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Jasmonate production through chlorophyll *a* degradation by Stay-Green in *Arabidopsis thaliana*

Abstract
Leaf color change through chlorophyll degradation is a characteristic symptom of senescence. Magnesium removal from chlorophyll *a* is the initial step in chlorophyll *a* degradation, in a reaction catalyzed by Stay-Green (SGR). *Arabidopsis thaliana* has three *SGR* homologs, *SGR1*, *SGR2*, and *SGR-like*. When *SGR1* is overexpressed, both chlorophyll *a* and *b* are degraded and leaves turn yellow. This process is visually identical to senescence, suggesting that *SGR1* overexpression affects various physiological processes in plants. To examine this possibility, gene expression associated with chlorophyll metabolism and senescence was analyzed following dexamethasone-inducible *SGR1* introduction into Arabidopsis. When *SGR1* was overexpressed following 18 h of dexamethasone treatment, genes involved in chlorophyll degradation were upregulated, as were senescence-associated genes. These observations suggested that chlorophyll *a* degradation promotes senescence. As jasmonate is the plant hormone responsible for senescence and was expected to be involved in the regulation of gene expression after dexamethasone treatment, the level of jasmonoyl-isoleucine, the active form of jasmonate, was measured. The jasmonoyl-isoleucine level increased slightly after 10 h of *SGR1* overexpression, and this increase became significant after 18 h. These observations suggest that jasmonate is produced through chlorophyll *a* degradation and affects the promotion of senescence.

Key words
Arabidopsis; Stay-Green; chlorophyll degradation; senescence; jasmonate

Introduction
Land plants and green algae contain chlorophyll *a* and *b* as photosynthetic pigments, which are degraded during senescence. In this process, chlorophyll *b* is converted to chlorophyll *a* to enter the degradation pathway (Fig. 1). The formyl group at C-7 of chlorophyll *b* is reduced to a hydroxymethyl group by chlorophyll *b* reductase, producing 7-hydroxymethyl chlorophyll *a* (Kusaba et al., 2007). There are two isozymes of chlorophyll *b* reductase, Non-Yellow Coloring 1 (NYC1) and NYC1-like. The hydroxymethyl group of this intermediate is reduced to a methyl group to produce chlorophyll *a* in a reaction catalyzed by 7-hydroxymethyl chlorophyll *a* reductase (Meguro et al., 2011). The first step in chlorophyll *a* degradation is the removal of the central Mg by Stay-Green (SGR, also known as NYE1) to produce pheophytin *a* (Shimoda et al., 2016). The phytyl group of pheophytin *a* is hydrolyzed by pheophytinase (PPH) to produce pheophorbide *a* (Schelbert et al., 2009), and then pheophorbide *a* oxygenase (PAO) opens the chlorin ring of pheophorbide *a* to generate red chlorophyll catabolite (Pruzinska et al., 2003). This molecule is further modified to increase its solubility, following which it is transferred to the vacuole (Kräutler, 2016). Hydrolysis of the phytyl group of chlorophyll *a* by chlorophyll dephytylase, an enzyme distinct from PPH, also occurs in green tissue (Lin et al., 2016). Although PPH and chlorophyll dephytylase both serve to remove the phytyl group, their substrates and expression patterns are different. All the genes involved in chlorophyll degradation in chloroplasts have been identified (Kuai et al., 2018). In addition to senescent leaves, chlorophyll degradation also occurs in response to biotic and abiotic stresses, as well as during fruit ripening and seed maturation (Kuai et al., 2018). Under these conditions, chlorophyll is degraded by the same pathway as during senescence. Formation of the isocyclic fifth ring, the last step in chlorophyll biosynthesis to be characterized, was recently demonstrated (Chen et al., 2018) and all the chlorophyll

biosynthesis steps have now been established. Consequently, it is now possible to characterize chlorophyll metabolism in its entirety.

SGR catalyzes the committed step in chlorophyll *a* degradation. The *SGR* gene was identified from a mutant showing a stay-green phenotype during senescence (Hörtensteiner, 2009; Kusaba et al., 2013). Arabidopsis has three *SGR* homologs, *SGR1*, *SGR2*, and *SGR-like* (*SGRL*), with *SGR1* and *SGR2* showing a high degree of sequence homology. The *SGR1*-deficient mutant, but not that of *SRG2*, shows the stay-green phenotype (Ren et al., 2007; Wu et al., 2016), indicating that *SGR1* is the major enzyme involved in chlorophyll *a* degradation during senescence. While *SGRL*, which is expressed in green tissue (Rong et al., 2013; Sakuraba et al., 2014), removes Mg from chlorophyll *a* and chlorophyllide *a*, SGR1 and SGR2 remove Mg only from chlorophyll *a* (Shimoda et al., 2016). SGRL may function by coupling with chlorophyll dephytylase in green tissue (Lin et al., 2016). SGR cannot remove Mg from chlorophyll *b*, possibly because chlorophyll *b* binds Mg tightly (Saga and Tamiaki, 2012), suggesting that chlorophyll *b* has to be converted to chlorophyll *a* during degradation to loosen the Mg for the SGR reaction.

Senescence involves the mobilization of nutrients from leaves to growing tissues for reproduction (Havé et al., 2017). For nutrient mobilization, chlorophyll must be degraded for two reasons. First, since chlorophylls that are not incorporated into the photosystem are potential photosensitizers, chlorophyll-related phototoxicity must be suppressed to maintain cell viability for efficient nutrient mobilization during senescence (Hörtensteiner, 2006; Li et al., 2017). Second, before the apoproteins of chlorophyll-protein complexes are degraded, chlorophyll must be removed and catabolized because chlorophyll-protein complexes are resistant to attack by proteases. When some of the chlorophyll is removed from chlorophyll-binding proteins, they become susceptible to proteolysis. Mutant plants defective in chlorophyll degradation cannot degrade chlorophyll-binding proteins, indicating the importance of chlorophyll degradation (Kusaba et al., 2007; Park et al., 2007).

Jasmonate and its derivatives (collectively, jasmonates) are plant hormones that induce senescence-associated gene expression, as well as leaf senescence (Kim et al., 2015). Jasmonate is synthesized from α-linolenic acid released from galactolipids of chloroplast membranes by lipase (Browse, 2009). 12-Oxo-phytodienoic acid, the precursor of jasmonate, is produced through the dioxygenation of α-linolenic acid by lipoxygenase and the successive activities of allene oxide synthase and allene oxide cyclase. All the enzymes involved in generating 12-oxo-phytodieneoic acid are located in the chloroplast. 12-Oxo-phytodienoic acid is then transferred into peroxisomes and converted to jasmonic acid (JA). Jasmonoyl-isoleucine (JA-Ile), the receptor-active form of jasmonate, is generated from JA by jasmonoyl-isoleucine synthetase. JASMONATE ZIM-DOMAIN (JAZ) proteins bind and repress MYC transcription factors when JA-Ile levels are low. In the presence of JA-Ile, CORONATINE INSENSITIVE 1 binds to JAZ proteins, resulting in JAZ ubiquitination and degradation. JAZ protein degradation induces the formation of the transcription preinitiation complex with RNA polymerase II and synthesis of various transcription factors, such as the bHLH-type transcription factors MYC2, MYC3, and MYC4 (Howe et al., 2018). In addition, these three MYC transcription factors redundantly regulate the expression of downstream genes, although their tissue-specific expression patterns are somewhat different (Fernández-Calvo et al., 2011). MYC proteins are positive regulators of chlorophyll catabolic genes (Zhu et al., 2015), indicating that jasmonate is involved in the regulation of chlorophyll degradation. When *SGR* is overexpressed, both chlorophyll *a* and *b* are degraded. Since chlorophyll *b* is not a substrate for SGR, chlorophyll *b* conversion to chlorophyll *a* must be activated after *SGR* overexpression. Indeed, a previous study reported that *NYC1* is induced after *SGR* overexpression (Sato et al., 2018), indicating that chlorophyll *a* degradation affects nuclear gene expression. The thylakoid membrane and envelope were also found to be disorganized

and plastoglobules accumulate after *SGR* overexpression (Sato et al., 2018). These structures are similar to chloroplasts in the senescent leaf. These observations raise the question of how chlorophyll *a* degradation in the chloroplast organizes the entire chlorophyll degradation process and promotes senescence. Jasmonate is the plant hormone that activates the chlorophyll catabolic pathway and promotes senescence. In this study, we investigated *SGR*-overexpressing plants and found that jasmonate signaling was induced through chlorophyll degradation.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana (ecotype Col-0) was used for the experiments. The plants were grown for 3–4 weeks at 23°C. To suppress diurnal changes in gene expression, the plants were grown under continuous light conditions (80 µmol photons m^{-2} s^{-1}). To induce *SGR1* expression chemically in Arabidopsis, *SGR1* was expressed under the control of the pOp6 promoter and the synthetic transcription factor LhGR (Craft et al., 2005; Wielopolska et al., 2005). *SGR1* cDNA with a C-terminal FLAG tag was introduced into a binary vector, pOpON, which was constructed from the pOpOff2 vector, as previously described (Shimoda et al., 2016). This construct was transferred into wild type plants and two independent transgenic lines (line #3 and line #19) were selected for use in this study. To introduce inducible *SGR1* into the JA-defective *allene oxide synthase* (*aos*) mutant (Park et al., 2002), transformants harboring inducible *SGR1* were crossed with *aos* mutants and an *aos*-deficient line was isolated. To recover male fertility in the *aos* mutants, 2 mM JA with 0.01% Triton X-100 was applied to the floral buds. To induce *SGR1*, plants were sprayed with 10 µM dexamethasone (DEX) supplemented with 0.015% Silwet L-77. Mock treatment consisted of a Silwet L-77 solution.

Pigment analysis

Pigments were extracted from leaf disks (0.5 cm^2) with 100% acetone and then separated and quantified by HPLC using a C8 column (Water Symmetry C8, Waters) with an eluent (methanol: acetonitrile: acetone [1:2:1 v:v:v]) at a flow rate of 1 mL min^{-1} at 40°C. The pigments were monitored with a diode array detector (SPD-M 10AVP; Shimadzu) with 663-nm absorbance for chlorophyll *a* and 646-nm absorbance for chlorophyll *b* (Shimoda et al., 2016). Pigment content was normalized by leaf area.

RNA isolation and quantitative RT-PCR

Total RNA was extracted from leaf tissues using Plant RNA Purification Reagent (Invitrogen). The cDNA was synthesized using a PrimeScriptRT Reagent Kit with gDNA eraser (Takara). Quantitative RT-PCR analysis was performed using SsoAdvanced Universal SYBR Green Supermix (BioRad) and a MyiQ2 (BioRad). The sequences of the primers for *ACT2* (AT3G18780), *SGR1* (AT4G22920), *SGR2* (AT4G11910), *NYC1* (AT4G13250), *PPH* (AT5G13800), *PAO* (AT3G44880), *ORE1* (AT5G39610), *SAG12* (AT5G45890), *SAG13* (AT2G29350), *MYC2* (AT1G32640), *MYC3* (AT5G46760), *MYC4* (AT4G17880), *JAZ1* (AT1G19180), *JAZ3* (AT3G17860), *JAZ8* (AT1G30135), *JAZ10* (AT5G13220), *AOS* (AT5G42650), *LOX2* (AT3G45140), and ascorbate peroxidase 1 (*APX1*) (AT1G07890) used for quantitative RT-PCR are shown in Supplementary Table S1. Transcriptome analysis was performed by Macrogen using the Arabidopsis Gene Expression Microarray kit (4X44K, V3) (Agilent) with RNA extracted from leaf tissues of plants treated with DEX for 0, 10, and 18 h. Two plants were used for the 18 h DEX treatment to determine the average gene expression profiles. Gene-enrichment and functional annotation analysis of the significant probe list was performed using DAVID (http://david.abcc.ncifcrf.gov).

Jasmonate quantification

Jasmonate was quantified as previously described (Sato et al., 2009). Briefly, approximately 200 mg of mock- or DEX-treated leaves were harvested after 0, 10, and 18 h. The leaves were frozen in liquid nitrogen and the plant materials extracted in ethanol. The mixture was filtered and the crude extract was put onto a Bond Elut C18 cartridge column (Agilent) to remove pigments. The elution was analyzed with a UPLC MS/MS system. Deuterium-labeled JA and JA-Ile were used as the internal standards for quantification. Stereoisomers were not identified using this system.

Results

Chlorophyll degradation and chlorophyll metabolic gene expression through *SGR* overexpression

DEX-inducible *SGR1* was introduced into wild type Arabidopsis. When *SGR1* was overexpressed *via* DEX treatment, chlorophyll was degraded (Fig. 2). Chlorophyll *a* contents decreased after 10 h of DEX treatment in line #3 and after 18 h in line #19. Line #3 responded to DEX treatment more rapidly than line #19. Chlorophyll *a* contents showed a greater reduction than those of chlorophyll *b*, resulting in a decrease in the chlorophyll *a*/*b* ratio after 10 h of DEX treatment in line #3 and after 18 h in line #19. SGR removes Mg from chlorophyll *a* but not from chlorophyll *b*. Consequently, chlorophyll *b* must be converted to chlorophyll *a* before degradation (Fig. 1). This substrate specificity of SGR explains why chlorophyll *b* degradation was not as significant as that of chlorophyll *a*. *NYC1* was reportedly induced and chlorophyll *b* degraded after 30 h of *SGR* overexpression (Sato et al., 2018). This suggests that chlorophyll *a* degradation by SGR changes the expression of a variety of genes since the transcription factors involved in the regulation of *NYC1* expression also directly upregulate several genes (Kuai et al., 2018). A comprehensive gene expression analysis was performed with DEX-treated plants from line #19 (Supplementary Table S2). In this study, to understand the primary plant response to DEX treatment, gene expression was analyzed for up to 18 h of DEX treatment. To suppress diurnal changes in gene expression, plants were grown under continuous light conditions. mRNA was extracted from leaves after 10 h or 18 h of DEX treatment and the gene transcription levels were compared to those in a non-treated plant. To understand the gene expression profiles, gene-set enrichment analysis was performed with the DAVID functional annotation tool. The results based on Gene Ontology and KEGG annotations suggested that stress- and plant hormone-related genes were differentially expressed under DEX treatment (Supplementary Figs. S1 and S2). The expression of genes involved in chlorophyll biosynthesis decreased, whereas that of genes involved in chlorophyll degradation increased (Supplementary Table S2). These results were subsequently confirmed by quantitative RT-PCR analysis. *NYC1*, *PPH*, and *PAO* mediate chlorophyll degradation and regulation of their expression is dependent on the developmental stage or environmental conditions. The mRNA levels of these genes were examined (Fig. 3). *ACT2* was used to normalize the mRNA levels. The *NYC1* mRNA levels increased after 18 h of DEX treatment, whereas those of *PPH* and *PAO* increased after 10 h and increased significantly after 18 h. The expression of the *SGR2* gene was also increased under DEX treatment. Since the ORE1 transcription factor regulates the expression of *NYC1* and *PAO* (Qiu et al., 2015), *ORE1* mRNA levels were also examined. The results showed that *ORE1* expression increased after 10 h of DEX treatment and increased further after 18 h. The overall plant responses to DEX treatment were similar to those observed during the senescence process; consequently, DEX treatment was expected to induce senescence. To investigate whether senescence was induced with DEX treatment, expression of the senescence marker genes *Senescence associated gene 12* (*SAG12*) and *SAG13* (Kim et al., 2015) were analyzed. *SAG12* expression was not detected at 0 or 10 h in mock- or DEX-treated leaves, but was detected after 18 h of DEX treatment. *SAG13* was also induced as a result of DEX treatment.

These observations indicate that SGR-mediated chlorophyll *a* degradation alters the expression of genes involved in the chlorophyll metabolic pathway and promotes senescence.

Jasmonate production through chlorophyll *a* degradation

Chlorophyll degradation through SGR overexpression is visually identical to that during senescence. Since jasmonate is a senescence-related plant hormone (Schippers et al., 2007), this suggests that jasmonate is produced through chlorophyll *a* degradation. Transcriptome analysis also suggested that jasmonate was produced (Supplementary Table S2). To examine whether jasmonate is involved in the regulation of gene expression associated with SGR overexpression, jasmonate-related gene expression was analyzed by quantitative RT-PCR. *MYC2*, *MYC3*, and *MYC4* are closely related transcription factors that regulate many jasmonate-responsive genes in Arabidopsis (Howe et al., 2018). *MYC2* and *MYC3* levels were induced by DEX treatment, whereas that of *MYC4* decreased (Fig. 4). JAZ proteins are involved in jasmonate signaling through the repression of MYC transcription factor activity. Although JAZ proteins repress the transcription of jasmonate-responsive genes (Thines et al., 2007), JAZ expression levels are induced by jasmonate. Rapid synthesis of new JAZ proteins may reduce the jasmonate response after signal transmission. The Arabidopsis genome encodes 13 JAZ proteins that are classified into five groups (Caitlin et al., 2015). The expression of *JAZ1*, *JAZ3*, *JAZ8*, and *JAZ10*, which belong to different groups within this classification, was examined (Fig. 4), and all were found to be induced by DEX treatment. The expression profiles of these jasmonate-related genes suggested that jasmonate was produced following DEX treatment. The levels of 13-lipoxygenase, encoded by *LOX2*, and of allene oxide synthase, encoded by *AOS*, both of which are involved in jasmonate synthesis (Ellinger et al., 2010), were also found to be upregulated (Fig. 4). Next, JA and JA-Ile levels were also measured (Fig. 5). These jasmonate levels were very low before DEX treatment; however, after 10 h of DEX treatment, JA and JA-Ile levels both increased, with the increase becoming significant after 18 h. These results showed that chlorophyll *a* degradation induced jasmonate production and resulted in wide-ranging changes in gene expression.

Chlorophyll degradation and gene expression after DEX treatment under dark conditions

In this study, DEX-treated plants were maintained under light conditions because transient overexpression of *SGR* in leaves of *Nicotiana benthamiana* plants does not result in efficient chlorophyll degradation under dark conditions (Bell et al., 2015). We examined the effects of light conditions on chlorophyll degradation. When plants were placed under dark conditions for 24 h after DEX treatment, chlorophyll degradation was not observed (Fig. S3). The rapid chlorophyll degradation allowed us to observe plant responses to chlorophyll degradation extensively because chlorophyll degradation is synchronized in DEX-treated leaf cells. However, under light conditions, chlorophyll degradation intermediates elicit light stress through the production of reactive oxygen species (ROS). Ascorbate peroxidase is a hydrogen peroxide scavenger. To examine whether DEX-treated plants are under oxidative stress, *APX1* mRNA levels were quantified. *APX1* was induced after DEX treatment, suggesting that H_2O_2 was produced through chlorophyll degradation with DEX treatment. To confirm that the gene expression shown in Figs. 3 and 4 was induced through chlorophyll *a* degradation and not solely through light stress, DEX-treated plants were placed under light conditions for 4 h after DEX treatment and then in the dark for a further 20 h. Exposure to light for 4 h is needed to achieve the response to DEX treatment, possibly because the induction of several biological processes required for the expression of the target genes under DEX treatment requires energy. Chlorophyll was not significantly degraded during the first 4 h (Fig. 6A), indicating that the effect of light stress was minimal. After an additional 20 h of incubation in the dark, chlorophyll was degraded in the DEX-treated leaves (Fig. 6A). The mRNA levels of the genes involved in chlorophyll degradation and plant hormone-related factors were examined before and after incubation in the dark. The mRNA levels of PPH did not increase and were not

different between mock- and DEX-treated plants (Fig. 6B). The mRNA level of PAO exhibited a similar profile to that of PPH, although there were some differences between the two transgenic lines (Fig. 6B). In contrast, DEX treatment resulted in increased *JAZ1* and *SAG13* mRNA levels during incubation in the dark, suggesting that chlorophyll degradation by SGR promotes senescence independently of light stress, at least partly.

Discussion

SGR-induced upregulation of chlorophyll catabolic genes through chlorophyll *a* degradation

Tight regulation of chlorophyll biosynthesis and degradation is crucial as the intermediates of these pathways are potential sources of oxidative stress. In Arabidopsis, SGR was shown to have catalyzed most of the chlorophyll *a* when it was overexpressed for 30 h (Shimoda et al., 2016), suggesting that once SGR starts to degrade chlorophyll *a*, all the steps involved in the chlorophyll degradation pathway must be activated. In a previous study, *NYC1* was found to be induced by *SGR* (Sato et al., 2018). In this study, a comprehensive analysis of gene expression profiles was undertaken (Supplementary Table S2). Since chlorophyll *a* degradation was observed after 18 h of DEX treatment (Fig. 2), gene expression was analyzed for up to 18 h of DEX treatment to determine the primary response of plants to chlorophyll *a* degradation. Numerous genes involved in chlorophyll biosynthesis were downregulated, whereas those involved in chlorophyll degradation were upregulated (Supplementary Table S2). Senescence-associated genes were also upregulated (Supplementary Table S2), suggesting that chlorophyll *a* degradation triggered leaf senescence. The gene expression profiles are different between developmental senescence and artificially induced senescence under dark treatment in Arabidopsis (Buchanan-Wollaston et al., 2005). The gene expression profiles after DEX treatment (Supplementary Table S2) could not be clearly categorized as either developmental or dark-induced senescence. For example, during leaf senescence, genes related to nitrogen mobilization are upregulated. Glutamate decarboxylase 1 (AT5G17330) and glutamate dehydrogenase 2 (AT5G07440), both of which are involved in nitrogen mobilization, are upregulated during developmental senescence and dark-induced senescence, respectively (Buchanan-Wollaston et al., 2005). However, these two genes were both upregulated after DEX treatment, suggesting that developmental senescence processes are likely involved in senescence triggered by *SGR1* overexpression. Gene expression was also examined by quantitative RT-PCR. The results showed increased expression of *NYC1*, *PPH*, and *PAO*, genes that are involved in chlorophyll degradation (Fig. 3), indicating that DEX-treated plants regulate the chlorophyll metabolic pathway to reduce chlorophyll levels. In a previous study, NYC1 mRNA and protein levels reportedly increased when *SGR* was overexpressed in Arabidopsis, resulting in chlorophyll *b* degradation in wild type plants (Sato et al., 2018). This shows that an increase in NYC1 mRNA levels is accompanied by an increase in NYC1 activity. Changes in *PPH* and *PAO* mRNA levels also result in changes in their respective activities. After 10 h of DEX treatment, *PPH* and *PAO* were induced, but *NYC1* expression was not detected. These data suggest that chlorophyll *a* was degraded more rapidly than chlorophyll *b*, consistent with the observation that SGR1 only catalyzes chlorophyll *a*, and that the chlorophyll degradation pathway must be activated to suppress the accumulation of the intermediate molecule of chlorophyll degradation.

Jasmonate production through chlorophyll degradation

Production of the jasmonates JA and JA-Ile was observed following SGR overexpression (Fig. 5). Although chlorophyll catabolite is not a jasmonate precursor, chlorophyll degradation can lead to increased jasmonate levels. When chlorophyll is degraded, so are the chlorophyll-protein complexes. Subsequently, the thylakoid membrane is destabilized and is also degraded (Sato et al., 2018), leading to free fatty acid production. When α-linolenic acid is available, jasmonate is synthesized by preexisting enzymes (Wasternack, 2007), indicating

that jasmonate production occurs rapidly after chlorophyll degradation. In this study, jasmonate was found to accumulate after 10 h of DEX treatment. Although the recorded reduction in chlorophyll levels in line #19 was still not significant after 10 h, a low level of chlorophyll degradation would be sufficient to supply α-linolenic acid for jasmonate synthesis. The thylakoid lipid content in leaf tissue is reported to be 3.79 μmol g^{-1} FW (Yin et al., 2012), and α-linolenic acid is the major fatty acid in thylakoid lipids. After 10 h of DEX treatment, JA and JA-Ile levels increased from 0.33 to 4.80 nmol g^{-1} FW and 0.056 to 0.131 nmol g^{-1} FW in line #3; and from 0.28 to 3.24 nmol g^{-1} FW and 0.015 to 0.073 nmol g^{-1} FW in the line #19, respectively (Fig. 5). These jasmonate levels represent less than 0.2% of the thylakoid lipid content. A very small amount of thylakoid degradation supplies enough α-linolenic acid to serve as substrate for jasmonate synthesis. The undamaged leaves of wounded plants were reported to accumulate 0.1 to 0.2 nmol g^{-1} FW of JA-Ile and express JAZ5 and JAZ7 (Koo et al., 2009). In this study, JA-Ile levels were approximately 0.1 nmol g^{-1} FW after 10 h of DEX treatment. This concentration may be enough to induce jasmonate-related genes.

SGR1 was induced in the *aos* mutant, which has defects in jasmonate biosynthesis (Park et al., 2002), because the jasmonate biosynthesis mutant is a useful tool for investigating whether jasmonate is involved in promoting senescence when *SGR1* is overexpressed. The expression levels of *PPH* and *PAO* were examined (Supplementary Fig. S4). The results showed that neither gene was completely suppressed, suggesting that other signaling molecules, like ethylene, may be involved in regulating the expression of these genes. Extensive studies using plant hormone mutants are required to investigate this possibility.

Numerous proteins present in the chloroplast are encoded in the nuclear genome, and coordination of chloroplast metabolism and nuclear gene expression is essential for plant development (Souza et al., 2017). In this study, the chlorophyll degradation pathway was activated when chlorophyll *a* degradation was induced, indicating signal transfer from the chloroplast to the nucleus. Jasmonate may be the corresponding chloroplast signaling molecule because jasmonate synthesis begins in the chloroplast (Souza et al., 2017). Tetrapyrrole derivatives and ROS are also known to be involved in interorganellar communication (Souza et al., 2017). Other components in addition to jasmonate remain to be identified.

Gene expression under dark conditions

Transcriptome analysis of Arabidopsis constitutively overexpressing turf grass *SGR* showed that *NYC1* and plant hormone-related genes were upregulated (Teng et al., 2016). These observations suggest that *SGR* expression accelerates senescence in Arabidopsis. However, it is not clear whether chlorophyll *a* degradation is the primary factor involved in the gene expression changes because constitutive *SGR* overexpression has been reported to induce stress in Arabidopsis and rice (Mur et al., 2010; Park et al., 2007). In our study, chlorophyll *a* degradation was transiently induced by 18 h of DEX treatment under light conditions. The effect of chlorophyll *a* degradation on gene expression can be examined clearly under these conditions compared to plants that constitutively overexpress *SGR*. Furthermore, incubation in the dark for 20 h after light incubation, which are conditions that minimize light stress, was also examined. Although neither *PPH* nor *PAO* expression was enhanced by DEX treatment under dark conditions, the mRNA levels of both *JAZ1* and *SAG13* were increased. These results suggest that light stress is not the only factor affecting the expression of the genes shown in Figs. 3 and 4.

Potential involvement of ROS and stress-related plant hormones in gene expression under light conditions

As described above, when DEX-treated plants were incubated under dark conditions, the expression levels of the analyzed genes (Fig. 6) were lower than those incubated under light conditions. This suggests that factors other than jasmonate are involved in regulating gene

expression under light conditions in DEX-treated plants. ROS are the candidate factors because chlorophyll catabolites can produce ROS under light conditions. The observed upregulation of *APX1*, which is associated with the ROS scavenging system (Fig. 4), also suggests that ROS are produced and affect the gene expression profiles. In addition to jasmonate, other plant hormones are also involved in regulating stress- and senescence-associated gene expression in DEX-treated plants. Ethylene is known to be produced under oxidative stress (Chamnongpol et al., 1998; Langebartels et al., 1991) and induces chlorophyll catabolic gene expression (Qiu et al., 2015; Yin et al., 2016), while ABA is a plant hormone involved in stress responses and its precursors are synthesized from carotenoids in chloroplasts (Dejonghe et al., 2018). ABA is reported to affect chlorophyll catabolic gene expression (Delmas et al., 2013; Gao et al., 2016; Liang et al., 2014; Nakajima et al., 2012). The microarray analysis (Supplementary Table S2) showed changes in the expression levels of ethylene- and ABA-responsive genes. Although ROS are not required for SGR-induced jasmonate synthesis, plant hormones like ethylene and ABA may be produced through ROS generation and may also affect the gene expression profiles.

Conclusion

In this study, we propose that jasmonate produced through SGR-mediated chlorophyll degradation affects gene expression associated with senescence. The early phase of the senescence process is reversible, whereas in the late phase, the transition between senescing and dead tissues is rapid and irreversible (Thomas, 2013). Jasmonate, a positive regulator of *SGR*, was produced when *SGR* was overexpressed, and this positive feedback may be involved in the irreversible late phase of senescence. Although only jasmonate was analyzed in this study, other plant hormones like ethylene and ABA that are produced through oxidative stress can also affect this positive feedback. A comprehensive picture of the positive feedback loop of senescence promotion remains to be elucidated.

Figure legends

Fig. 1 Chlorophyll degradation pathway in the chloroplast.
Chlorophyll *b* must be converted to chlorophyll *a* for degradation. Green tissue may have an additional dephytylase- and SGRL-catalyzed pathway from chlorophyll *a* to pheophorbide *a* *via* chlorophyllide *a*. HCAR, 7-hydroxymethyl chlorophyll *a* reductase; NOL, NYC1-like; RCCR, red chlorophyll catabolite reductase.

Fig. 2 Chlorophyll degradation by DEX treatment.
(A) Plant phenotypes. Two genetically independent transformants (line #3 and line #19) were grown for 3–4 weeks. Plants (line #19) were treated with mock or DEX for 18 h. Chlorophyll contents (B) and the chlorophyll *a*/*b* ratio (C) after mock or DEX treatment. Chlorophyll was extracted from leaves at 0, 10, and 18 h after mock or DEX treatment. Chlorophyll contents were normalized by leaf area. The error bars represent the S.D. using three biological replicates. * $p < 0.05$, ** $p < 0.01$ (unpaired *t*-test).

Fig. 3 Chlorophyll degradation- and senescence-associated gene expression after DEX treatment.
Two genetically independent transformants (line #3 and line #19) were grown for 3–4 weeks. Total RNA was extracted from mock- and DEX-treated leaves at 0, 10, and 18 h. The mRNA levels were examined by quantitative RT-PCR using specific primer sets. The mRNA level in each sample was normalized to *ACT2*. The error bars represent the S.D. using three biological replicates. * $p < 0.05$, ** $p < 0.01$ (unpaired *t*-test).

Fig. 4 Jasmonate-related gene expression after DEX treatment.
Two genetically independent transformants (line #3 and line #19) were grown for 3–4 weeks. Total RNA was extracted from mock- and DEX-treated leaves at 0, 10, and 18 h. The mRNA levels were examined by quantitative RT-PCR using specific primer sets. The mRNA level in each sample was normalized to *ACT2*. The error bars represent the S.D. using three biological replicates. * $p < 0.05$, ** $p < 0.01$ (unpaired *t*-test).

Fig. 5 Jasmonate production after DEX treatment.
JA and JA-Ile production after DEX treatment. Two genetically independent transformants (line #3 and line #19) were grown for 3–4 weeks. JA and JA-Ile were extracted with ethanol from mock- and DEX-treated leaves after 0, 10, and 18 h. After filtration, the extracts were concentrated and analyzed by UPLC MS/MS. The error bars represent the S.D. using three biological replicates. * $p < 0.05$, ** $p < 0.01$ (unpaired *t*-test).

Fig. 6 DEX treatment under dark conditions.
(A) Chlorophyll contents after mock or DEX treatment. Two genetically independent transformants (line #3 and line #19) were grown for 3–4 weeks. Mock- and DEX-treated plants were incubated under light conditions for 4 h, and then for 20 h under dark conditions. Chlorophyll contents were normalized by leaf area. The error bars represent the S.D. using three biological replicates. (B) Gene expression after mock or DEX treatment. Mock- and DEX-treated plants were incubated under light conditions for 4 h and for 20 h under dark conditions. The mRNA levels were examined by quantitative RT-PCR using specific primer sets. The mRNA level in each sample was normalized to *ACT2*. The error bars represent the S. D. using three biological replicates. * $p < 0.05$ (unpaired *t*-test).

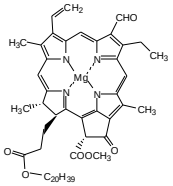
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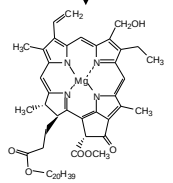
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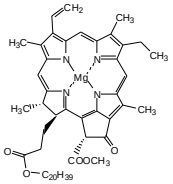
Chlorophyll *b*

↓ NYC1, NOL



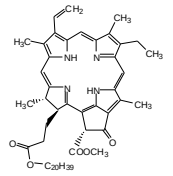
7-Hydroxymethyl chlorophyll *a*

↓ HCAR



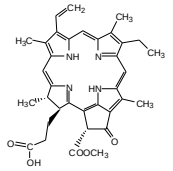
Chlorophyll *a*

↓ SGR



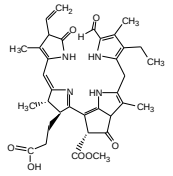
Pheophytin *a*

↓ PPH



Pheophorbide *a*

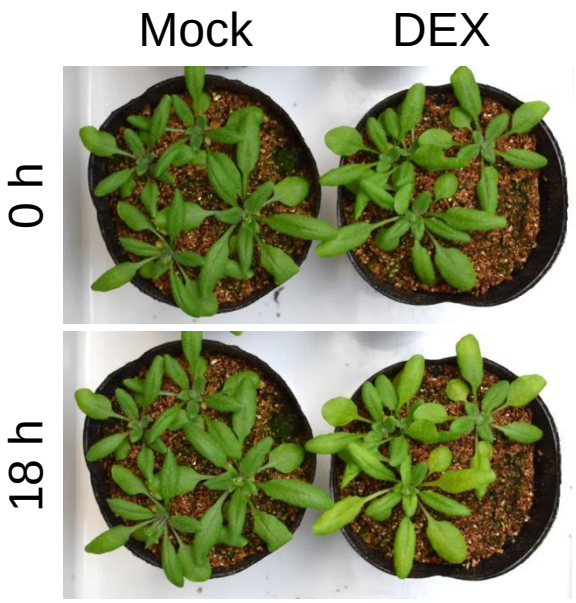
↓ PAO



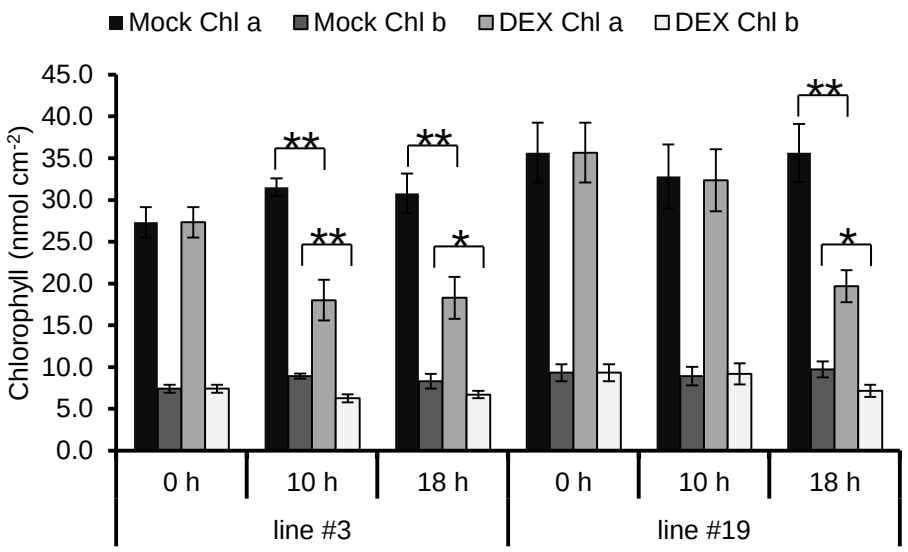
Red chlorophyll catabolite

↓ RCCR

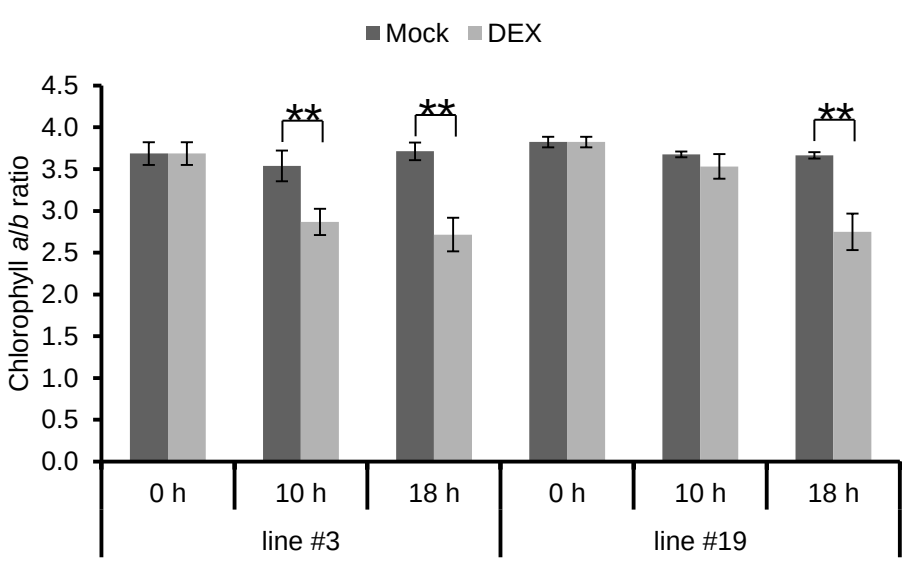
A

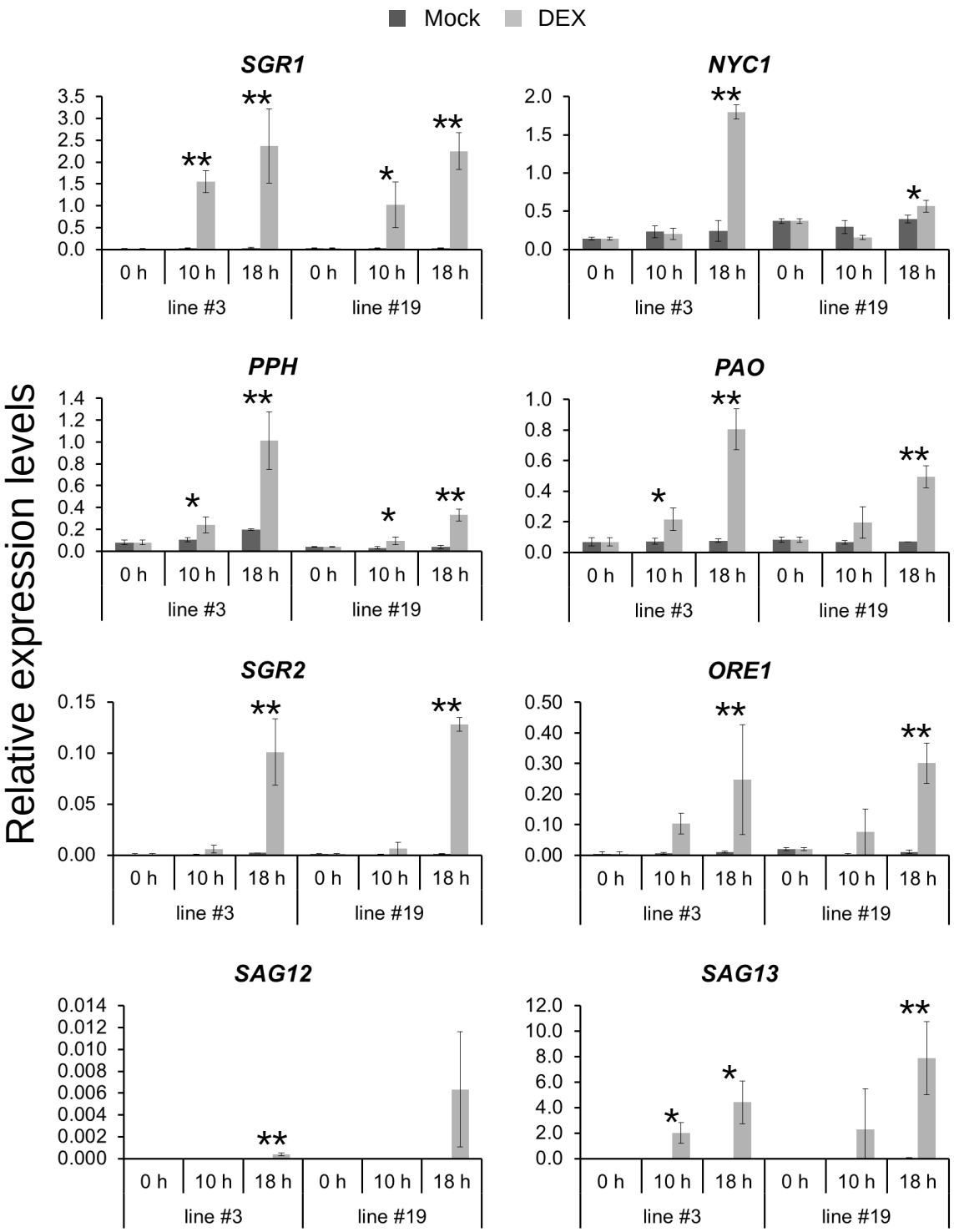

Mock
DEX
0 h
18 h

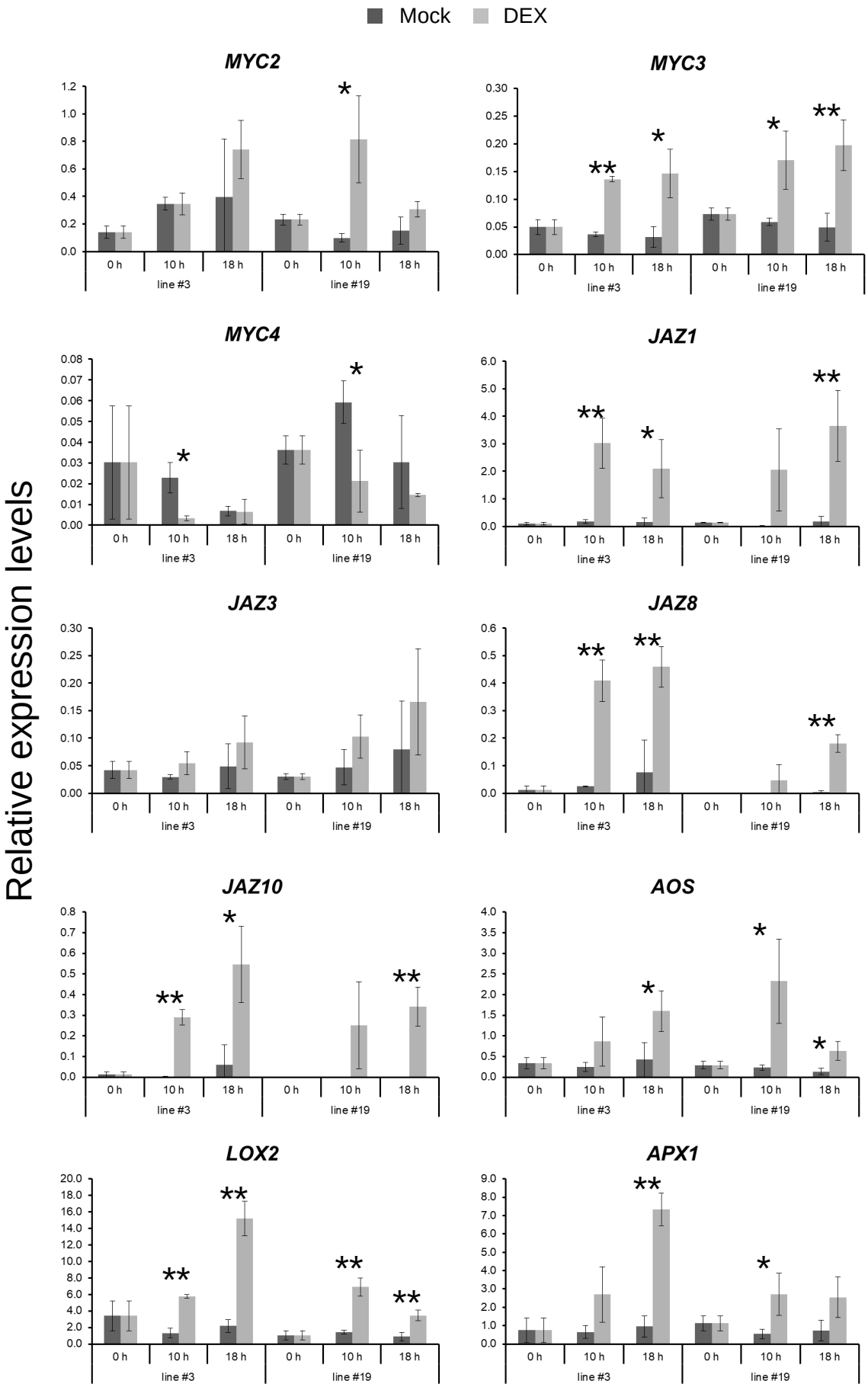
B

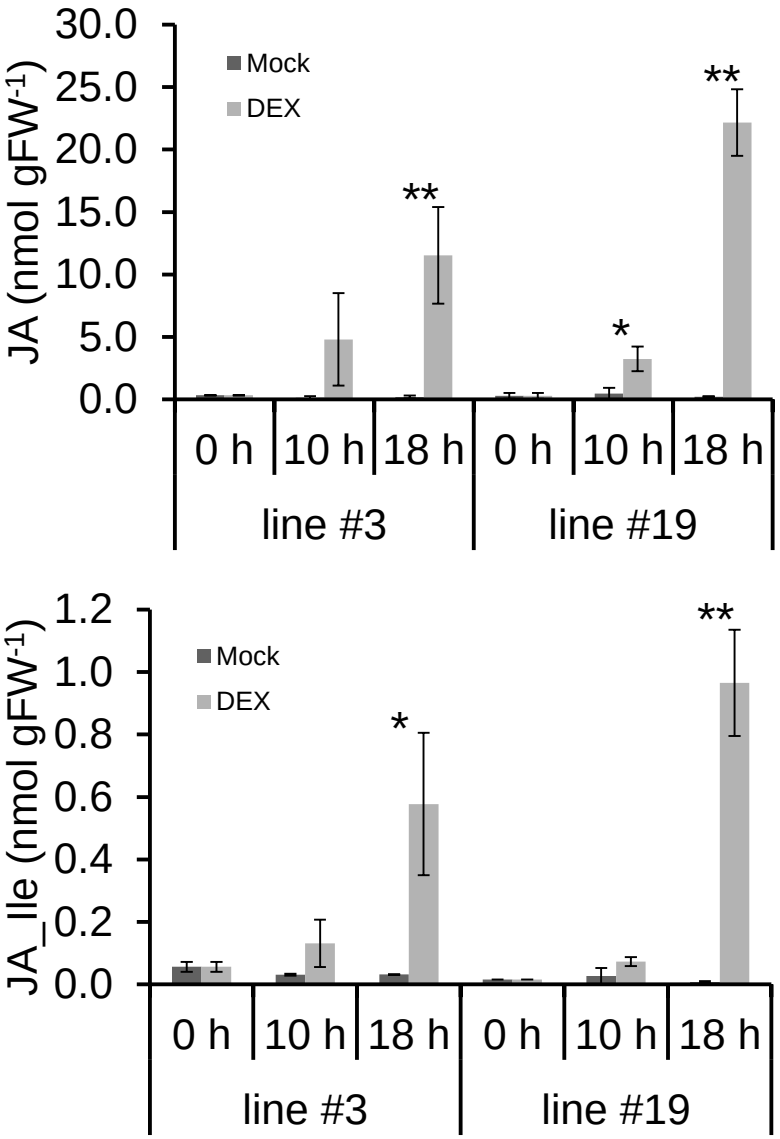

Mock Chl a
Mock Chl b
DEX Chl a
DEX Chl b
Chlorophyll (nmol cm-2)
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
**
**
**
*
**
*
0 h
10 h
18 h
0 h
10 h
18 h
line #3
line #19

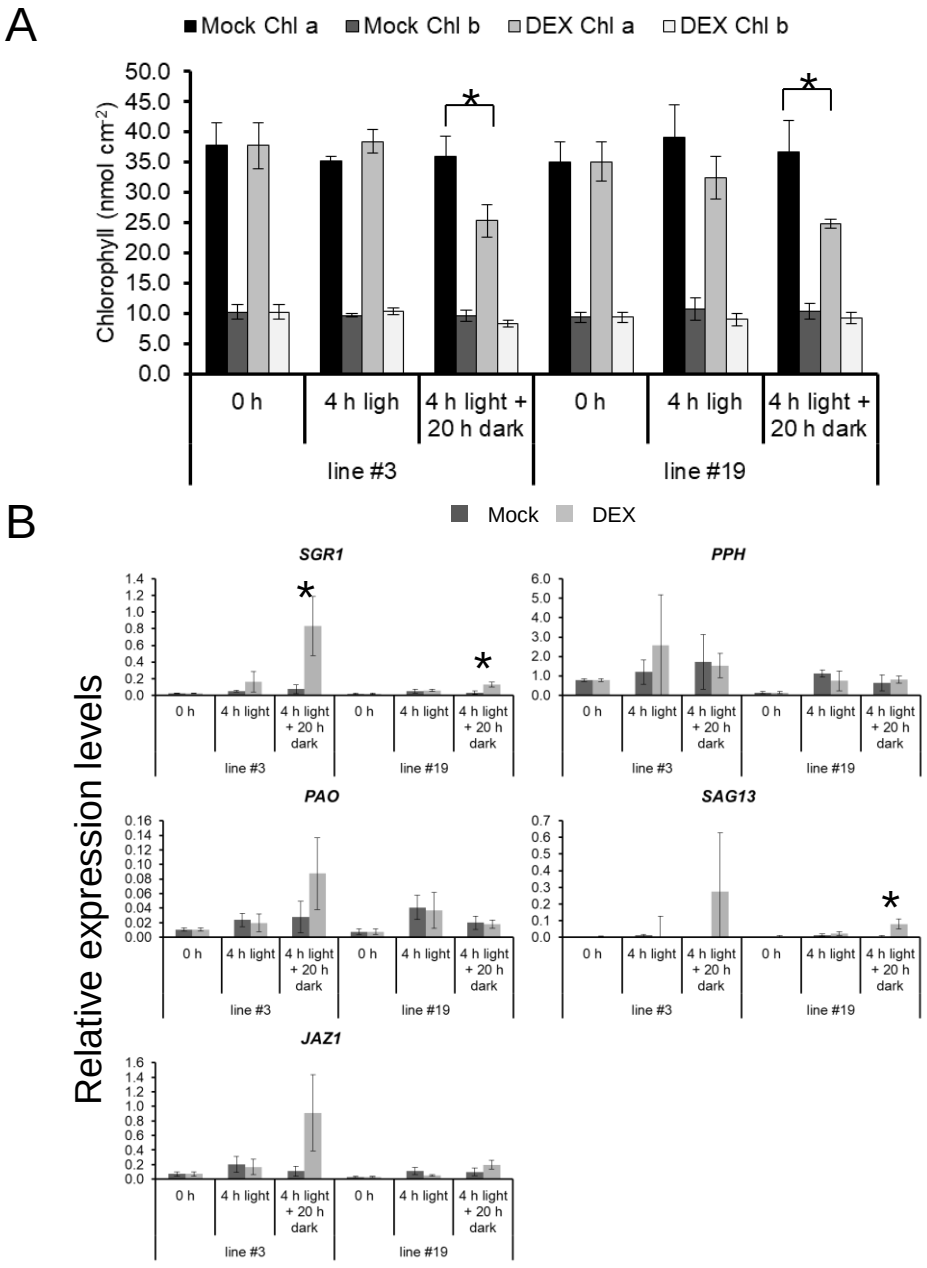
C


Mock
DEX
Chlorophyll a/b ratio
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
**
**
0 h
10 h
18 h
0 h
10 h
18 h
line #3
line #19


Mock
DEX
Relative expression levels
SGR1
NYC1
PPH
PAO
SGR2
ORE1
SAG12
SAG13
0 h
10 h
18 h
line #3
line #19


Mock
DEX
Relative expression levels
MYC2
MYC3
MYC4
JAZ1
JAZ3
JAZ8
JAZ10
AOS
LOX2
APX1
0 h
10 h
18 h
line #3
line #19


30.0
25.0
20.0
15.0
10.0
5.0
0.0
JA (nmol gFW-1)
Mock
DEX
**
*
**
0 h
10 h
18 h
0 h
10 h
18 h
line #3
line #19
1.2
1.0
0.8
0.6
0.4
0.2
0.0
JA_Ile (nmol gFW-1)
Mock
DEX
*
**
0 h
10 h
18 h
0 h
10 h
18 h
line #3
line #19


A
Mock Chl a
Mock Chl b
DEX Chl a
DEX Chl b
Chlorophyll (nmol cm-2)
50.0
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
*
*
0 h
4 h ligh
4 h light + 20 h dark
0 h
4 h ligh
4 h light + 20 h dark
line #3
line #19
B
Mock
DEX
Relative expression levels
SGR1
PPH
PAO
SAG13
JAZ1
0 h
4 h light
4 h light + 20 h dark
line #3
line #19