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A NAP-AAO3 Regulatory Module Promotes Chlorophyll Degradation via ABA Biosynthesis in *Arabidopsis* Leaves[W][OPEN]

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Chlorophyll degradation is an important part of leaf senescence, but the underlying regulatory mechanisms are largely unknown. Excised leaves of an *Arabidopsis thaliana NAC-LIKE, ACTIVATED BY AP3/PI* (*NAP*) transcription factor mutant (*nap*) exhibited lower transcript levels of known chlorophyll degradation genes, *STAY-GREEN1* (*SGR1*), *NON-YELLOW COLORING1* (*NYC1*), *PHEOPHYTINASE* (*PPH*), and *PHEIDE a OXYGENASE* (*PaO*), and higher chlorophyll retention than the wild type during dark-induced senescence. Transcriptome coexpression analysis revealed that abscisic acid (ABA) metabolism/signaling genes were disproportionately represented among those positively correlated with *NAP* expression. ABA levels were abnormally low in *nap* leaves during extended darkness. The ABA biosynthetic genes *9-CIS-EPOXYCAROTENOID DIOXYGENASE2*, *ABA DEFICIENT3*, and *ABSCISIC ALDEHYDE OXIDASE3* (*AAO3*) exhibited abnormally low transcript levels in dark-treated *nap* leaves. NAP transactivated the promoter of *AAO3* in mesophyll cell protoplasts, and electrophoretic mobility shift assays showed that NAP can bind directly to a segment (−196 to −162 relative to the ATG start codon) of the *AAO3* promoter. Exogenous application of ABA increased the transcript levels of *SGR1*, *NYC1*, *PPH*, and *PaO* and suppressed the stay-green phenotype of *nap* leaves during extended darkness. Overexpression of *AAO3* in *nap* leaves also suppressed the stay-green phenotype under extended darkness. Collectively, the results show that NAP promotes chlorophyll degradation by enhancing transcription of *AAO3*, which leads to increased levels of the senescence-inducing hormone ABA.

INTRODUCTION

Senescence is the final stage of leaf development and leads to whole-organ death. With various regulated changes at the physiological, biochemical, and molecular levels, leaf senescence is mainly an age-dependent process, although it can be triggered by external abiotic and biotic stresses, such as lack of water or nutrients, high or low light intensity, and pathogen infection (Lim et al., 2007). Senescence of organs, and the resulting degradation of cellular macromolecules, provides valuable resources for biosynthesis of new organs.

Initiation and progression of leaf senescence is affected by various plant hormones. Auxin (Kim et al., 2011), gibberellic acid (Yu et al., 2009), and cytokinin (Richmond and Lang, 1957; Gan and Amasino, 1995) suppress leaf senescence. By contrast, ethylene promotes senescence, and inhibitors of ethylene biosynthesis or mutations that disrupt ethylene signaling delay senescence (Zacarias and Reid, 1990; Reid and Wu, 1992; Lim et al., 2007). Compared with nonsenescing leaves, senescing leaves of *Arabidopsis thaliana* have higher concentrations of both jasmonic acid (JA) and salicylic acid (SA) (He et al., 2002; Breeze et al., 2011). Furthermore, transcript levels of many genes involved in JA or SA biosynthesis and signaling are upregulated in senescing leaves of *Arabidopsis* (van der Graaff et al., 2006). Therefore, ethylene, JA, and SA, together with abscisic acid (ABA; see below), are considered to be senescence-promoting.

ABA is a sesquiterpenoid derived from carotenoids, and almost all of its biosynthetic genes have been identified through the isolation of mutants (Nambara and Marion-Poll, 2005). Key enzymes controlling ABA production include 9-*cis*-epoxycarotenoid dioxygenases (NCEDs), which are involved in xanthophyll cleavage (Tan et al., 2003), and ABSCISIC ALDEHYDE OXIDASE3 (AAO3), which is responsible for the final step in ABA biosynthesis (Seo et al., 2004). ABA DEFICIENT3 (ABA3) is a sulfurase involved in the biosynthesis of the molybdenum cofactor that is required for AAO3 activity (Finkelstein, 2013). ABA plays a central role in plant responses to stress conditions (Chandler and Robertson, 1994). ABA also regulates various developmental processes, including seed maturation and dormancy, organ abscission, and flower and leaf senescence (Cutler et al., 2010). It has long been known that exogenously applied ABA can induce senescence-associated mRNAs and reduce chlorophyll content in detached leaves (Becker and Apel, 1993; Weaver et al., 1998; Yang et al., 2002). Recent transcriptomic analysis showed that many genes involved in ABA biosynthesis, metabolism, and signaling are upregulated during leaf senescence, while chlorophyll *a* and *b* content declines (van der Graaff et al., 2006).

During leaf senescence, degreening or yellowing due to chlorophyll degradation is a visible marker of macromolecule degradation and nutrient remobilization. Plant mutants in which leaf degreening is delayed compared with the wild type are called stay-green mutants. Based on the temporal changes of leaf

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chlorophyll content and photosynthetic capacity, five distinct types of stay-green phenotype are defined (Thomas and Howarth, 2000). Type C mutants are defective in chlorophyll degradation but not photosynthesis or other physiological functions. Several type C stay-green mutants and their genetic lesions have been characterized. Rice (*Oryza sativa*) *NON-YELLOW COLORING1* (*NYC1*) and *NYC1-like* encode two subunits of chlorophyll *b* reductase (Sato et al., 2009). Mutation of the *PAO* gene, encoding pheide a oxygenase, in *Arabidopsis* resulted in abnormal chlorophyll retention in leaves during dark-induced senescence (Pruzinská et al., 2005). A pheophytinase (PPH) was identified in *Arabidopsis* to be an enzyme for porphyrin-phytol hydrolysis, and a *pph* mutant accumulated abnormally high amounts of phein during senescence (Schelbert et al., 2009). In addition, there are a number of *STAY-GREEN* (*SGR*) genes encoding chloroplast proteins that are highly conserved in plant species, such as meadow fescue (*Festuca pratensis*; Thomas et al., 1989), pea (*Pisum sativum*; Sato et al., 2007; Aubry et al., 2008), *Arabidopsis* (Ren et al., 2007), rice (Park et al., 2007), tomato (*Solanum lycopersicum*), and bell pepper (*Capsicum annuum*; Barry et al., 2008). SGR proteins are thought to destabilize chlorophyll-protein complexes as a prerequisite to degradation of both chlorophyll and apoprotein (Hörtensteiner, 2009). These proteins/enzymes constitute part of the pathway for chlorophyll degradation, which includes chlorophyll-protein complex destabilization, phytol group removal, porphyrin ring opening, and subsequent steps (Matile et al., 1999; Hörtensteiner, 2006).

Chlorophyll is a double-edged sword for plant cells: It is essential for photosynthesis and growth but can become phytotoxic when overexcited by light energy, resulting in the production of reactive oxygen species and cell death (Tanaka and Tanaka, 2006). Therefore, the biosynthesis and degradation of chlorophyll are tightly regulated during plant development. Although the enzymology of chlorophyll degradation is now quite well understood (Hörtensteiner and Kräutler, 2011), relatively little is known of how the process is regulated at the transcriptional or posttranscriptional levels. Recent genetic studies identified a number of putative regulatory genes involved in leaf senescence in *Arabidopsis*, encoding the putative transcription factors WRKY53 (Miao et al., 2004), NAP/ANAC029 (Guo and Gan, 2006), ORE1/ANAC092 (Kim et al., 2009), ORS1/ANAC059 (Balazadeh et al., 2011), and EIN3 (Li et al., 2013), a cytokinin receptor (AHK3) (Kim et al., 2006), a protein receptor kinase (PRK1) (Lee et al., 2011), and the nucleus-targeted protein S40-3 (Fischer-Kilbienski et al., 2010). Mutations in these genes result in stay-green phenotypes under natural conditions and/or prolonged darkness, via unknown mechanisms.

NAP is a NAC (named after NAM, ATAF1, 2, and CUC2) family transcription factor. Loss-of-function *nap* mutants in *Arabidopsis* exhibited delayed leaf senescence, and detached leaves showed a stay-green phenotype during prolonged darkness (Guo and Gan, 2006). At-NAP was reported to regulate silique senescence and to be required for ethylene stimulation of respiration (Kou et al., 2012). Very recently, Os-NAP, an ortholog of At-NAP, was reported to be a positive regulator of senescence and nutrient remobilization in rice (Liang et al., 2014). Upregulation of Os-*NAP* (in the gain-of-function mutant *ps1-D* or by overexpression) accelerated senescence, while knockdown of Os-*NAP* by RNA interference (RNAi) delayed senescence and chlorophyll degradation (Liang et al., 2014). Here, we show that At-NAP promotes chlorophyll degradation in *Arabidopsis* via induction of the ABA biosynthetic gene, *AAO3*, which leads to increased levels of the senescence-promoting hormone ABA.

RESULTS

nap Mutant Leaves Exhibit Abnormally High Chlorophyll Concentrations and Low Expression of Chlorophyll Degradation Genes during Extended Darkness

Under normal growth conditions, leaf chlorophyll concentrations were equal in the *nap* mutant and the wild type (Figure 1A, time zero). Extended darkness led to a gradual decrease in chlorophyll concentration in excised leaves of both the wild type and *nap* mutant, although the decrease was significantly less in the mutant (Figure 1A). After 6 d of darkness, chlorophyll levels in *nap* leaves were nearly four times higher than those in the wild type, indicating a partial defect in chlorophyll degradation in the mutant.

To gain a mechanistic understanding of the impaired chlorophyll degradation in *nap*, transcript levels of genes involved in chlorophyll degradation were measured by quantitative RT-PCR (qRT-PCR). Dark treatment led to a massive increase in *NAP* transcript in the wild type but not the *nap* mutant (Figure 1B). Prior to dark treatment, leaves of the wild type and *nap* plants had similar levels of transcript of each of the genes involved in chlorophyll breakdown, *SGR1*, *NYC1*, *PPH*, *PaO*, *CLH1*, and *CLH2* (Figure 1B). Prolonged darkness led to significant increases in transcript levels of *SGR1*, *NYC1*, *PPH*, and *PaO* in the wild type but significantly smaller increases in these in the mutant (Figure 1B). After 3 d of darkness, transcript levels of *SGR1*, *NYC1*, *PPH*, and *PaO* in *nap* were 31, 41, 51, and 47%, respectively, of those in the wild type. These results indicated that NAP plays a role, either direct or indirect, as a positive regulator of chlorophyll degradation genes during dark-induced leaf senescence. Furthermore, abnormally low expression of chlorophyll degradation genes in the *nap* mutant may account for its stay-green phenotype in the dark.

Interestingly, transcript levels of *CLH1* and *CLH2*, which are supposed to remove the phytol group during chlorophyll degradation, decreased significantly in both *nap* and wild-type leaves in response to dark treatment (Figure 1B). These results are consistent with a previous report that *CLH1* and *CLH2* are not essential for senescence-related chlorophyll breakdown (Schenk et al., 2007).

NAP Expression Is Induced during Natural Senescence and by ABA and Ethylene Treatments

Several transcriptomic studies have shown that *NAP* transcript levels increase during natural senescence (Buchanan-Wollaston et al., 2005; van der Graaff et al., 2006) and that the gene is preferentially expressed in senescent leaves and sepals (Zimmermann et al., 2004; Winter et al., 2007). qRT-PCR showed that *NAP* transcript levels in *Arabidopsis* rosette leaves 4 and 5 (combined)

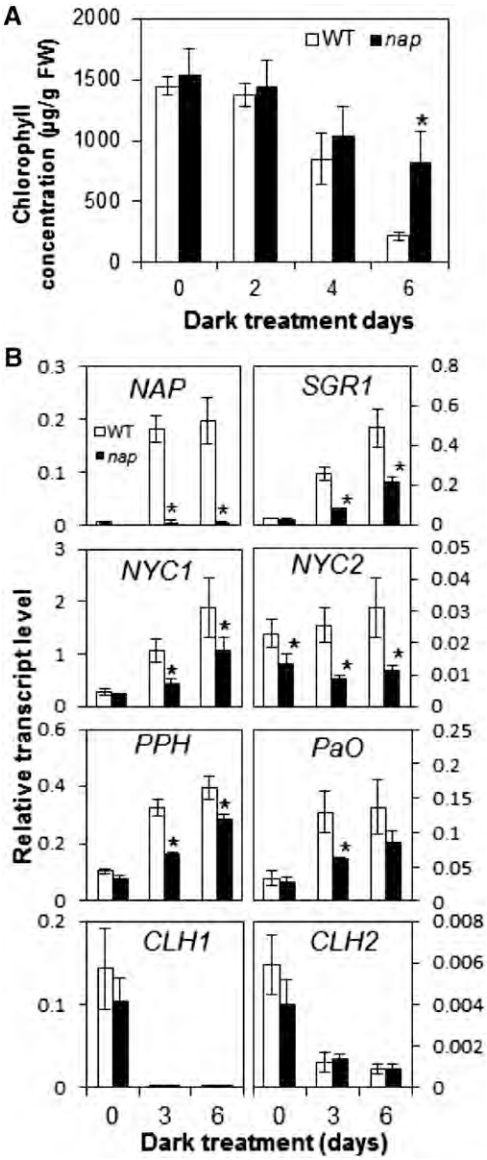

Figure 1. Chlorophyll Degradation in Wild-Type and *nap* Leaves during Extended Darkness.

(A) Chlorophyll concentration in wild-type and *nap* leaves. Values are means ±SD (n = 6).

(B) Relative transcript levels of *NAP* and chlorophyll degradation-related genes in wild-type and *nap* leaves. Transcript levels are expressed relative to that of *UBQ10* in each sample, and values are means ± SD (n = 3). Asterisk indicates significant difference from the wild type at $P < 0.05$, using the Student's t test.

increased 4- and 7-fold between days 10 and 30 or 40, respectively (Figure 2A). Under the growth conditions used in these experiments, plants began bolting around day 20, and leaves 4 and 5 started yellowing around day 30.

Several senescence-promoting hormones were tested for their effects on *NAP* expression in detached leaves under light, including ABA, ethylene (via the precursor 1-aminocyclopropane-1-carboxylic acid [ACC]), methyl jasmonate (MeJA), and SA. *NAP* transcript levels increased in water-treated controls within 4 h of leaf excision (Figure 2B). Treatment of leaves with ABA, but not MeJA or SA, resulted in significantly higher *NAP* transcript levels compared with those in water-treated controls at all three time points: 4, 8, and 24 h (Figure 2B). Additionally, ACC treatment increased *NAP* transcript levels above that of the control only after 24 h.

ABA Metabolism Genes Are Coexpressed with *NAP*

To investigate further the role of NAP in chlorophyll degradation, we performed coexpression analysis using ATTED-II (version 7.1) (http://atted.jp) (Obayashi et al., 2011). We selected the 300 genes with transcript levels most strongly correlated with those of *NAP* (Supplemental Data Set 1A). MapMan analysis (Thimm et al., 2004) (Pathways: Regulation_overview) of these genes revealed four major functional groups of genes associated with

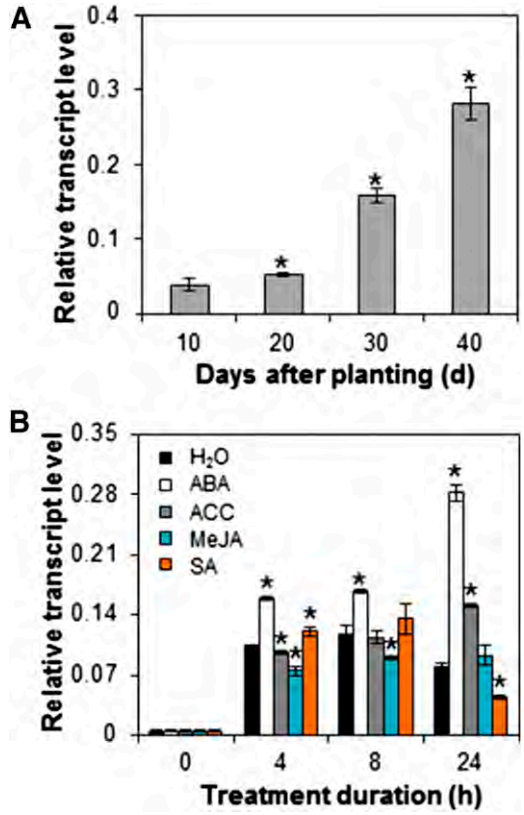

Figure 2. Age- and Hormone-Induced Expression of *NAP* in Leaves.

(A) *NAP* transcript levels in rosette leaves 4 and 5 (combined) of plants grown in soil for up to 40 d. Error bars indicate the SD of three biological replicates. Asterisk indicates significant difference compared with day 10 at $P < 0.05$, using the Student's t test.

(B) *NAP* transcript levels in wild-type rosette leaves subjected to different treatments. Excised leaves were incubated under light and treated with water (negative control), ABA, ACC (precursor of ethylene), MeJA, or SA. Asterisk indicates significant difference from water control at $P < 0.05$, using the Student's t test.

NAP expression: transcription factors (35 genes), protein degradation (32 genes), ABA metabolism/signaling (six genes), and protein modification (six genes) (Supplemental Figure 1). We were particularly intrigued by the ABA-related group for three reasons: *NAP* expression can be induced by ABA treatment (Figure 2B); ABA can induce chlorophyll degradation (Weaver et al., 1998; Yang et al., 2002); and loss of *NAP* function retards chlorophyll degradation (Figure 1A). Therefore, we set out to clarify the relationship between NAP, ABA, and chlorophyll degradation.

nap Leaves Have Abnormally Low Levels of ABA under Dark Treatment

To test whether net ABA production was affected in *nap* leaves during dark incubation, we determined the concentration of ABA in detached leaves of both the wild type and *nap* mutant. In wild-type leaves, ABA concentration increased from 0.185 to 3.58 nmol/g fresh weight (FW) during 6 d of darkness (Figure 3). Although ABA concentration in *nap* leaves was the same as that of wild-type leaves prior to dark treatment, ABA levels increased less in the mutant in the dark and were approximately half the level measured in the wild type after 6 d of darkness (Figure 3). This result indicates that ABA production was lower in *nap* leaves than in the wild type during dark treatment or that ABA degradation was higher in the mutant.

nap Leaves Exhibit Abnormally Low Transcript Levels of Several ABA Biosynthesis Genes during Extended Darkness

Transcript levels of eight genes for ABA biosynthesis, *ABA1/ZEP*, *NCED2*, *NCED3*, *NCED5*, *NCED9*, *ABA2*, *ABA3*, and *AAO3* (Seo et al., 2009), were measured by qRT-PCR. No significant differences in transcript levels of any of these genes were found between wild-type and *nap* leaves prior to dark treatment (Figure 4). However, during extended darkness, transcript levels of *NCED2*, *ABA3*, and *AAO3* increased significantly more in the wild type than in *nap* leaves (Figure 4). This was not the case for the other five genes (Supplemental Figure 2). The transcript levels of *NCED2*, *ABA3*, and *AAO3* in *nap* were ~46, 64, and 40%, respectively, of those in the wild type after 3 d of darkness. Thus, loss of *NAP* function muted the induction of ABA biosynthesis genes in leaves under extended darkness.

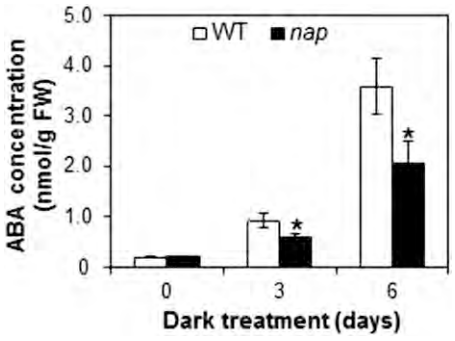

Figure 3. Levels of ABA in Wild-Type and *nap* Leaves during Extended Darkness.

The data represent mean values of three replicates ± SD. Each replicate included four to six leaves. Asterisk indicates significant difference from the wild type at $P < 0.05$, using the Student's *t* test.

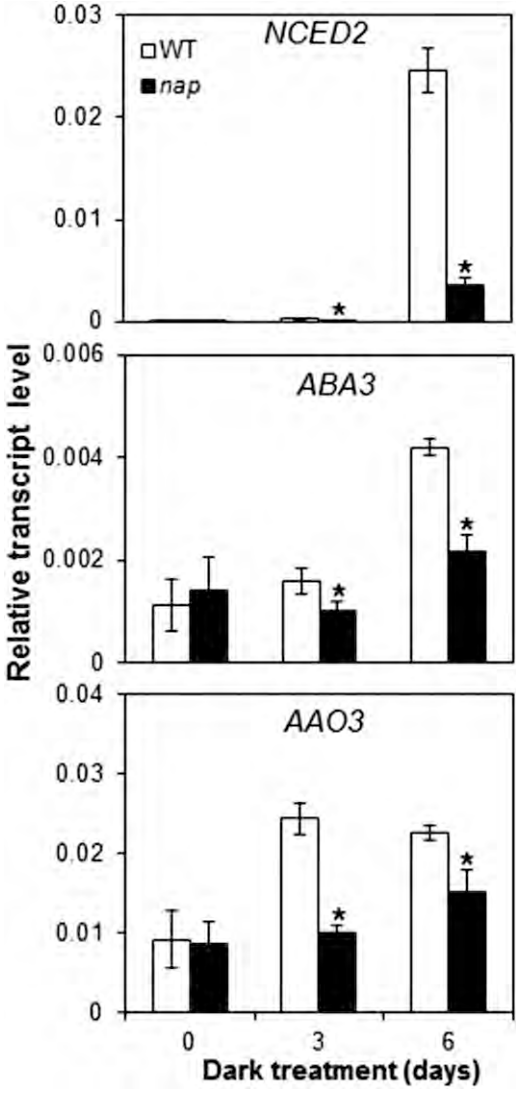

Figure 4. Transcript Levels of ABA Biosynthesis Genes *NCED2*, *ABA3*, and *AAO3* in Wild-Type and *nap* Leaves during Dark Treatment.

Error bars indicate the SD of three biological replicates. Asterisk indicates significant difference from the wild type at $P < 0.05$, using the Student's *t* test.

NAP Binds Directly to the *AAO3* Promoter and Transactivates Gene Expression

Although *NCED2*, *ABA3*, and *AAO3* transcript levels were lower in *nap* than in wild-type leaves during dark treatment (Figure 4), only *AAO3* was among the six ABA metabolism/signaling related genes that were coexpressed with *NAP* (Supplemental Table 1). Significantly, a protein phosphatase 2C gene (At5g59220, *SAG113*)

that was previously reported to be a direct target of NAP (Zhang and Gan, 2012) was also among the six ABA-related genes coexpressed with *NAP*, and transcript levels of *AAO3* exhibited higher correlation with those of *NAP* than did those of *SAG113* (Supplemental Table 1).

To establish a direct regulatory connection between NAP and *AAO3*, first we performed luciferase (LUC)-based transactivation assays. Coexpression of *NAP* with an *AAO3* promoter-*LUC* reporter gene in *Arabidopsis* mesophyll protoplasts increased luciferase activity by 240% (Figure 5A), indicating that NAP binds to the *AAO3* promoter to activate transcription. To verify this, we performed electrophoretic mobility shift assays (EMSAs) using purified NAP protein and synthetic *AAO3* promoter segments.

NAP was expressed as a fusion protein with thioredoxin and His6 added to the N terminus (Trx-NAP). Next, we confirmed that the Trx-NAP fusion protein binds to a 32-bp segment of the *SAG113* promoter (P_{113}-S, AGTGTTAGACTTTGATTGGTGC**ACG**TAAGTGT), as described previously for NAP (Zhang and Gan, 2012) (Supplemental Figure 3A). A 9-bp core sequence of P_{113}-S (underlined above) was reported to be crucial for NAP binding (Zhang and Gan, 2012). Using sequence variants in the core sequence of P_{113}-S, we found that the ACG triplet (in bold letters in P_{113}-S above) were absolutely necessary for Trx-NAP binding (Supplemental Figure 3B).

There are six ACGs and eight CGTs in the positive strand of the 1228-bp *AAO3* promoter sequence that was used in the transactivation assays. Four fragments between 300 and 350 bp that contained ACG or CGT were amplified from the *AAO3* promoter for use in EMSA assays, i.e., P1, −1231 to −924; P2, −899 to −548; P3, −529 to −189; and P4, −323 to −8, relative to the translation start codon, ATG (Supplemental Figure 4). A 330-bp fragment of *SAG113* promoter, P_{113}-L: −427 to −98, which contains P_{113}-S, was used as the positive control. EMSA showed that the P4 fragment bound strongly to the Trx-NAP, as did the P_{113}-L fragment (Figure 5B). P2 and P3 fragments also showed some binding. Binding of Trx-NAP to the labeled P4 fragment was inhibited by a 200-fold excess of unlabeled fragment. Similar results were obtained for P_{113}-L (Figure 5C).

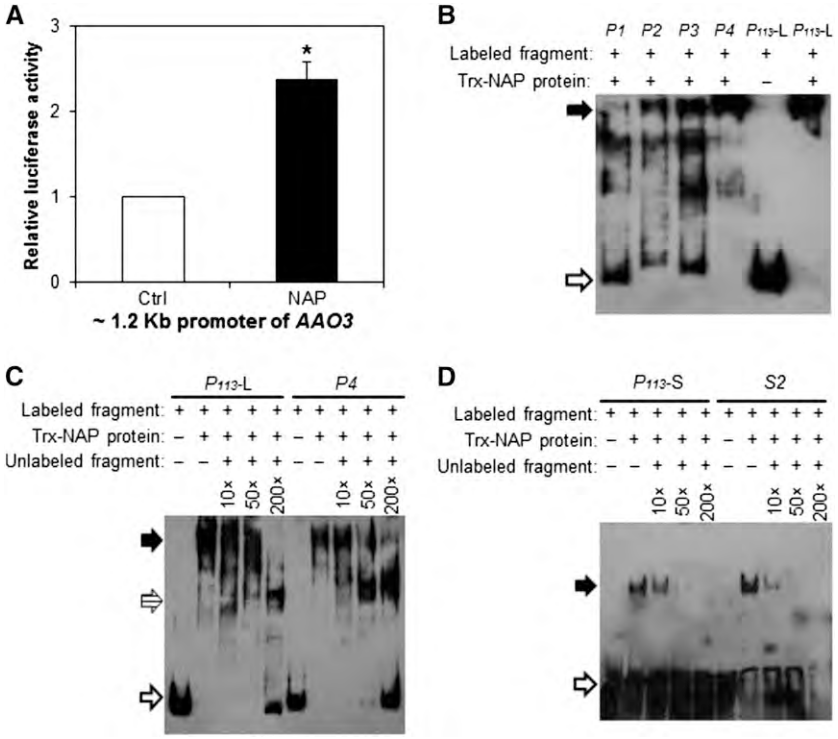

Figure 5. Interactions between NAP and the *AAO3* Promoter.

(A) Transactivation of 1.2 kb of the *AAO3* promoter by NAP in *Arabidopsis* mesophyll cell protoplasts. The P_{AAO3}:*FLuc* construct was cotransformed with the *35S*:*NAP* construct (omitted in control experiments). A *35S*:*RLuc* construct was used to normalize for transformation efficiency. Error bars indicate the SD of three biological replicates. Asterisk indicates significant difference from control at $P < 0.05$, using the Student's *t* test.

(B) EMSA. Purified Trx-NAP protein was mixed with each of four distinct fragments of the *AAO3* promoter, P1 ~ P4. Free P4 fragment was shifted to the same extent as the positive control P_{113}-L fragment by Trx-NAP.

(C) Competition EMSA. Excess (10- to 200-fold) unlabeled P4 abolished binding of Trx-NAP to labeled P4 fragment. The same pattern was observed for the control P_{113}-L.

(D) Competition EMSA. Excess (10- to 200-fold) unlabeled S2 abolished the specific binding of Trx-NAP to labeled S2. The same pattern was observed for the P_{113}-S control fragment. The sequence of S2 is AGATGTG**CGT**GAAAGAGGCGCAACTATAAGAG (Supplemental Figure 4).

Solid, open, and striped arrows in EMSA figures indicate protein-bound probe, free probe, and nonspecific binding, respectively.

To determine more precisely which sequences in the P4 fragment of the *AAO3* promoter bind to the NAP protein, we synthesized four segments, each of which contained one or two CGT sequences: S1, −267 to −236; S2, −196 to −165; S3, −93 to −62; S4, −78 to −47, relative to the start ATG. Trx-NAP bound to S2 but to none of the other segments (Supplemental Figure 5). The specificity of Trx-NAP binding to S2 was confirmed by EMSA competition with unlabeled S2 DNA (Figure 5D). Again, similar results were obtained with the positive control fragment, P_{113}-S. Collectively, the EMSA results indicated that NAP can bind specifically to segment S2 (−196 to −165) of the *AAO3* promoter.

ABA Suppresses the Stay-Green Phenotype of *nap* Leaves Subjected to Prolonged Darkness

Next, we examined the effect of exogenously-applied ABA on chlorophyll degradation in *nap* leaves during extended darkness. Excised leaves of *nap* treated with ABA solution (either 4 or 10 μM) showed wild-type-like yellowing after 6 d of darkness, in contrast to the water-treated *nap* control. The chlorophyll concentration in ABA-treated *nap* leaves was 194 μg/g FW, similar to that of the water-treated wild-type and in contrast with 755 μg/g FW in water-treated *nap* leaves (Figure 6A). Furthermore, ABA treatments increased the transcript levels of *SGR1*,

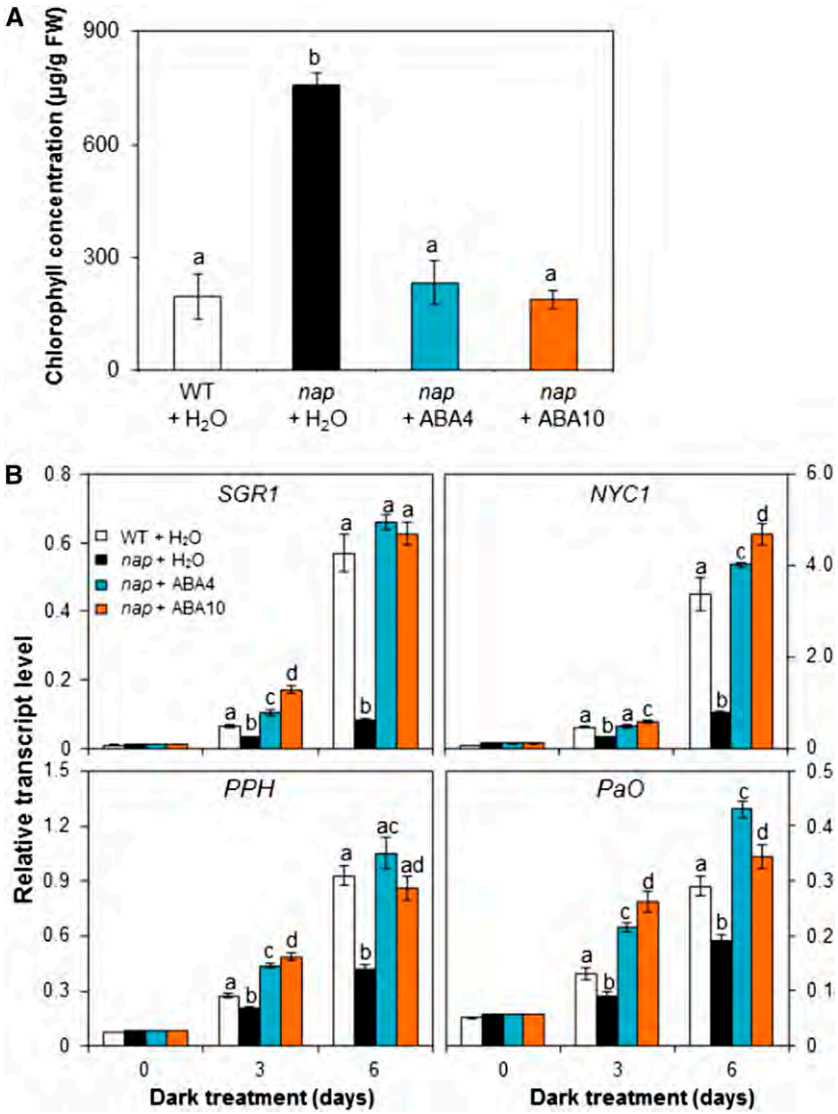

Figure 6. Effects of ABA on Chlorophyll and Gene Transcript Levels in *nap* Leaves under Extended Darkness.

(A) Leaf chlorophyll concentration after 6 d of darkness in the wild type or *nap* exposed to water or ABA. Values are means ± SD (n = 4).
(B) Transcript levels of the chlorophyll degradation genes *SGR1*, *NYC1*, *PPH*, and *PaO* in wild-type and *nap* leaves exposed to water or ABA following dark treatment. Error bars indicate the SD of three biological replicates. Different letters above columns in both panels indicate significant differences based on Tukey's test ($P < 0.05$).

NYC1, *PPH*, and *PaO* in dark-treated *nap* leaves to levels equal to or above those in wild-type leaves, while transcript levels of these genes were substantially lower in water-treated *nap* leaves during dark treatment (Figure 6B). In summary, application of ABA to detached *nap* leaves increased both the expression of chlorophyll degradation genes and the degradation of chlorophyll.

aao3 Mutant Leaves Exhibit a Stay-Green Phenotype during Extended Darkness

To test the involvement of *AAO3* in leaf senescence, we obtained a T-DNA insertion mutant of *AAO3*, with the T-DNA in the second intron of the gene (Supplemental Figure 6). After 6 d of dark treatment, *aao3* leaves showed a stay-green phenotype similar to that of *nap* (Figure 7A). Consistent with this, chlorophyll concentration in *aao3* leaves after dark treatment was the same as in *nap* and significantly higher than in wild-type leaves (Figure 7B).

Overexpression of *AAO3* in *nap* Leaves Suppresses the Stay-Green Phenotype

To test whether reduced expression of *AAO3* in *nap* can account for the stay-green phenotype of the mutant leaves under prolonged darkness, *AAO3* was constitutively expressed in the *nap* background. Three independent *AAO3*/*nap* transgenic lines were selected based on their wild-type-like levels of *AAO3* transcript after 6 d of dark treatment (Figure 8A). Leaves of all three *AAO3*/*nap* transgenic lines exhibited wild-type-like yellowing after 6 d of darkness. Chlorophyll concentrations in *AAO3*/*nap* leaves were ~240 μg/g FW, similar to that of the wild type, while it was 708 μg/g FW in *nap* (Figure 8B). Measurements of transcript levels by qRT-PCR revealed that *AAO3* overexpression in the *nap* mutant resulted in wild-type-like levels of the chlorophyll degradation genes, *SGR1*, *NYC1*, *PPH*, and *PaO* (Supplemental Figure 7).

Overexpression of *AAO3* in the *nap* mutant resulted in significant increases in ABA concentration in detached leaves of all three independent transgenic lines after 3 and 6 d of darkness (Figure 8C). Levels of ABA in the *AAO3*/*nap* lines were the same as the wild type at day 3 of darkness and intermediate between the wild type and the *nap* mutant at day 6 (Figure 8C).

Progression of senescence in intact wild-type, *nap*, and *AAO3*/*nap* plants was also investigated. Under normal growth conditions, *nap* rosette leaves showed delayed senescence compared with the wild type, while overexpression of *AAO3* in *nap* partially restored the wild-type phenotype (Figures 9A and 9B; Supplemental Figure 8A). Additionally, when intact plants were subjected to 6 d of prolonged darkness, without water stress, leaves of wild-type and *AAO3*/*nap* plants wilted and some turned gray-green (Supplemental Figure 8BC). Leaves of *nap* plants were less affected by this treatment.

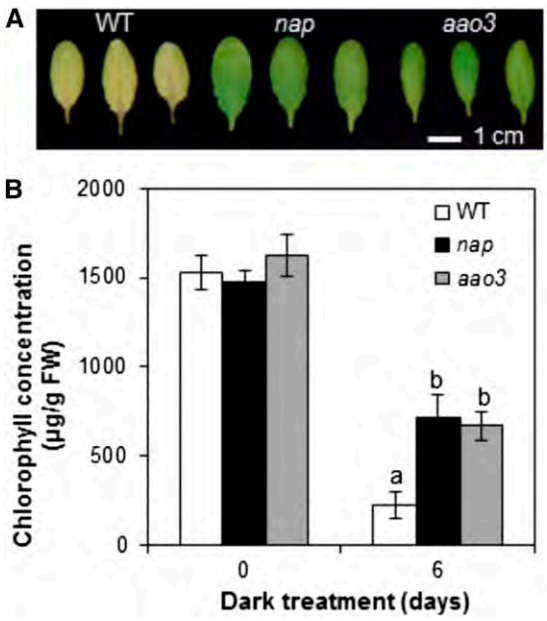

Figure 7. Effect of Darkness on Detached Leaves of the Wild Type, *nap*, and *aao3*.

(A) Phenotype of wild-type, *nap*, and *aao3* leaves after 6 d of dark treatment.

(B) Chlorophyll concentration in leaves prior to and after dark treatment. Values are means ± SD (n = 4). Different letters above bars indicate significant differences based on Tukey's test ($P < 0.05$).

DISCUSSION

Chlorophyll degradation is a conspicuous and important aspect of leaf senescence. Delayed or accelerated leaf degreening has been used often to identify senescence-related mutants. Although several transcription factors have been implicated in leaf degreening, exactly how they affect chlorophyll degradation was unknown. In this study, we investigated the mechanism of action of NAP during leaf degreening in *Arabidopsis*. Our results indicate that NAP positively regulates the expression of the *AAO3* gene involved in ABA biosynthesis, which increases ABA levels and induces the expression of genes involved in chlorophyll degradation.

ABA Biosynthesis Is Abnormally Low in *nap* Leaves during Dark Incubation

Almost all plant hormones have been shown to affect leaf senescence (Jibran et al., 2013). In general, ABA, ethylene, JA, and SA promote, whereas cytokinin, auxin, and gibberellic acid suppress leaf senescence. Recently, gene network analysis revealed that leaf senescence involves extensive crosstalk among different hormones and responses to environmental cues, together with developmental signals (Li et al., 2012).

NAP transcription in *Arabidopsis* was more sensitive to ABA treatment than to other hormones (Figure 2B). Coexpression and MapMan analysis revealed that ABA metabolism/signaling genes were coexpressed with *NAP* (Supplemental Figure 1 and Supplemental Data Set 1B). Furthermore, we found that ABA production in *nap* leaves was lower than in the wild type during extended darkness (Figure 3). Given that exogenously applied ABA reduces chlorophyll content in detached leaves (Becker and Apel, 1993; Weaver et al., 1998; Yang et al., 2002), we hypothesized that retarded chlorophyll degradation in *nap* leaves may be

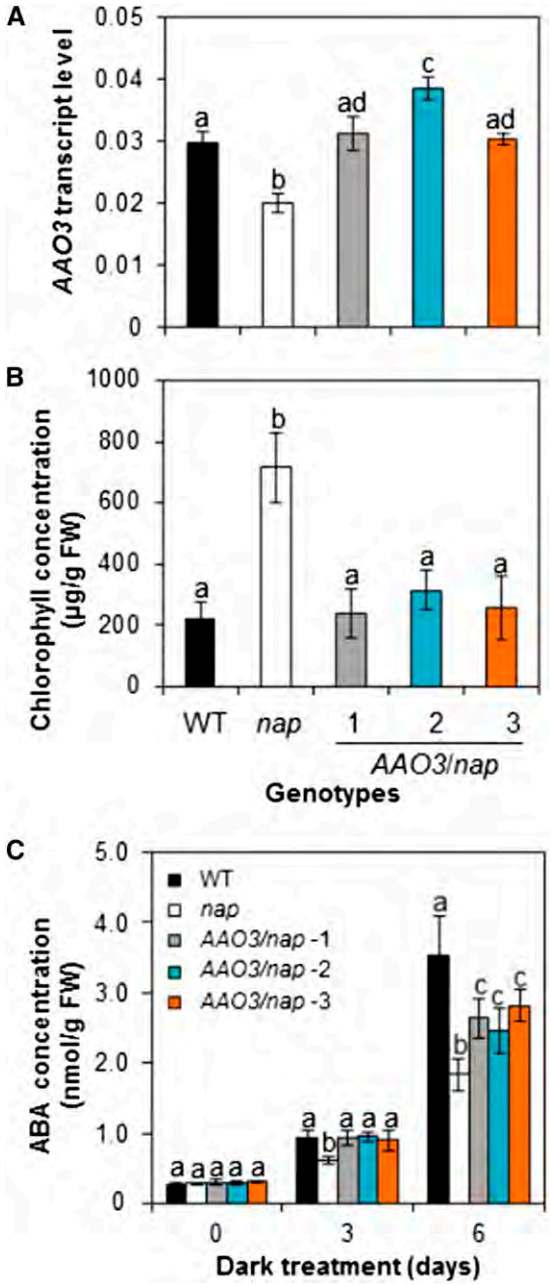

Figure 8. *AAO3* Overexpression Suppresses the *nap* Stay-Green Phenotype.

(A) Relative transcript levels of *AAO3* in wild-type, *nap*, and three *AAO3* overexpression lines of *nap* after 6 d of dark treatment. Error bars indicate the SD of three biological replicates.

(B) Chlorophyll concentration in leaves of the various genotypes after 6 d of dark treatment. Values are means ± SD (n = 4).

(C) ABA concentration in wild-type, *nap*, and *AAO3/nap* leaves subjected to 0, 3, and 6 d of darkness. Error bars indicate the SD of three biological replicates. Different letters above columns in all three panels indicate significant differences based on Tukey's test ($P < 0.05$).

related to impaired ABA biosynthesis or signal transduction. In support of this hypothesis, application of ABA to *nap* mutant leaves led to increased transcript levels of the four chlorophyll degradation genes, *SGR1*, *NYC1*, *PPH*, and *PaO*, and increased chlorophyll degradation, which matched that of the wild-type control (Figure 6).

The enzymatic steps of ABA biosynthesis, from zeaxanthin to active ABA, have been elucidated through the isolation of mutants (Nambara and Marion-Poll, 2005; Seo et al., 2009). We found that three ABA biosynthesis genes, *NCED2*, *ABA3*, and *AAO3*, had lower transcript levels in *nap* than in wild-type leaves during extended darkness (Figure 4). *NCED2* is one of five *NCED* genes encoding 9-*cis* epoxycarotenoid dioxygenase, which is involved in xanthophyll cleavage (Tan et al., 2003), while *AAO3* encodes an abscisic aldehyde oxidase responsible for the final step in ABA biosynthesis. *ABA3* encodes a sulfurase involved in molybdenum cofactor biosynthesis, which is required for AAO3 activity (Finkelstein, 2013). Low expression of these three genes in *nap* leaves indicated that NAP may affect multiple steps of ABA biosynthesis during dark-induced leaf senescence.

AAO3 Is a Direct Target of NAP

Six ABA metabolism/signaling related genes are coexpressed with *NAP* (Supplemental Table 1), including *AAO3* and *SAG113*, which encodes a protein phosphatase 2C involved in ABA signaling (Zhang et al., 2012). Previously, it was shown that NAP protein can bind to a 9-bp core sequence of the *SAG113* promoter (Zhang and Gan, 2012). Our transient transactivation and EMSA results demonstrated that NAP can transactivate the *AAO3* promoter and bind directly to the S2 segment (−196 to −162) of the promoter (Figure 5). Thus, like *SAG113*, *AAO3* appears to be a direct target of NAP transcriptional regulation. Importantly, overexpression of *AAO3* in *nap* leaves restored expression of the chlorophyll degradation genes *SGR1*, *NYC1*, *PPH*, and *PaO* (Supplemental Figure 7) and suppressed the stay-green phenotype of the mutant (Figure 8B). These data indicate that *AAO3* acts "downstream" of NAP, in a regulatory sense, consistent with the conclusion that it is a direct target of NAP activity. Various steps of ABA biosynthesis and catabolism, and upstream metabolism such as carotenoid biosynthesis, are potential points of control to modulate endogenous ABA level (Nambara and Marion-Poll, 2005). Our work has shown that *AAO3* is one such control point. In fact, AAO3 appears to be a primary control point for ABA biosynthesis and chlorophyll catabolism, given that *AAO3* overexpression in the *nap* mutant significantly increased ABA concentrations and chlorophyll turnover in leaves (Figure 8C; Supplemental Figure 7). However, the fact that ABA production was not completely restored to wild-type levels in *AAO3/nap* leaves (Figure 8C) indicates that other steps in ABA biosynthesis may be compromised in *nap*. In the future, it would be interesting to determine whether NAP directly regulates *NCED2* and *ABA3*, given that transcript levels of these two genes were abnormally low in *nap* leaves during dark treatment (Figure 4).

This work establishes NAP as a transcription factor that regulates *AAO3* expression. However, it is unlikely to be the only TF that can fulfill this role, as *AAO3* expression eventually increases

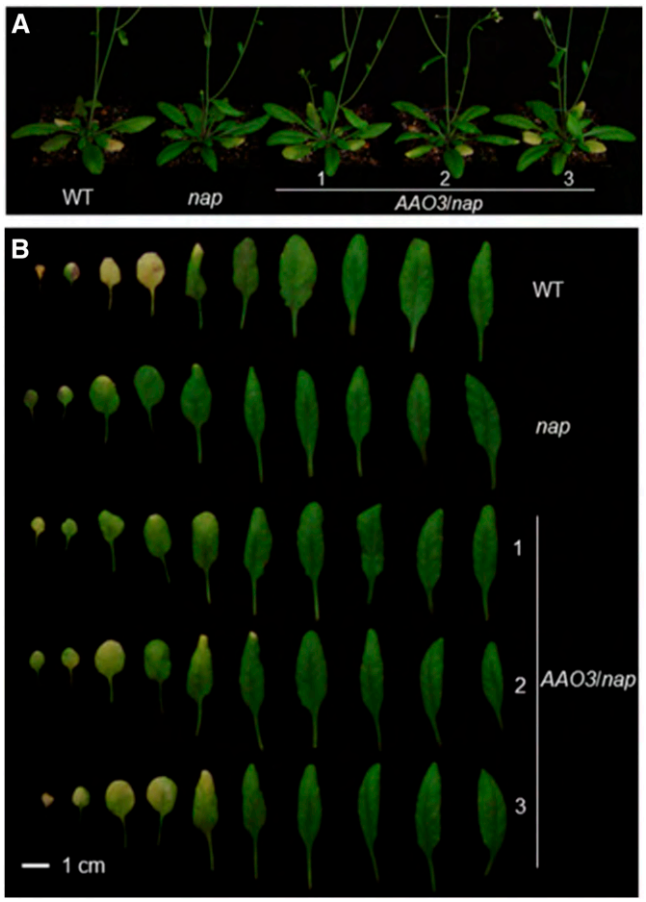

Figure 9. Progression of Senescence in Intact Wild-Type, *nap*, and *AAO3/nap* Plants Grown in Soil under Normal Conditions for 28 d.

Whole rosettes **(A)** and detached rosette leaves **(B)** arranged from oldest to youngest, left to right.

in *nap* leaves during extended darkness, despite the absence of NAP (Figure 4). Other dark-induced NAC transcription factors, such as ORE1/ANAC092 (Kim et al., 2009) and ORS1/ANAC059 (Balazadeh et al., 2011), may substitute for NAP to induce *AAO3* expression under these conditions.

Based on the results presented here, we propose the following model for the role of NAP in dark-induced chlorophyll degradation (Figure 10). Dark treatment induces *NAP* transcription (Figure 1B) by an unknown mechanism. NAP activates *AAO3* transcription by binding to a specific sequence in its promoter (Figure 5). The subsequent increase in AAO3 protein and activity relieves a bottleneck in ABA production, which increases ABA levels in leaves (Figure 3). This, in turn, leads to increased expression of chlorophyll degradation genes and eventually chlorophyll degradation (Figure 6). Although it remains unknown how ABA induces the expression of chlorophyll degradation genes during extended darkness, it is salient to note the role of the B3 domain transcription factor, *ABSCISIC ACID INSENSITIVE3*, in seed degreening (Delmas et al., 2013). It will be interesting to see if similar transcription factors play a role in leaf degreening in response to environmental or developmental cues, via the pathway defined in part here. Also of interest for future research is the potential role in chlorophyll degradation of the protein phosphatase 2C gene, *SAG113*, another direct target of NAP (Zhang and Gan, 2012). Finally, given that ABA can induce *NAP* gene expression (Figure 2B), a positive feedback loop appears to exist by which NAP increases its own expression via induction of ABA biosynthesis. Recently, a putative ortholog of At-NAP, Os-NAP, was characterized as a transcriptional activator of senescence in rice (Liang et al., 2014). Knockdown of Os-*NAP* by RNAi delayed senescence and chlorophyll degradation, similar to the phenotype of *Arabidopsis nap* leaves. In contrast to the results presented here for *Arabidopsis* NAP, however, rice NAP appears to repress ABA biosynthesis, as ABA concentrations in rice leaves are lower in a gain-of-function *ps1-D* mutant and higher in *NAP*-RNAi plants than in the wild type. Several ABA biosynthesis

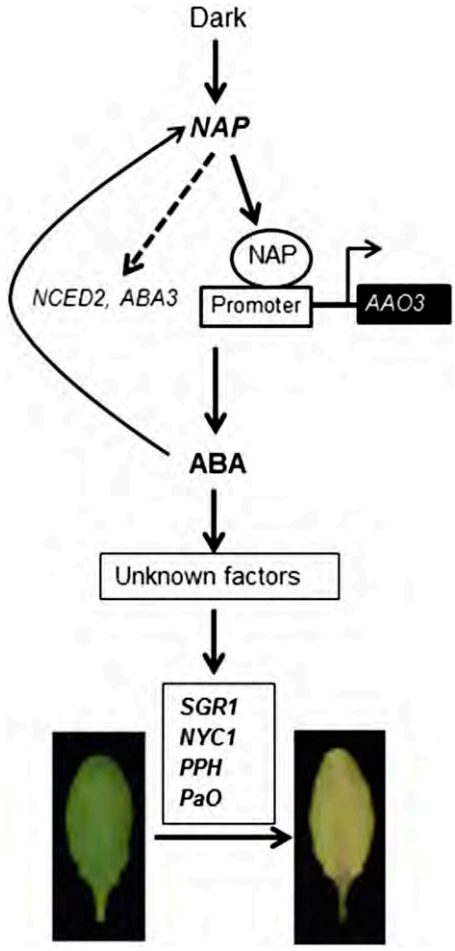

Figure 10. Model of NAP Role in Dark-Induced Chlorophyll Degradation.

Extended darkness induces *NAP* expression. NAP activates *AAO3* transcription via specific binding to its promoter. NAP also induces *NCED2* and *ABA3* transcription. The resulting increase in ABA biosynthesis promotes transcription of chlorophyll degradation genes, including *SGR1*, *NYC1*, *PPH*, and *PaO*, via unknown intermediates. The dashed line indicates unconfirmed regulation. The curved arrow indicates the induction of *NAP* expression by ABA (Figure 2B).

genes of rice, i.e., *NCED1*, *NCED3*, *NCED4*, and *ZEP*, were found to be downregulated in the *ps1-D* mutant (Liang et al., 2014). Considerable differences between developmental senescence and dark-induced senescence have been found at the molecular level (Buchanan-Wollaston et al., 2005; van der Graaff et al., 2006), which may account for some of the differences observed in our study of dark-induced senescence in *Arabidopsis* and the study of developmental senescence in rice. Nonetheless, overexpression of *AAO3* in the *nap* mutant partially restored the defect in developmental senescence of the *nap* mutant (Figure 9), consistent with our model in which NAP promotes senescence via induction of *AAO3* and ABA biosynthesis in *Arabidopsis* (Figure 10).

Elucidating the regulatory networks that control deconstruction of macromolecules during leaf senescence is important not only because of their biological significance for nutrient use efficiency, which affects reproductive success for instance, but also because of their agricultural significance to yield and quality in annuals and perennials and for nutrient remobilization and storage in other organs prior to shoot regrowth in perennials. For example, postharvest senescence of leafy vegetables, especially during storage or transportation in the dark, results in loss of nutritive quality and consumer acceptability (Kader, 2005; Lers, 2012). Conversely, removal of macronutrients, including N, P, and K, from shoots during annual senescence of switchgrass (*Panicum virgatum*; Yang et al., 2009) will be an important feature of sustainable bioenergy crops that provide fixed-carbon for biofuel production with minimal losses of soil nutrients. Thus, knowledge gained in this area has the potential to affect agriculture in several important ways.

METHODS

Plant Materials and Treatments

Arabidopsis thaliana seeds of ecotype Columbia-0 wild type, mutants of *nap* (SALK_005010C; Guo and Gan, 2006) and *aao3* (SALK_072361C; Seo et al., 2004), and *35S:AAO3/nap* were surface-sterilized with 75% (v/v) ethanol. After vernalization at 4°C for 2 d, seeds were germinated and grown on half-strength Murashige and Skoog medium containing 0.8% agar and 1% sucrose for 7 d (16 h light and 8 h dark) with white light illumination (120 μmol/s/m^2) at 22°C and 55% relative humidity. Seedlings were then transferred to Metro-Mix 350 soil (Sun Gro Horticulture) and grown further under the same conditions.

Rosette leaves were detached from plants 3 to 4 weeks after transferring to soil and placed on wet filter paper in continuous darkness at room temperature for up to 6 d to induce senescence. *Arabidopsis* plants were grown in soil under normal conditions (see above) to observe natural senescence. A subset of plants were subjected to the same growth conditions but then deprived of light to induce senescence. For hormonal treatment, detached rosette leaves were placed on filter paper wetted with 10 μM ABA, 25 μM ACC, 50 μM MeJA, and 100 μM SA, respectively and kept under dim light (40 μmol/s/m^2). The treated leaves were collected at designated time intervals for total RNA extraction.

Measurement of Chlorophyll Concentration

Leaves were collected and pulverized in liquid N_2. Chlorophyll was extracted from powdered samples with 80% acetone in water, and chlorophyll concentration was calculated after measuring the absorption at 663 and 646 nm (Porra, 2002).

Endogenous ABA Determination

Leaves were collected, weighed, and immediately frozen in liquid nitrogen. Frozen leaves were pulverized and ABA was extracted as described previously (González-Guzmán et al., 2002). Quantitative determination of endogenous ABA was performed by the competitive ELISA method using a Phytodetek ABA test kit (Agdia).

Protoplast Isolation and Transactivation Assays

Arabidopsis mesophyll protoplasts were prepared according to a published protocol (Yoo et al., 2007). Transactivation assays were performed as described previously (Wu et al., 2012). The effector construct (*35S:NAP*) was made by replacing the *GUS* gene with the coding sequence of *NAP* downstream (3′) of the *35S* promoter in *pCAMBIA3301* (Cambia). The reporter construct was prepared by cloning *AAO3* promoter sequence (~1.2 kb) into the *pGL4.10* vector (Promega) upstream of the Firefly Luciferase coding region (FLuc). The reporter construct, *35S:NAP* plasmid, and *35S*-driven Renilla Luciferase vector (*35S:RLuc*, used as the internal control) were cotransformed into protoplasts by polyethylene glycol-mediated transfection (Hayashimoto et al., 1990). The activity of FLuc and RLuc were assayed using a Dual Luciferase Reporter Assay System (Promega). Target promoter activity was expressed as FLuc/RLuc and normalized to the value obtained from protoplasts transformed with only the promoter-*Fluc* and *35S:RLuc* plasmids (no effector).

Protein Expression and EMSA

The coding region of *NAP* was PCR amplified and cloned into the *pET32a* vector (Novagen) via *Bam*HI and *Xho*I sites. The primer sequences used are listed in Supplemental Table 2. The resulting construct was transformed into *Escherichia coli* strain BL21(DE3). Protein expression was induced in a 500-mL culture using 0.5 mM isopropyl-β-D-thiogalactopyranoside, and cells were collected 16 h after induction at 28°C. The thioredoxin-polyhistidine-tagged NAP protein (Trx-NAP) was purified using Ni-NTA agarose (Qiagen) according to the manufacturer's instructions. The resulting protein was checked for size and purity by SDS-PAGE and Coomassie Brilliant Blue staining. Protein concentration was determined using a *RC DC* protein assay kit, based on the Lowry assay (Bio-Rad).

Based on the reported NAP binding site in the *SAG113* promoter (Zhang and Gan, 2012), the necessity of nine nucleotides in core binding sequence was tested by site mutations. The fragments of ~300 to 350 bp containing putative NAP binding region were amplified with 5′ biotin labeling from promoter region of *AAO3* for EMSA. To further refine the NAP binding site in the identified *AAO3* promoter fragment, forward and reverse 32-bp primers were synthesized with 5′ biotin labeling. The complementary primers were annealed by heating to 95°C followed by slow cooling to room temperature. Biotin-labeled DNA and Trx-NAP fusion protein were used for EMSA. The binding reaction, electrophoresis, transfer to nylon membrane, and detection of biotin-labeled DNA were performed using a LightShift Chemiluminescent EMSA kit (Thermo Scientific).

Plasmid Constructs for Plant Transformation

The open reading frame of *AAO3* was PCR amplified with additional *Bam*HI and *Pml*I restriction sequences and cloned into *pCAMBIA3301* vector via the *Bgl*II and *Pml*I sites, resulting in a *35S:AAO3* construct. This construct was transferred by the freeze-thaw method (Chen et al., 1994) into *Agrobacterium tumefaciens* strain C58, which was then used to transform *Arabidopsis* homozygous *nap* mutants, following the floral dip method (Clough and Bent, 1998). Transgenic seedlings were selected by spraying with 10 mg/L glufosinate-ammonium solution and confirmed by PCR. Phenotypic analysis was performed in the T2 generation.

Real-Time qRT-PCR Analysis

Total RNA was extracted from leaves using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. After treatment with DNase I (Ambion), 2 μg purified RNA was used for synthesis of first-strand cDNA by Superscript III reverse transcriptase (Invitrogen). The cDNA was used as template for qRT-PCR analysis. qRT-PCR was performed using an ABI PRISM 7000 instrument (ABI Applied Biosystems) and SYBR green (Czechowski et al., 2004). Transcript levels of target genes were normalized to that of the housekeeping gene *UBQ10* (At4g05320) using the equation of $2^{-\Delta CT}$, where C_T is the threshold cycle for each gene in every sample. Primer sequences are listed in Supplemental Table 2.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *NAP* (At1g69490), *SGR1* (AT4g22920), *NYC1* (AT4g13250), *PPH* (AT5g13800), *PaO* (AT3g44880), *NCED2* (At4g18350), *ABA3* (At1g16540), *AAO3* (At2g27150), *SAG113* (At5g59220), and *UBQ10* (At4g05320).

Supplemental Data

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

Supplemental Figure 1. MapMan Analysis of 300 Genes Coexpressed with *NAP* to Identify Processes Associated with *NAP*.

Supplemental Figure 2. Relative Transcript Levels of Five ABA Biosynthesis-Related Genes in Wild-Type and *nap* Leaves during Extended Darkness.

Supplemental Figure 3. Preliminary EMSA Using Trx-NAP Protein and Mutated P_{113}-S Fragments.

Supplemental Figure 4. The Promoter Region of *AAO3*.

Supplemental Figure 5. Trx-NAP Binding to Four Segments of *AAO3* Promoter Region P4 Was Tested by EMSA.

Supplemental Figure 6. Identification of the *aao3* Mutant.

Supplemental Figure 7. Low Transcript Levels of the Chlorophyll Degradation Genes *SGR1*, *NYC1*, *PPH*, and *PaO* in *nap* Leaves Were Restored to Wild-Type-Like Levels in *AAO3*/*nap* Leaves Subjected to 6 d of Dark Treatment.

Supplemental Figure 8. Leaf Phenotypes of Wild-Type, *nap*, and *AAO3*/*nap* Plants.

Supplemental Table 1. The Correlation Values of Six ABA Metabolism/Signaling-Related Genes That Were Coexpressed with *NAP*.

Supplemental Table 2. Sequences of the Oligonucleotide Primers Used in This Work.

Supplemental Data Set 1A. 300 Genes Coexpressed with *NAP*, Predicted by ATTED-II.

Supplemental Data Set 1B. The Top Processes Represented by Genes That Are Coexpressed with *NAP*.

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AUTHOR CONTRIBUTIONS

J.Y. and M.U. designed the research. J.Y. and E.W. performed the research. J.Y. and M.U. wrote the article.

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