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

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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乙烯-resistant 1 (etr1) and乙烯-insensitive 2 (em2) 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 monoxygenases (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 Wintemans 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'-GAAAAAGGGTTCTTGTGCATGTGAAACTCC reverse primer, 5'-CAAACCTCCATACCGGAGTTTACACATGC-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 BamHI and PstI before ligation to a BamHI–PstI 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 mono-oxygenase (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).]
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

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 (Fv/Fm) 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.
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-expressing 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

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**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 (Fv/Fm) 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 F1 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 F1 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 F1 leaves. As shown in Fig. 4D, the loss of total chlorophyll content in 35S::iaaL 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
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 a-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 (SAGs), 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 NAC11/ANAC021 (At1g56010) and NAC6/ANAC092 (At5g39610) are up-regulated during the senescence process (Balazadeh et al., 2008).
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. UBOQ10 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-ax 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. 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.

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