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Strigolactones regulate sepal senescence in Arabidopsis

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Abstract

Flower sepals are critical for flower development and vary greatly in life span depending on their function post-pollination. Very little is known about what controls sepal longevity. Using a sepal senescence mutant screen, we identified two Arabidopsis mutants with delayed senescence directly connecting strigolactones with senescence regulation in a novel floral context that hitherto has not been explored. The mutations were in the strigolactone biosynthetic gene \textit{MORE AXILLARY GROWTH1} (\textit{MAX1}) and in the strigolactone receptor gene \textit{DWARF14} (\textit{AtD14}). The mutation in \textit{AtD14} changed the catalytic Ser97 to Phe in the enzyme active site, which is the first mutation of its kind \textit{in planta}. The lesion in \textit{MAX1} was in the haem–iron ligand signature of the cytochrome P450 protein, converting the highly conserved Gly469 to Arg, which was shown in a transient expression assay to substantially inhibit the activity of \textit{MAX1}. The two mutations highlighted the importance of strigolactone activity for driving to completion senescence initiated both developmentally and in response to carbon-limiting stress, as has been found for the more well-known senescence-associated regulators ethylene and abscisic acid. Analysis of transcript abundance in excised inflorescences during an extended night suggested an intricate relationship among sugar starvation, senescence, and strigolactone biosynthesis and signalling.

Keywords: Arabidopsis, \textit{AtD14}, darkness, \textit{MAX1}, mutants, sepal senescence, strigolactones, sugar starvation.

Introduction

Senescence typically occurs in mature cells of tissues after their growth phase has ceased to enable efficient recycling of nutrients to new growing sinks such as seeds (Thomas, 2013). At the whole-plant level, senescence is considered critical for plant fitness, enabling plants to survive optimally in their given environments. The ability to senesce requires a change in competency of the tissue that happens during ageing (Jing et al., 2005; Fracheboud et al., 2009). Nevertheless, imposition of stress can make tissues senesc early. Prolonged darkness is a stress that results in carbon deprivation and early senescence...
of chlorophyllous tissues. This has been observed in individually covered attached leaves (Weaver and Amasino, 2001; Law et al., 2018), and in sepal of immature inflorescences of broccoli and Arabidopsis (Page et al., 2001; Trivellini et al., 2012). Research on the precocious senescence of these tissues has revealed that the signalling key to their degreening is similar to that occurring naturally in leaves in planta as they senesce in an age-dependent manner and in response to canopy shading. For example, the phytochrome-interacting factor genes PIF4 and PIF5 that have important roles in the shade response of canopy leaves (Sakuraba et al., 2014) were first identified to regulate the precocious degreening of harvested immature inflorescences of Arabidopsis held in the dark (Trivellini et al., 2012). Similarly, mutations in EIN2 and ORESARA1/ANAC092 that alter the timing of sepal senescence of dark-held inflorescences (Trivellini et al., 2012) were previously identified in Arabidopsis as components of the feedforward control of age-related leaf senescence (Kim et al., 2009).

Phytohormones have long been known to have key roles in senescence regulation. Ethylene, salicylic acid, abscisic acid, jasmonic acid, and brassinosteroid promote the onset or progression of senescence, whereas cytokinin, gibberellic acid, and auxin delay the process (Gan and Amasino, 1997; Lim et al., 2007). The hormones do not work alone, but rather in concert with each other to control senescence progression. For example, ethylene, abscisic acid, and jasmonates interact to control the timing and progression of leaf senescence in Arabidopsis (Kim et al., 2011).

More recently, strigolactones (SLs), which are well known for their function in regulating seed germination in parasitic plants (Toh et al., 2012; Wang and Bouwmeester, 2018), plant shoot branching (Gomez-Roldan et al., 2008; Umehara et al., 2008), and stress responses such as to drought and high salinity (Bu et al., 2014; Ha et al., 2014), were reported to regulate natural- and dark-induced leaf senescence (Hamiaux et al., 2012; Yamada et al., 2014; Ueda and Kusaba, 2015). SL biosynthesis starts with the conversion of all-trans-β-carotene into carlactone (CL), a common precursor of all SLs (Alder et al., 2012; Seto et al., 2014; Wang and Bouwmeester, 2018). In Arabidopsis, this requires the sequential activities of the carotenoid isomerase DWEB27 (D27) and two carotenoid cleavage dioxygenases CCD7/MAX3 and CCD8/MAX4 (Alder et al., 2012). Following this, the MORE AXILLARY GROWTH1 (MAX1) cytochrome P450 monoxygenase (Booker et al., 2005) oxidizes CL into carlactonoc acid (CLA) (Abe et al., 2014), which is further converted by downstream enzymes to other SL-like compounds (Brewer et al., 2016). The bioactive SL is perceived by the D14 receptor, an α/β-fold hydrolase (Arite et al., 2009), which hydrolyses the SL then interacts with the F-box protein MAX2/D3 to trigger SL signalling and response (Yao et al., 2016).

Very little is known about how sepal senescence is regulated, with most research on flowers having focused on petals (Rogers, 2013). To identify key regulators of sepal senescence, we systematically evaluated a population of mutant Arabidopsis plants derived from seeds treated with ethyl methanesulfonate (EMS). By using an Arabidopsis inflorescence degreening assay (Hunter et al., 2018), we previously identified three independent mutations in Chl b reductase that resulted in a delayed degreening phenotype (Jibran et al., 2015). Here we report on the characterization of a further two mutants with delayed senescence that highlight the role of SLs in controlling the life span of a floral organ.

Materials and methods

Plant growth conditions and mutant analysis

EMS mutants of Arabidopsis thaliana Landsberg erecta (Ler-0) were selected and the causal mutations determined as described in Jibran et al. (2015), Hunter et al. (2018), Supplementary Fig. S1, and Supplementary Table S1. Mutants were backcrossed twice to wild-type (WT) Ler-0 prior to analysis and genetic complementation. Seeds were germinated and grown in a temperature-controlled growth chamber set at 21 °C with 65% relative humidity and under a 16 h light/8 h dark cycle (long day) unless otherwise stated. For in planta assays, plants were grown in a temperature-controlled growth cabinet (Contherm Model CAT 630, Wellington, New Zealand), at 20–22 °C with 60% relative humidity and a 16 h light/8 h dark long-day photoperiod (~180 µE with metal halide lamps). For long-day treatments, inflorescences were placed in a container, covered with transparent film, and incubated in the growth chamber. For SL treatments, inflorescences were treated with the racemic mixture sa-GR24, which contains a synthetic SL analogue (Chiralix, Nijmegen, The Netherlands). This was dissolved in pure DMSO and diluted to final concentrations of 5 µM sa-GR24 and 1% (v/v) DMSO. Mock treatments for controls were 1% (v/v) DMSO.

Chlorophyll analysis

Chlorophyll (Chl) was extracted from single inflorescences according to Jibran et al. (2015) with the following changes: fresh samples were used and samples were incubated in the dark at 4 °C for 4 d after adding ethanol.

RNA isolation and RT-qPCR analysis

Total RNA was isolated from inflorescences using the Quick-RNA™ MiniPrep kit (Zymo Research, Irvine, CA, USA) with on-column DNase treatment. cDNA was synthesized from RNA (500 ng) using iScript™ Reverse Transcription Supermix for quantitative reverse transcription–PCR (RT–qPCR; Bio-Rad, Hercules, CA, USA). The cDNA template was diluted 10 times for RT–qPCR analysis. The RT–qPCR was prepared using a LightCycler® 480 SYBR Green I Master kit (Roche Diagnostics, Mannheim, Germany) and the PCR was performed with a LightCycler® 480 Instrument II (384-well; Roche Diagnostics) on three biological replicates, each with four technical replicates for each biological replicate. Primers were designed using QuantPrime online software (Arvidsson et al., 2008) and are listed in Supplementary Table S2. The Ct value was calculated using the algorithm of “Abs Quanti/2nd Derivative Max” present in LightCycler® 480 Software (version 1.5). Data were normalized to the reference gene PP2AA3 (Atig13320), which was confirmed to be stable for development and environmental conditions (Czechowski et al., 2005), senescence (Jibran et al., 2015), and SL treatment (Supplementary Fig. S2), and relative transcript abundance changes were calculated using the ΔΔCt method (Livak and Schmittgen, 2001; Dvange and Bertone, 2009).
nCounter analysis

Transcriptional analysis was performed using the nCounter Analysis System (NanoString, Seattle, WA, USA) (Geiss et al., 2008). Two sets of gene-specific probes (along with a reporter probe and a capture probe) were designed by NanoString Support (see Supplementary Table S3). Total RNA (300 ng) was hybridized using the nCounter PlexSet-24 Reagent Pack according to the ‘PlexSet™ Reagents User Manual’. After hybridization, samples were vertically pooled and were placed on the automated nCounter Prep Station (NanoString) for purification and immobilized in the cartridge. This cartridge was then transferred to the nCounter Digital Analyzer for data collection. Data analysis was performed with nSolver™ 4.0 Analysis Software according to the user manual. All samples passed quality control. The background thresholding was set to ‘12’ according to the count value of the internal negative control. Positive control normalization was carried out by using the geometric mean of the top three positive counts. Reference gene normalization was calculated using the geometric mean of counts for the three reference genes PP2AAs1/At1g13320, ACT2/At1g18780, and MON1/At2g28390 (Czechowski et al., 2005).

In silico analysis

Sequence alignments were performed using Geneious desktop software (Kearse et al., 2012). The three-dimensional (3D) structure of AtD14 was obtained from the SL-induced AtD14–D3–ASK1 complex (PDB: 5HZG) (Yao et al., 2016). The homology model of MAX1 was calculated using the I-TASSER On-line Server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) (Zhang, 2008; Roy et al., 2010; Yang et al., 2015). The 3D images were prepared with CCP4MG (McNicholas et al., 2011).

Plasmid constructions

For transient expression assays, the Arabidopsis Col-0-based SL biosynthetic genes (D27, MAX3, MAX4, and MAX1) were cloned as described in Zhang et al. (2014). The Ler-0-based MAX1 gene and genetic variants (MAX1-WT, MAX1-G469R, or MAX1-G469A) were cloned using the same protocol but with the primers listed in Supplementary Table S4.

Transient expression in leaves of Nicotiana benthamiana

Enzymatic characterization of MAX1 and the genetic variants was carried out as described in Zhang et al. (2014) except that Agrobacterium tumefaciens was reseeded in 50 mM MES (Duchefa, Haarlem, The Netherlands)–KOH buffer (pH 5.6) containing 2 mM NaH₂PO₄ (Merck, Darmstadt, Germany), 100 μM acetoxyringone (Sigma–Aldrich, St. Louis, MO, USA), and 0.5% (w/v) glucose (MP Biomedical, France) to a final OD₆₀₀ of 0.5. Instead of OsD27, OsCGD7, and OsCGD8, we used AtD27, MAX3, and MAX4, which were co-infiltrated with AtMAX1 and genetic variants to study the conversion of CL to CLA. Infiltration was performed using 4-week-old N. benthamiana plants which were soil grown in pots in a plant house with artificial light to make a photoperiod of 16 h light at 25 °C and 8 h dark at 22 °C. For each gene combination, six individual plants were used as biological replicates.

Analysis of carlactone and carlactonoic acid in N. benthamiana

CL and CLA were detected using ultra-high performance-LC/MS/ MS (UHPLC-LC-MS/MS) and CLA conjugates with UPLC-LC-quadrupole-time-of-flight-MS (UHPLC-qTOF-MS). For both analyses, 200 mg of fine-ground N. benthamiana leaves were extracted in 2 ml of ethyl acetate, using GR24 (5 pmol) as internal standard. Samples were vortexed and centrifuged for 20 min at 2000 g at 4 °C. The supernatant was dried in vacuo. Prior to mass analysis, samples were reconstituted in 100 μl of 25% acetonitrile/water (v/v) and filtered using a micro-spin 0.2 μm nylon membrane filter (Thermo Fisher Scientific, Waltham, MA, USA).

Targeted analysis of CL and CLA was performed using an Acquity UPLC system (Waters, Milford, MA, USA) coupled to a Xevo® TQ-XP triple-quadrupole mass spectrometer (Waters MS Technologies, Manchester, UK) with electrospray interface. Samples were injected onto a reverse-phase UPLC® Acquity BEH C18 column (2.1×100 mm, 1.7 μm, Waters) at 45 °C. Retention of analytes was controlled by gradient elution of 15 mM formic acid in water (A) and 15 mM formic acid in acetonitrile (B) at a flow rate of 0.4 ml min⁻¹. The 10 min linear gradient started by isocratic elution at 0–0.5 min with 5% B, increased to 60% B in 1.5 min, and to 90% B in the next 5.3 min. The column was washed for 1.5 min with 90% B and equilibrated for initial conditions for 1.5 min. The eluate was introduced in the electrospray interface ion source of the triple quadrupole MS analyser, operating in both positive and negative mode with the following conditions: capillary voltage, 1.2 kV; ion source/desolvation temperature, 150/600 °C; desolvation/cone gas flow, 1000/150 l h⁻¹; cone voltage, 20–25 V; and collision energy, 18–25 eV. MS data were recorded in multiple reaction monitoring mode (MRM) of four characteristic transitions for each of the compounds. The MassLynx™ software package (version 4.2, Waters) was used to operate the instrument, and acquire and process MS data.

Detection and quantification of carlactonoic acid conjugates by UHPLC-qTOF-MS

The N. benthamiana leaf extracts were analysed by UHPLC-qTOF-MS consisting of an Agilent 1290 liquid chromatograph coupled to a Bruker Daltonics microTOF-Q mass spectrometer (Bremen, Germany). The liquid chromatogram was equipped with a KINETEX® XB-C18 column (2.1 mm×100 mm, 2.6 μm; Phenomenex). Mobile phase A consisted of 5% (v/v) acetonitrile in water and 0.1% (v/v) formic acid, whereas mobile phase B consisted of 95% (v/v) acetonitrile and 0.1% (v/v) formic acid. The gradient was 0–3 min (isocratic at 95% A), 3–35 min (increase to 100% B), 40–41 min (decrease to 95% A), and column equilibration for 9 min at initial conditions (95% A). The chromatographic run lasted 40 min with a flow rate of 0.2 ml min⁻¹. The mass spectrometer was operated in negative mode. The mass spectrometer’s settings were: dry gas flow rate, 8 l min⁻¹ at 220 °C; capillary voltage, 3.8 kV; collision energy, 10 eV; and collision radiofrequency, 1200 Vpp. The qTOF was operated with the m/z range set from 50 Da to 1500 Da. The injection volume was 10 μl. Acquisition of LC-MS data was performed using Bruker DataAnalysis 4.3.

Statistical analysis

Statistical analysis was performed with GenStat 17th Edition (a VSNI product: https://www.vsni.co.uk/software/genstat/). One-way ANOVA [Fisher’s protected least significant difference (LSD) test P<0.05] was used to determine the statistical significances for the Chl data, RT-qPCR data, and nCounter data for a period of 72 h of dark treatment in WT Ler-0. A linear mixed model was used to determine the differences for the data of 6 h and 18 h treatments in both WT and max1-5/ds13 with rac-GR24 or 1% DMSO treatment. Comparisons among means were made using LSDs at P=0.05 (5% LSD). CL and CLA data were analysed using one-way ANOVA. To equalize the variances, the variables were log transformed prior to analysis. Comparisons among means were made using 5% Fisher’s LSDs.

Accession numbers

Gene and protein accession numbers are listed in Supplementary Table S5.
Results

Two EMS mutants exhibit delayed dark-induced senescence of excised immature inflorescences

We identified two EMS mutants with immature inflorescences that when detached and held in the dark exhibited delayed sepal degreening compared with the WT. They were designated delayed inflorescence senescence (dis) 9 and dis15 (Fig. 1A). We confirmed that their immature inflorescences retained more Chl than the WT at day 3 of dark incubation (Fig. 1B). Transcripts of senescence markers SAG12 (Grbic, 2003) and ANAC092 (Balazadeh et al., 2010) were not detected in the freshly harvested inflorescences, and their increased transcript abundance was suppressed in the two mutants compared with the WT at 72 h of dark incubation (Fig. 1C, D). This suggested that the delayed degreening of the mutants resulted from slower progression of senescence.

Both mutants backcrossed to the Ler-0 WT segregated ~3:1 (WT:mutant) for their delayed degreening trait (Supplementary Table S6), indicating that a single locus was responsible for their dis phenotype. These two mutants were also shorter and had more flowering stalks compared with the WT (Supplementary Fig. S3), traits that co-segregated with their dis phenotype. This suggested that the two DIS loci control senescence of excised immature inflorescences in the dark and flowering stem elongation and branching in planta.

dis9 and dis15 also exhibit delayed sepal degreening in planta

We hypothesized that the DIS loci in both mutants would also control sepal degreening during plant development because genes that are key for regulating dark-induced leaf senescence

Fig. 1. Characterization of two EMS mutants displaying delayed degreening of excised dark-incubated inflorescences. (A) Degreening of dark-incubated inflorescences. Inflorescences were excised and placed in water and incubated in the dark for 5 d. Three biological replicates are shown. (B) Total chlorophyll retention (% of day 0 value) at day 3 of dark incubation. Data are means ±SE (n=6). Letters represent significant differences between Ler-0 and dis mutants in one-way ANOVA (Fisher’s protected LSD test P<0.05). (C) Transcript abundance of ANAC092 in Ler-0 and dis mutants at 0 and 72 h. (D) Transcript abundance of SAG12 in Ler-0 and dis mutants at 0 and 72 h. Transcript abundance was normalized to that of PP2AA3 and expressed relative to Ler-0 at 0 h. Data are means ±SE (n=3). Letters represent significant differences between Ler-0 and dis mutants at 72 h dark incubation for each gene comparison in one-way ANOVA (Fisher’s protected LSD test P<0.05). Means with the same letter denote not significantly different between dis9 and dis15.
also regulate natural senescence (J. Kim et al., 2018). We grew plants for 4–5 weeks in long-day conditions to allow the floral organs to develop and observed the colour of the sepals when they started to abscise. In four independent experiments, the sepals of the mutants were always green when they abscised, whereas in comparable WT plants they were yellow (Fig. 2A, B; Supplementary Fig. S4). The delayed sepal yellowing also occurred in the detached inflorescences that were incubated in long-day conditions (Fig. 2C; Supplementary Fig. S4). This indicated that in addition to affecting the timing of dark-induced degreening, the DIS loci also controlled sepal degreening in planta and in detached inflorescences held in long days.

dis9 and dis15 have point mutations in AtD14 and MAX1, respectively

Both mutants were crossed to Col-0 for mapping purposes, and their causal mutations were identified using a combination of high resolution melting (HRM)-based mapping and whole-genome sequencing analysis. The dis9 mutation was identified as a C to T transition at position 290 downstream of the translation start site (TSS) of the Arabidopsis D14 gene (AtD14, AT3G03990) (Supplementary Fig. S1A), encoding a α/β-fold hydrolase protein that functions as an SL receptor (Arite et al., 2009; Yao et al., 2016). The mutation causes a substitution of Ser to Phe at position 97 (S97F) of AtD14. The dis15 mutation was identified as a G to A transition at position 1405 downstream of the TSS of the coding sequence of the MAX1 gene (AT2G26170) (Supplementary Fig. S1B). This mutation resulted in a substitution of Gly to Arg at position 469 (G469R) in the MAX1 cytochrome P450 monoxygenase (Booker et al., 2005) that is involved in SL biosynthesis converting CL to CLA (Abe et al., 2014). The dis9 and dis15 degreening phenotypes were complemented by WT AtD14 and MAX1 genomic regions, respectively (Supplementary Figs S5, S6). These two mutants were therefore renamed d14-6/dis9 and max1-5/dis15, respectively.

Highly conserved amino acids are substituted in AtD14 and MAX1

The D14-S97F substitution in d14-6/dis9 occurred in the Ser–His–Asp catalytic triad responsible for hydrolase activity of the receptor (Abe et al., 2014). The importance of the Ser97 residue for hydrolase activity is supported by its high conservation in orthologues from other species (Arite et al., 2009; Gao et al., 2009; Liu et al., 2009; Hamiaux et al., 2012; de Saint Germain et al., 2016; Zheng et al., 2016) and the paralogue AtKAI2 (Waters et al., 2012) (Fig. 3A). It is also supported by the finding that replacing Ser with a non-nucleophilic residue abolishes activity of the receptor protein expressed in vitro (Abe et al., 2014) and prevents formation of a covalently linked intermediate molecule (CLIM) in the active site of the protein (Yao et al., 2016) (Fig. 3B). Therefore, the SL-defective phenotype of d14-6/dis9 strongly suggested that substitution

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**Fig. 2.** Delayed sepal degreening of dis9 and dis15 in planta and in detached immature inflorescences held in long-day conditions. (A) Inflorescences attached to plants. Inflorescences from the primary bolts of 4.5-week-old wild-type and dis plants were photographed. Two biological replicates with representative abscising sepals (in white squares and magnified) are shown. (B) Degreening of sepals in planta. Flowers of 5-week-old plants were harvested when their sepals were just starting to abscise. Each flower of the seven biological replicates is from an independent plant. (C) Sepal degreening of excised inflorescences. Inflorescences were harvested from the primary bolts of 4.5-week-old plants that had their first flower opened on the same day. The inflorescences with removed opened buds were placed in water and incubated for 3 d. Three biological replicates are shown. Representative sepals are indicated by red arrows.
Strigolactones promote sepal senescence of Ser97 to Phe (a non-nucleophilic amino acid) also caused loss of receptor activity in planta.

The MAX1-G469R substitution in max1-5/dis15 occurred in the last residue of the Cys haem–iron ligand signature [FW]-[SGNH]-x-[GD]-{F}-[RKHPT]-{P}-C-[LIVMFAP]-[GAD], which is highly conserved in the cytochrome P450 superfamily (Prosite: https://prosite.expasy.org/PDOC00081). This G469 residue is invariant in all MAX1 functional orthologues studied thus far (Fig. 3C) (Yoneyama et al., 2018). However, as the ligand signature [GAD] indicates, Gly (G) can be replaced by Ala (A) or Asp (D). This occurs at very low frequency in the wider cytochrome P450 protein family, with G replaced by A in 3.4% or by D in 0.18% of the 1087 predicted cytochrome P450 proteins that have the Cys haem–iron ligand...
pattern (according to the Prosite database) (Supplementary Fig. S7). We modelled the 3D structure of AtMAX1 on the most closely related cytochrome P450 (human cytochrome P450 CYP3A4) (Yano et al., 2004) with a protein crystal structure available (sequence identity of 28% and E-value of 7e-51). The model showed that G469 is in the haem pocket, packed against the haem group and close to the haem–iron ligand Cys (Cys467) (Fig. 3D).

G469R substitution in max1-5/dis15 disrupts enzyme activity of MAX1

To confirm the loss of activity of MAX1-G469R suggested by in silico prediction, we used transient expression in N. benthamiana, as developed to study the function of SL biosynthetic enzymes (Zhang et al., 2014). Arabidopsis MAX1-WT, MAX1-G469R, and MAX1-G469A were transiently expressed with the upstream enzyme-encoding genes of the CL biosynthetic pathway (AtD27, AtMAX3, and AtMAX4), then the substrate (CL) and product (CLA) of MAX1 were measured. As expected, transient expression of AtD27, AtMAX3, and AtMAX4 resulted in the production of CL and not CLA (Fig. 4A). When co-expressed with MAX1-WT (either the Ler-0 or Col-0 version) or MAX1-G469A, CL was significantly reduced and some CLA was detected. However, when co-expressed with MAX1-G469R, the amount of CL did not decrease although CLA was produced in similar amounts to that produced by MAX1-WT (Fig. 4A). The lack of a decrease in CL suggested that MAX1-G469R had reduced enzymatic activity. We considered that the absence of a difference in CLA production in the different treatments was likely to be caused by conjugation (e.g. glycosylation) of CLA by endogenous N. benthamiana enzymes, since we had observed this several times previously in N. benthamiana (e.g. in the transient production of geranic acid that was glycosylated with one or two hexoses; Dong et al., 2013). If CLA conjugation occurs efficiently, the amounts of free CLA would remain low and not reflect the rate of conversion of CL to CLA. Thus, we investigated whether N. benthamiana leaves expressing MAX1-WT accumulated CLA conjugates using LC-qTOF-MS analysis. Indeed, N. benthamiana leaves expressing the CL pathway genes together with MAX1-WT accumulated CLA–dihexose and CLA–hexose conjugates (Fig. 4B; Supplementary Fig. S8). When MAX1-G469A was substituted for MAX1-WT, conjugate formation was not significantly different from that in the pathway with MAX1-WT (Fig. 4B; Supplementary Fig. S8). However, when MAX1-G469R was substituted for MAX1-WT, conjugate production decreased 36- and 15-fold, respectively. To confirm that the G469R mutation was affecting enzyme activity rather than exerting its effect through transcriptional changes, mRNA abundance of MAX1-WT and MAX1-G469R was analysed. There was no difference in...
expression between MAX1-WT and MAX1-G469R when they were expressed in N. benthamiana (Supplementary Fig. S9). Thus, we concluded that the lack of CL conversion and reduced CLA conjugate production in N. benthamiana upon co-infiltration of the CL pathway with MAX1-G469R was caused by reduced activity of the MAX1-G469R enzyme.

SL biosynthetic and response genes are up-regulated by 24 h of dark incubation in inflorescences

The SL biosynthetic pathway is thought to be induced by senescence signalling (Ueda and Kusaba, 2015). To test this, we used nCounter technology to compare the timing of transcriptional changes in selected senescence marker and SL pathway genes in excised WT inflorescences every 24 h over a period of 3 d of dark treatment.

The transcript abundance of early stage senescence markers, namely ANAC092 and the Chl degradation gene SGR1 (Park et al., 2007), significantly increased at 24 h (Fig. 5A), suggesting senescence in the inflorescences had already initiated by this time. Increased transcript abundance of the late stage senescence-specific marker gene SAG12 at 48 h indicated that at 2 d of dark incubation senescence was well advanced.

The transcript abundance changes of three SL biosynthetic genes, MAX1, MAX3, and MAX4, was used to estimate when SL biosynthesis was initiating in the dark-held inflorescences. MAX1 transcript abundance did not significantly change during the first 24 h of dark treatment, but then significantly and substantially increased to be highest at 72 h (Fig. 5B). MAX3 transcript abundance was slightly increased at 24 h, suggesting that SL production in the tissue was just starting. From 24 h onwards, MAX3 transcript abundance increased in concert with both early senescence markers SGR1 and ANAC092. RT-qPCR analysis of MAX4 revealed a pattern of transcript accumulation that was very similar to that of MAX3, suggesting co-regulation and the beginning of SL synthesis by 24 h (Supplementary Fig. S10A).

We examined changes in the transcript abundance of the SL signalling genes AtID14, SMXL6, SMXL7, and SMXL8. Transcript abundance of the first three genes increased significantly at 24 h of dark treatment (Fig. 5C, D), whereas SMXL8 transcript abundance decreased to be undetectable at 24 h (Fig. 5D). The nCounter results for MAX3, SMXL6/8, ANAC092, and SAG12 were confirmed by RT-qPCR analysis (Supplementary Fig. S10). Overall, the results from the transcript profiling of the inflorescence suggested that, by 24 h of dark incubation, SL biosynthesis has been initiated, SL signalling is occurring, and senescence has started. Thus, earlier time points were investigated to determine the order of pathway activation.

SL signalling, but not biosynthetic, genes respond rapidly to the light–dark transition

At 24 h of darkness, the inflorescence tissue had been exposed to 8 h of regular and 16 h of extended night. Holding tissue

in extended darkness leads to acute carbon starvation caused by exhaustion of starch reserves (Usadel et al., 2008) that can lead to precocious senescence. To test whether SL biosynthesis and response were associated with carbon deprivation–based signalling, we compared the timing of expression of transcriptional markers of tissue carbon status (SnRK1-related genes AKI1β1 and bZIP63) (Bläsing et al., 2005; Usadel et al., 2008; Li et al., 2009; Mair et al., 2015) with that of SL-associated genes in both the regular and the early extended night.

In Ler-0 controls during the first 6 h into the regular night, transcript abundance of AKI1β1 and bZIP63 increased in the detached WT inflorescences held in the dark (Fig. 6A) consistent with the genes being markers of reduced carbohydrate status. Key senescence–regulatory genes ANAC092 (Kim et al., 2009) and AtNAP (Guo and Gan, 2006) were up-regulated at 3 h (Fig. 6B) probably because of their known induction by reduced sugar status (Bläsing et al., 2005; Usadel et al., 2008) and/or the circadian clock (H. Kim et al., 2018; Song et al., 2018). There was no increase in transcript abundance of MAX1 6 h into the regular night, and MAX3 transcripts were not detected at this time (Fig. 6C). Transcript abundance of SMXL7 also did not change during the regular night (Fig. 6D). However, at 3 h into the dark period, SMXL6 and SMXL8 were up- and down-regulated, respectively (Fig. 6D).

In order to determine the effect of SL on the expression of the above genes, we treated the inflorescences with rac-GR24. Rac-GR24 is a racemic mixture of two enantiomers, GR24SDS a synthetic canonical SL, and GR24int-SDS that induces karrin (KAR) signalling (Kramm et al., 2019). However, GR24int-SDS is probably not relevant for the dark-induced degreening phenotype since defects in KARRIKIN INSENSITIVE 2 (KAI2), a KAR-specific receptor, do not delay dark-induced leaf senescence (Ueda and Kusaba, 2015). In the mock-treated max1-1/5/dis15 mutant, the two MAX and three SMXL genes exhibited similar expression patterns to that of the WT, although their abundance was lower (Fig. 6C, D). The reduced expression of the three SMXL genes was reversed when the mutant was treated with 5 μM rac-GR24 for 3 h (Fig. 6D). Intriguingly, AtNAP was up-regulated by rac-GR24 at 3 h of treatment (Fig. 6B), suggesting that it is also an SL-inducible gene. Thus, based on transcription, the increased transcript abundance of sugar-related genes did not induce expression of SL biosynthetic genes in the WT inflorescences during the normal night. In the max1-1/5/dis15 mutant, both SL signalling genes and AtNAP respond rapidly to rac-GR24 treatment, indicating that these genes were SL inducible.

GR24 induces the transcript abundance of senescence-related genes in max1-1/5/dis15 during an extended night

We next determined the effect of extended darkness (i.e. darkness that surpassed the anticipated night period) on
carbon status markers, senescence markers, and SL biosynthesis and signalling genes. In the WT, at 4 h of extended night (i.e. 12 h of dark treatment), transcript abundance of \( \text{AKIN} \beta_1 \) and \( b\text{ZIP}63 \) was substantially increased (Fig. 7A). This was consistent with the WT inflorescences experiencing carbon starvation, as has been reported for rosette leaves exposed to a 4 h extended night (Usadel et al., 2008). Transcript abundance of \( \text{ANAC092} \) and \( \text{AtNAP} \) was also significantly increased at this time (Fig. 7B). However, in \( \text{Ler} \) controls, transcript abundance of \( \text{MAX1} \) was not increased by the 4 h night extension, and \( \text{MAX3} \) abundance remained undetectable, suggesting that SL biosynthesis was still not occurring. By 10 h of extended night (18 h of dark treatment), \( \text{MAX1} \) transcript abundance had still not changed, but that of \( \text{MAX3} \) had increased, suggesting that SL biosynthesis had started (Fig. 7C). The three \( \text{SMXL} \) genes were differentially expressed during the extended night (Fig. 7D). \( \text{SMXL8} \) transcript counts were almost undetectable at both 12 h and 18 h; \( \text{SMXL6} \) transcript abundance was increased at 12 h but then declined; and \( \text{SMXL7} \) started to increase at 18 h in a pattern similar to \( \text{MAX3} \).

We then determined how the patterns of expression of the above genes were affected by SL deficiency by examining their transcript accumulation in the \( \text{max1-5}/\text{dis15} \) mutant. Overall, in the mock-treated \( \text{max1-5}/\text{dis15} \) mutant, the patterns of accumulation of carbon status-related genes, senescence marker...
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...genes, and SL biosynthesis and signalling genes in the mutant were very similar to that seen in the WT over the 10 h extended night (Fig. 7), suggesting that their pattern of regulation was not controlled by SL. Interestingly, when the mutant was treated with rac-GR24, the transcript abundance of sugar-related genes AKINβ1 and bZIP63 was suppressed significantly at 12 h but not at 18 h (Fig. 7A). In contrast, the transcript abundance of the two senescence-related genes ANAC092 and AtNAP was elevated at 12 h (4 h of extended night) by rac-GR24, and so was MAX1 (Fig. 7B, C). All three SMXL genes were up-regulated by rac-GR24 at both time points (Fig. 7D), as observed during the regular night.

Taken together, the nCounter profiling study has highlighted a temporal sequence of events whereby markers of carbon deprivation and senescence regulation first increased, followed within hours by markers for SL production. Further, GR24 treatment of the max1-5/dis15 mutant indicated that SL acts to promote transcription of senescence-controlling genes and to suppress transcription of SnRK1-related genes.

Discussion

Mutations in SL biosynthesis and receptor proteins define functionally important amino acids

The finding with an EMS screen of two independent mutations in the SL pathway that significantly affected sepal senescence progression emphasizes the importance of this hormone...
in the floral death process in addition to its more explored roles in plant development (Gomez-Roldan et al., 2008; Umehara et al., 2008; Kapulnik et al., 2011; Ruyter-Spira et al., 2011; Rasmussen et al., 2012; Toh et al., 2012).

MAX1 encodes a CYP711A1 protein of the cytochrome P450 superfamily (Booker et al., 2005). The MAX1-G469R substitution occurred at the last residue in the highly conserved Cys haem–iron ligand signature, which is just two amino acids C-terminal to the absolutely conserved Cys at position 467. To date, no crystal structure of MAX1 has been reported. However, a 3D model based on the closest homologous structure, the human microsomal P450 CYP3A4, revealed that Gly469 packs against the haem cofactor in the binding pocket and is close to the haem–iron ligand Cys467 (Fig. 3D). The G469R substitution presumably causes loss of function by disrupting the steric structure of this pocket because there is not enough space to accommodate Arg, which has one of the largest side chains, compared with Gly that has the smallest.

The G469 residue is invariant in MAX1 orthologues and its closely related proteins in Metazoa, Bacteria, and Archaea (Fig. 3C; Supplementary Fig. S7; Challis et al., 2013), whereas in the wider cytochrome P450 family in rare instances this...
residue is replaced by Ala. Our transient expression assay in N. benthamiana, and successful genetic complementation of the max1-5/dis15 mutant with MAX1-G469A (Supplementary Fig. S6) demonstrated that the Ala substitution did not reduce MAX1 function, suggesting that this small non-polar amino acid does not introduce steric clashes in the wider family of cytochrome P450 proteins either. This is consistent with the equivalent Gly to Ala substitution not affecting activity of the Arabidopsis cytochrome P450 CYP83B1, a modulator of auxin homeostasis (Barlier et al., 2000; Bak et al., 2001). The high content of CL and strongly reduced production of CL hexose conjugates in the leaves infiltrated with the MAX1-G469R construct are consistent with accumulation of CL pre-

by Ala.

Thus, G469 is an important hydrophobic substrate-binding pocket containing a Ser–His–
tunia (PhDAD2) and rice (OsD14) show that they have a

Zhou et al. (2013). Unlike the classical hormone receptors that non-covalently and reversibly bind to hormone molecules, the crystal structure of the SL-induced AtD14–D3–ASK1 complex reveals that AtD14 binds to SL and hydrolyses it into a CLIM (Yao et al., 2016). It is under debate whether this hydrolysis is required for signalling to occur (Seto et al., 2019). Experiments with the F-box protein D3, a rice orthologue of Arabidopsis MAX2, showed that SL triggers signalling by enabling AtD14 to bind to MAX2 to recruit repressors (e.g. Arabidopsis SMXL6/7/8) for degradation through the 26S proteasome (Jiang et al., 2013; Zhou et al., 2013; Wang et al., 2015). In the d14-6/dis9 mutant, Ser97 in the catalytic triad was replaced by Phe. This produced phenotypes similar to the null mutant d14-1, suggesting loss of activity of D14 (Waters et al., 2012; Ueda and Kusaba, 2015). It is also consistent with mutation of this residue to another non-nucleophile residue, Ala (atd14:S97A), abolishing hydrolyase activity in vitro of the expressed protein (Abe et al., 2014). Thus, it is probable that the in planta S97F mutation we identified causes complete loss of D14 activity by affecting its hydrolyase activity.

SLs regulate dark-induced inflorescence senescence in association with a change of carbon status during extended night

Our results indicated that SLs hastened Arabidopsis sepal senescence in planta and under energy deprivation conditions. Previously we showed that dark treatment of detached inflorescences was associated with reduced soluble sugar content and transcriptional changes of sugar-related genes (Trivellini et al., 2012). Thus, it was plausible that SLs would interact with sugar signalling to control dark-induced inflorescence senescence, which would be consistent with reports of crosstalk between SLs and sugar regulation of shoot branching and seedling establishment in Arabidopsis (Li et al., 2016; Otori et al., 2017), and the finding that SL-induced senescence of bamboo leaves is suppressed by exogenous sugar treatment (Tian et al., 2018).

SL and sugar interaction during normal night

In the inflorescences, genes that control sugar-dependent transcriptional response (Bläsing et al., 2005; Baena-González et al., 2007; Usadel et al., 2008), namely the SnRK1-related genes AKNB1 (a subunit of SnRK1) (Li et al., 2009) and bZIP63 (one of the direct targets of SnRK1) (Mair et al., 2015), were up-regulated during the regular night, consistent with sugar status reducing as the night progressed. To determine whether this normal diurnal reduction in sugar content was associated with changes in SL content, we used the approach of Li et al. (2018) and measured transcript abundance changes of the SL biosynthetic genes because of the difficulty in measuring SLs in Arabidopsis (Seto et al., 2014; Lv et al., 2018), although we acknowledge that the involvement of post-transcriptional factors cannot be discounted. SLs did not appear to be synthesized in response to the sugar decline, since MAX1 abundance was unchanged and MAX3 counts were below the threshold for detection (Fig. 6A).

Mashiguchi et al. (2009) and Brewer et al. (2016) had suggested that such an interpretation could be confounded by SL negative feedback on SL biosynthetic genes. However, we did not find evidence for SL negative feedback since: (i) transcript abundance of the SL biosynthetic genes was not higher in the max1-5/dis15 mutant and Ler-0 WT; and (ii) treatment of the inflorescences with the SL analogue did not suppress their expression. This absence of negative feedback agrees with the findings of Bainbridge et al. (2005) on MAX4.

SL and sugar interaction during extended night

The extended night commences when the regular night ends. In Arabidopsis rosettes, a 4 h extended night leads to acute carbon deprivation and transcriptional reprogramming by SnRK1s (Baena-González et al., 2007; Usadel et al., 2008). Consistent with this, we found that AKNB1 and bZIP63 transcripts increased to their highest abundance in both the WT and max1-5/dis15 at 12 h (4 h into extended night) (Fig. 7). Transcripts of the senescence-regulating genes ANAC092 and NAP continued to increase at 18 h and this corresponded to the time when MAX3 and SMXL7 started to increase. We used SMXL6, SMXL7, and SMXL8 to determine SL response because the delayed senescence phenotype in both mutants was tightly linked to altered branching and these SMXL genes are functionally redundant in controlling Arabidopsis shoot branching (Soundappan et al., 2015; Wang et al., 2015). The expression pattern of SMXL7 correlated best with the SL biosynthetic genes and senescence marker genes.
SMXL7 also has higher transcript abundance than SMXL6 and SMXL8 in senescent leaves (Stanga et al., 2013), which suggests that this SMXL may have a more important role in senescence than the others.

In summary, two novel mutants have highlighted a connection between the SL pathway and floral organ senescence in planta, and in response to carbon limiting conditions. Our analyses indicated an intricate relationship among sugar starvation, senescence, and SL biosynthesis and signalling in excised dark-held inflorescences. Here we propose a model (Fig. 8) in which sugar shortage resulting from prolonged darkness triggers senescence initiation and progression in the inflorescence, and this associates with transcriptional changes of senescence-related transcription factor genes such as NAP and ANAC092 (Trivellini et al., 2012). SLS may not have a major role in the inflorescence during the normal night but are synthesized during the extended night, perhaps in response to sustained low sugar content and consequent senescence initiation. Then SLS play an important role in promoting senescence progression, perhaps by activation of key senescence regulators.

Supplementary data

The following supplementary data are available at JXB online.

Fig. S1. Identification of causal mutations in dis9 and dis15 mutants.

Fig. S2. Stability of PP2A reference gene expression in response to GR24 treatment and in a strigolactone-deficient mutant.

Fig. S3. The dwarf and bushy phenotypes of dis9 and dis15 in planta.

Fig. S4. Delayed sepal degreening of dis9 and dis15 in planta and in detached inflorescences held in long-day conditions.

Fig. S5. Genetic complementation of dis9.

Fig. S6. Complementation of dis15 with MAX1-WT and MAX1-G469A.

Fig. S7. Sequence alignment of Arabidopsis MAX1 with its putative orthologues, related proteins, and other cytochrome P450 proteins with the haem–iron ligand signature.

Fig. S8. Identification of CLA conjugates in N. benthamiana leaves infiltrated with different combinations of SL biosynthetic genes.

Fig. S9. Transcript abundance of MAX1 in leaves of N. benthamiana infiltrated with constructs harbouring MAX1-WT, -G469R, or -G469A.

Fig. S10. Transcript abundance changes of SL pathway and senescence-related genes.

Table S1. HRM primers for fine mapping of dis9.

Table S2. Primers for RT-qPCR analysis.

Table S3. Probes for nCounter analysis.

Table S4. Primers for MAX1-related constructs used in agroinfiltration of N. benthamiana.

Table S5. Gene and protein accession numbers.

Table S6. Pearson’s $\chi^2$ test for F$_2$ progenies of dis9 and dis15.

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**Author contributions**

XX, DAH, and PPD: conceptualization and design; XX, RJ, and AE: mutant screening; HB: overseeing transient expression, LC-MS, and qTOF analysis; XX, YW, LD, and KF: data analysis; XX and AR-GM: statistics; XX and AH: nCounter analysis; AS-S: crystal structure modelling; HB and KF: LCMS and qTOF method sections; XX, DAH, and PPD: writing; DAB: review and editing. All authors read and approved the final version.

**Conflict of interest**

The authors declare no conflict of interest.

**Data availability**

All data supporting the findings of this study are available within the paper and within its supplementary data published online.

**References**


Rogers HJ. 2013. From models to ornamentals: how is flower senescence regulated? Plant Molecular Biology 82, 563–574.


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