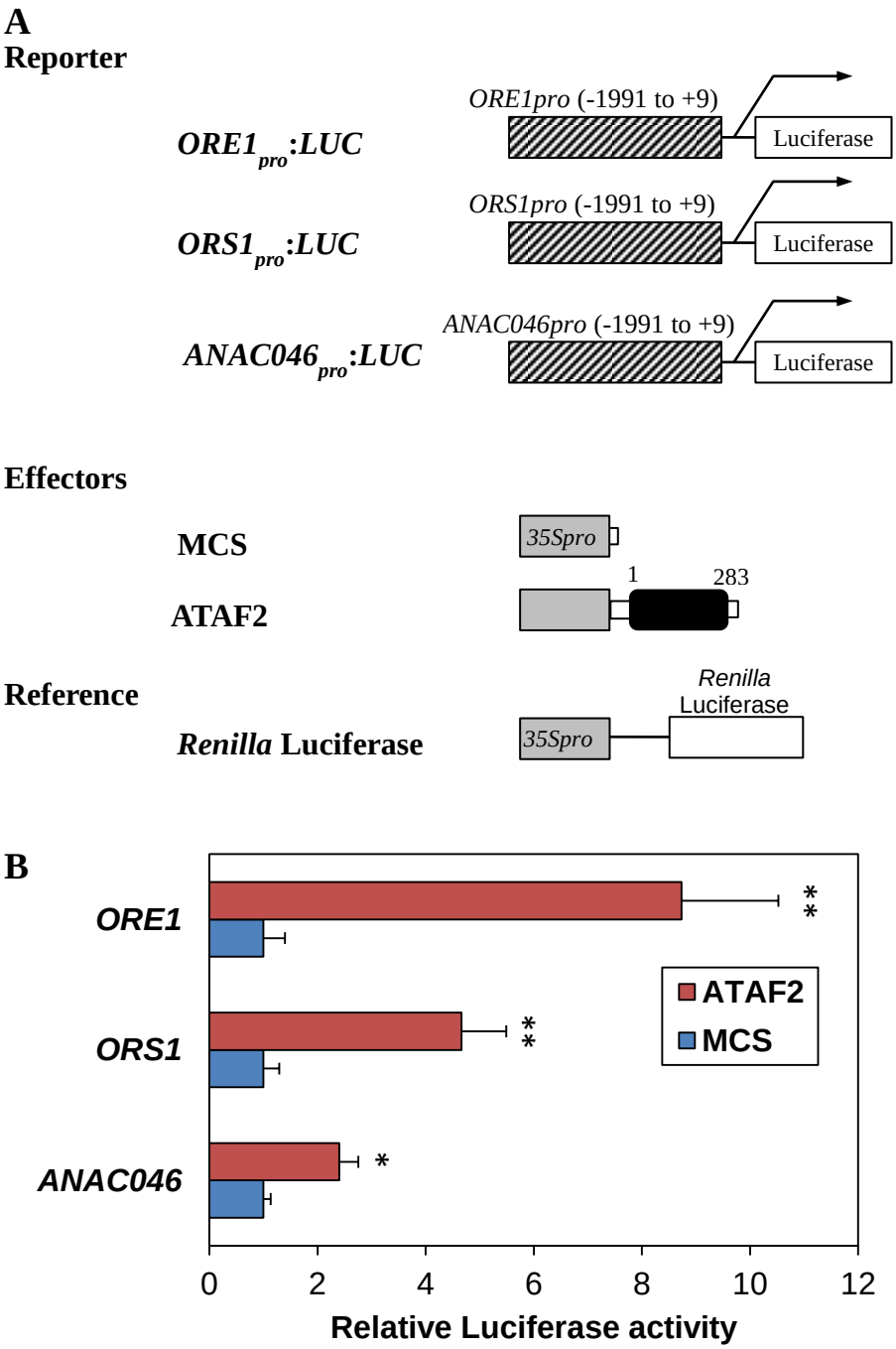


Figure 11. ATAF2 transcriptionally activates senescence regulatory transcriptional factors.

(A) Schematic diagram of the effector and reporter constructs. The effector constructs contain the MCS and ATAF2 driven by the *35S* promoter. The reporter constructs contained the promoters of *ORE1*, *ORS1*, and *ANAC046* with a min pro (B) Results of the transient transfection assay. Firefly luciferase activities were normalized by *Renilla* luciferase activities. Error bars indicate SD (n = 4). Asterisks indicate statistically significant differences (Students t-test **p < 0.01; *p < 0.05) compared to the MCS vector control.

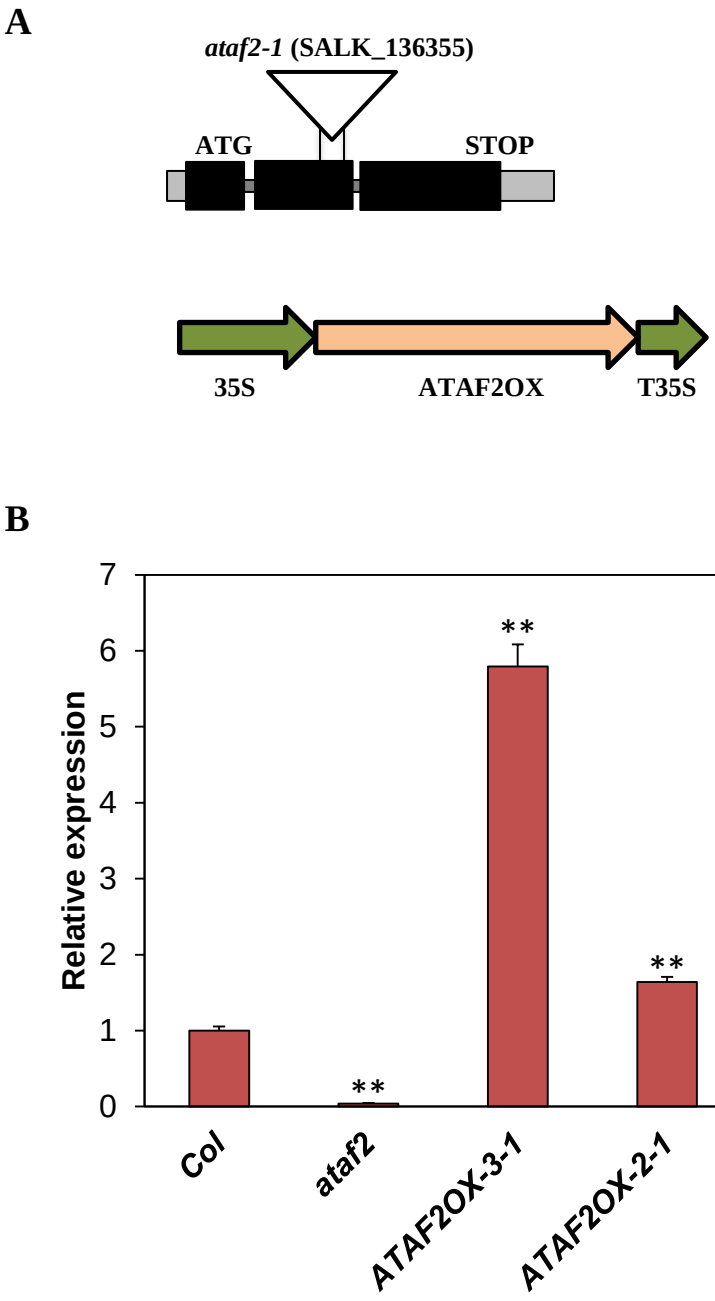


Figure 12. (A) Schematic representation of a T-DNA insertion *ataf2* mutant line of *Arabidopsis*. Schematic representation of the *ATAF2* overexpression construct. (B) Relative expression level of *ataf2, ATAF2OX-3-1* and *ATAF2OX-2-1* compared to control. Error bars indicate SD (n = 4). Asterisks indicate statistically significant differences (Students t-test **$p < 0.01$) compared to the control.

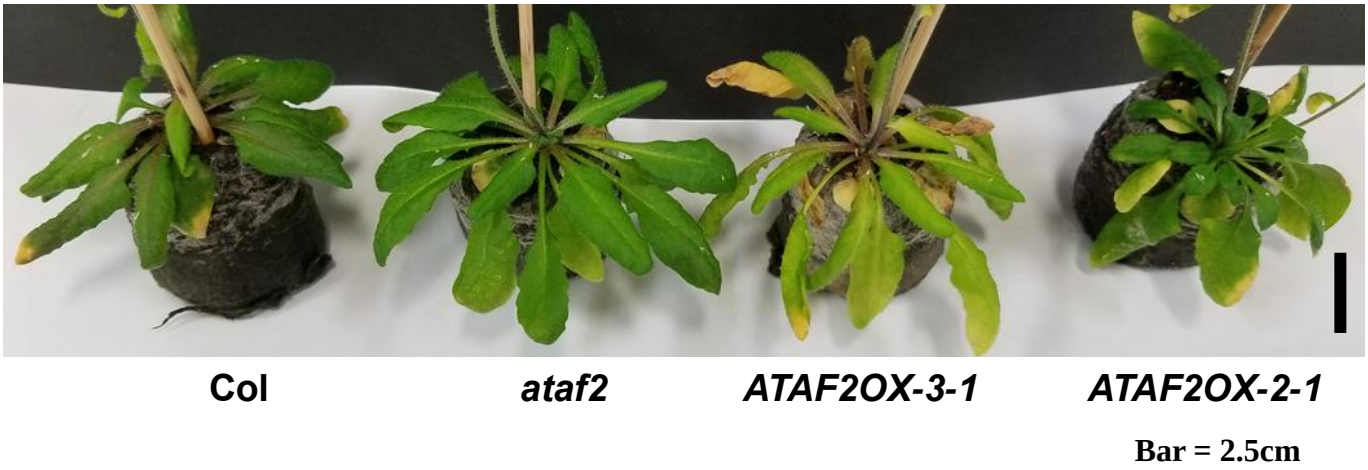


Figure 13. *ATAF2* overexpression causes senescence accelerating phenotype. Whole-plant phenotype of Col, *ataf2*, *ATAF2OX-3-1* and *ATAF2OX-2-1* at 45 d after germination under the long day condition. The scale bar represents 2.5 cm.

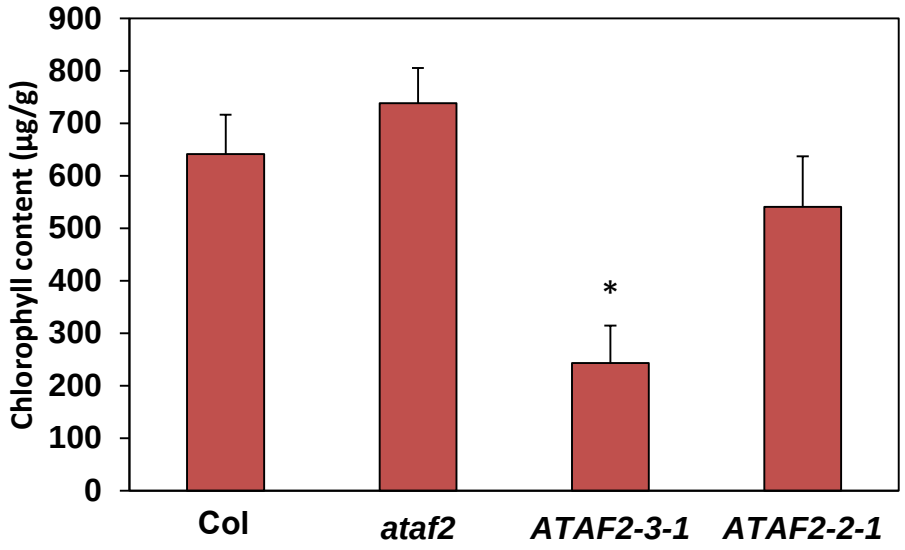


Figure 14. Chlorophyll contents of 45-day-old plants. 7th leaves were used. Error bars indicate SD (n = 4). Asterisks indicate statistically significant differences (Students t-test *p < 0.05) compared to the control.

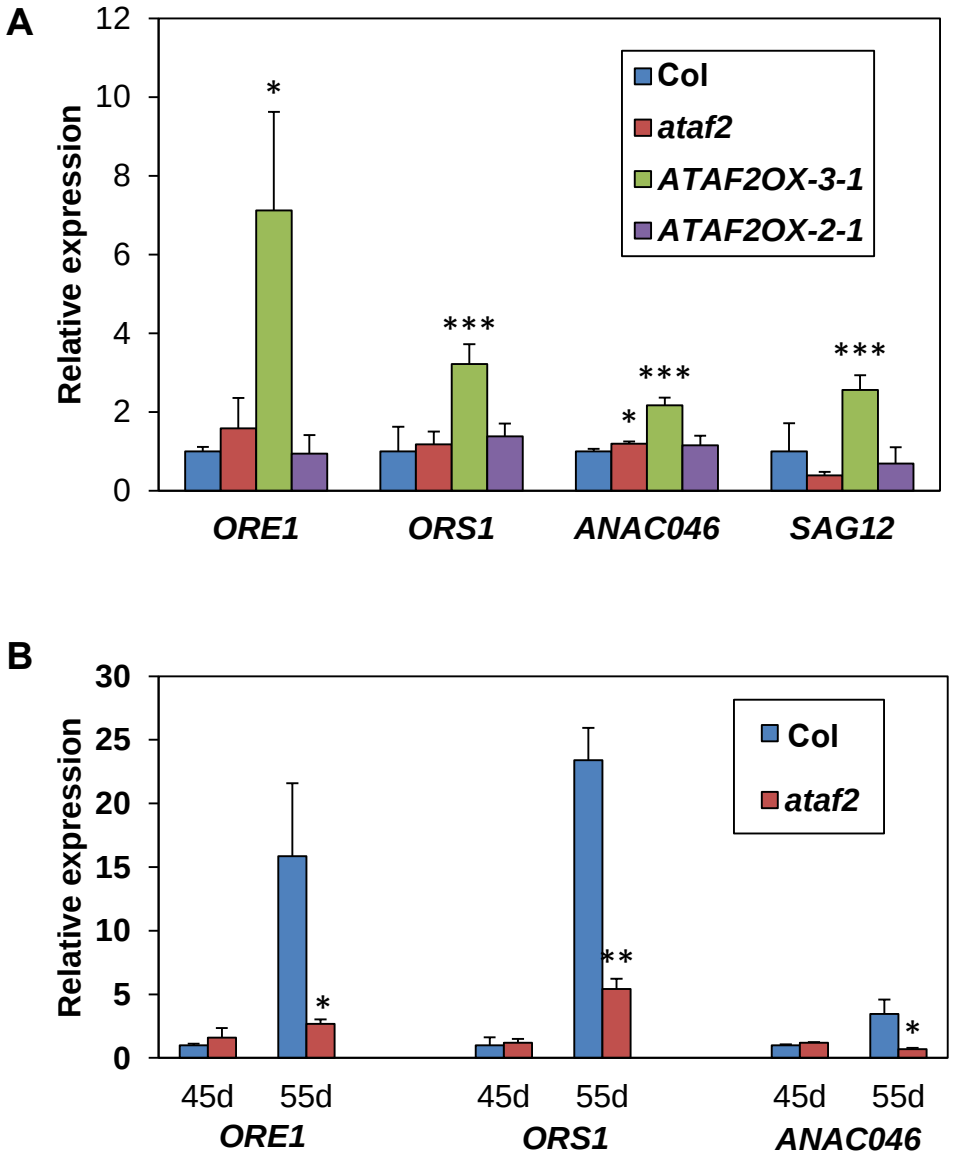


Figure 15. Expression of senescence associated gene *SAG12* and senescence transcriptional factors *ORE1*, *ORS1,* and *ANAC046.* (A) *SAG12* expression level in *ataf2* and control at 45-day- and 55-day-old plants. (B) *ORE1, ORS1,* and *ANAC046* expression level in *ataf2, ATAF2OX-3-1* and *ATAF2OX-2-1* at 45-day-old plants. Error bars indicate SD (n = 3). Asterisks indicate statistically significant differences (Students t-test ***p < 0.001 *p < 0.05) compared to the control.

Figure 16. The *ataf2* and *ATAF2OX-3-1* have senescence delaying, and accelerating phenotype, respectively, under dark condition. 5th leaf of 4-weeks old plants was incubated in moisture filter papers for 5 days in continuous light- or continuous dark-condition. Error bars indicate SD (n = 4). The scale bar represents 1 cm.

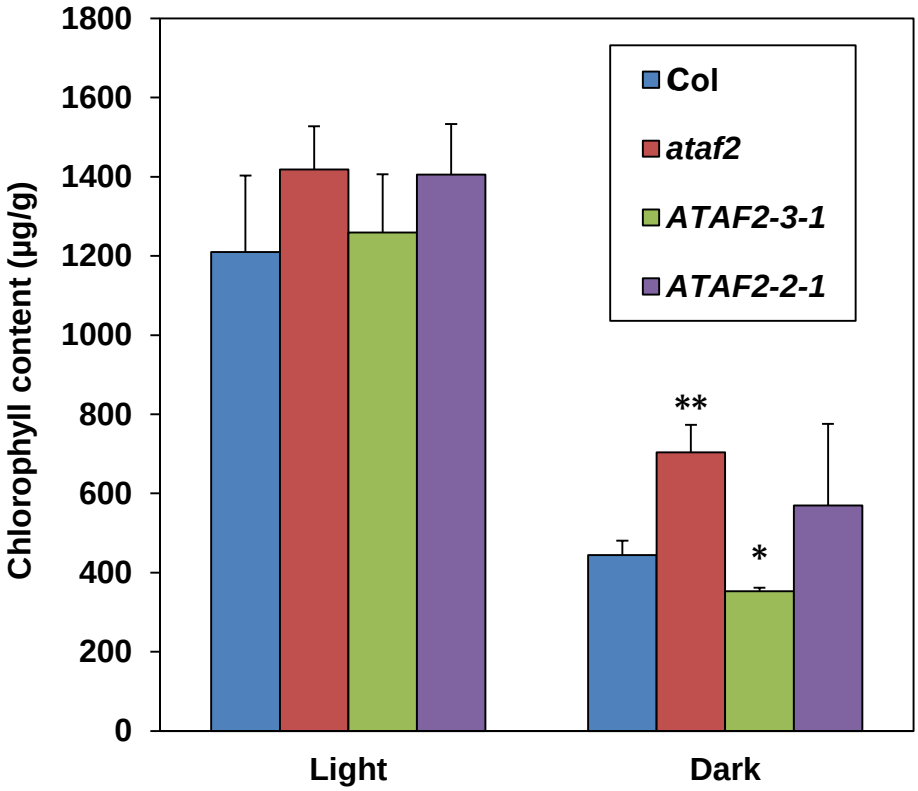


Figure 17. Chlorophyll content of the 5th leaves incubated under continuous light or dark condition. 4-weeks-old-plant were used. Error bars indicate SD (n = 4). Asterisks indicate statistically significant differences (Students t-test **$p < 0.01$ *$p < 0.05$) compared to the control.

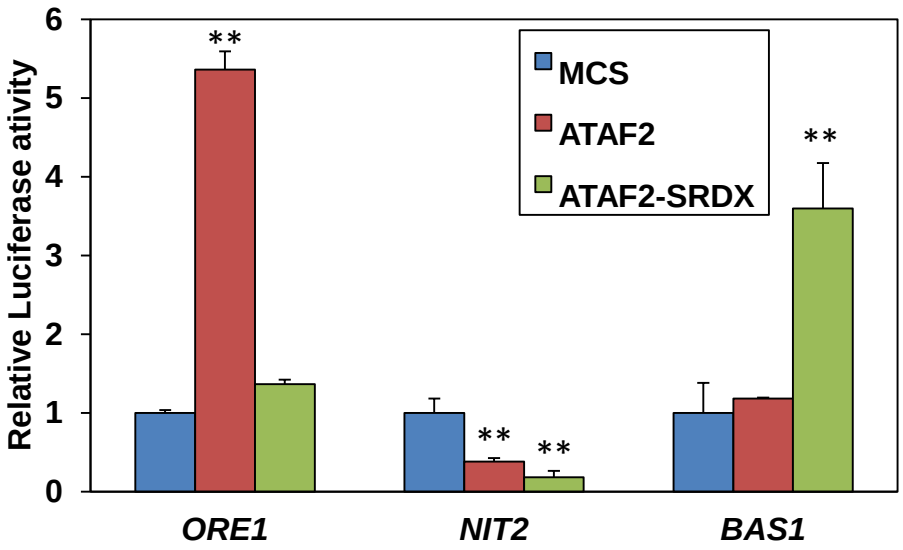


Figure 18. ATAF2 has bifunctional activities. (A) Schematic diagrams of the constructs. The reporter constructs contain the promoter of *ORE1*, *NIT2*, or *BAS1* with the firefly luciferase reporter gene. The effector constructs contain the MCS, *ATAF2*, or *ATAF2-SRDX* driven by the *35S* promoter. (B) Results of the transient transfection assay. Firefly luciferase activities were normalized by *Renilla* luciferase activities. Error bars indicate SD (n = 4). Asterisks indicate statistically significant differences (Students t-test **$p < 0.01$) compared to the MCS.

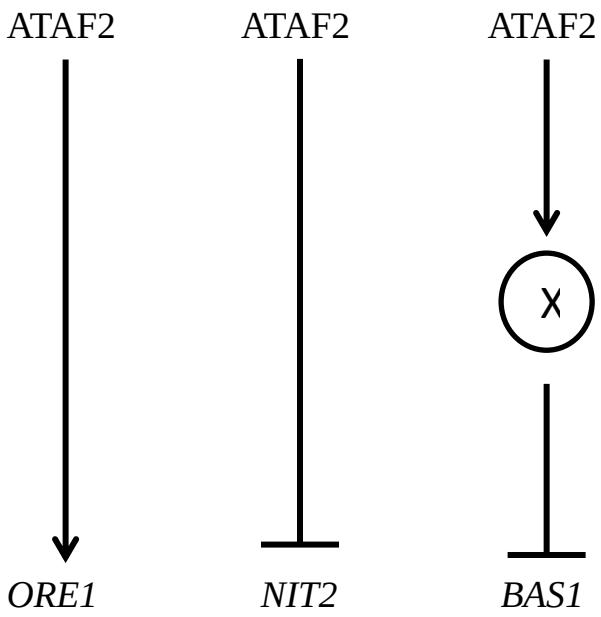


Figure 19. Schematic diagram of the transcriptional activities of ATAF2 accoding to the transient expression assays. ATAF2 activates *ORE1* expression and represses *NIT2* expression. In addition, ATAF2 upregulates unknown factors that negatively regulate *BAS1* expression (X).

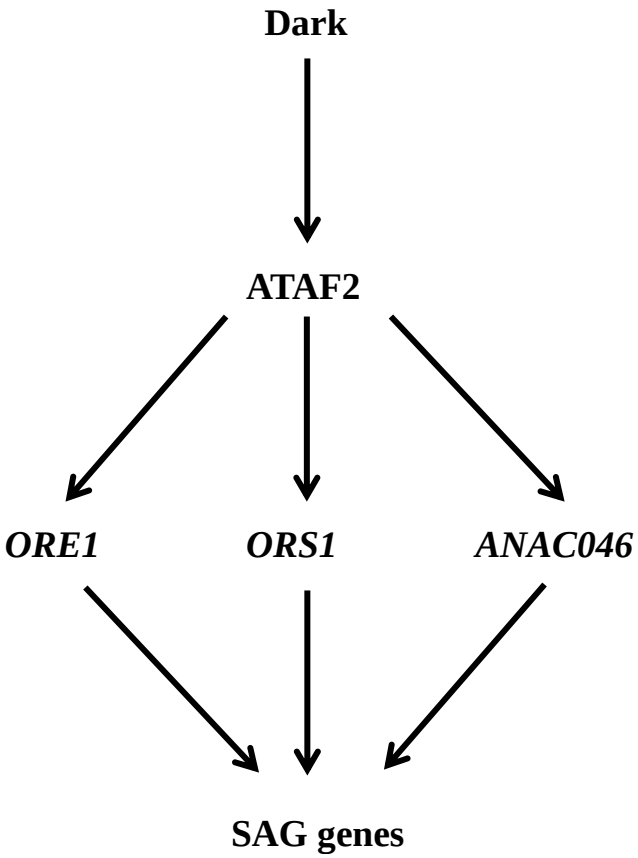


Figure 20. Schematic model of regulation of senescence by NAC domain transcription factors.

Chapter 4

VNI2 inhibits ATAF2 by forming protein complex, resulting in retardation of senescence

4.1 Introduction

Responses to environmental stress responses are complex mechanisms by which plants process to survive in adverse conditions. NAC domain transcription factors, one of the plant-specific families, have been known to be associated with not only their roles in stress and senescence but also in physiological development such as cell proliferation, flowering time, secondary cell wall biosynthesis (Souer et al. 1996; Kim et al. 2006; Yamaguchi et al. 2008).

Among the NAC transcription factor genes identified in Arabidopsis, *VNI2* is of particular interest as it is influenced by both developmental and environmental cues (Yamaguchi et al. 2010b; Yang et al. 2011). VNI2 was originally isolated as VND7 interacting protein, and represses transcriptional activation activities of VND7 (Yamaguchi et al. 2010b). Promoter analysis demonstrated that *VNI2* expression was observed in many types of tissues and organs including phloem, guard cells, roots besides xylem vessels (Yamaguchi et al. 2010b). Consistent with the data, public database GENEVESTIGATOR also shows that *VNI2* is expressed in almost all parts of plants and different stress conditions (https://www.genevestigator.com/gv/index.jsp). These expression profiles suggested a broad range of VNI2 function (Yamaguchi et al. 2010b; Yang et al. 2011). Further studies reported that VNI2 stability is regulated by the PEST motif via proteasome-mediated proteolysis (Yamaguchi et al. 2010b). Yang et al. (2011) have studied with respect to the leaf senescence and integration of abscisic acid signals, and provided important roles of VNI2 in abiotic stress responses.

I showed that ATAF2 is isolated as an interacting protein with VNI2 in chapter 2. In addition, in chapter 3, I found that ATAF2 upregulates some of principle senescence regulators such as *ORE1*, *ORS1*, and *ANAC046*, and promotes senescence. Furthermore, *VNI2*, as well as *ATAF2*, are upregulated in dark condition (Delessert et al. 2004; Yang et al. 2011; Peng et al. 2015). Light condition is an external environmental factor which influences plant growth such as photosynthesis and senescence. Senescence is a well-programmed process but pre-mature senescence can be occurred due to the unfavourable environmental condition. Since VNI2 and ATAF2 make protein a complex, and both are known to be involved in the regulation of senescence and stress, in this chapter, I focused to elucidate the VNI2-ATAF2 interaction during senescence process. I performed the transient assay to investigate how VNI2 regulates ATAF2.

Then, I also tried to obtain evidence for the relationship between VNI2 and ATAF2 by using genetic approaches.

4.2 Materials and methods

Vector construction

The coding sequences of (CDSs) of NAC domain transcription factors and promoter fragments were amplified by polymerase chain reaction (PCR) with the gene specific primer sets (Table S1). Effector constructs were prepared as described in chapter 2 materials and methods. The amplified fragments were subcloned into the pENT/D-TOPO vector (Life Technology) to generate the entry clones. The promoter fragments contained vectors were integrated into the pAGL Gateway destination vector containing the firefly *luc* (Endo et al. 2015). *ATAF2* cDNA contained pENT/D-TOPO vector was integrated to pH2GW7 to transformation of plants.

Plant materials

The *Arabidopsis thaliana* wild type (Columbia – 0 (Col)) and T-DNA insertion plants were obtained from Arabidopsis biological resource center (SALK_136355C, SALK_143793). Plants were grown in Jiffy-7 soil pots (Sakata Seed) under a 16-h-light/8-h-dark cycle. Multiple mutant lines were generated by crossing.

Expression analysis

RNA from 5^{th} or 7^{th} leaves of wild-type plants, transgenic plants or T-DNA insertion lines was extracted using RNeasy mini prep kit (Qiagen). cDNA was synthesized from 400 ng total RNA using SuperScript II reverse transcriptase (Invitrogen). qPCR reactions were performed with 7300 read Time PCR system (Applied Biosystems) and the primer sets shown in Supplemental Table S1.

Dual luciferase transient expression assay

Experiment was carried out according materials and method in chapter 2.

Chlorophyll analysis

Chlorophyll was extracted with *N*,*N*-dimethylformamide from *Arabidopsis* leaves. The extracts were spectrophotometrically measured at wavelengths of 646.8 and 663.8 nm (Porra et al. 1989).

ATAF2 overexpression construct

The resultant plasmids were electroporated into *Agrobacterium tumefaciens* strain *GV3101/pMP90*, which was used to transform *Atabidopdis vni2*. The T1 generation of transgenic seedlings was selected on GM medium supplemented with 20 µg/mL hygromycin under continuous light 15 days. The plants were transferred to antibiotic-free medium for 7 days and then planted in Jiffy 7 pots under continuous light.

4.3 Results

VNI2 represses the transcriptional activation activity of ATAF2

In the previous chapters, I showed that ATAF2 is a transcriptional activator and upregulates senescence regulators, such as *ORE1*, *ORS1*, and *ANAC046* (Figure 11). To investigate whether VNI2 is able to change the expression of the senescence regulator *ORE1* transactivated by ATAF2, I constructed effector plasmids containing the *35S* promoter linked to *VNI2* or *ATAF2* cDNAs, and delivered them into *Arabidopsis* protoplasts with a reporter plasmid containing an *ORE1* promoter (Figure 21A). Results showed that VNI2 repressed *luc* expression transactivated by ATAF2 (Figure 21B), indicating that VNI2 inhibits transcriptional activation activity of ATAF2.

VNI2 modulates senescence by depending on ATAF2

To examine whether VNI2 is involved in the senescence by forming a protein complex with, and controlling ATAF2, the genetic approach was carried out. I observed the senescence phenotype of *vni2*, *ataf2*, and *vni2 ataf2* mutants (Figure 22) together with wild-type plants under the long day condition. Consistent with previous data (Yang et al. 2011), *vni2* mutants exhibited accelerated senescence phenotype (Figure 23). On the other hand, *ataf2* mutants did not show any difference compared to wild-type (Figure 23). Interestingly, senescence acceleration phenotype of *vni2* was abolished under *ataf2* mutant backgroud, suggesting that VNI2 regulates senescence depending on ATAF2 function (Figure 23). Then, I tried to quantify the senescence by measuring the chlorophyll content using the 7^{th} leaves (Figure 24). Convincing the above phenotype, *vni2* had lowest chlorophyll content and *vni2 ataf2* had intermediate chlorophyll content (Figure 24). *ataf2* did not show any significant difference compared to wild-type (Figure 24). In *vni2* mutants, ATAF2 seems to be able to upregulate senescence regulators such as *ORE1* without inhibition of VNI2. Under the *ataf2* background, senescence is neither accelerated nor delayed possibly due to the functional redundancy of ATAF2 homologs. In the *vni2 ataf2* background, ATAF2 was absent to accelerate senescence via *ORE1* even though missing of the VNI2 transcriptional repressor. These data suggested that *VNI2* has a genetic interaction with *ATAF2*, and regulates senescence by inhibiting ATAF2 function.

I further examined the VNI2 and ATAF2 interaction by generating *ATAF2* overexpression lines under the *vni2* background (*ATAF2OXvni2-1*) (Figure 25). As I expected, *ATAF2OXvni2-1* line showed the highest senescence accelerating phenotype compared to wild-type (Figure 25).

Expression levels of a senescence marker gene are altered in *vni2* and *vni2 ataf2*

To further examine the senescence phenotypes of *vni2*, *ataf2*, and *vni2 ataf2* together with wild-type, senescence marker gene *SAG12* expression was measured. In 30 days plants, *SAG12* expression was almost undetectable (Figure 26A). However, *SAG12* was expressed in 55-day-old plants, suggesting that senescence was progressing at this age (Figure 26A). As I expected, *SAG12* gene expression was significantly higher in *vni2* line than that in wild-type while *ataf2* had the lowest *SAG12* expression. *vni2 ataf2* showed intermediate expression level indicating that the senescence accelerating phenotype in *vni2* mutants is depending on the existence of ATAF2 (Figure 26A). In addition, we examined the *ORE1* expression pattern under these plants (Figure 26B). High *ORE1* expression was detected in wild-type as well as *vni2* while it was much lower in *ataf2* at 55 days after germination (DAG). These data suggested that lack of *ataf2* ceased to induce *ORE1* expression, consistent with the transient assay data in Figure 21. It is noteworthy that the intermediate expression level was detected in *vni2 ataf2* (Figure 26B).

VNI2 and ATAF2 regulates dark-induced senescence

To determine the biological roles of VNI2-ATAF2 complex with respect to the light, I performed the dark-induced senescence assay. The 5th leaves from the 4-week-old *vni2*, *ataf2*, and *vni2 ataf2* mutant lines together with wild-type plants were incubated in moistured filter papers for 5 days under the light or dark condition (Figure 27). No phenotypic changes were observed among lines under the light condition (Figure 27). By contrast, under the dark condition, leaves from *vni2* mutants showed senescence accelerating phenotype while those from *ataf2* showed senescence delay phenotype (Figure 27). The intermediate phenotype was observed in the *vni2 ataf2*. Furthermore, I examined the chlorophyll content (Figure 28). The lowest and highest chlorophyll contents were observed in *vni2* and *ataf2* mutants, respectively (Figure 28). These data strongly suggest that VNI2 and ATAF2 regulate senescence in response to the dark condition.

4.4 Discussion

VNI2 has been characterized as a transcriptional repressor (Yamaguchi et al. 2010). Consistent with the previous data, my data has shown that VNI2 effectively repressed *ORE1* expression transactivated by ATAF2 (Figure 21). *ORE1* is known as a principle senescence regulator. In young plants, the *ORE1* expression is downregulated by the ethylene signalling pathway and *miRNA164* (Kim et al. 2009; Kim et al. 2014). They further implied that there are many routes to ensure leaf senescence and the associated cell death upon ageing additionally to the EIN2-mediated pathway. ATAF1, a close homolog to ATAF2, directly upregulates *ORE1* expression (Garapati et al. 2015a). Thus, ATAF2 may also directly regulate *ORE1*. VNI2 was reported as a senescence mediator via *COR* and *RD* genes in ABA-dependent manner (Yang et al. 2011). However, ATAF2 does not have any effects on ABA. This data suggested that VNI2 delays senescence via inhibiting ATAF2 under ABA-independent pathway.

What is the biological meaning of the VNI2-ATAF2 protein complex during regulation of senescence? Once senescence is induced, this program is irreversible to the end. VNI2 is known to be an unstable protein (Yamaguchi et al 2010b). Therefore, it is possible that VNI2 makes temporary inhibition before unnecessarily onsetting senescence. In addition, *VNI2* expression is gradually being increased when the plant is getting old (Yang et al. 2011). Similarly, the *ATAF2* expression is also detected in older parts of the plants (Dellesert et al. 2005; eFP browser, public database). Senescence is a well-programmed process. The main task of the senescence is nutrient remobilization. Importantly, accelerated senescence process has a negative effect on plant life cycle since macronutrients catabolism, detoxification and transporting to young vegetative parts would be partially inefficient due to lack of time (Gregersen et al. 2013). Thus, senescence rate is also important to the overall productivity of the plant (Richards 2000; Long et al. 2006). Thus, it is also possible that VNI2 inhibits ATAF2 to keep sufficient time for the nutrient recycling process. In addition, the *ATAF2* expression is induced in a dark condition (Peng et al. 2015). Similarly, the *VNI2* expression is also elevated in response to dark (Yang et al. 2011). It is known that dark condition accelerates leaf senescence (Biswal et al. 1984; Liebsch and Keech. 2016). It seems to be important to inhibit pre-mature senescence due to dark condition for a short time. Besides, efficient nutrient mobilization required enough time. Thus, it is possible that

ATAF2 promotes senescence in response to a low light condition, and VNI2 represses ATAF2 to fine-tune the senescence progress for efficient nutrient reallocation.

I showed that natural senescence is accelerated in *vni2* mutants as the previous report (Yang et al. 2011; Figure 26). The expression level of *SAG12* and *ORE1* were also highly elevated in *vni2* (Figure 23). This senescence accelerating phenotype seems to be because of the release of inhibition of ATAF2 function from VNI2 in the mutants. In contrast, *ataf2* and *vni2 ataf2* did not show a clear difference compared to wild-type (Figure 23). The expression level of senescence regulator *ORE1* in *ataf2* was lower than wild type, but delayed senescence phenotype was not observed in *ataf2*, suggesting that *ORE1* expression upregulated by ATAF2 though it is not sufficient for promotion of natural senescence. Interestingly, there was no significant difference in *ORE1* expression between wild-type plants and *vni2* mutants though there was a significant difference in expression levels of *SAG12*, a downstream target of ORE1 (Figure 26A). In addition, senescence accelerating phenotype was abolished under the *vni2 ataf2* background (Figure 23), suggesting that VNI2 controls senescence at least partially dependent on ATAF2.

Dark-induced senescence data showed that *vni2* had senescence accelerating phenotype while *ataf2* showed senescence delaying phenotype (Figure 27). Since both VNI2 and ATAF2 are induced under the dark condition, this protein interaction might govern the dark-induced senescence. Particularly, even though there was no significant contribution to show a phenotype in *ataf2* under the natural senescence, mutation of *ataf2* clearly showed delay senescence phenotype under the dark condition (Figure 27), suggesting that ATAF2 is able to contribute to induce senescence in response to the dark condition. *vni2 ataf2* had shown intermediate phenotype, providing two insights. One is that acceleration of senescence in *vni2* mutants requires ATAF2 similar to the natural senescence condition (Figure 23). Second is that the delay senescence phenotype by lack of *ataf2* is at least partly recovered by simultaneous mutation of *vni2*. This strongly suggested that VNI2 inhibits not only ATAF2 but also its homologs such as ATAF1. In *vni2 ataf2* double mutants, the homologs of ATAF2 would be released from inactivation by VNI2, resulting in higher expression levels of senescence-associated genes than *ataf2* single mutants. These overall data suggested that VNI2-ATAF2 protein interaction has significant roles in respect to senescence.

4.5 Figures

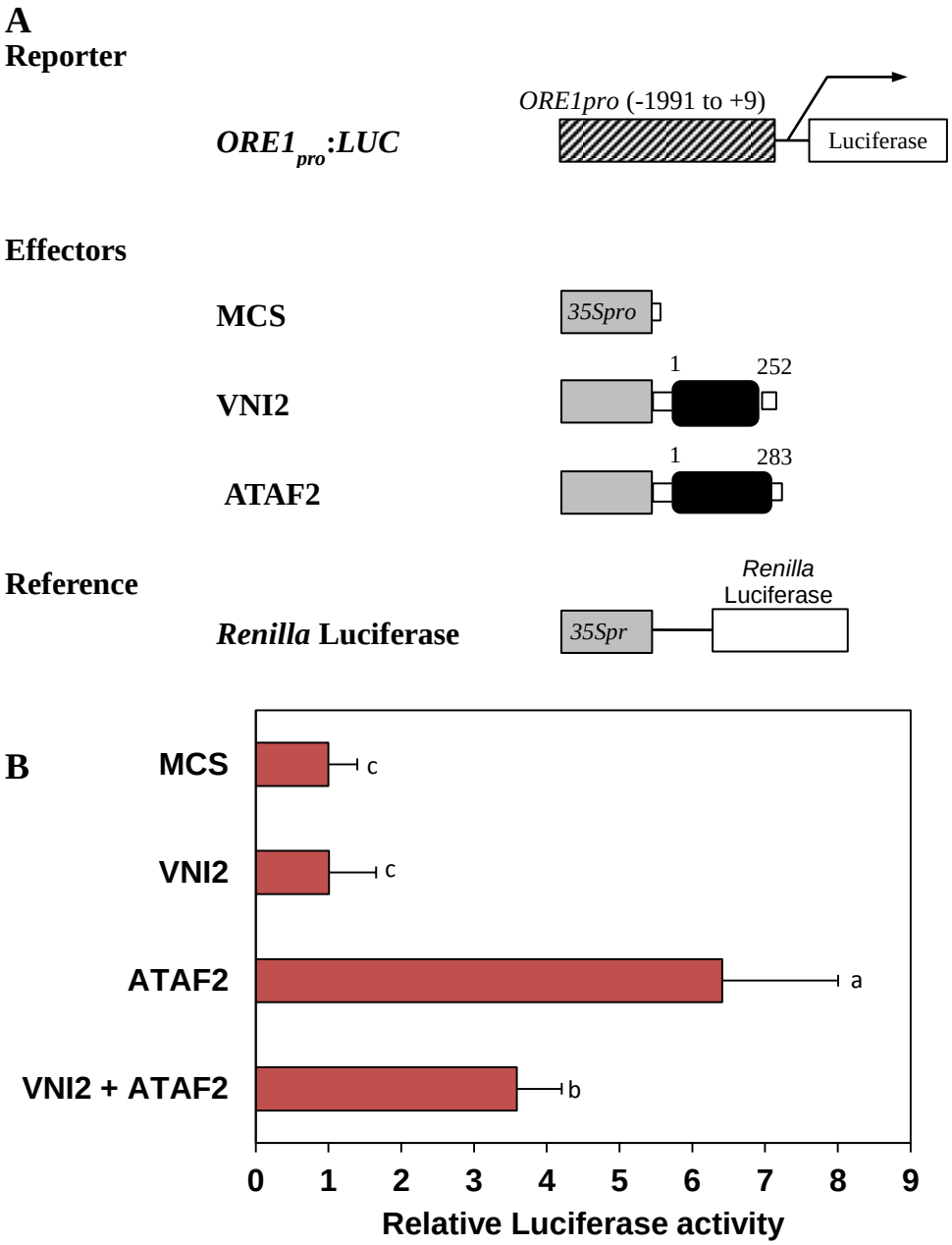


Figure 21. VNI2 inhibits the transcriptional activation activity of ATAF2. (A) Schematic diagrams of the effector and reporter constructs. The reporter construct contains the promoter of *ORE1* with a min pro. Upstream from the firefly luciferase reporter gene. The effector constructs contained *ATAF2* and *VNI2* downstream of *35S* promoter. (B) Results of the transient

transfection assay. Firefly luciferase activities were normalized by *Renilla* luciferase activities. Error bars indicate SD (n = 4). $p < 0.05$ (Tukey`s HSD).

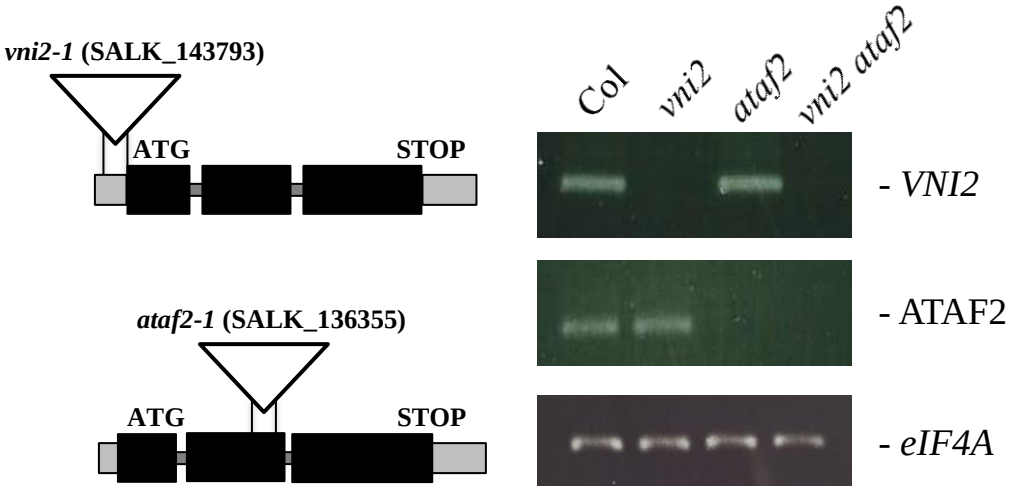


Figure 22. Schematic representation of a T-DNA insertion and over expression lines. T-DNA insertion *vni2, ataf2* mutant lines of *Arabidopsis*. RT-PCR analysis of *VNI2* and *ATAF2.*

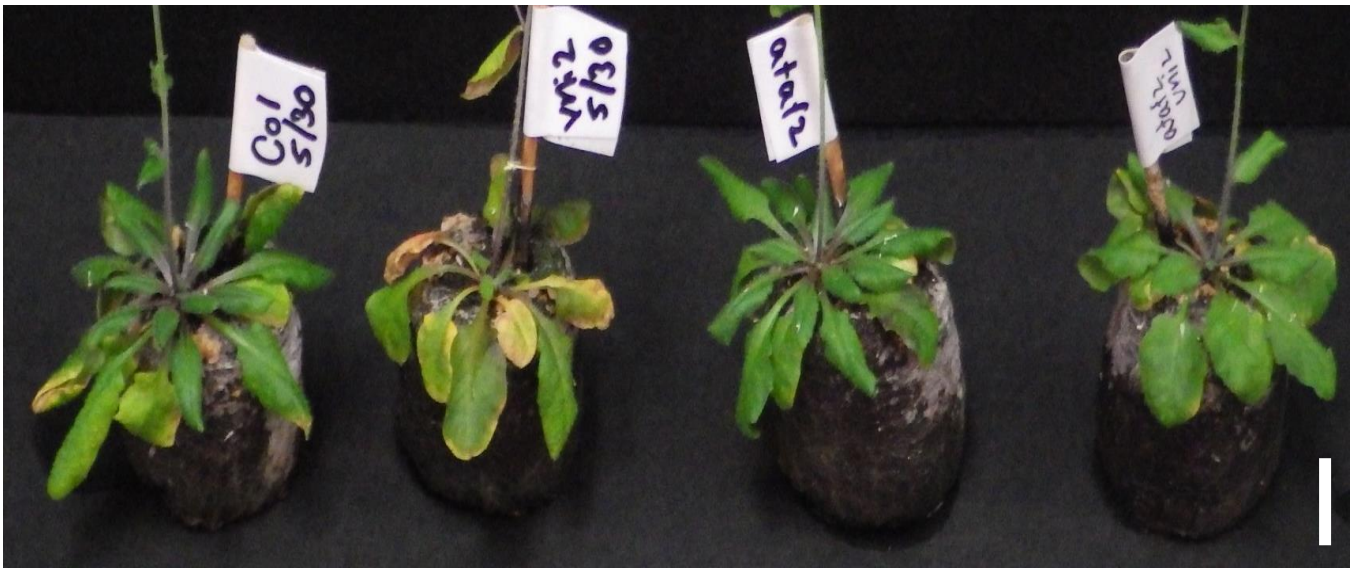


Figure 23. Natural senescence of fifty-five-day-old plants under the long-day condition. The scale bar represents 2.5 cm.

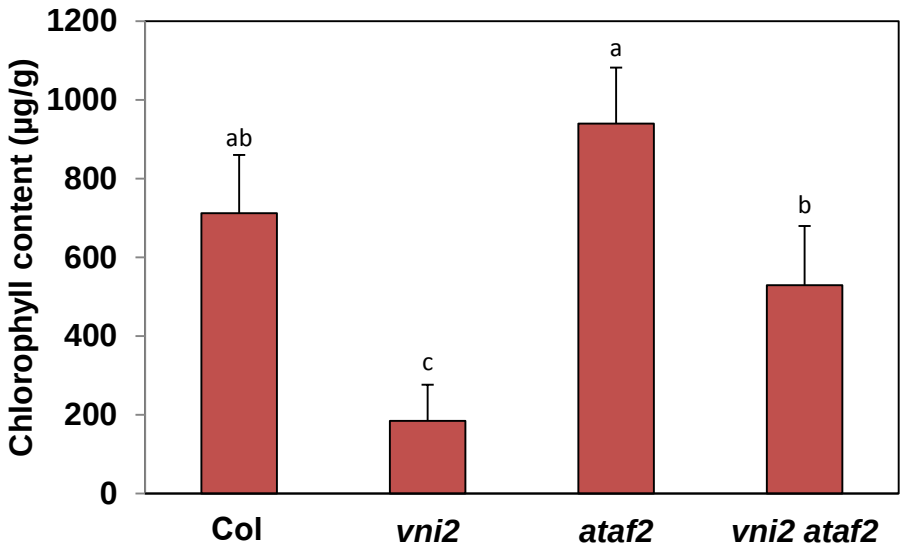


Figure 24. Chlorophyll content of 55-day-old plants. Seventh leaves were used. Error bars indicate SD (n = 3). $p < 0.05$ (Tukey`s HSD).

Col ***vni2*** ***ataf2*** ***vni2ataf2*** ***ATAF2vni2-3-1*** ***ATAF2vni2-5-1*** ***ATAF2vni2-6-1***

Col ***ataf2*** ***ATAF2OX-3-1*** ***ATAF2OX-2-2*** ***ATAF2OX-5-2***

Bar = 2.5cm

Figure 25. *ATAF2* overexpression under the *vni2* background shows highest senescence acceleration compared to control. Forty-five-day-old plants under long day condition were used. The scale bar represents 2.5 cm.

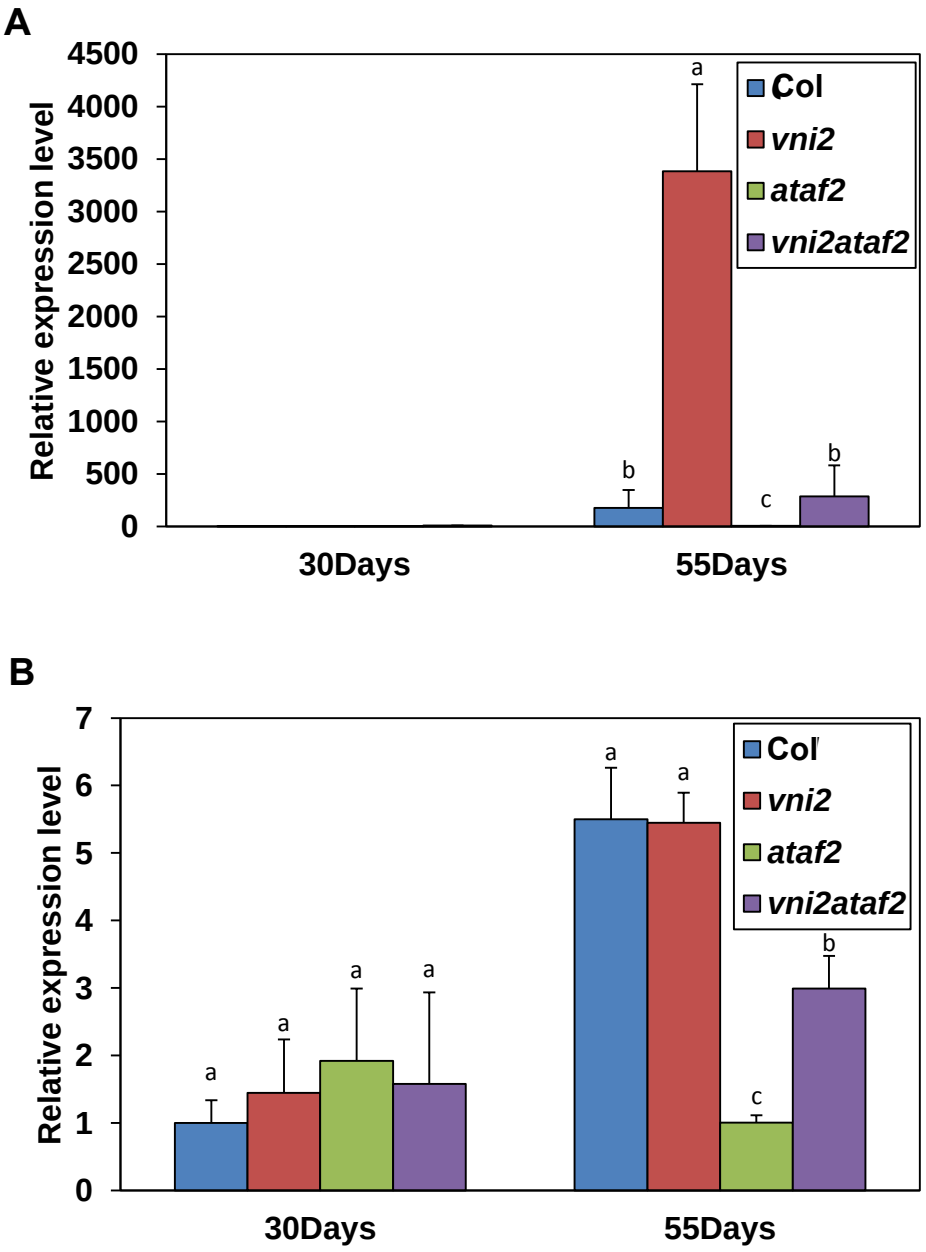


Figure 26. Expression of senescence associated gene *SAG12* and senescence transcriptional factors *ORE1*. (A) *SAG12* expression level in seventh leaves of thirty-day- and 55-day-old plants. (B) *ORE1* expression level in 7th leaves of 30-day- and 55-day-old plants. Error bars indicate SD (n = 3). $p < 0.05$ (Tukey`s HSD).

Figure 27. VNI2 has ATAF2 dependent function in senescence. Fifth leaves from four-week-old plants incubated on moisture filter papers for 5 days under continuous light- or dark-condition. The scale bar represents 1 cm.

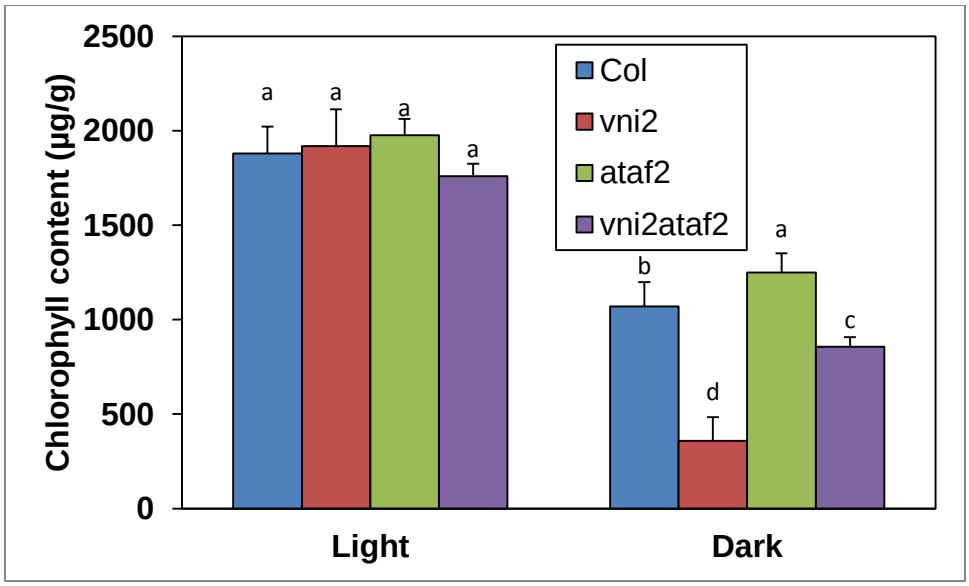


Figure 28. *vni2* and *ataf2* show lowest and highest chlorophyll content and highest chlorophyll content respectively. Fifth leaves incubated under continuous light or dark condition. Error bars indicate SD (n = 4). $p < 0.05$ (Tukey`s HSD).

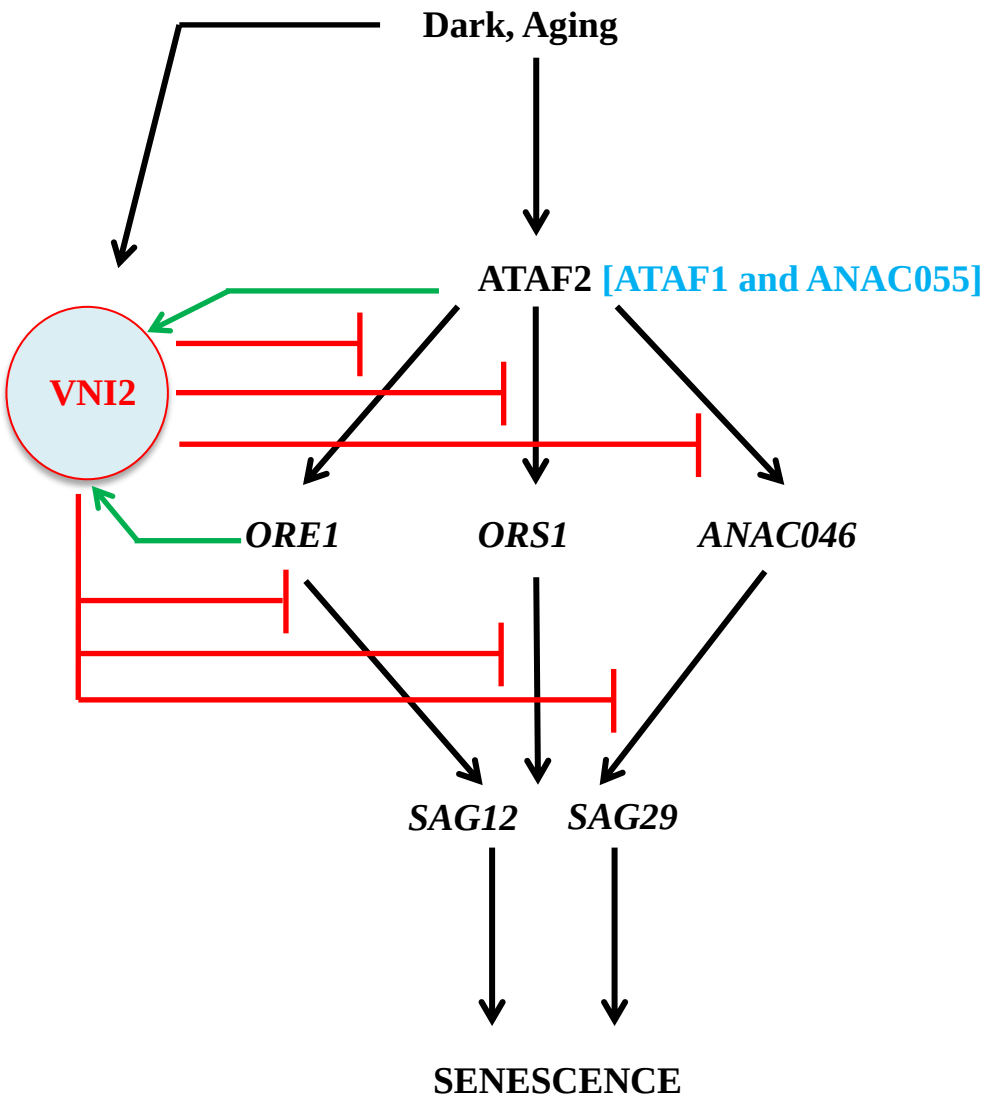


Figure 29. Schematic model of VNI2-ATAF2 senescence regulation depending on dark.

Chapter 5

General Discussion

An NAC domain transcription factor, VNI2, is originally identified as an interacting factor with VND7, which is another NAC domain transcription factor, and a master regulator for xylem vessel formation (Yamaguchi et al. 2010b). It was also reported that VNI2 efficiently bound with other VND proteins (Yamaguchi et al. 2010). Overexpression of these *VND* genes induced transdifferentiation of various types of cells into xylem vessel elements (Kubo et al. 2005; Yamaguchi et al. 2010a; Endo et al. 2015). Endo et al. (2015) demonstrated that VND1 to VND7 regulate *VND7* expression suggesting the existence of a positive feedback regulation for the promotion of the cell differentiation. Recently, it was found that VNI2 also inhibited transcriptional activation activities of VND1, VND2, and VND3 (Yamada et al. unpublished data). Furthermore, there is also a negative feedback loop to control xylem vessel differentiation. Namely, overexpression of *VND* genes upregulates *VNI2* expression (Ohtani et al. 2011). These data indicate that VNI2 plays a role in repression of xylem vessel differentiation through regulating and regulated by VND proteins, members of NAC domain transcription factors (Figure 30).

Here, I demonstrated that VNI2 inhibits ATAF2 function by forming the protein complex, and regulates senescence. Observation of *vni2 ataf2* double mutants suggested that related homologs of ATAF2 may be also regulated by VNI2. It has been reported that ATAF1 and ORE1 upregulate *VNI2* expression (Garapati et al. 2015a). Taken together with these data suggested that VNI2 is associated with senescence by regulating and regulated by several NAC domain transcription factors, such as ATAF2 and its homologs, and ORE1 (Figure 30).

It is known that some transcription factors have several distinct roles during biological processes by forming complexes with different proteins, such as DELLA family and PHYTOCHROME-INTERACTING FACTOR (PIF) proteins (de Lucas et al. 2008; Feng et al. 2008; Yoshida et al. 2014). It is known that NAC domain transcription factors form homo- or heterodimer with other transcription factors. However, it has not been reported that NAC domain protein has various biological functions by interaction with different proteins. Thus, my obtaining data provided a new insight that NAC domain proteins can form heterodimers with different NAC domain transcription factors, and give rise to various distinct functions. Since *VNI2* is expressed in different kinds of tissues, it seems that VNI2 has more different functions (Yamaguchi et al.

2010b). Indeed, I have isolated a number of NAC domain transcription factors as interacting proteins with VNI2, including ANAC102 (Nagahage, master thesis, 2015). Thus, further studies of VNI2 interaction is important to unveil the biological roles of VNI2 more deeply.

It is well known that stress conditions might induce premature senescence of leaves that leads to a shortage of assimilates, ultimately causing an acceleration in the whole-plant maturation process (Gan 2003). Stress-induced senescence is caused by dark/shade, heat/light, drought, low nitrogen supply and biotic stress (Chaves et al. 2002; Buchanan-Wollaston et al. 2005; Robert-Seilaniantz et al. 2011; Gregersen, 2011; Suzuki et al. 2012). It is assumed that a main signal for the dark-induced senescence could be the rapid reduction in sugar levels, leading to a more significant switch to lipid degradation to supply an energy source (Graham and Eastmond, 2002; Van Doorn. 2004). *ATAF2* is repressed under the sucrose condition (Delessert et al. 2005). Interestingly, *ATAF2*, *VNI2*, and *ORE1* are highly expressed in petals (Figure 25). There is some suggestion that senescence in petal could be induced by sugar starvation (van Doorn, 2004). *ATAF2* overexpression strongly upregulates a transcription factor, *ATB2*, known to have a complex function during light and sucrose (Delessert et al. 2005). In addition, ATAF2 homolog ATAF1 has a function in trehalose metabolism in energy starvation (Garapati et al. 2015b). The induction of this pathway has previously been reported in plants exposed to a long night period, and in sugar-starved suspension culture (Thimm et al. 2004). These results suggested that ATAF2 has a role related to energy starvation induce senescence. Then, it is possible that VNI2 control ATAF2 function for efficient nutrient remobilization.

The yield of the plants has been increased by extending the photosynthetic period of a plant. So early senescence is causing to reduce yield quantity as well as quality. By using the knowledge of my study might be helpful to understand pre-mature senescence due to low light condition and energy starvation. When plants grow in indoor due to aesthetic beauty, natural air purification and humidity, the light condition might be the main constraint. Low light condition induces the senescence of the plant if it lost the main carbon source to live. By using the knowledge of this study, low light induced senescence could be controlled. In addition, I could unveil the important biological role of VNI2.

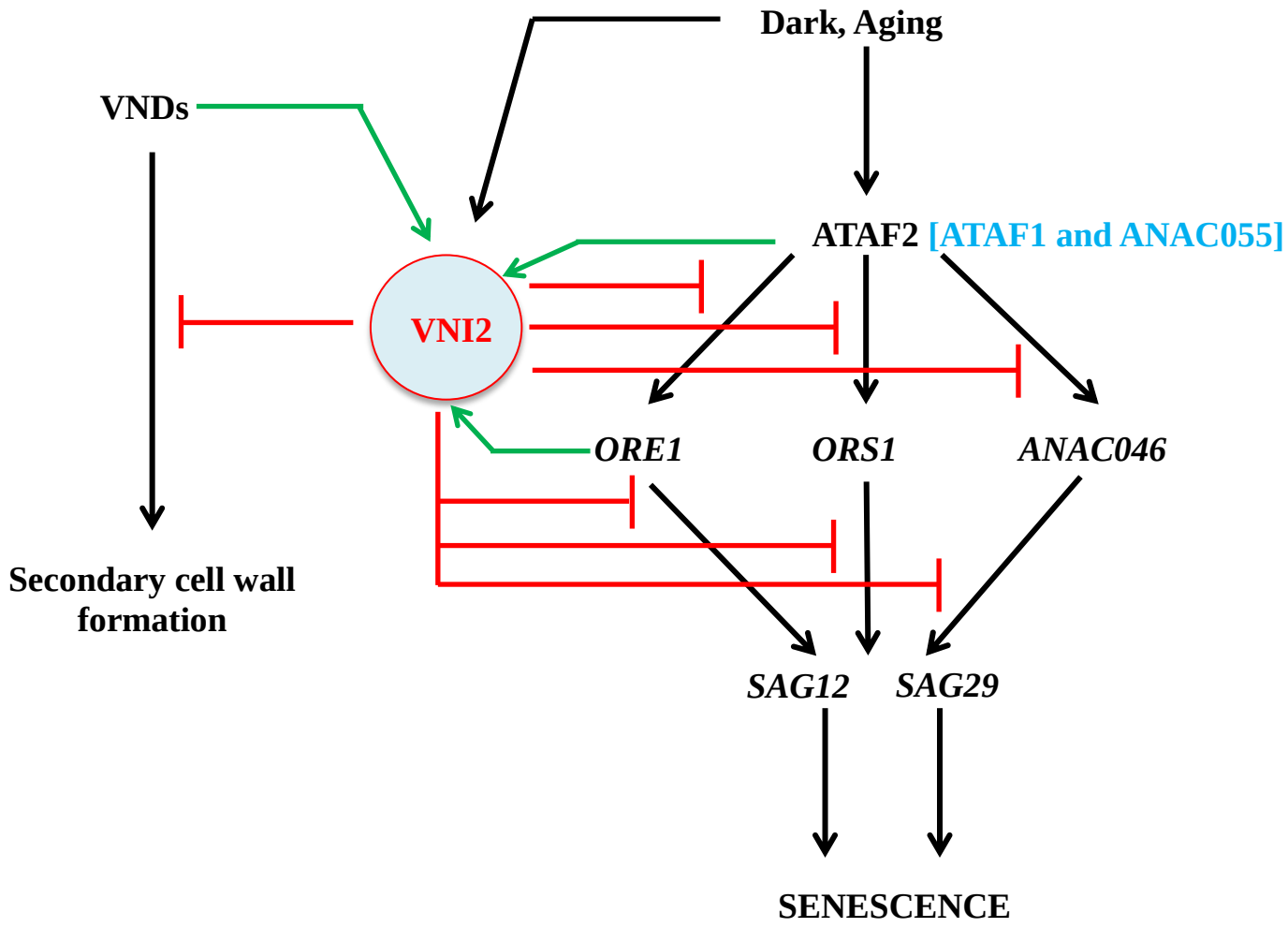


Figure 30. Schematic model of VNI2 biological roles in senescence and xylem vessel differentiation.

Supplementary table

Table S1. Oligonucleotides used in this study

Name	Forward primer	Reverse primer
ATAF2	CACCATGAAGTCGGAGCTAAATTTACCA	TTACCCCTGTGGAGCAAAACTCCAATTC
ATAF2- SRDX	CACCATGAAGTCGGAGCTAAATTTACCA	TTAAGCGAAACCCAAACGGAGTTCTAGATCCAGATCTAGCCCCTGTGGAGCAAAA
VNI2	CACCATGGATAATGTCAAACTTGTTAAGAATGGT	TCATCTGAAACTATTGCAACTACTGGTCTC
VND7	CACCATGGATAATATAATGCAATCGTCAAT	TTACTGAACCGGGGCAAGCTCGGA
ORE1 promoter	CACCTATCTCTATTAACGGTTACCATCAAAATTT	GTAATCCATTTTATCCTAATAGGGTTTCTA
NIT2 promoter	CACCTTCACTTTCATCAGCATAATTTTCA	AGTTGACATTTTTTCTGTTTTAACTTGAGC
BAS1 promoter	CACCTATATATAAGTTTTAAAAAACGTGTATTGA	TTCCTCCATTGTCGGAACACCAACAAAGAA
SAG12 promoter	CACCTAGCTTAATTATAGCATTGATTTCTAAATT	TAAAGCCATTGTTTTAGGAAAGTTAAATGA
AtNAP promoter	CACCAAACAAAGATGCTAGAGCTTTTGTTTGCGT	TACTTCCATGATTTTCAGACAATTTAGAAA
SWEET15 promoter	CACCACCAAAGTTGCTTCAATAGTAATTAGAGAT	GACTCCCATTTTCTATAGCAATTGAGAAAA
COR promoter	CACCCTTCGGAACAACAACAAGAGTTATTATGCT	CATCGCCATGAGAGAGATCTTTAAGATGTG
ANAC055 promoter	CACCTTCGAATTTCATTGGCGCACTTTGATTTTA	GAGACCCATTTTTCCTATCACCTCTTTCTC
XTH15	CACCTGCCGGCTGGAATAGATA	TGGACCCATTTGGGTTTGGTTGATAG

promoter	GAGATCGAAGAG	AAAT
GLYI7 promoter	CACCAATACATTTTCCCACAAG TGACAGAGCAAA	GTCCTTCATTTTTTCTTTCTTACCCAG AGA
BGLU44 promoter	CACCTGTGGTCAATGTGTCTAT GATTTGGGTTGT	GTGTCTCATGGTGAGTGAGGTTTGAC CGTT
AT5G57655 promoter	CACCACGTACGACTGCTTTAAC TCACCTATAAAA	TTTCTTCATAGTTTTCAACGAACCTTG AAC
NCED3 promoter	CACCATGTTTCTTTTGTATTAA ATTATTAGACGG	AGAAGCCATTTTTCAAGTGTGTTCAAT CAG
ERD1 promoter	CACCATTTGAATAAAGAAGAG AGACGTGGTCGGA	CACCTCCATGATCGAATTCGTCGTCG GTGA
AT1G68907 promoter	CACCTCTTTGAACAGGAGGATG AGAGAACA	TTTCTCCATTACTAATTGTAACCTCAA AAT
AT3G26540 promoter	CACCTTCGTGAACCATTTGAAT ATCAATGC	CACTGCCATGGTTCATGACCTCAAAG GTTG
UGT1 promoter	CACCTCATATGGAAGACGAAA ACATTGGTTCTCC	TGGCGCCATTTTTTTTCTACTTGTCTT AGC
SESA2 promoter	CACCTGTTACATTGCTGCTCTC TAAAAAGTCATG	GTTTGCCATTTTTTTGCTATTTGTGTTT GT
PR1 promoter	CACCTATCCGTTCGCATTAAAG TTAAATAGATTG	AAAATTCATTTTTCTAAGTTGATAATG GTT
PDF1.2 promoter	CACCACGACGTTGGACTGTTTC ATCATATCCCAT	CTTAGCCATGATGATTATTACTATTTT GTT
ANAC047 promoter	CACCTTAACTAGATCATGTGGG TGTTTATGATTC	GCTTATCATTGTTTAAAGGAATGATAT TTT
ORS1 promoter	CACCCCAGTCTTAGCTAGTTTG TTTTATTTAATT	GTAATCCATTTCAGCGATTAGATATA ATCA
ANAC046 promoter	CACCTTAAATCCCCAACTTTTA ATATACTCCCTT	TTCCACCATCTATATATATGTATGCTT GAT
Multiple cloning site	CACCTAGTGGATCCCCCGGGCT GCAGGAATTCGATATCAAGCTT	CATCACGAGGTCGACGGTATCGATAA GCTTGATATCGAATTCCTGCAGCCCG

(MCS)	ATCGATACCGTCGACCTCGTGATG	GGGGATCCACTAGGTG
ATAF2 real time PCR	TCTTCTACGCAGGGAAAGCTCC	AGCCATTGTCGTGGTCCTCG
VNI2 real time PCR	TTTGCAGAGCTGATCCTTGG	CGGTTCCCATTTGGGTATTT
eIF4a real time PCR	TGACCACACAGTCTCTGCAA	ACCAGGGAGACTTGTTGGAC
SAG12 real time PCR	GTGTCTCGGCATTATCTAGC	CTTTTGGATTGGTCTTTTTG
ORE1 real time PCR	CTTACCATGGAAGGCTAAGATGGG	TCGGGTATTTCCGGTCTCTCAC
ORS1 real time PCR	CCTTGGAAGGCTAAGCTTGG	ATCAGCGGAGGTAAACCAAC
ANAC046 real time PCR	GGTTTCGGTTTCACCCTACTGATG	ATGGCTCGTTCTTGTTGAGGTC

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