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Research article

Transgenic poplar expressing *Arabidopsis YUCCA6* exhibits auxin-overproduction phenotypes and increased tolerance to abiotic stress

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ABSTRACT

YUCCA6, a member of the YUCCA family of flavin monooxygenase-like proteins, is involved in the tryptophan-dependent IAA biosynthesis pathway and responses to environmental cues in *Arabidopsis*. However, little is known about the role of the YUCCA pathway in auxin biosynthesis in poplar. Here, we generated transgenic poplar (*Populus alba* × *P. glandulosa*) expressing the *Arabidopsis YUCCA6* gene under the control of the oxidative stress-inducible *SWPA2* promoter (referred to as SY plants). Three SY lines (SY7, SY12 and SY20) were selected based on the levels of *AtYUCCA6* transcript. SY plants displayed auxin-overproduction morphological phenotypes, such as rapid shoot growth and retarded main root development with increased root hair formation. In addition, SY plants had higher levels of free IAA and early auxin-response gene transcripts. SY plants exhibited tolerance to drought stress, which was associated with reduced levels of reactive oxygen species. Furthermore, SY plants showed delayed hormone- and dark-induced senescence in detached leaves due to higher photosystem II efficiency and less membrane permeability. These results suggest that the conserved IAA biosynthesis pathway mediated by YUCCA family members exists in poplar.

1. Introduction

Poplar, a perennial plant, has many uses and environmental benefits, such as providing raw materials, reducing erosion, moderating climate conditions, and helping to maintain biodiversity (Harfouche et al., 2011). However, with the increasing demand for both forest products and conservation of the ecosystem, it has become increasingly urgent to develop poplar with improved growth, wood quality, and tolerance to abiotic stress for use in reforestation.

Auxin is a central, key regulator of a variety of plant physiological and developmental processes (Hooley, 1998). Although auxin has been studied for over 100 years, its biosynthesis, transport and signaling pathways are still not well understood. Several interconnecting pathways have been proposed to synthesize auxin in plants, including four tryptophan (Trp)-dependent routes and a Trp-independent pathway (Bartel, 1997; Mano and Nemoto, 2012). Trp-dependent auxin biosynthesis involves the conversion of Trp to indole-3-acetaldoxime, indole-3-acetaide, indole-3-phyruvic acid (IPA), and tryptamine (TAM), which has been clearly demonstrated to be important for cell division and differentiation, apical dominance, root and fruit growth and development, flowering, senescence, and other developmental processes (Zhao et al., 2002; Mashiguchi et al., 2011).

The YUCCA family of flavin monooxygenase (FMO)-like proteins catalyzes the rate-limiting step in the TAM pathway (Won et al., 2011). In addition, recent studies have revealed that YUCCAs also function in the IPA pathway in *Arabidopsis* (Mashiguchi et al., 2011). Eleven *YUCCA* members play essential roles in auxin biosynthesis and plant development in *Arabidopsis* (Zhao et al., 2001; Cheng et al., 2006). *YUCCA* genes are important for auxin biosynthesis in other plant species, such as potato, petunia, tomato, rice, maize,

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strawberry, and poplar (Tobeña-Santamaria et al., 2002; Expósito-Rodríguez et al., 2007; Yamamoto et al., 2007; Ye et al., 2009; LeClere et al., 2010; Liu et al., 2012; Kim et al., 2013). Although it is clear that *YUCCA* genes play a critical role in maintaining auxin levels in plants, the cellular and biochemical characteristics of these family members, as well as their specific functions, have remained elusive. Recent biochemical studies in recombinant *Arabidopsis* YUCCA6 have shown that YUCCA6 contains oxidized flavin adenine dinucleotide (FAD), which is reduced by nicotinamide adenine dinucleotide phosphate (NADPH); reduced YUCCA6 then reacts with IPA to generate IAA (Dai et al., 2013). Overexpression of *Arabidopsis YUCCA6* results in increased plant height and longevity, enhanced drought tolerance (in both *Arabidopsis* and potato), and delayed leaf senescence (in *Arabidopsis*) (Kim et al., 2013). Transgenic strawberry overexpressing *FvYUC6* (strawberry *YUCCA6*-like gene) also exhibits typical auxin-overproduction phenotypes and delayed flowering time (Liu et al., 2014). Genes highly homologous to *Arabidopsis YUCCA* genes have also been identified in poplar. Twelve *YUCCA* genes from *Populus trichocarpa* (*PtYUCCA*) are expressed in a tissue-specific manner in shoot tip, immature and mature leaf, young root, stem, and bark tissues (Ye et al., 2009). *PtYUCCA* genes are downregulated under hormone or abiotic stress treatments in most plant tissues (Ye et al., 2009). However, little is known about the regulation of YUCCA-mediated auxin biosynthesis in poplar. Unraveling the mechanisms underlying auxin biosynthesis and its correlation with environmental cues in trees would greatly accelerate current tree breeding programs.

In this study, we developed transgenic poplar (*Populus alba* × *Populus glandulosa*) expressing the *Arabidopsis YUCCA6* gene under the control of the oxidative stress-inducible *SWPA2* promoter using *Agrobacterium tumefaciens*-mediated transformation. Our results provide evidence suggesting that the YUCCA pathway of IAA biosynthesis exists in poplar.

2. Materials and methods

2.1. Plant materials and growth conditions

Hybrid poplar, *P. alba* × *P. glandulosa* was used in this study. The plants were sub-cultured monthly by transferring stem cuttings with double nodes to fresh rooting medium (RM), i.e., 1 × Murashige and Skoog (MS) medium containing 0.2 mg/L IBA (Murashige and Skoog, 1962). After 1 month of culture on RM, rooted plantlets were transplanted to soil and grown in a growth chamber at 25 °C, 60% relative humidity under a light intensity of 150 μmol m^{-2} s^{-1}, and a 16/8 h (light/dark) photoperiod.

2.2. Generation of transgenic poplar plants

To construct the plant expression vector pCAMBIA2300-*SWPA2-YUCCA6*, *YUCCA6* containing *Eco*RI and *Pst*I sites (kindly provided by Prof. Dae-Jin Yun, Gyeongsang National University, Jinju, Korea) was ligated into the corresponding sites of a modified pCAMBIA2300 binary vector under the control of the oxidative stress-inducible *SWPA2* promoter (Kim et al., 2003, 2011b), which harbors the *nptII* gene for use as a selectable marker. The construct was introduced into poplar plants by *Agrobacterium tumefaciens* (strain *EHA105*)-mediated transformation, as described by Choi et al. (2005). The transformed calli were selected on callus induction medium (CIM) comprising 1 × MS medium containing 1.0 mg/L 2,4-dichlorophenoxyacetic (2,4-D), 0.1 mg/L benzylaminopurine (BAP), and 0.01 mg/L 1-naphthylacetic acid (NAA) supplemented with 50 mg/L kanamycin. Kanamycin-resistant shoots were regenerated from calli via transfer to shoot induction medium (SIM) comprising Woody Plant Medium (Lloyd and McCown, 1980) containing 1.0 mg/L zeatin, 0.1 mg/L BAP, and 0.01 mg/L NAA. Regenerated shoots were transferred to RM for rooting, and the rooted plantlets were transplanted to pots and grown in a growth chamber.

2.3. Gene expression analysis

Total RNA was extracted from the indicated plant tissues using a GeneAll Ribospin Plant™ kit (GeneAll, Seoul, Korea) according to the manufacturer's instructions. For cDNA synthesis, 2 μg of total RNA was reverse transcribed using an RT-PCR kit (TOPscript™ RT Dry MIX). The reaction mixture was diluted in sterile water to a total volume of 100 μL, and 2 μL was used for real-time quantitative RT-PCR. The gene-specific primers are listed in Supplementary Table 1. All quantitative RT-PCR analysis was performed in a CFX96 Touch Real-time PCR Detection System (DNA Engine Opticon 2, MJ Research, USA) using Ever-Green 20 fluorescent dye (BioFACT, Seoul, Korea). The following program was used for PCR: 44 cycles of PCR (95 °C for 20 s, 60 °C for 20 s, and 72 °C for 20 s) after an initial denaturation step for 5 min at 95 °C. All reactions were repeated at least three times.

2.4. Southern blot analysis

Genomic DNA from poplar was extracted from leaves using the protocol described by Kim and Hamada (2005), separated on a 0.8% agarose gel after restriction digestion with *Eco*RI (Roche, Manheim, Germany), transferred to a Zeta-probe GT membrane (Bio-Rad, CA, USA), and hybridized with a ^{32}P-labeled probe designed based on the *YUCCA6* cDNA fragment, a divergent sequence of *PtYUCCA* cDNA.

2.5. Extraction and measurement of free IAA contents

Endogenous IAA from the indicated tissues was extracted as described by Pan et al. (2010). Ground tissues were incubated in extraction solvent (2-propanol/H_2O/concentrated HCl, 2:1:0.002, v/v/v) with a sample:solvent ratio of 1:10 (mg/μL) on a shaker at 100 rpm for 30 min at 4 °C; 1 mL dichloromethane was then added to each sample. After shaking for 30 min at 4 °C, the sample was centrifuged at 13,000 g for 5 min at 4 °C and the lower phase was concentrated using a nitrogen evaporator with nitrogen flow and re-dissolved in 0.1 mL methanol for IAA measurements.

Free IAA contents were measured using a Phytodetek™ IAA Test kit (Agdia, CA, USA) according to the manufacturer's instructions. The assay uses the competitive antibody binding method. Antibody-coated microwells were filled with alkaline phosphatase-labeled IAA-tracer and plant extract. The hormone in the plant sample competed with the tracer for antibody binding sites. After incubation at 4 °C for 3 h, the contents of the wells were expelled, and the wells were refilled with substrate solution. After incubation at 37 °C for 60 min, the absorbance values at 405 nm were measured using an iMark™ Microplate Reader (Bio-Rad, CA, USA). Calculation of sample IAA concentrations followed the standard curve. Each experiment was performed with at least three biological replicates and two technical replicates.

2.6. Drought stress treatment and measurement of relative water contents

Poplar stem cuttings with double nodes were cultured in fresh RM for 1 month, and the rooted plantlets were transplanted to pots. Three-month-old plants grown under well-watered conditions were subjected to water deficiency for 6 days; photographs were taken before and 6 days after re-watering. The 3rd–5th intact, fully expanded leaves from the top were collected and used for relative

water content (RWC) measurements as described by Ma et al. (2006). In brief, excised leaves were immediately weighed, and the saturated fresh weight was measured after incubation in water at 20 °C for 6 h. Leaves were dried at 80 °C for 48 h, and the dry weights were measured after 2 h of equilibration at room temperature. The formula RWC (%) = (fresh weight − dry weight) × 100/(turgid weight − dry weight) was used to measure RWC.

2.7. Methyl viologen (MV) treatment and ion leakage analysis

MV treatment was performed using detached leaves, i.e., the 2^{nd}–8^{th} leaves of 2-month-old poplar plants grown in culture dishes. The detached leaves were floated on 0.4% (w/v) sorbitol solution containing 1 μM MV, incubated in the dark for 12 h to allow for MV diffusion, and subjected to continuous light (150 μmol m^{-2} s^{-1}) at 25 °C. The extent of cellular damage was quantified based on ion leakage, which is a measure of membrane disruption. Ion leakage was measured as described by Bowler et al. (1991) and Kwon et al. (2002) using an ion conductivity meter (model 455C, Istek Co., Seoul, Korea) at 0, 6, 12, 24, and 48 h during treatment. The values were compared with the total conductivity of the solution after autoclaving at 121 °C for 15 min.

2.8. Quantification and detection of hydrogen peroxide (H_2O_2)

To quantify H_2O_2 levels, the roots, shoots, leaves, and petioles of 2-month-old poplar plants cultured in a culture dish were ground well and incubated in a 1 mg/mL solution of 3,3-diaminobenzidine (DAB)-HCl (pH 3.8) for 6 h at 25 °C under continuous light conditions according to the methods of Kim et al. (2011b), followed by decolorizing in boiling ethanol (95%) for 20 min. After cooling, H_2O_2 contents were spectrophotometrically measured according to Loreto and Velikova (2001). H_2O_2 accumulation in detached leaves of plants was visualized by DAB staining under 150 μmol m^{-2} s^{-1} light at 25 °C for 6 h.

2.9. Hormone- and dark-induced senescence assay

The hormone- and dark-induced senescence assay was carried out according to Weaver Louis et al. (1998) and Buchanan-Wollaston et al. (2005). In brief, the 2^{nd}–8^{th} (from top to bottom) detached leaves from 2-month-old non-transgenic (NT) and SY plants grown in culture dishes were incubated in 3 mM MES (PH 5.7) solution for the designated times with and without the designated concentrations of 1-aminocylopropane-1-carboxylic acid (ACC) and methyl jasmonate (MeJA) or abscisic acid (ABA) for hormone treatment or sealed with aluminum foil for dark treatment.

2.10. Analysis of photosynthetic activity

Photosynthetic activity of 2^{nd}–8^{th} (from top to bottom) detached leaves from 2-month-old NT and SY plants grown in culture dishes were estimated based on chlorophyll fluorescence determination of photochemical yield (Fv/Fm), which represents the maximal yield of the photochemical reaction on photosystem II (PSII), using a portable chlorophyll fluorescence meter (Handy PEA, Hansatech, England) after 30 min of dark adaption.

3. Results

3.1. SY plants display auxin-overproduction morphological phenotypes

To examine whether the YUCCA auxin biosynthesis pathway is functionally conserved in poplar, we generated transgenic poplar plants (*P. alba* × *P. glandulosa*) expressing *YUCCA6* under the control of the oxidative stress-inducible *SWPA2* promoter (referred to as SY plants) by *Agrobacterium*-mediated transformation (Fig. 1A). Ten independent SY plants were confirmed by genome PCR analysis using *AtYUCCA6* and *SWPA2* promoter-specific primers (Supplementary Fig. S1A), and three lines (SY7, SY12 and SY20) were selected for further characterization by RT-PCR (Supplementary Fig. S1B) and Southern blot analysis (Fig. 1B). As shown in Fig. 1B, lines SY7, SY12, and SY20 contained double, double, and single copy insertions of *AtYUCCA6*, respectively.

To evaluate the morphological phenotypes of NT and SY plants, poplar stem cuttings (NT, SY7, SY12, and SY20) with double nodes were cultured in culture dishes for 2 months (Supplementary Fig. S2A, B and C). For the pot experiment, two rooted transgenic plantlets (SY7 and SY12) were transplanted to pots. SY20 was not included, since it failed to root in pots, as it exhibited bushy, hairy roots with severe defects in main root development (data not shown). Three-month-old SY plants exhibited typical auxin-overproduction phenotypic alterations such as narrow leaves with downward curvature, elongated petioles, and long internodes (Fig. 2A and D). Compared to NT plants, SY plants displayed rapid shoot growth but short, hairy roots (Fig. 2B and C), and the angles between the petioles and stems in SY plants presented a larger to smaller gradient from top to bottom (Fig. 2E).

As expected, the transcript levels of *AtYUCCA6* in different tissues of culture dish-grown SY plants were elevated. SY plants contained 52.6–219.7% more free IAA contents than NT plants in various tissues including root, shoot, leaf, and petiole tissue (Fig. 3A

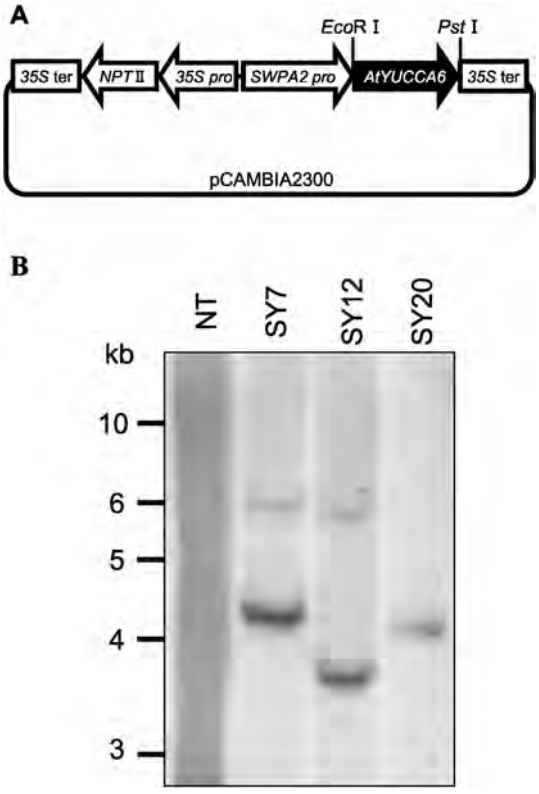

Fig. 1. Generation of *AtYUCCA6*-overexpressing transgenic poplar plants (SY plants). (A) Schematic diagram of the oxidative stress-inducible *SWPA2* promoter:*AtYUCCA6* construct. (B) Southern blot analysis of SY plants; the integration and gene copy number of the construct in SY plants were confirmed using a ^{32}P-labeled probe designed based on the *YUCCA6* cDNA fragment, which is a divergent sequence of *PtYUCCA* cDNA.

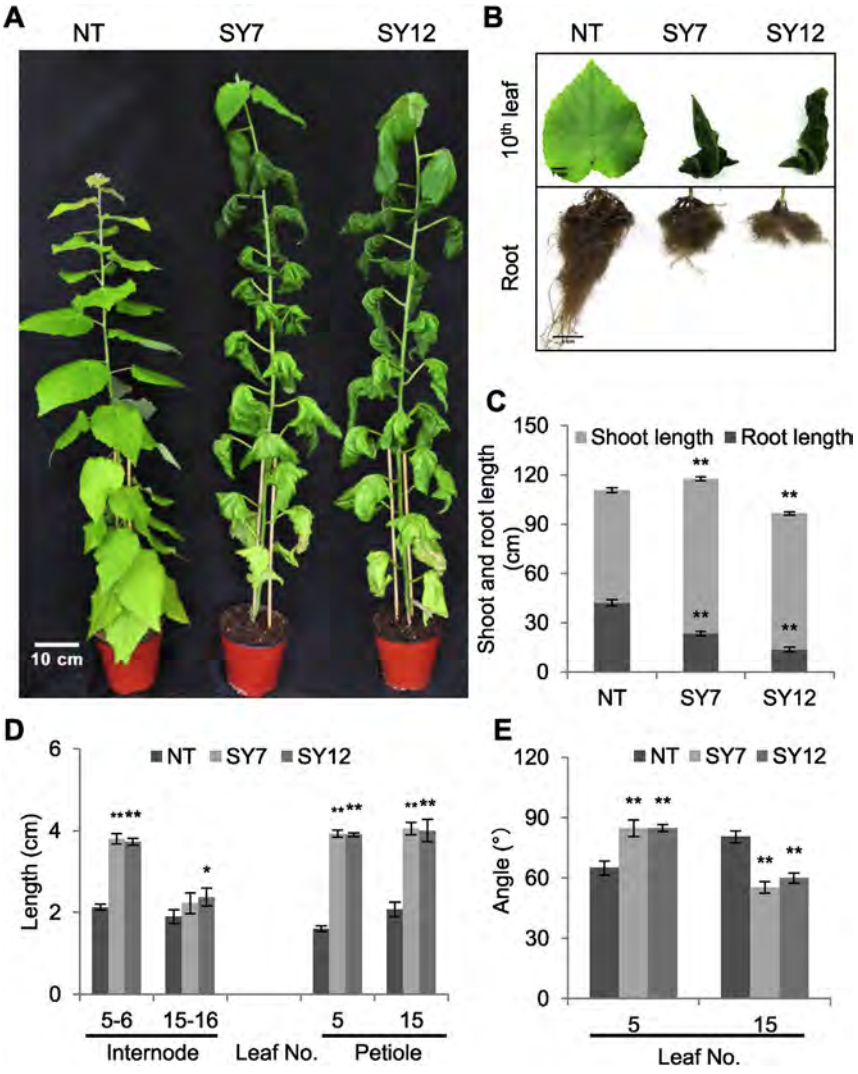

Fig. 2. SY plants exhibit auxin-overproduction morphological phenotypes. (A) Three-month-old SY plants display auxin-overproduction phenotypes. (B) Third leaf and root morphology of NT and SY plants grown in pots for 3 months. (C–D) Comparison of shoot and root length, internode and petiole length, and petiole angle in NT and SY plants. Petiole length and angle were determined from the 5th and 15th leaves, while internode length was measured from the 5th–6th and 15th–16th leaves. Leaves were counted sequentially from the top intact, fully expanded leaf. Data represent the mean ± SD of three biological replicates. Bars labeled with asterisks show significant differences from that of NT at $^{*}P < 0.05$ or $^{**}P < 0.01$ by t-test.

and B). Quantitative RT-PCR analyses of the early auxin-response genes *IAA1*, *IAA2*, *IAA5*, and *IAA6* further demonstrated that the phenotypes of SY plants were positively correlated with the intracellular auxin levels and transcript levels of auxin-response genes (Fig. 3C).

3.2. SY plants exhibit increased tolerance to drought stress

To determine whether *AtYUCCA6* overexpression increases drought stress tolerance in poplar, poplar stem cuttings (NT, SY7, SY12, and SY20) with double nodes were cultured in polyethylene glycol (PEG)-infused plates. PEG is an inert, non-ionic reagent containing virtually impermeable chains that is frequently used to induce water stress and to maintain uniform water potentials throughout the experimental period (van der Weele et al., 2000). SY plants exhibited less loss (21.3–34.7%) in root fresh weight than NT plants, which barely rooted under the −0.5 MPa water potential present in the PEG-infused plates (Supplementary Fig. S3A and B). Furthermore, when 3-month-old NT and SY (SY7, SY12) plants were subjected to water deficiency for 6 days, NT plants wilted more rapidly than SY plants under drought conditions; this difference in wilting symptoms became even more pronounced at 6 days after re-watering (Fig. 4A). The RWC of SY plants was higher than that of NT plants, which is consistent with their drought-resistant phenotypes (Fig. 4B). Thus, we conclude that overexpression of *AtYUCCA6* confers drought stress tolerance to poplar.

3.3. SY plants exhibit increased oxidative stress tolerance

To evaluate the oxidative stress tolerance of SY plants, we incubated detached leaves in 1 μM methyl viologen (MV), a typical reactive oxygen species (ROS)-generating chemical (Babbs et al., 1989), and quantified the loss of cytoplasmic solutes based on electrical conductance. After 24 h of MV treatment, detached leaves of NT plants displayed almost complete cellular damage (approximately 95.8% ion leakage), whereas the SY plants displayed less membrane damage (approximately 12.9–56.0% ion leakage) (Fig. 5A and B). Increased levels of *YUCCA6* transcript were observed

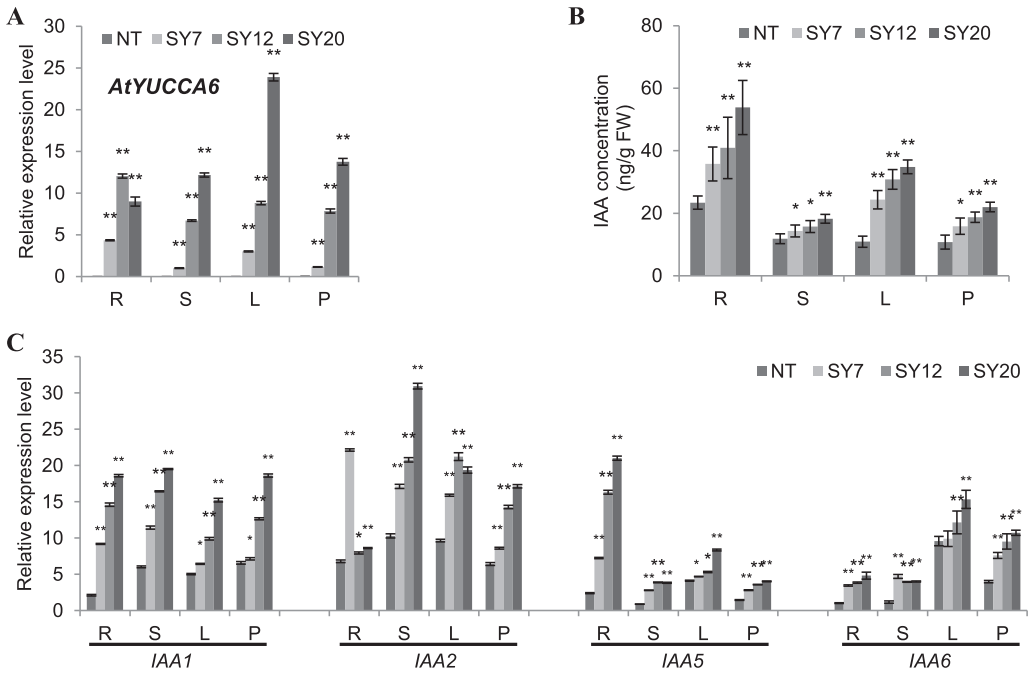

Fig. 3. SY plants exhibit increased auxin contents and transcript levels of early auxin-responsive genes. (A–D) Expression levels of *AtYUCCA6* (A), free IAA concentrations (B), and various auxin-responsive *IAA* gene (C) transcripts were measured using quantitative real-time PCR in roots, shoots, leaves, and petioles of NT and SY plants. Transcript levels of *AtYUCCA6* and various *IAA* genes were normalized to the poplar *actin* gene as an internal control. R, root; S, shoot; L, leaf; P, petiole. Leaves were counted sequentially from the top intact, fully expanded leaf. Data represent three independent experiments. Asterisks indicate a significant difference from that of NT at $^{*}P < 0.05$ or $^{**}P < 0.01$ by t-test.

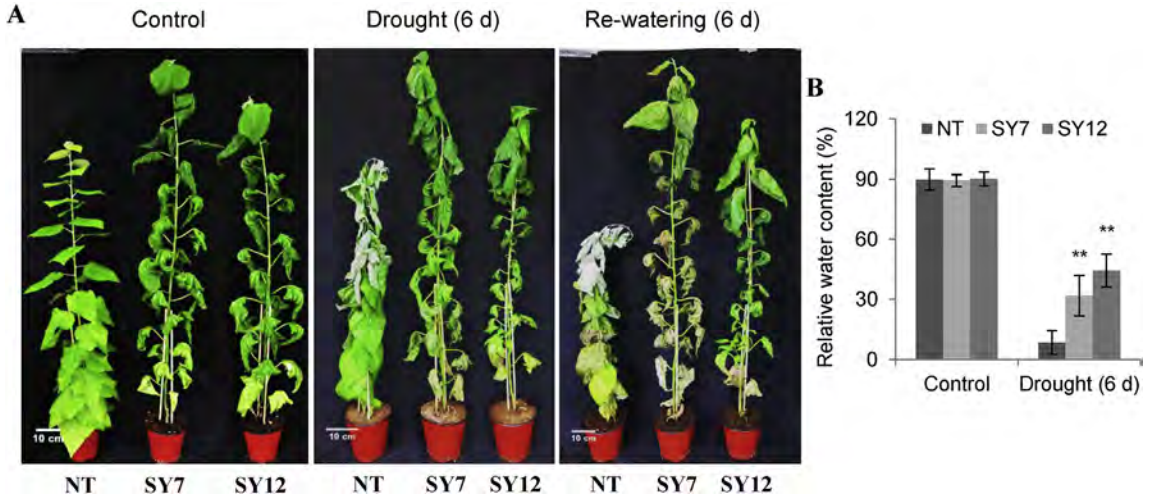

Fig. 4. SY plants exhibit increased resistance to drought stress. (A) Phenotypes of 3-month-old NT and SY plants after withholding water for 0 and 6 days, and re-watering for 6 days. (B) Relative water contents of NT and SY plants after 6 days of water withholding. Data represent the mean ± SD of three biological replicates. Asterisks indicate a significant difference from that of NT at $^{*}P < 0.05$ or $^{**}P < 0.01$ by t-test.

in SY plants during MV treatment, but not in NT plants (Fig. 5C).

We also investigated the correlation between *AtYUCCA6* overexpression and ROS content in 2-month-old NT and SY (SY7, SY12 and SY20) plants grown in culture dishes. H_2O_2 accumulation in detached leaves of NT and SY plants was visualized by DAB staining under MV stress treatment for 24 h. The detached leaves of SY plants displayed less H_2O_2 accumulation than NT plants (Fig. 5D). Furthermore, the roots, shoots, leaves, and petioles of SY plants had significantly lower H_2O_2 levels than NT plants (Fig. 5E). Taken together, these results suggest that *AtYUCCA6* overexpression increases tolerance to MV-mediated oxidative stress in transgenic poplar.

3.4. SY plants exhibit delayed hormone- and dark-induced senescence in detached leaves

We investigated whether elevated auxin levels lead to the senescence phenotype in SY plants. Detached leaves from 2-month-old NT and SY (SY7, SY12 and SY20) plants grown in culture dishes were incubated in 3 mM MES (PH 5.7) solution with or without 50 μM 1-aminocylopropane-1-carboxylic acid (ACC) and 50 μM of MeJA or 100 μM abscisic acid (ABA) for hormone treatment (16 h light/8 h dark photoperiod) or subjected to dark treatment for 8 days. The ion leakage and Fv/Fm (senescence indicators) were measured in detached leaves from NT and SY plants (Fig. 6A). SY

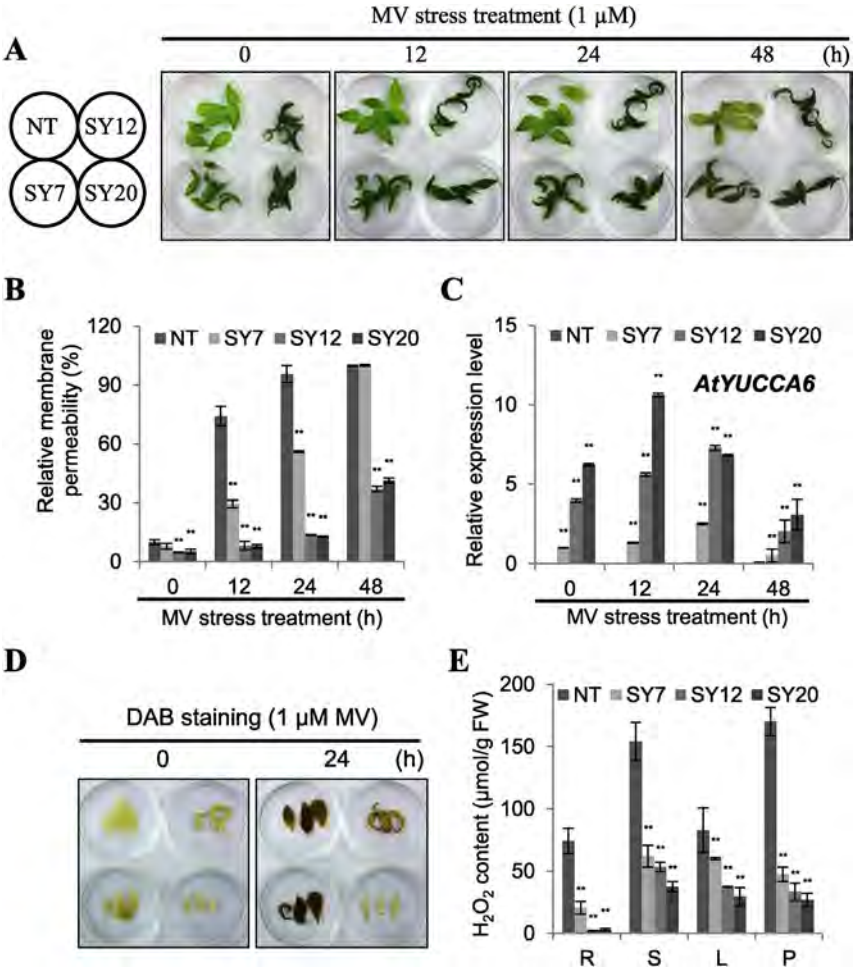

Fig. 5. SY plants display oxidative stress tolerance. (A–C) 2nd–8th leaves of 2-month-old culture dish-grown NT and SY plants treated with 1 μM methyl viologen (MV) at 25 °C under a light intensity of 150 μmol photons m^{-2} s^{-1} for 48 h. (A) Visible differential damage in leaves collected from NT and SY plants during MV treatment; (B) analysis of ion leakage in NT and SY plants in response to MV treatment. The electrical conductivity of the MV solution was compared with the total conductivity of the solution following tissue destruction. Detached leaves collected from the 2nd–8th leaves of plants grown in culture dishes for 2 months; (C) expression levels of *AtYUCCA6* during MV treatment. Transcript levels of *AtYUCCA6* were measured by quantitative real-time PCR and normalized to the levels of poplar *actin* transcript. (D) DAB staining for reactive oxygen species (ROS) accumulation in detached leaves subjected to MV stress. The 3rd–5th leaves from 2-month-old NT and SY plants grown in culture dishes were treated with 1 μM MV under 150 μmol photons m^{-2} s^{-1} light at 25 °C for 12 h (E) H_2O_2 contents were measured in roots, shoots, leaves, and petioles of 2-month-old NT and SY plants grown in culture dishes. R, root (whole root); S, shoot (stem from 3rd–7th internode); L, leaf (2nd–8th leaves); P, petiole (3rd–7th leaves). Leaves were counted sequentially from the top intact, fully expanded leaf. Data represent the means of three biological repeats. Asterisks indicate a significant difference from that of NT at $^{*}P < 0.05$ or $^{**}P < 0.01$ by t-test.

plants exhibited higher photosystem II efficiency and less membrane permeability than NT plants (Fig. 6B and C). These results reveal that the extent of senescence in detached leaves is negatively correlated with intracellular auxin levels.

4. Discussion

Auxin is crucial for plant viability and developmental processes. Auxin metabolism (including biosynthesis, transport and degradation or conversion) is precisely monitored by multiple metabolic pathways, which maintain homeostasis during growth and development in all higher plants (Rosquete et al., 2012). Disturbed auxin homeostasis causes several alterations, such as abnormal morphology and changes in physiological and biochemical characteristics. In particular, some evidence indicates that auxin plays a central role in determining root architecture (Overvoorde et al., 2010). The involvement of *YUCCA* in auxin biosynthesis has been demonstrated in plants ranging from mosses to monocots and dicots (Zhao et al., 2001; Yamamoto et al., 2007; Gallavotti et al., 2008; Eklund et al., 2010; Kim et al., 2013; Landberg et al., 2013). Although 12 *YUCCA*-like genes have been identified in poplar with high sequence homology and similar motifs to the *Arabidopsis YUCCA* genes, their functional conservation in relation to auxin has not been clearly elucidated (Ye et al., 2009). In the current study, we successfully developed transgenic poplar plants expressing *Arabidopsis YUCCA6* under the control of the oxidative stress-inducible *SWPA2* promoter (SY plants). The results suggest that the YUCCA pathway of IAA biosynthesis also exists in poplar. For instance, the expression of *AtYUCCA6* in poplar produced typical auxin overproduction phenotypic alterations, including narrow leaves with downward curvature, elongated petioles and internodes, increased plant height, and short, hairy roots (Fig. 2A, B and C). Interestingly, the leaf phenotype and the angles between petioles and stems from the top to bottom of SY plants (Fig. 2A and E) are consistent with the notion that the direction of auxin distribution is commonly followed by “polar auxin transport” (Friml and Palme, 2002). Moreover, both the free IAA contents and transcript levels of early auxin-response genes increased in all organs of SY plants (Fig. 3B and C). Specifically, the transcript levels of auxin-response genes and the IAA contents in SY20 plant were higher than those of SY7 and SY12

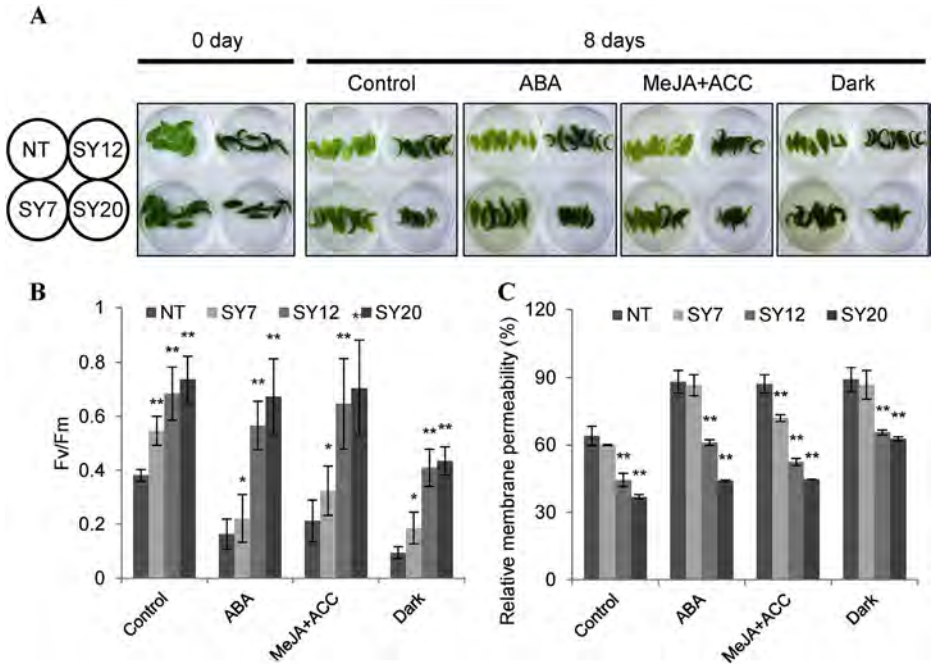

Fig. 6. SY plants exhibit delayed hormone-induced and dark-induced senescence in assays using detached leaves. (A) 2nd–8th detached leaves from 2-month-old NT and SY plants grown in culture dishes were incubated in 3 mM MES (PH 5.7) solution under a 16 h light/8 h dark photoperiod with and without (control) 100 μM ABA, or 50 μM ACC and 50 μM MeJA, or dark treatment for 8 days. (B) Photosystem II efficiency (Fv/Fm) and (C) total chlorophyll contents of NT and SY plants. Data represent the mean ± SD of three biological repeats. Asterisks indicate a significant difference from that of NT at $^{*}P < 0.05$ or $^{**}P < 0.01$ by t-test.

(Fig. 3B and C). Even though SY20 vigorously rooted in culture dishes (Supplementary Fig. S2A, B and C), this line failed to acclimate to soil conditions since it developed only bushy, hairy roots without main root elongation. We therefore analyzed the soil-grown phenotypes of SY7 and SY12.

ROS, such as super oxide radical anions (O_2 •−), H_2O_2, and hydroxyl radicals (OH •), are inherent to plants because they are constantly produced by aerobic processes in chloroplasts, mitochondria, and peroxisomes. Various stresses including drought, salt, and high intensity light induce the overproduction of ROS, which are highly reactive and toxic, causing damage and cell death (Gill and Tuteja, 2010). ROS levels are primarily maintained by ROS scavenging antioxidant defense machinery. Enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR), while non-enzymatic antioxidants include glutathione (GSH), arachidonic acid (AA) (both water soluble), carotenoids, and tocopherols (lipid soluble) (Gill et al., 2011; Gill and Tuteja, 2010; Mittler et al., 2004). In the current study, SY plants showed relatively low ROS contents compared to NT plants (Fig. 5A and D). The reduced levels of ROS observed in SY plants may have been caused by the activation of ROS scavenging or a decrease in ROS production, which may represent a major reason for the drought stress tolerance of the SY plants, as well as transgenic *Arabidopsis* and potato plants overexpressing *YUCCA6* (Kim et al., 2013). However, the relationship between IAA regulation and ROS metabolism in SY plants is still not well understood. NADPH-dependent thioredoxin reductase plays an essential role in the direct reduction of ROS levels and acquiring stress tolerance in plants (Cha et al., 2014). YUCCA family flavin-dependent monooxygenase is involved in auxin biosynthesis in plants, and recent studies have revealed that YUCCA6 contains oxidized FAD, which is readily reduced by NADPH and shows significant NADPH oxidase activity. YUCCA6 appears to play a thioredoxin reductase-like role via similar catalytic mechanisms.

Leaf senescence is organ-level senescence, but it is important for plant growth and reproduction. Leaf senescence usually involves many biochemical changes, including degradation of proteins and RNA and a decline in photosynthetic activity (Lim et al., 2007). Some hormones are involved in senescence, as are well-known senescence accelerators, such as ethylene, jasmonic acid, and ABA (Woo et al., 2001; He et al., 2002; van der Graaff et al., 2006; Lim et al., 2007). Decreased light intensity or darkness can also accelerate senescence by reducing the expression of light-dependent genes and the levels of photosynthetic proteins and chlorophyll (Buchanan-Wollaston et al., 2005). Overexpression of *YUCCA6* in poplar produced delayed hormone- and dark-induced senescence in detached leaves (Fig. 6A, B and C), which is consistent with the finding that transgenic *Arabidopsis* overexpressing *YUCCA6* exhibits delayed hormone- and dark-induced senescence (Kim et al., 2011a). While the mechanism remains unknown, a recent experiment revealed that aging is likely to be a multifactorial process, and there is significant evidence suggesting that the generation of ROS and the corresponding response to oxidative stress are key factors in aging (Finkel and Holbrook, 2000). Although the role of auxin in regulating leaf senescence remains elusive, previous studies have shown that auxin delays senescence by reducing chlorophyll loss and protein degradation, and (directly or indirectly) suppressing the transcription of senescence-associated genes (Ellis et al., 2005; Kim et al., 2011a).

Promoters function as molecular switches and terminal points of stress response cascades, which are important for the transcriptional regulation of a dynamic network of genes controlling various biological processes, such as abiotic responses, hormone responses and developmental processes (Yamaguchi-Shinozaki and Shinozaki, 2005). We previously cloned the *SWPA2* promoter, a strong oxidative stress-inducible peroxides promoter, from sweet potato (*Ipomoea batatas*) (Kim et al., 2003). Compared to the constitutive *CaMV35S* promoter, transgenic potato plants

expressing *AtNDPK2* under the control of the stress-inducible *SWPA2* promoter exhibit a more efficient response to oxidative stress but with a less negative effect on plant development. Moreover, the *SWPA2* promoter has been successfully used to produce transgenic poplar, potato, sweet potato, rice, and alfalfa (Liu et al., 2007; Tang et al., 2008; Kim et al., 2009, 2011b; Li et al., 2014; Wang et al., 2014). In this study, the expression of *YUCCA6* under the control of the *SWPA2* promoter was induced by MV stress in SY plants (Fig. 5C), suggesting that the *SWPA2* promoter functions well in regulating the expression of *YUCCA6* in response to stress conditions in SY plants.

Poplar is an important perennial crop plant. Genetic engineering of trees can potentially be used to develop poplar with improved growth, wood quality, and tolerance to saline and drought stress (Harfouche et al., 2011; RA Sedjo 2001, Tang et al., 2007). In this study, we successfully generated transgenic poplar expressing *Arabidopsis YUCCA6*. The SY plants displayed auxin-overproduction phenotypes, with increased free IAA contents and elevated transcript levels of early auxin-response genes. Furthermore, the SY plants showed enhanced tolerance to drought and MV-mediated oxidative stress, suggesting that the correlation between auxin regulation and ROS metabolism is mediated by thioredoxin reductase-like YUCCA6 in SY plants. In addition, the SY plants exhibited delayed hormone- and dark-induced senescence in detached leaves, suggesting that auxin levels are negatively correlated to the extent of senescence. In conclusion, these results indicate that the conserved IAA biosynthesis pathway mediated by *YUCCA* genes probably exists in poplar. The possible applications of SY poplar plants remain to be studied through further biological characterization of these plants under various soil conditions.

Author contributions

S.S. Kwak and Q.B. Ke: conceived and designed the experiments. Q.B. Ke, Z. Wang, C.Y. Ji and H.B. Li: performed the experiments. H.B. Li, J.C. Jeong, B. Xu and H.S. Lee: analyzed the data. X.P. Deng and H.S. Lee: contributed reagents/materials/analysis tools. Q.B. Ke and S.S. Kwak: wrote the paper.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2015.05.003.

References

Babbs, C.F., Pham, J.A., Coolbaugh, R.C., 1989. Lethal hydroxyl radical production in paraquat-treated plants. Plant Physiol. 90, 1267–1270.
Bartel, B., 1997. Auxin biosynthesis. Annu. Rev. Plant Biol. 48, 51–66.
Bowler, C., Slooten, L., Vandenbranden, S., De Rycke, R., Botterman, J., Sybesma, C., Van Montagu, M., Inzé, D., 1991. Manganese superoxide dismutase can reduce cellular damage mediated by oxygen radicals in transgenic plants. EMBO J. 10, 1723.
Buchanan-Wollaston, V., Page, T., Harrison, E., Breeze, E., Lim, P.O., Nam, H.G., Lin, J.F., Wu, S.H., Swidzinski, J., Ishizaki, K., 2005. Comparative transcriptome analysis reveals significant differences in gene expression and signalling pathways between developmental and dark/starvation-induced senescence in *Arabidopsis*. Plant J. 42, 567–585.
Cha, J.-Y., Kim, J.Y., Jung, I.J., Kim, M.R., Melencion, A., Alam, S.S., Yun, D.-J., Lee, S.Y., Kim, M.G., Kim, W.-Y., 2014. NADPH-dependent thioredoxin reductase A (NTRA) confers elevated tolerance to oxidative stress and drought. Plant Physiol. Biochem. 80, 184–191.
Cheng, Y., Dai, X., Zhao, Y., 2006. Auxin biosynthesis by the YUCCA flavin monooxygenases controls the formation of floral organs and vascular tissues in *Arabidopsis*. Genes. Dev. 20, 1790–1799.
Choi, Y., Noh, E.W., Lee, H.S., Han, M.S., Lee, J.S., Choi, K.S., 2005. An efficient and novel plant selectable marker based on organomercurial resistance. J. Plant Biol. 48, 351–355.
Dai, X., Mashiguchi, K., Chen, Q., Kasahara, H., Kamiya, Y., Ojha, S., DuBois, J., Ballou, D., Zhao, Y., 2013. The biochemical mechanism of auxin biosynthesis by an *Arabidopsis* YUCCA flavin-containing monooxygenase. J. Biol. Chem. 288, 1448–1457.
Eklund, D.M., Thelander, M., Landberg, K., Ståldal, V., Nilsson, A., Johansson, M., Valsecchi, I., Pederson, E.R., Kowalczyk, M., Ljung, K., 2010. Homologues of the *Arabidopsis thaliana SHI/STY/LRP1* genes control auxin biosynthesis and affect growth and development in the moss *Physcomitrella patens*. Development 137, 1275–1284.
Ellis, C.M., Nagpal, P., Young, J.C., Hagen, G., Guilfoyle, T.J., Reed, J.W., 2005. AUXIN RESPONSE FACTOR1 and AUXIN RESPONSE FACTOR2 regulate senescence and floral organ abscission in *Arabidopsis thaliana*. Development 132, 4563–4574.
Expósito-Rodríguez, M., Borges, A.A., Borges-Pérez, A., Hernández, M., Pérez, J.A., 2007. Cloning and biochemical characterization of *ToFZY*, a tomato gene encoding a flavin monooxygenase involved in a tryptophan-dependent auxin biosynthesis pathway. J. Plant Growth Regul. 26, 329–340.
Finkel, T., Holbrook, N.J., 2000. Oxidants, oxidative stress and the biology of ageing. Nature 408, 239–247.
Friml, J., Palme, K., 2002. Polar auxin transport-old questions and new concepts? Plant Mol. Biol. 49, 273–284.
Gallavotti, A., Barazesh, S., Malcomber, S., Hall, D., Jackson, D., Schmidt, R.J., McSteen, P., 2008. Sparse inflorescence1 encodes a monocot-specific *YUCCA-like* gene required for vegetative and reproductive development in maize. Proc. Natl. Acad. Sci. U. S. A. 105, 15196–15201.
Gill, S.S., Khan, N.A., Anjum, N.A., Tuteja, N., 2011. Amelioration of cadmium stress in crop plants by nutrients management: morphological, physiological and biochemical aspects. Plant Stress 5, 1–23.
Gill, S.S., Tuteja, N., 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol. Biochem. 48, 909–930.
Harfouche, A., Meilan, R., Altman, A., 2011. Tree genetic engineering and applications to sustainable forestry and biomass production. Trends Biotechnol. 29, 9–17.
He, Y., Fukushige, H., Hildebrand, D.F., Gan, S., 2002. Evidence supporting a role of jasmonic acid in *Arabidopsis* leaf senescence. Plant Physiol. 128, 876–884.
Hooley, R., 1998. Auxin signaling: homing in with targeted genetics. Plant Cell 10, 1581–1583.
Kim, J., Baek, D., Park, H.C., Chun, H.J., Oh, D.-H., Lee, M.K., Cha, J.-Y., Kim, W.-Y., Kim, M.C., Chung, W.S., 2013. Overexpression of *Arabidopsis YUCCA6* in potato results in high-auxin developmental phenotypes and enhanced resistance to water deficit. Mol. Plant 6, 337–349.
Kim, J., Murphy, A.S., Baek, D., Lee, S.-W., Yun, D.-J., Bressan, R.A., Narasimhan, M.L., 2011a. *YUCCA6* over-expression demonstrates auxin function in delaying leaf senescence in Arabidopsis thaliana. J. Exp. Bot. 62, 3981–3992.
Kim, K.-Y., Kwon, S.-Y., Lee, H.-S., Hur, Y., Bang, J.-W., Kwak, S.-S., 2003. A novel oxidative stress-inducible peroxidase promoter from sweetpotato: molecular cloning and characterization in transgenic tobacco plants and cultured cells. Plant Mol. Biol. 51, 831–838.
Kim, S.-H., Hamada, T., 2005. Rapid and reliable method of extracting DNA and RNA from sweetpotato, *Ipomoea batatas (L). Lam*. Biotechnol. Lett. 27, 1841–1845.
Kim, Y.H., Lim, S., Yang, K.-S., Kim, C.Y., Kwon, S.-Y., Lee, H.-S., Wang, X., Zhou, Z., Ma, D., Yun, D.-J., 2009. Expression of *Arabidopsis NDPK2* increases antioxidant enzyme activities and enhances tolerance to multiple environmental stresses in transgenic sweetpotato plants. Mol. Breed. 24, 233–244.
Kim, Y.H., Kim, M.D., Choi, Y.I., Park, S.C., Yun, D.-J., Noh, E.W., Lee, H.-S., Kwak, S.-S., 2011b. Transgenic poplar expressing *Arabidopsis NDPK2* enhances growth as well as oxidative stress tolerance. Plant Biotechnol. J. 9, 334–347.
Kwon, S.-Y., Jeong, Y.J., Lee, H.-S., Kim, J.S., Cho, K.Y., Allen, R.D., Kwak, S.-S., 2002. Enhanced tolerances of transgenic tobacco plants expressing both superoxide dismutase and ascorbate peroxidase in chloroplasts against methyl viologen-mediated oxidative stress. Plant, Cell Environ. 25, 873–882.
Landberg, K., Pederson, E.R., Viaene, T., Bozorg, B., Friml, J., Jönsson, H., Thelander, M., Sundberg, E., 2013. The moss Physcomitrella patens reproductive organ development is highly organized, affected by the two *SHI/STY* genes and by the level of active auxin in the *SHI/STY* expression domain. Plant Physiol. 162, 1406–1419.
LeClere, S., Schmelz, E.A., Chourey, P.S., 2010. Sugar levels regulate tryptophan-dependent auxin biosynthesis in developing maize kernels. Plant Physiol. 153, 306–318.
Li, H.B., Wang, Z., Ke, Q.B., Ji, C.Y., Jeong, J.C., Lee, H.-S., Lim, Y.P., Xu, B.C., Deng, X.P., Kwak, S.-S., 2014. Overexpression of *codA* gene confers enhanced tolerance to abiotic stresses in alfalfa. Plant Physiol. Biochem. 85, 31–40.
Lim, P.O., Kim, H.J., Gil Nam, H., 2007. Leaf senescence. Annu. Rev. Plant Biol. 58, 115–136.
Liu, H.-Y., Yu, X., Cui, D.-Y., Sun, M.-H., Sun, W.-N., Tang, Z.-C., Kwak, S.-S., Su, W.-A., 2007. The role of water channel proteins and nitric oxide signaling in rice seed germination. Cell. Res. 17, 638–649.
Liu, H., Xie, W.F., Zhang, L., Valpuesta, V., Ye, Z.W., Gao, Q.H., Duan, K., 2014. Auxin

biosynthesis by the *YUCCA6* flavin monooxygenase gene in woodland strawberry. J. Integr. Plant Biol. 56, 350–363.
Liu, H., Ying, Y.-Y., Zhang, L., Gao, Q.-H., Li, J., Zhang, Z., Fang, J.-G., Duan, K., 2012. Isolation and characterization of two *YUCCA* flavin monooxygenase genes from cultivated strawberry (*Fragaria× ananassa Duch.*). Plant Cell Rep. 31, 1425–1435.
Lloyd, G., McCown, B., 1980. Commercially-feasible micropropagation of mountain laurel, Kalmia latifolia, by use of shoot-tip culture. Comb. Proc. Int. Plant Propagators' Soc. 30, 421–427.
Loreto, F., Velikova, V., 2001. Isoprene produced by leaves protects the photosynthetic apparatus against ozone damage, quenches ozone products, and reduces lipid peroxidation of cellular membranes. Plant Physiol. 127, 1781–1787.
Ma, Q.-Q., Wang, W., Li, Y.-H., Li, D.-Q., Zou, Q., 2006. Alleviation of photoinhibition in drought-stressed wheat (*Triticum aestivum*) by foliar-applied glycinebetaine. J. Plant Physiol. 163, 165–175.
Mano, Y., Nemoto, K., 2012. The pathway of auxin biosynthesis in plants. J. Exp. Bot. 63, 2853–2872.
Mashiguchi, K., Tanaka, K., Sakai, T., Sugawara, S., Kawaide, H., Natsume, M., Hanada, A., Yaeno, T., Shirasu, K., Yao, H., 2011. The main auxin biosynthesis pathway in *Arabidopsis*. Proc. Natl. Acad. Sci. U. S. A. 108, 18512–18517.
Mittler, R., Vanderauwera, S., Gollery, M., Van Breusegem, F., 2004. Reactive oxygen gene network of plants. Trends Plant Sci. 9, 490–498.
Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant 15, 473–497.
Overvoorde, P., Fukaki, H., Beeckman, T., 2010. Auxin control of root development. CSH Perspect. Biol. 2, a001537.
Pan, X., Welti, R., Wang, X., 2010. Quantitative analysis of major plant hormones in crude plant extracts by high-performance liquid chromatography–mass spectrometry. Nat. Protoc. 5, 986–992.
Rosquete, M.R., Barbez, E., Kleine-Vehn, J., 2012. Cellular auxin homeostasis: gatekeeping is housekeeping. Mol. Plant 5, 772–786.
Sedjo, R.A., 2001. Biotechnology in forestry. Resourc. Future 145, 10–12.
Tang, L., Kim, M.D., Yang, K.-S., Kwon, S.-Y., Kim, S.-H., Kim, J.-S., Yun, D.-J., Kwak, S.-S., Lee, H.-S., 2008. Enhanced tolerance of transgenic potato plants overexpressing nucleoside diphosphate kinase 2 against multiple environmental stresses. Transgenic Res. 17, 705–715.
Tang, W., Newton, R.J., Li, C., Charles, T.M., 2007. Enhanced stress tolerance in transgenic pine expressing the pepper CaPF1 gene is associated with the polyamine biosynthesis. Plant Cell Rep. 26, 115–124.
Tobeña-Santamaria, R., Bliek, M., Ljung, K., Sandberg, G., Mol, J.N., Souer, E., Koes, R., 2002. FLOOZY of petunia is a flavin mono-oxygenase-like protein required for the specification of leaf and flower architecture. Genes. Dev. 16, 753–763.
van der Graaff, E., Schwacke, R., Schneider, A., Desimone, M., Flügge, U.-I., Kunze, R., 2006. Transcription analysis of *Arabidopsis* membrane transporters and hormone pathways during developmental and induced leaf senescence. Plant Physiol. 141, 776–792.
van der Weele, C.M., Spollen, W.G., Sharp, R.E., Baskin, T.I., 2000. Growth of *Arabidopsis thaliana* seedlings under water deficit studied by control of water potential in nutrient-agar media. J. Exp. Bot. 51, 1555–1562.
Wang, Z., Li, H.B., Ke, Q.B., Jeong, J.C., Lee, H.-S., Xu, B.C., Deng, X.P., Lim, Y.P., Kwak, S.-S., 2014. Transgenic alfalfa plants expressing AtNDPK2 exhibit increased growth and tolerance to abiotic stresses. Plant Physiol. Biochem. 84, 67–77.
Weaver Louis, M., Susheng, Gan, Betania, Quirino, Amasino Richard, M., 1998. A comparison of the expression patterns of several senescence-associated genes in response to stress and hormone treatment. Plant Mol. Biol. 37, 455–469.
Won, C., Shen, X., Mashiguchi, K., Zheng, Z., Dai, X., Cheng, Y., Kasahara, H., Kamiya, Y., Chory, J., Zhao, Y., 2011. Conversion of tryptophan to indole-3-acetic acid by TRYPTOPHAN AMINOTRANSFERASES OF *ARABIDOPSIS* and YUCCAs in *Arabidopsis*. Proc. Natl. Acad. Sci. U. S. A. 108, 18518–18523.
Woo, H.R., Chung, K.M., Park, J.-H., Oh, S.A., Ahn, T., Hong, S.H., Jang, S.K., Nam, H.G., 2001. ORE9, an F-box protein that regulates leaf senescence in *Arabidopsis*. Plant Cell 13, 1779–1790.
Yamaguchi-Shinozaki, K., Shinozaki, K., 2005. Organization of cis-acting regulatory elements in osmotic-and cold-stress-responsive promoters. Trends Plant Sci. 10, 88–94.
Yamamoto, Y., Kamiya, N., Morinaka, Y., Matsuoka, M., Sazuka, T., 2007. Auxin biosynthesis by the *YUCCA* genes in rice. Plant Physiol. 143, 1362–1371.
Ye, X., Kang, B-g, Osburn, L.D., Li, Y., Max, C.Z.-M., 2009. Identification of the flavin-dependent monooxygenase-encoding *YUCCA* gene family in *Populus trichocarpa* and their expression in vegetative tissues and in response to hormone and environmental stresses. Plant Cell Tiss. Org. 97, 271–283.
Zhao, Y., Christensen, S.K., Fankhauser, C., Cashman, J.R., Cohen, J.D., Weigel, D., Chory, J., 2001. A role for flavin monooxygenase-like enzymes in auxin biosynthesis. Science 291, 306–309.
Zhao, Y., Hull, A.K., Gupta, N.R., Goss, K.A., Alonso, J., Ecker, J.R., Normanly, J., Chory, J., Celenza, J.L., 2002. Trp-dependent auxin biosynthesis in *Arabidopsis*: involvement of cytochrome P450s CYP79B2 and CYP79B3. Genes. Dev. 16, 3100–3112.