Journal of Experimental Botany, Vol. 62, No. 11, pp. 3981–3992, 2011
doi:10.1093/jxb/err094 Advance Access publication 21 April, 2011
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Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

YUCCA6 over-expression demonstrates auxin function in delaying leaf senescence in *Arabidopsis thaliana*

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Received 13 January 2011; Revised 4 March 2011; Accepted 9 March 2011

Abstract

The *Arabidopsis thaliana* YUCCA family of flavin monooxygenase proteins catalyses a rate-limiting step in *de novo* auxin biosynthesis. A *YUCCA6* activation mutant, *yuc6-1D,* has been shown to contain an elevated free IAA level and to display typical high-auxin phenotypes. It is reported here that *Arabidopsis* plants over-expressing *YUCCA6,* such as the *yuc6-1D* activation mutant and *35S:YUC6* transgenic plants, displayed dramatic longevity. In addition, plants over-expressing *YUCCA6* exhibited classical, delayed dark-induced and hormone-induced senescence in assays using detached rosette leaves. However, plants over-expressing an allele of *YUCCA6*, that carries mutations in the NADPH cofactor binding site, exhibited neither delayed leaf senescence phenotypes nor phenotypes typical of auxin overproduction. When the level of free IAA was reduced in *yuc6-1D* by conjugation to lysine, *yuc6-1D* leaves senesced at a rate similar to the wild-type leaves. Dark-induced senescence in detached leaves was accompanied by a decrease in their free IAA content, by the reduced expression of auxin biosynthesis enzymes such as *YUCCA1* and *YUCCA6* that increase cellular free IAA levels, and by the increased expression of auxin-conjugating enzymes encoded by the *GH3* genes that reduce the cellular free auxin levels. Reduced transcript abundances of *SAG12*, *NAC1*, and *NAC6* during senescence in *yuc6-1D* compared with the wild type suggested that auxin delays senescence by directly or indirectly regulating the expression of senescence-associated genes.

Key words: *Arabidopsis thaliana*, auxin, leaf senescence, longevity, YUCCA6.

Introduction

Senescence is the age-dependent end of the life span. In plants, it is characterized by the visible yellowing of leaves that accompanies the mobilization of leaf nutrients to the reproductive structures. The yellowing of senescing leaves is correlated with biochemical changes such as a loss of chlorophyll contents, the degradation of proteins and RNA, and a decline in photosynthetic activity. Because accelerated leaf senescence curtails carbon assimilation, it stunts plant growth and reduces yield (Gay and Thomas, 1995; Thomas and Howarth, 2000). As the final stage of plant development, senescence has a crucial impact on agriculture, especially in crops where crop yield is enhanced by longer growth periods. Many studies have shown that senescence proceeds in a highly organized manner. It is an active process characterized by drastic catabolic changes and executed by both programmed gene expression and hormonal signals (Gan, 2007).

Plant hormones play key roles in responses to senescence. Senescence is accelerated by the hormones ethylene, abscisic acid (ABA), and jasmonic acid (JA) that mediate plant responses to biotic and abiotic stresses. Exogenous ethylene enhances visible leaf yellowing (Grbić and Bleecker, 1995;Weaver *et al.*, 1998) and several ethylene biosynthesis genes are up-regulated in senescing leaves (van der Graaff *et al.*, 2006). Ethylene-insensitive mutants such as *ethylene-resistant 1* (*etr1*) and *ethylene-insensitive 2* (*ein2*) display delayed leaf senescence (Bleecker *et al.*, 1988; Chao *et al.*, 1997). Similarly, the exogenous application of ABA accelerates senescence (Weaver *et al.*, 1998; Zeevaart and Creelman, 1988) and the level of ABA also increases during senescence (Gepstein and Thimann, 1980). In addition, exogenous methyl jasmonic acid (MeJA) has been reported to accelerate leaf senescence. The JA-insensitive mutant, *coronatine insensitive 1* (*coi1*) fails to display JA-dependent senescence (He *et al.*, 2002). Elevated cytokinin levels accompany delayed senescence, and endogenous cytokinin levels decrease during leaf senescence (Noodén *et al.*, 1990). Ectopic overproduction of cytokinins has been shown to delay leaf senescence in tobacco, petunia, cassava, and lettuce (Gan and Amasino, 1995; McCabe *et al.*, 2001; Chang *et al.*, 2003; Rivero *et al.*, 2007; Zhang *et al.*, 2010). The induction of the senescence programme is characterized by up-regulation of a set of signature genes that are referred to as Senescence Associated Genes (SAGs) that include genes encoding specific catabolic enzymes and transcription factors. Accordingly, leaf senescence induced by the hormones ABA, JA, and ethylene is characterized by the induction of the expression of some SAGs whereas the delay of senescence by cytokinin is characterized by the reduced expression of some SAGs (Weaver *et al.*, 1998; Gepstein *et al.*, 2003).

Auxins function in multiple aspects of plant development and growth, including apical dominance, vascular differentiation, and shoot elongation. However, the role of auxin in regulating senescence is not as clear as that of cytokinins, ABA, and ethylene. Several early studies revealed that exogenous application of auxin delayed leaf blade abscission in bean (Shoji *et al.*, 1951; Sacher, 1957). The exogenous application of auxin represses the transcription of *SAG12*, a well-studied senescence-response gene (Noh and Amasino, 1999), and mutation of the *ARF2* and *ARF1* repressors of auxin-responsive transcription can delay senescence and *SAG12* expression (Ellis *et al.*,2005; Okushima *et al.*, 2005; Lim *et al.*, 2010). On the other hand, auxin can stimulate the biosynthesis of senescence-promoting hormones such as ethylene and ABA (Hansen and Grossmann, 2000; Vandenbussche *et al.*, 2003). Further, the concentration of free IAA in senescing leaves of *Arabidopsis* was 2-fold higher than in non-senescing leaves (Quirino *et al.*, 1999), which suggests either that auxin has a senescence-promoting effect or that it accumulates as a consequence of senescence.

The major auxin in plants is indole-3-acetic acid (IAA). Auxin exerts its effects both locally and distally. Local effects are determined by the rate of its local biosynthesis, conjugation, and degradation as well as transport to that site and they have a profound role in plant development and responses to the environment. Localized auxin biosynthesis, in particular, has been recognized as being important (Zhao, 2008; Chandler, 2009). Genetic modulation of the auxin biosynthesis pathways shows that *de novo* auxin biosynthesis is important for the response to shade and for the development of various plant organs (Zhao, 2008; Cheng *et al.*, 2006, 2007; Tao *et al.*, 2008).

Auxin biosynthesis occurs via several interconnecting pathways. Of these, four are tryptophan-dependent. The four pathways involve the conversion of tryptophan to indole-3-acetamide, indole-3-pyruvic acid, indole-3-acetaldoxime, and tryptamine (Stepanova *et al.*, 2008; Tao *et al.*, 2008; Chandler, 2009). Flavin-containing monooxygenases (FMOs) catalyse the rate-limiting step in the tryptamine pathway (Zhao *et al.*, 2001; Kim *et al.*, 2007). The FMOs are encoded by the *YUCCA* genes that constitute a family with 11 members in *Arabidopsis*. Mutational analyses have shown that the YUCCA members have overlapping functions in the localized *de novo* auxin biosynthesis that is important for the development of various plant organs (Cheng *et al.*, 2006, 2007). It is reported here that a dominant activation mutant *yuc6-1D* and *35S:YUC6* transgenic plants display a delayed-senescence phenotype. Under normal growth conditions, the delayed-senescence phenotype manifests as an extended reproductive phase. Detached rosette leaves of the dominant activation mutant *yuc6-1D* and *35S:YUC6* lines exhibit classical, delayed dark-induced senescence that is strongly associated with elevated levels of auxin. Auxin-mediated inhibition of dark-induced senescence in detached leaves involves the control of expression of *SAG12* and the senescence-associated transcription factors *NAC1/ANAC021* and *NAC6/ANAC092*.

Materials and methods

Plant material and growth conditions

The background ecotype of *yuc6-1D* is Col-0 *gl1* and the ecotype of *yuc1-ox* is Col-0 (Zhao *et al.*, 2001; Kim *et al.*, 2007). The ecotype of *35S:iaaL* transgenic plant is Col-0. The *35S:YUC6* transgenic lines were generated in the Col-0 as well as the Col-0 *gl1* background. The generation and isolation of *35S:YUC6* were previously reported by Kim *et al.* (2007). Transgenic lines with single copy inserts and expressing *YUCCA6* at approximately the same level as the *yuc6-1D* mutant were selected for this work. Proper isogenic wild-type plants were used for each experiment. Plants were grown at 20–23 °C on MetroMix 360 (Scotts), under a 16/8 h light/dark cycle and approximately 125 μmol m^{-2} s^{-1} of light intensity in the greenhouse or growth chamber. For growth analyses, seedlings were grown under sterile conditions on Murashige and Skoog media plates containing 0.8% agar and 30 g l^{-1} sucrose. Seeds were surface-sterilized with 20% bleach for 5 min and subsequently washed five times with sterile distilled water. Seeds were cold-treated for 4 d at 4 °C and then plates were placed in a growth room at 22 °C under 80 μmol m^{-2} s^{-1} illumination on a 16/8 h light/dark cycle.

Dark-induced senescence assay

For the senescence assay, 3rd and 4th rosette leaves were detached from 3.5-week-old soil-grown plants. Detached rosette leaves were

incubated in 3 mM MES buffer (pH 5.7) for the designated times and sampled for analysing senescence markers including chlorophyll contents, SAG transcript levels, and photosynthetic activity. The photochemical efficiency of photosystem II (PSII) was measured using a portable plant efficiency analyser (Hansatech Instruments).

Chlorophyll contents and free IAA measurement

Leaf fresh weight was measured before chlorophyll extraction. Total chlorophyll was extracted with 95% ethyl alcohol after incubating samples at 70 °C for 1 h. Absorbance at 665 nm and 649 nm was measured using a spectrophotometer (Shimadzu, Japan). Total chlorophyll contents were calculated as reported by Wintermans and de Mots (1965).

Twenty detached dark-induced senescent leaves were pooled for free IAA measurement. Free IAA was measured as described in Kim *et al.* (2007).

RNA preparation and expression analysis

Total RNA was extracted from the designated tissues using Trizol (Invitrogen). After treatment with DNaseI (Invitrogen), 2 μg of total RNA was used for the synthesis of the first-strand cDNA using the Thermoscript ™ RT-PCR system and oligo dT as primers (Invitrogen). Quantitative PCR was performed in a StepOnePlus™ Real-Time PCR System (Applied Biosystems) using Fast SYBR® Green Master Mix (Applied Biosystems). The relative expression ratios of target genes were calculated in comparison to a reference gene (*UBQ10*) and ΔΔCt methods were used for a relative expression ratio. Primer sequences are shown in Supplementary Table S1 at *JXB* online.

Generation of mutated YUCCA6 *over-expression transgenic plants*

Site-directed mutagenesis of the *YUCCA6* ORF was performed with two primers in opposite orientations: forward primer, 5′-GAAAAAGGGTTCTTGTCGTCG**C**ATGTG**T**AAACTCC reverse primer, 5'-CAAACCTCCATACCGGAGTTT**A**CACAT**G**C-GACGAC. The shared complementary sequence is underlined and the mutated bases G611C and G617T are in bold. The *YUCCA6* ORF cloning–intermediate plasmid pGEM-T-*YUCCA6* was used as the template. The PCR product was transformed into *Escherichia coli* and correct mutagenesis was confirmed in the recovered plasmid by sequencing. Mutated *YUCCA6* ORF PCR products were digested with *Bam*HI and *Pst*I before ligation to a *Bam*HI–*Pst*I fragment from the pCAMBIA1300-PT vector. The construct was introduced into *Agrobacterium tumefaciens*-mediated (strain GV3101) and then into Col-0 *gl1* plants using the floral-dipping transformation method.

Results

YUCCA6 *over-expression plants exhibit delayed leaf senescence*

YUCCA6 (At5g25620) encodes a flavin-containing monooxygenase (FMO) (Kim *et al.*, 2007). It is one of 11 *Arabidopsis thaliana* YUCCA family members that have been reported to be involved in *de novo* auxin biosynthesis (Zhao *et al.*, 2001). The dominant *A. thaliana yuc6-1D* activation mutant contains elevated levels of free IAA due to the over-expression of *YUCCA6* and exhibits phenotypes which are typical in auxin-overproducing plants, for example, curled rosette leaves and long hypocotyls (Kim *et al.*, 2007). The *yuc6-1D* mutant also has a dramatically delayed senescence phenotype. As shown in Fig. 1, *yuc6-1D* and *35S:YUC6* plants show a prolonged life span. Five-month-old mutants even produce new shoots and flowers (Fig. 1A, B). The loss of total chlorophyll content in *yuc6-1D* leaves during the natural senescence process is delayed compared with that in wild-type leaves (Fig. 1C).

The role of auxin in senescence was investigated further by the classic, detached-leaf dark-induced senescence assay system (Miller and Huffaker, 1985; Buchanan-Wollaston *et al.*, 2005). Fully grown third and fourth rosette leaves from the wild type and *yuc6-1D* and *35S:YUC6* transgenic plants were detached and incubated under dark conditions. Total chlorophyll degradation and the loss of photosystem II efficiency during the dark treatment were measured as senescence indicators (Kim *et al.*, 2009). As shown in Fig. 2,

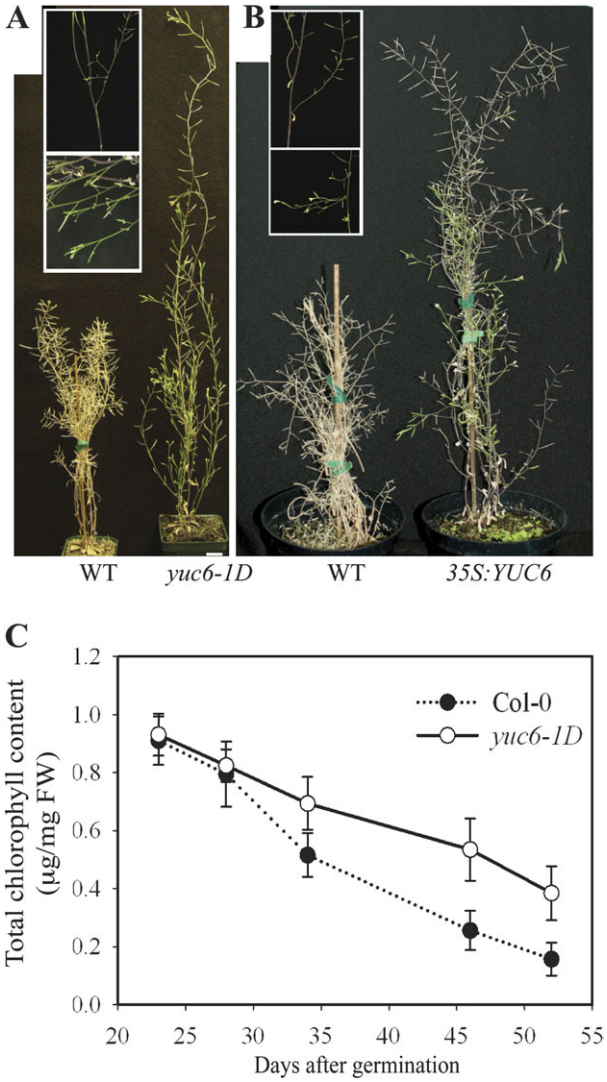

Fig. 1. *YUCCA6* over-expression plants display the staygreen phenotype. Five-month-old *yuc6-1D* (A) and *35S:YUC6* plants (B) are shown along with their wild-type (WT) controls. New shoots and flowers produced from 5-month-old *YUCCA6* over-expression lines are shown in the insets. (C) Total chlorophyll content of the 3rd and 4th rosette leaves of the wild type and *yuc6-1D* are compared. Data represent the mean ±SD (n=10–12).

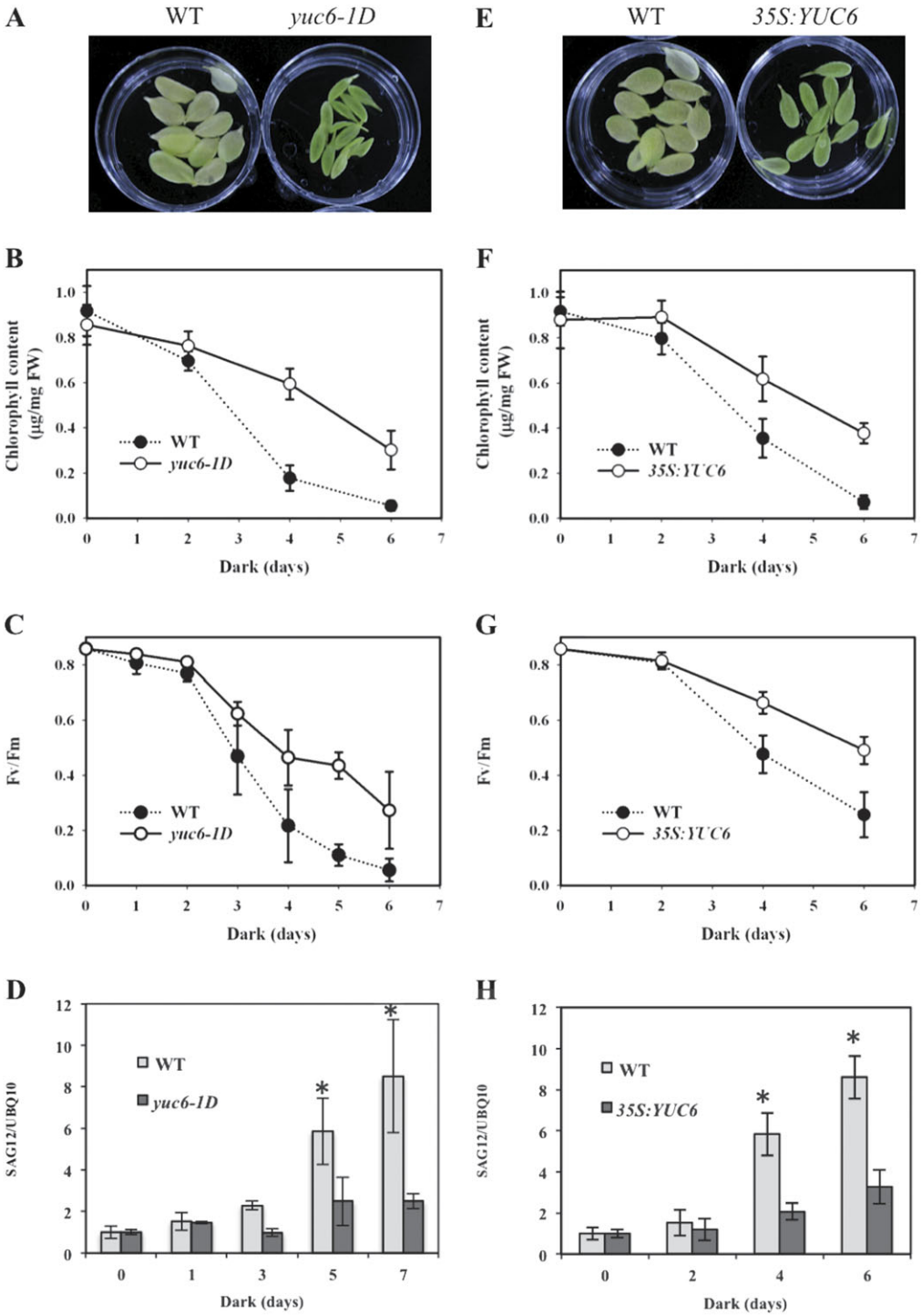

Fig. 2. Over-expression of *YUCCA6* is associated with a delay of dark-induced senescence in detached leaves. Third and fourth rosette leaves from 3.5-week-old wild type (WT), *yuc6-1D*, and *35S:YUC6* plants were detached and incubated in 3 mM MES buffer (pH 5.7) in the dark. (A, E) Leaves were photographed 4 d after incubation in the dark. (B, F) Total chlorophyll content of detached rosette leaves at different time points during dark incubation is shown. (C, G) Photosystem II efficiency (F_v/F_m) of dark incubated rosette leaves is depicted. The data in (B), (C), (F), and (G) represent the mean ±SD (n=12). (D, H) Shown are the mRNA levels of *senescence associated gene 12* (*SAG12*) normalized to *UBQ10* (At4g05320) mRNA levels over the dark-incubation period. mRNA levels were measured by real-time PCR. Data represent the mean ±SD of three biological repeats. Asterisks indicate P <0.05 from Student's t test.

loss of total chlorophyll content and photosystem II efficiency during dark-induced senescence was delayed in both *yuc6-1D* and *35S:YUC6* leaves compared with their isogenic wild-type controls. The transcript of *SAG12* (At5g45890), a well-characterized senescence marker which encodes a cysteine protease, increases specifically during senescence (Nam, 1997; Noh and Amasino, 1999). The accumulation of the *SAG12* transcript was found to be delayed in *yuc6-1D* and

35S:YUC6 leaves during dark-induced senescence compared with wild-type leaves (Fig. 2D, H). Together, these measurements show that over-expression of *YUCCA6* delays dark-induced leaf senescence.

Plants over-expressing mutated YUCCA6 *do not exhibit delayed leaf senescence or phenotypes typical of auxin hyper-accumulation*

Multiple sequence alignments of animal, yeast, and *A. thaliana* FMO genes revealed that YUCCA6 contains conserved binding sites for the cofactors NADPH and FAD. Previous reports have shown that Gly residues in these NADPH and FAD binding sites are required for cofactor binding and enzyme activity of FMOs from both *A. thaliana* and animals (Kubo *et al.*, 1997; Bartsch *et al.*, 2006). The conserved Gly residues in the NADPH binding motif of YUCCA6 were therefore changed to Ala (G204A) and Val (G206V) by site-directed mutagenesis (Fig. 3A). Mutated *YUCCA6* ORF under the control of the cauliflower mosaic virus 35S promoter was transformed into wild-type plants and several single-insertion homozygous lines were identified. Among these homozygous lines, two transgenic lines over-expressing mutated *YUCCA6* were chosen and named as *mYUC6-16* and *mYUC6-11*. Both show enhanced *YUCCA6* transcript expression levels similar to *yuc6-1D* plants and were used for further study (Fig. 3B).

One function of YUCCA6 is to produce free IAA by catalysing the rate-limiting step in tryptophan-dependent auxin biosynthesis. Accordingly, the *yuc6-1D* mutant that over-expresses *YUCCA6* has elevated free IAA levels. By extension, transgenic plants over-expresssing *mYUC6* should have the same IAA contents as untransformed control plants because mYUC6 is expected to be catalytically inactive. A reflection of the elevated free IAA levels in the *yuc6-1D* mutant is the up-regulation of over 30 auxin-responsive genes, including members of the AUX/IAA

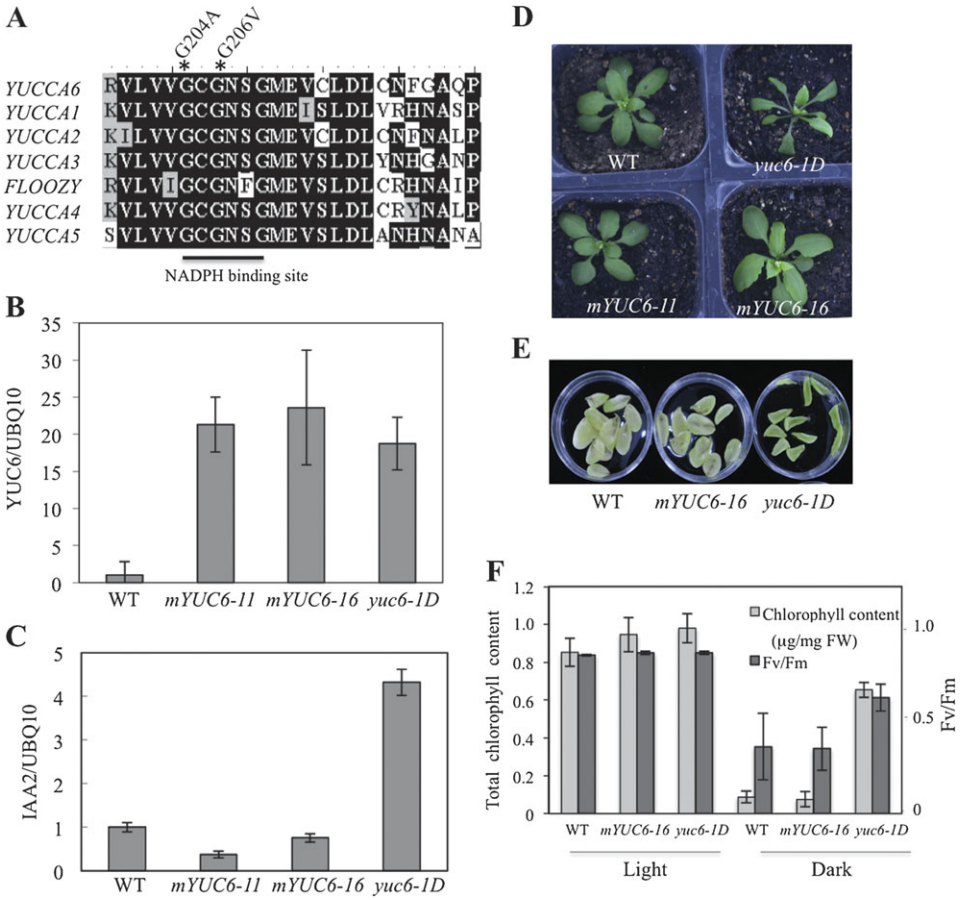

Fig. 3. Over-expression of mutated *YUCCA6* does not result in delayed dark-induced leaf senescence. (A) NADPH binding motif and conserved amino acids in *Arabidopsis* YUCCA family members are shown. Mutated amino acids are marked with asterisks. Glycine-204 and glycine-206 were changed to alanine and valine, respectively. (B, C) Shown are transcript levels of *YUCCA6* and *IAA2* in leaves of 3.5-week-old wild-type plants untransformed (WT) or transformed with *YUCCA6* having mutations in the NADPH binding site (*35S:mYUC6-11* and *35S:mYUC6-16*). Also shown for comparison are transcript levels of *YUCCA6* and *IAA2* in leaves of 3.5-week-old *yuc6-1D* mutant plants. Data represent the mean ±SD (n=3). (D) Shown are 4-week-old WT, *yuc6-1D, 35S:mYUC6-11*, and *35S:mYUC6-16* plants. (E) WT, *35S:mYUC6-16*, and *yuc6-1D* leaves were detached and photographed 4 d after dark incubation. (F) Shown are total chlorophyll contents and photosynthesis efficiencies (F_v/F_m) of untreated leaves (Light) and 4-d dark treated (Dark) WT, *35S:mYUC6-16*, and *yuc6-1D* leaves. Data represent the mean ±SD (n=8).

family of IAA-induced proteins, and the small auxin up RNA (SAUR) family (Kim *et al.*, 2007). The auxin-responsive gene *IAA2* (At3g23030), that was identified as a highly up-regulated gene in *yuc6-1D* by microarray analysis (Kim *et al.*, 2007), was therefore selected to indirectly compare auxin levels in leaves. As expected, expression of *IAA2* was highly increased in *yuc6-1D* leaves but not in leaves of *mYUC6-11* and *mYUC6-16* (Fig. 3C). Another indicator of auxin over-accumulation is the morphological phenotype of narrow and downward curled rosette leaves. This narrow and curled rosette leaf phenotype is observed in *Arabidopsis* lines over-expressing *YUCCA1*, *-2*, *-3*, *-4*, -5, and *-6* (Zhao *et al.*, 2001; Marsch-Martinez *et al.*, 2002; Woodward *et al.*, 2005; Kim *et al.*, 2007; Fig. 3D). However, *mYUC6-11* and *mYUC6-16* mutants did not display curled leaf morphology even though these mutants contain over-expressed mutated *YUCCA6* (Fig. 3B, D). The wild-type level of auxin-inducible gene expression and no high-auxin morphological phenotype in *mYUC6-11* and *mYUC6-16* plants suggest that over-expression of mutated *YUCCA6* could not elevate the auxin level. Dark-induced senescence was then tested with detached *mYUC6-16* rosette leaves. The total chlorophyll content and photosystem II efficiency of *mYUC6-16* and wild-type leaves that had been dark-treated for 4 d were comparable, whereas leaves of the *yuc6-1D* mutant retained more chlorophyll and greater photosystem II efficiency (Fig. 3E, F). These results suggest that the conserved NADPH binding site of FMOs that is required for catalytic activity is also required for YUCCA6 to function in auxin biosynthesis and delayed leaf senescence. This led to the hypothesis that the delayed leaf senescence phenotype of *yuc6-1D* is mediated by auxin.

Delay of dark-induced leaf senescence in yuc6-1D *is mediated by auxin*

To investigate whether elevated auxin levels in *yuc6-1D* actually result in a delayed senescence phenotype, *A. thaliana 35S:iaaL* transgenic plants that constitutively over-express *IAA-lysine synthetase* (*iaaL*) under the control of the CaMV 35S promoter (Romano *et al.*, 1991) were used. The *iaaL* gene encodes the *Pseudomonas savastanoi IAA-lysine* synthetase. In tobacco, constitutive overproduction of *iaaL* leads to a 20-fold reduction in free IAA with a concomitant increase in IAA-lysine resulting in morphological changes associated with auxin deprivation (Romano *et al.*, 1991). The phenotype of transgenic *Arabidopsis* plants that express *35S:iaaL* is also consistent with a reduction in free IAA (Jensen *et al.*, 1998). Therefore *yuc6-1D* was crossed with a *35S:iaaL* transgenic plant to eliminate the effects of excess auxin and phenotypes of the *yuc6-1D* parent and F_1 progeny (*35S:iaaL*×*yuc6-1D*) were compared. *yuc6-1D* plants exhibit long hypocotyls and increased expression of *IAA2* due to elevated auxin levels in *yuc6-1D* (Fig. 4A–C). Hypocotyls of F_1 progeny of the *35S:iaaL*×*yuc6-1D* plants failed to elongate as in *yuc6-1D*. Expression of the auxin-response gene *IAA2* in *35S:iaaL*×*yuc6-1D* was not as high as in *yuc6-1D* (P <0.01) also indicating that over-expressing the *iaaL* gene in *yuc6-1D* plants reduces the effects of the elevated auxin level observed in *yuc6-1D* plants (Fig. 4C). Finally, a dark-induced senescence assay was performed with *35S:iaaL*×*yuc6-1D* F_1 leaves. As shown in Fig. 4D, the loss of total chlorophyll content in *35S:iaaL*×*yuc6-1D* leaves during dark-induced senescence is not delayed as in *yuc6-1D,* while the loss of total chlorophyll content in *35S:iaaL* during dark-induced senescence is more than in wild-type plants. This suggests that elevated free auxin levels *in planta* protect against leaf senescence.

Auxin levels decrease during dark-induced senescence

Since auxin accumulation causes delayed senescence, it should follow that auxin levels decrease during dark-induced senescence. To test this hypothesis, free IAA levels were measured in dark-treated wild-type leaves and it was found that free IAA levels gradually decreased during dark-induced senescence (Fig. 5A). Transcript levels of the auxin-inducible gene, *IAA2*, decreased during dark-induced senescence in wild-type leaves, which is consistent with the observed decrease in free IAA content (Fig. 5A, B).

The free IAA pool is determined by multiple metabolic processes, which include *de novo* auxin biosynthesis, inactivation or storage of IAA by conjugation, and degradation of free IAA. Members of the *GH3* family encode IAA-amino synthetases that function in the storage or inactivation of IAA by conjugation to amino acids (Staswick *et al.*, 2005). As shown in Fig. 5C, the expression of *GH3.1* (At2g14960), *GH3.3* (At2g23170), *GH3.5* (At4g27260), and *GH3.6* (At5g54510) was gradually increased during dark-induced senescence, which is consistent with previous reports that also showed the increase of *GH3.2* (At4g37390), *GH3.5*, and *GH3.17* (At1g28130) transcripts during natural senescence and dark-induced senescence (Buchanan-Wollaston *et al.*, 2005; van der Graaff *et al.*, 2006). Considering that GH3 members are auxin-inducible genes, there appears to be a senescence-specific regulatory mechanism controlling *GH3* transcript accumulation. *Arabidopsis* YUCCA family members, such as YUCCA1 and YUCCA6, function in *de novo* auxin biosynthesis (Zhao, 2008). Therefore, transcript levels of *YUCCA1* (At1g21430) and *YUCCA6* were measured and it was found that they were decreased during dark-induced senescence (Fig. 5C). Thus both increased expression of the GH3 family of auxin-conjugating enzymes and decreased expression of auxin biosynthesis components (exemplified by *YUCCA1* and *YUCCA6*) contribute to the gradual reduction of free IAA levels during dark-induced senescence.

Plants over-expressing *YUCCA1*, similar to plants over-expressing *YUCCA6*, display phenotypes typical of plants with high levels of endogenous auxin indicating its involvement in auxin biosynthesis (Zhao *et al.*, 2001; Kim *et al.*, 2007). If it is really the free auxin level that determines the degree of senescence, it should follow that (i) over-expression of *YUCCA1*, that is down-regulated during senescence like *YUCCA6*, should result in delayed

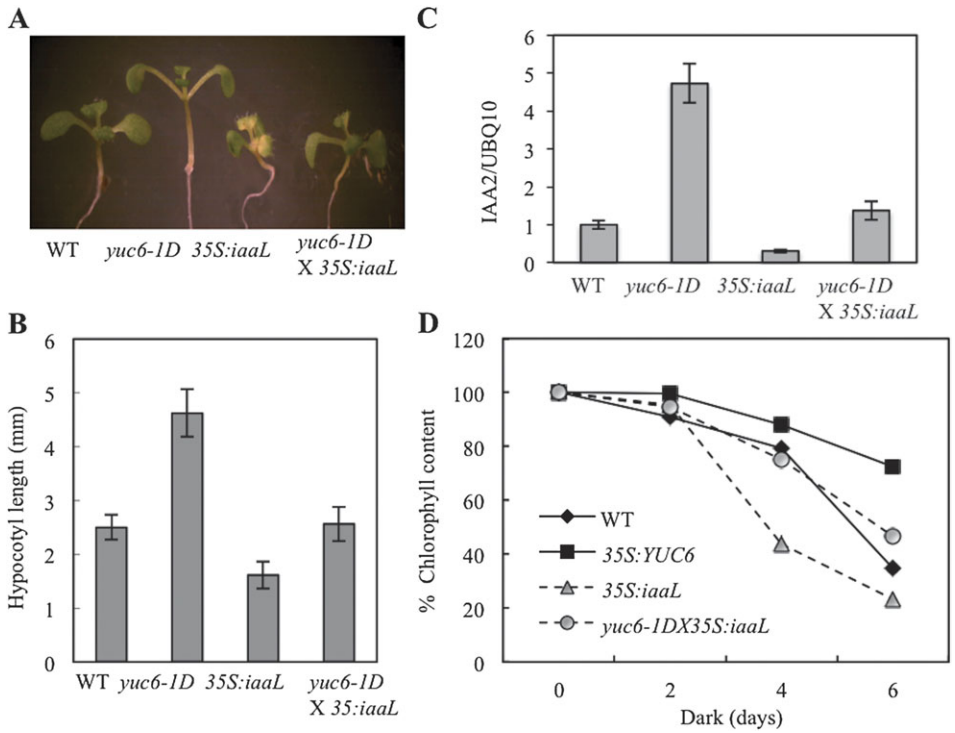

Fig. 4. The effects of excess auxin are eliminated in *35S:iaaL* transgenic plants. (A) Eight-day-old wild-type (WT), *yuc6-1D*, *35S:iaaL*, and *35S:iaaL* X *yuc6-1D* seedlings are shown. (B) Shown are lengths of hypocotyls of 8-d-old seedlings of the indicated lines. Data represent the mean ±SD (n=10–12). (C) Depicted are the relative expression levels of the auxin response gene *IAA2* in rosette leaves of 3.5-week-old plants that were measured by real-time PCR. *UBQ10* was used for internal control. Data represent the mean ±SD (n=3). (D) Shown are per cent total chlorophyll contents during dark-induced senescence in detached leaves of 3.5-week-old WT, *35S:YUC6*, *35S:iaaL*, and *35S:iaaL* X *yuc6-1D* leaves. The percentage total chlorophyll content was calculated from the ratio of the average of the chlorophyll content at the designated time to the average of the chlorophyll content at day zero (n=10–12).

senescence and (ii) exogenous application of auxin to wild-type leaves should delay dark-induced senescence. To test this hypothesis, rosette leaves of *yuc1-ox* which is a *YUCCA1* over-expression line were detached and incubated in the dark. As shown in Fig. 6A and B, *yuc1-ox* plants also showed delayed dark-induced senescence compared with the wild type. Furthermore, when the synthetic auxin α-naphthalene acetic acid (NAA) was exogenously applied to wild-type leaves, dark-induced senescence was delayed compared with mock-treated leaves (Fig. 6C, D). Considered together with data showing that *35S:iaaL*×*yuc6-1D* leaves tend to senesce at a rate similar to the wild type (Fig. 4D), these data support the hypothesis that elevated auxin delays dark-induced leaf senescence.

It has been reported that mature rosette leaves of the *yuc6-1D* mutant have a higher auxin content than those of wild-type plants (Kim *et al.*, 2007). Consequently, the free IAA content of detached *yuc6-1D* leaves, which also decreased during dark-induced senescence, remained proportionately higher than that of the wild type over the duration of the treatment (see Supplementary Fig. S1 at *JXB* online). The extent of senescence in detached leaves (Fig. 2A–D) was inversely correlated with intracellular auxin levels (see Supplementary Fig. S1 at *JXB* online).

YUCCA6 *over-expression affects the expression of senescence-associated genes*

Among hundreds of senescence-associated genes (*SAG*s), *SAG12* expression is regulated in a senescence-specific mode. Specifically, *SAG12* expression is limited to actively senescent tissues. *SAG12* expression was down-regulated during dark-induced senescence by over-expression of *YUCCA6* (Fig. 2D, H). It has been reported that application of auxin to wild-type *Arabidopsis* leaves during dark-induced senescence down-regulates the transcript level of *SAG12* compared with mock-treated leaves (Noh and Amasino, 1999). Therefore, down-regulation of *SAG12* expression during dark-induced senescence by over-expression of *YUCCA6* (Fig. 2D, H) is likely to be mediated by auxin.

Transcription factors are fundamental elements of the central regulatory networks for plant processes. It has been shown that the expression of 185 transcription factor genes changes during senescence (Balazadeh *et al.*, 2008). Specifically, NAC family transcription factors are shown to be involved in senescence (Guo *et al.*, 2005; Kim *et al.*, 2009). Among over 100 NAC family members, the transcripts of 20 NAC transcription factors including *A. thaliana NAC1*/*ANAC021* (At1g56010) and *NAC6*/*ANAC092* (At5g39610) are up-regulated during the senescence process (Balazadeh

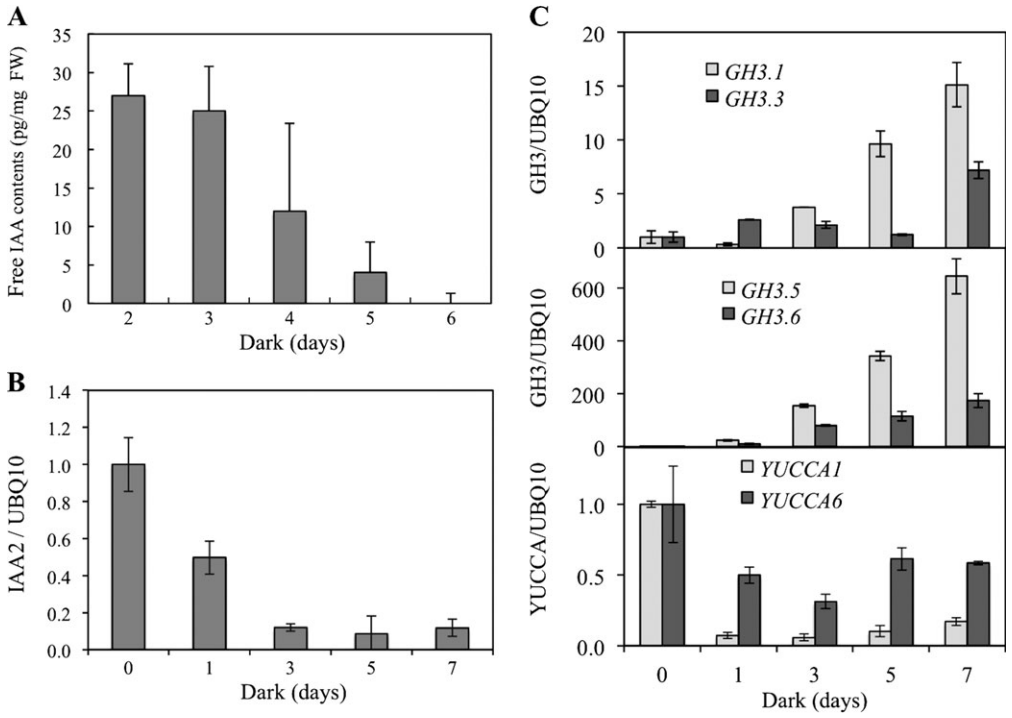

Fig. 5. Auxin level decreases during dark-induced senescence. All measurements were made using detached 3rd and 4th rosette leaves of 3.5-week-old wild-type plants. The leaves were incubated in 3 mM MES buffer (pH 5.7) in the dark to induce senescence. (A) Shown are the free IAA levels in the leaves over the dark treatment. Twenty leaves from each time point were pooled for IAA measurement. Data shown are the means ±SD from three biological and technical repeats. (B) Shown are expression levels of the auxin response gene (*IAA2*) during dark-induced senescence. (C) Shown are expression levels of the auxin conjugating enzymes (*GH3.1*, *GH3.3*, *GH3.5*, and *GH3.6*) and the auxin biosynthesis genes (*YUCCA1* and *YUCCA6*) during dark-induced senescence. Gene expression levels (B, C) were monitored by real-time PCR. *UBQ10* was used for internal control. Data represent the mean ±SD from three biological and technical repeats.

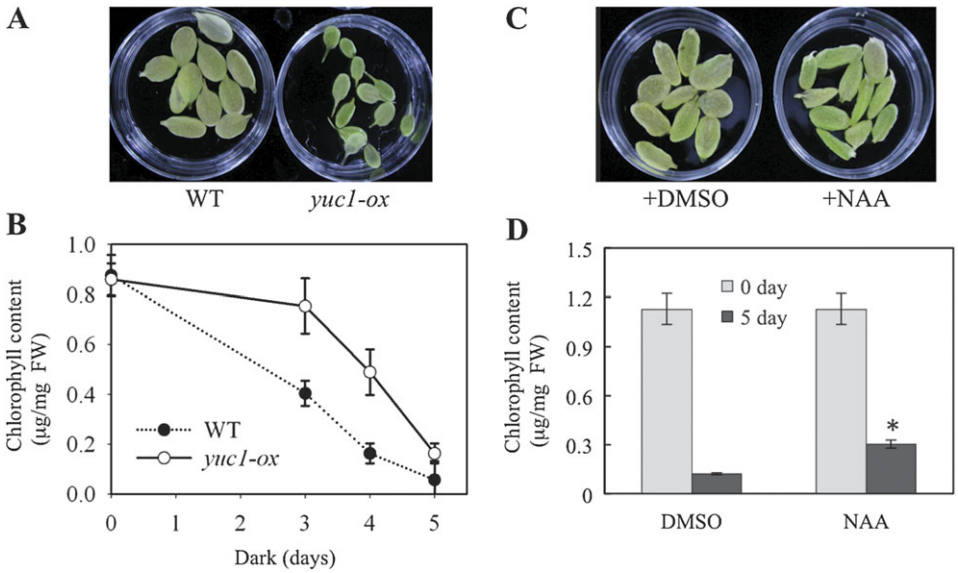

Fig. 6. Over-expression of *YUCCA1* and exogenous auxin treatment result in delayed dark-induced leaf senescence. All experiments were performed with detached 3rd and 4th rosette leaves of 3.5-week-old plants. (A) Shown are wild type (WT) and *yuc1-ox* detached leaves that were dark-treated for 4 d. (B) Chlorophyll contents from WT and *yuc1-ox* were measured at different time points during dark incubation. Data represent the mean ±SD (n=10–12). (C) Shown are detached WT leaves that were incubated in 3 mM MES buffer (pH 5.7) with DMSO or 20 μM NAA for 4 d under dark conditions. (D) Shown are total chlorophyll contents of detached WT leaves incubated as in (C) for 5 d. Data represent the mean ±SD (n=10). Asterisk indicates P <0.05 from Student's t test.

et al., 2008). Kim *et al.* (2009) showed that loss-of-function mutation in *NAC6* resulted in delayed leaf senescence and *NAC6* transcripts increased during senescence. Accordingly, it was found that the expression of *A. thaliana NAC1* and *NAC6* gradually increased in wild-type leaves during dark-induced senescence (Fig. 7B, D), paralleling the decrease in the free IAA content of the leaves (Fig. 5). Expression of *NAC1* and *NAC6* gradually increased in *yuc6-1D* leaves also during dark-induced senescence (Fig. 7B, D), albeit to a much lower extent compared with the wild type. *NAC1* and *NAC6* expression in leaves of a *35S:YUC6* transgenic plant also remained lower than in the wild type but was comparable with that of *yuc6-1D* during dark-induced senescence (Fig. 7A, C). These data provide further molecular evidence that over-expression of *YUCCA6*, and thereby elevated auxin, delays dark-induced leaf senescence. Since *NAC6* is known to control age-related senescence positively, *NAC6* transcripts are up-regulated during senescence and microarray analyses have shown that *NAC6* controls the expression of a large percentage of SAGs (Kim *et al.*, 2009; Balazadeh *et al.*, 2010), it is possible that elevated auxin delays senescence, in part through the regulation of levels of the transcription factor *NAC6*.

Discussion

Although half a century has passed since the first report of the retarding effect of auxin in leaf abscission (Addicott *et al.*, 1955; Thimann, 2000), few details of this function of auxin have since been revealed. As a critical hormone for plant growth and development, auxin homeostasis is closely controlled. The data presented here clearly show that the level of free IAA is controlled during senescence and an elevated auxin level delays leaf senescence.

The *yuc6-1D* mutant was initially identified from its strong apical dominance and extremely long lifespan (Kim *et al.*, 2007). The rosette leaves of *yuc6-1D* mutants also exhibited delayed senescence symptoms, although the prolonged life span after flowering is accomplished mainly by the continued production of new lateral shoots during the reproduction phase (Fig. 1). Biochemical analyses have shown that *Arabidopsis* YUCCA family proteins catalyse the rate-limiting step in auxin biosynthesis and YUCCA over-expression mutants exhibit phenotypes typical of elevated auxin levels (Zhao *et al.*, 2001; Cheng *et al.*, 2007; Kim *et al.*, 2007).

Detached vegetative rosette leaves of the *YUCCA* over-expression lines, *yuc6-1D*, *yuc1-ox*, and *35S:YUCCA6*, undergo senescence more slowly than wild-type leaves in response to dark treatment. This is manifested as greater chlorophyll retention, a more gradual decline of photosystem II efficiency, and reduced accumulation of transcripts of senescence marker genes such as *SAG12*, *NAC1*, and *NAC6* in dark-treated leaves of *YUCCA6* over-expression plants compared with the wild type (Figs 2, 7).

Free IAA content decreased systematically during dark-induced senescence in detached leaves and the protective effect of the *yuc6-1D* mutation on leaf senescence could be abrogated by a transgenic intervention that increased

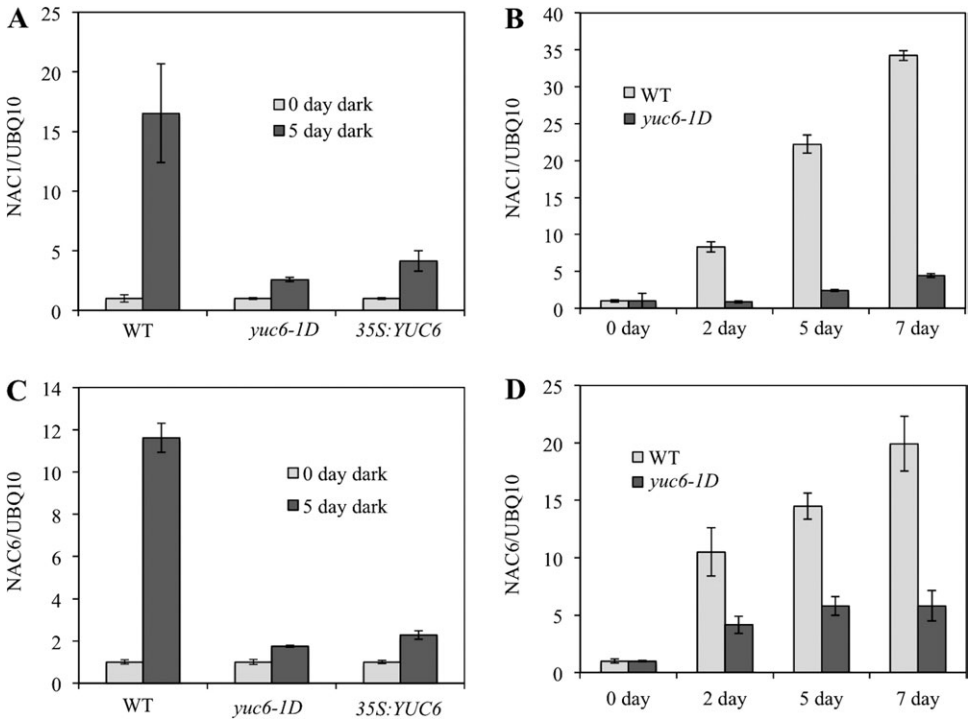

Fig. 7. Over-expression of *YUCCA6* suppresses the up-regulation of *NAC1/ANAC021* and *NAC6/ANAC092* during dark-induced senescence. Third and fourth rosette leaves of 3.5-week-old plants were detached and incubated in 3 mM MES buffer (pH 5.7) in the dark. (A) *NAC1* and (C) *NAC6* expression levels were measured in wild-type (WT), *yuc6-1D*, and *35S:YUC6* leaves with (grey) or without (white) 5-d dark treatment. Expression levels of (B) *NAC1* and (D) *NAC6* during dark treatment were measured in WT (white) and *yuc6-1D* (grey) during dark incubation. *UBQ10* was used for an internal control. Data represent the mean ±SD (n=3).

conjugation of free auxin *in vivo* (Figs 4, 5A). Transcript levels of the auxin response gene *IAA2* confirmed the change of free IAA levels in these experiments. The expression of *de novo* auxin biosynthetic enzymes such as *YUCCA1* dropped quickly, whereas the expression of auxin-conjugating enzymes *GH3.1, GH3.3, GH3.5*, and *GH3.6* gradually increased during the senescence process (Fig. 5) explaining the reduced free IAA level through the reduction of *de novo* auxin biosynthesis together with the induction of auxin conjugation. It should also be noted that significant differences occur during dark-induced senescence in detached leaves and natural senescence. For example, the decline in auxin content during dark-induced senescence in detached leaves could be due to altered light signalling or no import of auxins into the leaves. In this case, the decline in auxin content during dark-induced senescence (Fig. 5; see Supplementary Fig. S1 at *JXB* online) may have little to do with events during natural senescence. However, by minimizing variations, the dark-induced senescence assay allowed us to demonstrate that the auxin level correlates negatively with senescence. While the results presented here demonstrate that reduced levels of free auxin induce senescence in leaves, the mechanism by which reduced auxin content suppresses the accumulation of SAGs remains to be ascertained.

Many studies with hormone-response mutants suggest that plant hormones interact or cross-talk to co-ordinate growth, development, and stress responses. Cytokinin is a senescence-delaying hormone and induces the expression of genes encoding negative regulators of cytokinin response such as type A-ARRs (Taniguchi *et al.*, 1998). The expression of type A-ARRs is reduced in *yuc6-1D* leaves compared with the wild type (see Supplementary Fig. S2 at *JXB* online) suggesting that the delayed senescence phenotype of *yuc6-1D* does not result from an elevated level of cytokinin. Exogenously applied JA and ABA have been shown to promote natural and dark-induced leaf senescence (Pourtau *et al.*, 2004; Castillo and León, 2008), and it was also found that exogenous ABA and MeJA promoted a gradual decline in the total chlorophyll content and photosystem II efficiency in wild-type leaves (see Supplementary Fig. S3 at *JXB* online). However, the loss of total chlorophyll content and photosystem II efficiency induced by ABA and MeJA was less severe in *yuc6-1D* and *yuc1-ox* leaves than in the wild type (see Supplementary Fig. S3 at *JXB* online). Thus, elevated auxin retards ABA- and JA-induced leaf senescence, consistent with its protective effect on dark-induced senescence.

The recent results of Lim *et al.* (2010) show that mutation of *ARF2*, a gene encoding a repressor of auxin signalling, leads to delayed leaf senescence phenotypes which resemble those of the *yuc6-1D* mutant (Figs 1, 2; see Supplementary Fig. S3 at *JXB* online). The rosette leaves of *arf2* and *yuc6-1D* mutants exhibit delayed developmental senescence, and detached leaves exhibited delayed senescence in response to dark, ABA, and MeJA treatments. Reduced repression of auxin signalling in the *arf2* mutant was associated with increased sensitivity to auxin, which is mechanistically equivalent to an elevated auxin level *in vivo*. Thus the data of Lim *et al.* (2010), together with our results, support the notion that elevated auxin content and auxin signalling have a protective effect against leaf senescence in response to several triggers. Auxin plays key roles in plant development and growth. It is therefore possible that the delayed leaf senescence phenotype of the *YUCCA6* over-expression plants can be attributed wholly or partly to altered growth and development due to their high auxin content. However, there was no difference in the timing of leaf emergence or bolting time between *yuc6-1D*, *35S:YUC6*, and wild-type plants. Our observation that the exogenous application of auxin to detached leaves of wild-type plants delayed senescence and a previous report that the exogenous application of auxin represses transcription of the *SAG12* marker gene are consistent with the notion that auxin retards senescence (Noh and Amasino, 1999). However, interpretation of the results of a study of the effect of the exogenous application of auxin is always subject to uncertainties arising from the amount of auxin used and from contributions of different intracellular pools of auxin to the observed phenotype.

The *yuc6-1D* mutant exhibits strong apical dominance consistent with an elevated auxin content (Kim *et al.*, 2007). It has been known for a very long time that the primary site of auxin synthesis is the shoot apex and the downward transport of auxin in the shoot inhibits shoot branching (Leyser, 2003). However, prolonged life span after flowering is accomplished mainly by the continued production of new lateral shoots during the reproduction phase (Fig. 1). A few reports suggest that locally synthesized auxin promotes bud outgrowth. First, the auxin level rises in buds as they activate. Second, apically synthesized auxin does not get transported into buds (Morris, 1977). Lastly, the direct application of auxin to buds does not inhibit their outgrowth. Studies with *YUCCA* family members consistently show that local auxin synthesis is important and that *YUCCA* family members have unique as well as overlapping functions (Cheng *et al.*, 2006, 2007; Chandler, 2009; Sakata *et al.*, 2010). Our results show that *YUCCA1* transcript levels drop dramatically during senescence (Fig. 5C) and over-expression of *YUCCA1* delays dark-induced senescence in detached leaves (Fig. 6). However, expression of *YUCCA2* and *YUCCA4* are not significantly changed during senescence (data not shown), and the increase in life span accompanied by the continued production of new lateral shoots during the reproduction phase has not yet been reported in plants over-expressing other *YUCCA* genes. It is quite likely that YUCCA proteins contribute to unique auxin pools to elicit specific functions (Cheng *et al.*, 2006, 2007).

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Free IAA levels decrease during dark-induced senescence of *yuc6-1D* leaves but remain high compared with wild-type leaves.

Supplementary Fig. S2. Expression of Type-A *Arabidopsis response regulators* (*ARRs*) is down-regulated in *yuc6-1D* rosette leaves.

Supplementary Fig. S3. ABA- and methyl jasmonate-induced senescence is delayed in *yuc6-1D* and *yuc1-ox* plants compared with the wild type.

Supplementary Table S1. Quantitative RT-PCR primer sequences.

Acknowledgements

We thank Dr Harry Klee for allowing us to use *Arabidopsis thaliana 35S:iaaL* transgenic plants and Dr Mark Estelle for sending us seeds of the transgenic *35S:iaaL* plants. We also thank Dr Yunde Zhao for providing *yucca1-ox* seeds. This work was supported by the World Class University Program (grant no. R32-10148; to DJY), the Next-Generation BioGreen 21 Program (SSAC, to DJY), Rural Development Administration, Republic of Korea and from the National Science Foundation (NSF-IOS 0820648, to ASM).

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