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Abscisic acid receptor PYRABACTIN RESISTANCE-LIKE 8, *PYL8*, is involved in glucose response and dark-induced leaf senescence in *Arabidopsis*

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ABSTRACT

Abscisic acid (ABA) receptors in plants are thought to be involved in various cellular processes mediated by signal transduction pathways. There are about 14 ABA receptors in *Arabidopsis*, but only a few have been studied. In this study, we investigated the effect of the disruption and overexpression of an ABA receptor gene, *PYL8* (At5g53160) on plant responses to glucose (Glc) and dark-induced leaf senescence. Expression of *PYL8* was strongly reduced by Glc treatment. Overexpression of *PYL8* in *Arabidopsis* resulted in significantly reduced seed germination and cotyledon greening under high Glc conditions, while RNAi transgenic lines were more insensitive to Glc stress. Activities of two Glc-responsive genes, *Arabidopsis thaliana Hexokinase 1* (*AtHXK1*) and *ABA insensitive 5* (*ABI5*) were higher in *PYL8*-overexpressing plants than in the wild-type (WT) plants after Glc treatment, whereas the transcript levels of these genes in RNAi plants decreased. Furthermore, *PYL8*-overexpressing plants displayed increased yellowing, membrane ion leakage, and reduced chlorophyll content due to dark-induced senescence, and exhibited stronger expression of a group of senescence-inducible genes than did WT. The data show that *PYL8* plays essential roles in responses to both Glc and dark-induced senescence in *A. thaliana*.

1. Introduction

The plant hormone abscisic acid (ABA) is a crucial regulator of abiotic stress tolerance in plants, and it coordinates a complex regulatory network that enables plants to cope with abiotic stress [1]. A recent study on ABA signal transduction showed that the earliest events of the signaling pathway occur through a central signaling module composed of three protein classes: family of PYRABACTIN RESISTANCE 1 (PYR1)/PYR1-LIKE (PYL)/REGULATORY COMPONENTS OF ABA RECEPTORS (RCAR) ABA receptors, clade A of protein phosphatases type 2Cs (PP2Cs), and 3 ABA-activated protein kinases from the sucrose non-fermenting 1-related subfamily 2 (SnRK2) [1]. In this model, PYR/RCARs act as ABA receptors, PP2Cs act as negative regulators of the pathway, and SnRK2s act as positive regulators of downstream signaling [1,2]. Under normal conditions, clade A PP2Cs can interact with and dephosphorylate three SnRK2s/Open Stomata 1 (OST1), reducing their catalytic activities [3–5]. An increase in ABA levels in the plant cell leads to PYR/PYL/RCAR receptor-mediated inhibition of PP2C activity, which results in the activation of the three SnRK2s, and ultimately, of the ABA signaling pathway [3–5]. However, many of the cellular components and genes involved in ABA reception and downstream transduction have not yet been well characterized.

In plants, Glc has been shown to affect many processes, including germination, early seedling growth, flowering, and senescence [6]. Moreover, micro-array studies have shown that Glc treatment of *Arabidopsis* seedlings affected the expression of many genes [7]. Genetic analysis has shown that Glc signaling in plants is closely associated with plant hormone biosynthesis and signaling, in particular with that of ABA [8]. Four screenings for sugar response mutants, i.e., *sucrose uncoupled* (*sun*), *impaired sucrose induction* (*isi*), *glucose insensitive* (*gin*), and *sugar insensitive* (*sis*), identified ABA-deficient mutants and *ABA insensitive 4* (*abi4*) as sugar insensitive [9–12]. ABI4 encodes an Apetala 2 domain containing a transcription factor that binds a coupling element 1-like element present in many ABA- and sugar-regulated promoters [13]. These observations link sugar regulation to ABA signaling.

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Recently, the function of *PYL8* for the regulation of ABA has been elucidated; *PYL8* positively regulates ABA signaling during germination and abiotic stress responses [14]. To date, there is little data on the significance of the ABA receptor *PYL8* in Glc and senescence responses.

In this study, *PYL8* was shown to act as a signaling protein in Glc and leaf senescence responses. Overexpression of *PYL8* in transgenic *Arabidopsis* plants resulted in increased sensitivity to Glc during germination and seedling growth, whereas reduction in the expression of *PYL8* induced hyposensitivity to Glc. In addition, *PYL8* RNA interference (RNAi), *pyl8*, plants showed reduced chloritic leaf senescence. These results indicate that PYL8 potentially functions as a signaling component between Glc signaling and senescence response.

2. Materials and methods

2.1. Plant materials, growth conditions, and Glc induction

Arabidopsis (Col-0) plants were grown in growth chambers under intense light (120 μmol m^{-2} s^{-1}) at 22 °C, 60% relative humidity, and a 16-h day length. The plants were challenged with Glc by submersion of 10-day-old *Arabidopsis* seedlings in a solution containing 6% Glc. Samples were obtained at 0, 6, and 12 h of Glc treatment. In each case, the retrieved seedlings were promptly frozen in liquid nitrogen and stored at −80 °C.

2.2. Extraction of RNA, quantitative real-time PCR, and reverse transcription PCR

Total RNA was extracted from the frozen samples by using the Plant RNeasy Extraction Kit (Qiagen, Valencia, CA). To remove any residual genomic DNA, the RNA was treated with RNase-free DNase I, according to the manufacturer's instructions (Qiagen). The RNA was quantified accurately by spectrophotometric measurements, and 5 μg of total RNA was separated on 1.2% formaldehyde agarose gels to check the concentration and monitor the integrity. Quantitative real-time PCR (qPCR) was performed using a Rotor-Gene 6000 quantitative PCR apparatus (Corbett Research, Mortlake, NSW, Australia), and the results were analyzed using RG6000 1.7 software (Corbett Research). qPCR was performed using the SensiMix One-Step Kit (Quantance, London, UK). *ARABIDOPSIS ACTIN 1* (*ACT1*) (At2g37620) was used as the internal control. Quantitative analysis was performed using the Delta Delta C_T method [15]. Each sample was run in three independent experiments. The reaction primers are listed in Table S1.

Reverse transcription (RT)-PCR was used to measure *PYL8* expression levels in the transgenic plants. Five hundred nanograms of total RNA was used for RT-PCR, together with the gene-specific primers listed in Table S1. After 27 amplification cycles, 20 μL of each RT-PCR product was loaded onto a 1.2% (w/v) agarose gel in order to visualize the amplified DNAs.

2.3. Phenotype analysis and stress tests

For ABA cotyledon greening and seed germination tests, seeds were sown on Murashige and Skoog (MS) medium [16] supplemented with 0.5 μM ABA and maintained in a growth chamber. Cotyledon greening of each seedling was measured at 7 days. Experiments were conducted in triplicate for each line (50 seeds each). For the Glc stress tests, seeds were sown in MS medium supplemented with 6% Glc, maintained in a growth chamber, and assessed for percentage of seed germination or green cotyledon seedlings during 1–9 and 1–10 days, respectively. Experiments were conducted in triplicate for each line (50 seeds each). For the dark-induced senescence tests, plants were grown in the soil for 3 weeks. The fifth and sixth leaves were excised from the plants. To induce senescence, leaves were maintained on a sterile MS medium at 22 °C in the dark for 2–6 days.

2.4. Assay for leaf senescence

Chlorophyll a and b contents of leaves were determined by measuring the absorbance of extracts prepared using ethanol, according to the method described by Hendry and Grime [17]. Membrane ion leakage was determined by measuring electrolytes that leaked from the leaves [18]. Three leaves were immersed overnight in 25 ml of deionized water at 23 °C with gentle shaking, after which initial conductivity was recorded. Total conductivity was determined after boiling the leaves for 15 min. Conductivity was expressed as the percentage of initial conductivity versus total conductivity.

2.5. Statistical analysis

Statistical analyses were performed using Excel and SPSS. Analysis of variance was used to compare the statistical differences on the basis of Student's *t*-test, at a significant level of $0.01 < P < 0.05$ or $P < 0.01$.

3. Results and discussion

3.1. PYL8 is downregulated by Glc treatment

Previous studies have reported many examples of co-regulation of sugar and ABA in plants [8]. To determine whether *PYL8* could be associated with Glc response, the mRNA expression pattern of *PYL8* in 10-day-old *Arabidopsis* seedlings was initially assessed during Glc treatment by using qPCR. As shown in Fig. 1, *PYL8* transcripts were significantly reduced in response to Glc (6–12 h). The qPCR results revealed that *PYL8* mRNA levels were reduced 2.9- to 5.6-fold by Glc treatment (Fig. 1). The Glc-inducible gene, *AtHXK1* [7], was used as the control for the Glc treatment (Fig. 1). These results strongly suggest that *PYL8* is controlled by Glc.

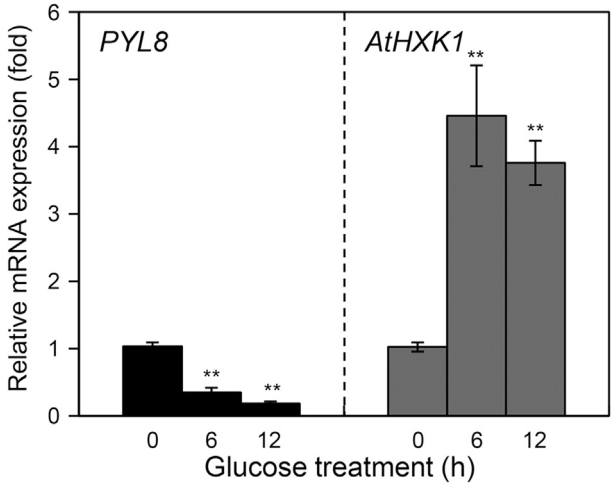

Fig. 1. Expression of the *PYL8* gene in *Arabidopsis* after Glc treatment. qPCR analysis showed that the expression of *PYL8* was reduced in response to Glc. Total RNA samples were obtained from 10-day-old plants treated with 6% Glc at the indicated times. Error bars indicate standard deviations of three independent biological samples. *Arabidopsis ACT1* was used as the internal control. Significant differences between the expression of *PYL8* in untreated 10-day-old *Arabidopsis* seedlings and those treated with Glc were indicated at $P < 0.01$ (**). *AtHXK1* was used as the control for the Glc treatment.

3.2. ABA response of the PYL8 transgenic plants

To investigate the *in vivo* function of *PYL8*, *PYL8* overexpression under the control of the 35S promoter was induced in *Arabidopsis*. Ten homozygous lines (T_3 generation) were obtained, and 2 lines (OX1-2 and OX3-6) that exhibited high levels of transgene expression (Fig. 2A) were selected for phenotypic characterization. To further evaluate the functional consequence of *PYL8* loss, *pyl8* RNAi lines were generated using the third exon cDNA sequence. *PYL8* expression was assessed using RT-PCR in two randomly selected *pyl8* RNAi lines (*ri1-4* and *ri2-3*). *PYL8* expression was knocked down in the RNAi lines (Fig. 2A).

Although, PYL8 has previously been elucidated for ABA response [1,14], it was of interest to test the ability of *PYL8* to function in our transgenic plants as a positive regulator in ABA response. To this end, we examined cotyledon greening in wild-type (WT), two *pyl8* RNAi (*ri1-4* and *ri2-3*), and two overexpressing lines (OX1-2 and 3–6) in the presence of either 0 μM ABA or 0.5 μM ABA. The germination rate among WT, *pyl8* RNAi, and *PYL8*-overexpressing plants was similar and not poor in MS medium (Figs. S1A and S1B). In addition, developmental processes were not affected in the transgenic plants (data not shown). To characterize the sensitivity of the *PYL8* transgenic lines to ABA stress, we evaluated the response to treatment with 0.5 μM ABA. As shown in Fig. S1, the relative reduction in cotyledon greening in the *PYL8*-overexpressing lines because of ABA treatment was more profound than that observed in the WT and *pyl8* RNAi plants at 7 days after germination. In the WT plants, cotyledon greening efficiency dropped to 26% relative to the untreated plants (100%), whereas cotyledon greening efficiency of *pyl8* RNAi (*ri1-4* and *ri2-3*) lines was 58.4% and 69.3%, respectively, of control levels with the experimental concentration of ABA (Fig. S1). These observations support the notion that PYL8 positively regulates ABA signaling during cotyledon greening.

3.3. Glc response of the PYL8 transgenic plants

There is strong evidence for cross-talk between Glc and ABA signaling pathways [8–13]. As a signaling molecule, ABA is an important component of Glc response pathways. Therefore, we decided to investigate the association between *PYL8* and Glc response. At 6% Glc, the germination rate of OX1-2 and OX3-6 seeds was significantly lower than that of WT seeds on day 4 after being sown in plates when exogenous Glc was applied, while the *pyl8* RNAi lines exhibited significantly higher germination rates than WT (Fig. 2B). To further correlate *PYL8* function with plant sensitivity to Glc during the post-germination stage, seeds of WT, *pyl8* RNAi, and *PYL8*-overexpressing plants were germinated on MS medium containing 6% Glc. Cotyledon greening efficiency of WT was only slightly below 53% at 7 days after germination. Only 36.5–38.7% of the OX1-2 and OX3-6 cotyledons expanded and turned green, in comparison with the 80.2–80.6% observed in *ri1-4* and *ri2-3* (Fig. 2C and D). These results indicated that *pyl8* RNAi was more likely to be insensitive to exogenous Glc than WT, while the *PYL8*-overexpressing plants were more sensitive. This indicated that PYL8 probably controls seed germination and cotyledon greening phenotypes through Glc signaling.

3.4. Reduced expression of AtHXK1 and ABI5 mRNA in the pyl8 RNAi lines

Proteins AtHXK1 and ABI5 act as key regulators in the response of *Arabidopsis thaliana* to Glc [19,20]. To determine whether Glc signaling is responsible for the induction of stress sensitivity in

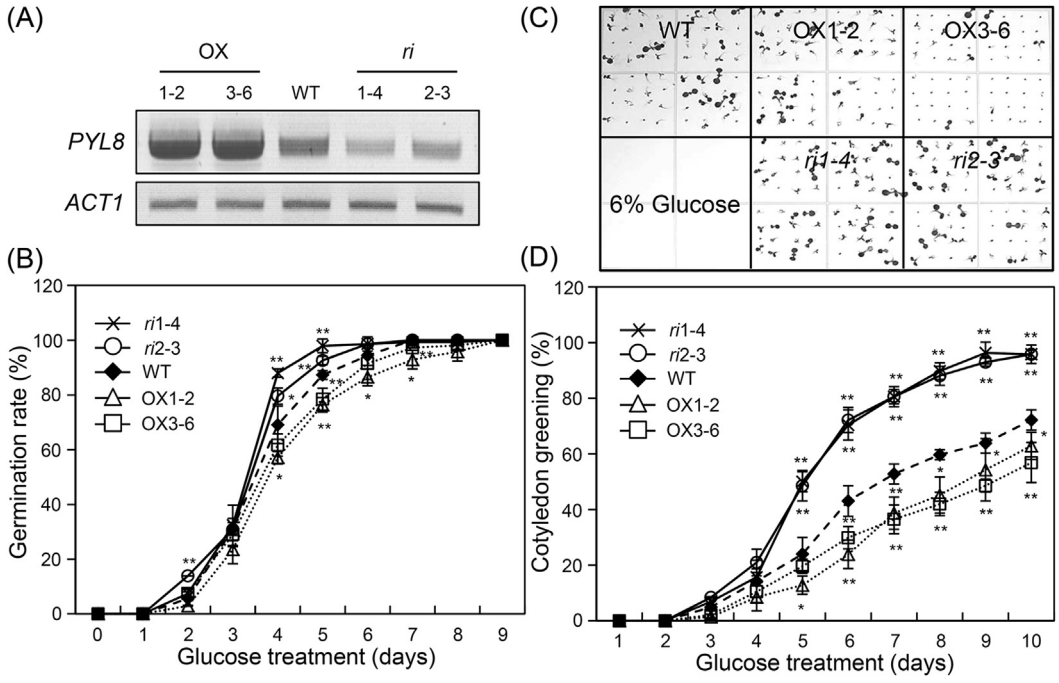

Fig. 2. Influence of *PYL8* transgenic lines on Glc sensitivity. (A) Expression levels of *PYL8* in WT, two independent *PYL8*-overexpressing (OX1-2 and OX3-6), and *pyl8* RNAi (*ri1-4* and *ri2-3*) transgenic lines determined using RT-PCR with the total RNA isolated from 10-day-old seedlings. *ACT1* was used as the internal control. (B) Germination sensitivity to Glc. Comparison of germination rates of WT, two *pyl8* RNAi, and two *PYL8*-overexpressing seeds after exposure to 6% Glc. Germination was defined as complete protrusion of the radicle, and germination was scored on the indicated days. Data represent mean ± standard deviation values of three independent experiments (50 seeds per point). Differences between WT and transgenic plants grown under the same conditions are significant at $0.05 > P > 0.01$ (*) or $P < 0.01$ (**). (C, D) Effect of Glc treatment on cotyledon greening. The photograph shows that *pyl8* RNAi plants exhibit better development and are greener than the WT and *PYL8*-overexpressing plants under Glc stress conditions (C). Seeds were sown in MS agar plates supplemented with 6% Glc and permitted to grow for 10 days, and seedlings with green cotyledons were counted (triplicates; n = 50 each) (D). Error bars represent standard deviations. Differences between WT and transgenic plants grown under the same conditions are significant at $0.05 > P > 0.01$ (*) or $P < 0.01$ (**).

PYL8-overexpressing plants, we assayed the expression of these genes by using qPCR to quantify the relative levels of *AtHXK1* or *ABI5* mRNA. As shown in Fig. 3, Glc-induced expressions of *AtHXK1* and *ABI5* were reduced in *pyl8* RNAi lines, in comparison with those in WT and *PYL8*-overexpressing plants. These observations suggest that *PYL8* enhances Glc-induced *AtHXK1* and *ABI5* expressions. Therefore, it is likely that *PYL8* participates in Glc stress responses through AtHXK1- or/and ABI5-mediated signaling pathway.

3.5. Overexpression of PYL8 leads to altered leaf greening

Pourtau et al. [21] demonstrated that ABI5, a basic leucine-zipper transcription factor, is involved in sugar responses during leaf senescence. Glc-inducible *AtHXK1* also participates in the regulation of leaf senescence [22]. Because *PYL8* enhances Glc-induced *AtHXK1* and *ABI5* expressions, it would be interesting to evaluate whether PYL8 is also associated with leaf senescence. To determine whether *PYL8* could be associated with senescence response, mRNA expression pattern of *PYL8* in excised leaves from 3-week-old *Arabidopsis* that have undergone dark-induced senescence was initially assessed during dark treatment by using RT-PCR. As shown in Fig. 4A, the *PYL8* transcript level was maximum at 2 days, but it rapidly declined thereafter and became virtually absent 6 days after dark treatment. The senescence-inducible gene,

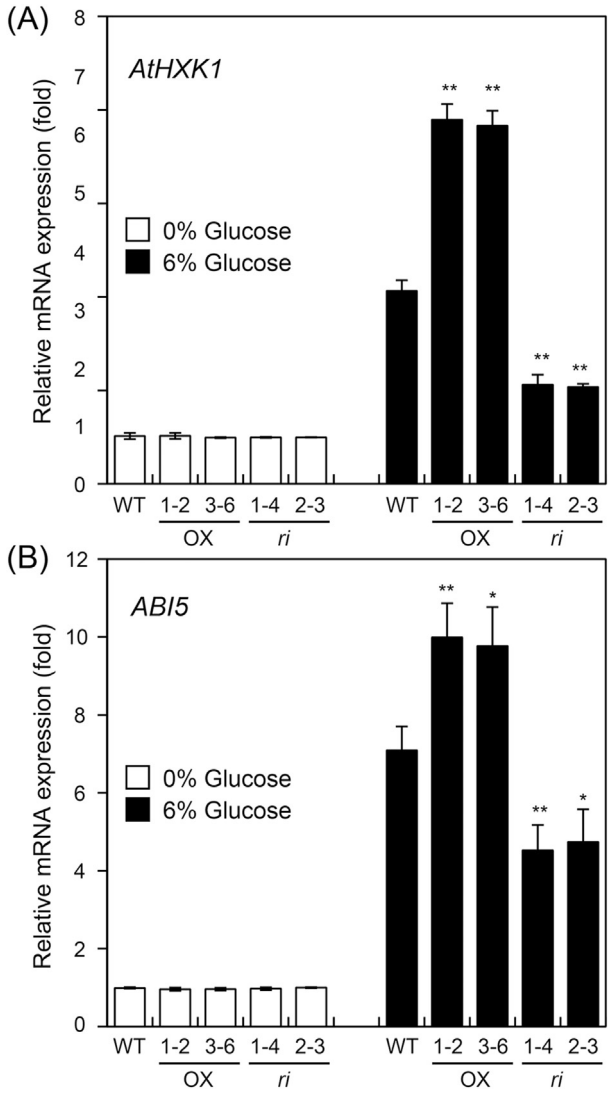

Fig. 3. Expression of Glc-inducible genes in *pyl8* RNAi and *PYL8*-overexpressing transgenic plants. mRNA levels were determined using qPCR with the total RNA from 10-day-old seedlings, which were exposed to 6% Glc with gentle shaking for 6 h. *ACT1* was used as the internal control. Differences between the expression of *AtHXK1* (A) or *ABI5* (B) in *Arabidopsis* seedlings not treated and treated with glucose stress are significant at $0.05 > P > 0.01$ (*) or $P < 0.01$ (**).

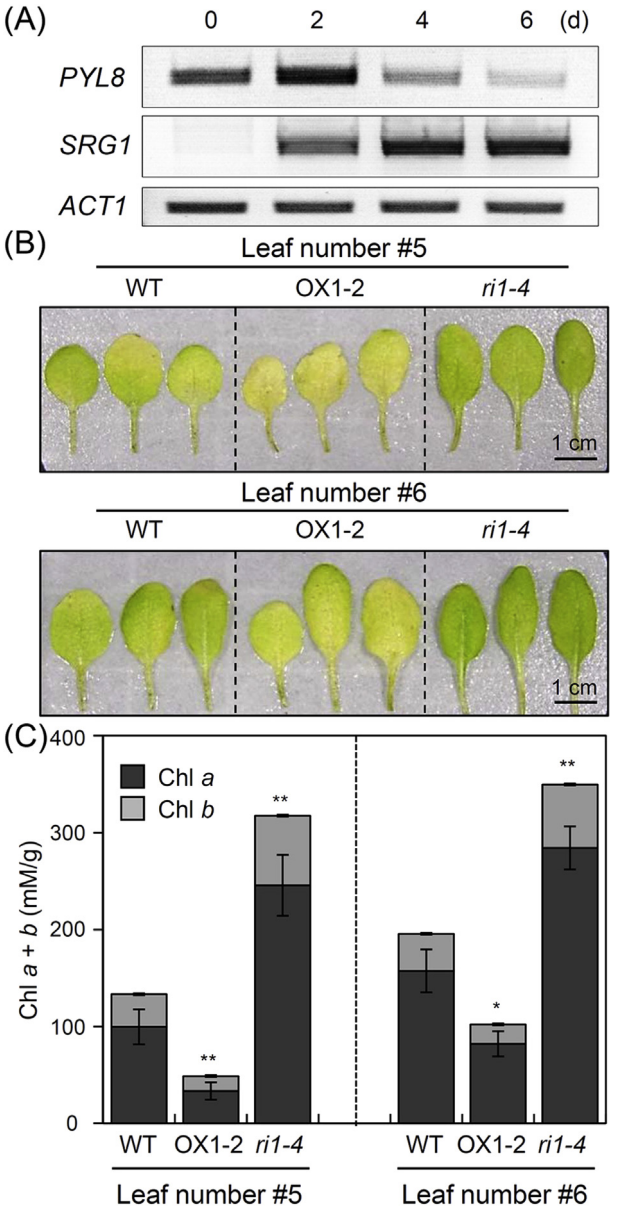

Fig. 4. Phenotypes of *PYL8* transgenic plants due to dark-induced senescence. (A) RT-PCR analysis of the expression changes of *PYL8* in response to dark-induced senescence. Total RNA samples were obtained from detached leaves of 3-week-old WT plants treated with darkness on the indicated days. *Arabidopsis ACT1* was used as the internal control. The *SRG1* gene was used as the control for the senescence treatment. (B) Rosette leaf numbers 5 and 6 were harvested from 3-week-old WT, *PYL8*-overexpressing (OX1-2), and *pyl8* RNAi (*ri1-4*) transgenic lines grown in the soil. The fifth and sixth leaves were excised from the plants, incubated on filter paper wetted with sterile MS medium in the dark for 6 days, and photographed. Yellowing of the rosette leaves was detected to a greater extent in *PYL8*-overexpressing lines than in WT and *pyl8* RNAi plants. (C) Quantification of chlorophyll $a + b$ content in leaves was performed 6 days after the plants were maintained in the dark. Values are the mean of three independent replicates of leaves of 100 mg fresh weight. Error bars represent standard deviation. The asterisk denotes a statistically significant difference when compared with WT [$0.05 > P > 0.01$ (*) or $P < 0.01$ (**)]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

senescence-related gene 1 (*SRG1*) [23] was used as a reference for the dark treatment (Fig. 4A). These results strongly suggest that *PYL8* is controlled by senescence.

Leaf yellowing is a convenient visible indicator, mainly reflecting chloroplast senescence in mesophyll cells. Experiments on dark-induced senescence in detached rosette leaf numbers 5 and 6 from 3-week-old plants grown in the soil for each construct were conducted. After 6 days in the dark, yellowing was detected in WT leaves, and it was slightly attenuated in *pyl8* RNAi leaves (Fig. 4B and C). In contrast, *PYL8*-overexpressing leaves showed accelerated yellowing, compared to WT and RNAi leaves (Fig. 4B and C). Therefore, these observations suggest that *PYL8* expression is associated with leaf senescence. To further examine the senescence-induced phenotype of the *PYL8*-overexpressing lines, chlorophyll content, which decreases with leaf senescence, was measured in the excised rosette leaf numbers 5 and 6 of 3-week-old transgenic plants that were maintained in the dark. The *PYL8*-overexpressing lines exhibited the lowest chlorophyll content during dark-induced senescence (Fig. 4C). The *pyl8* RNAi leaves showed significantly higher reservation ability with respect to this parameter than the WT leaves (Fig. 4C). Senescence also results in the disruption of plasma membrane integrity, which is the final step in cell death. This can be conveniently quantified by ion leakage. It was found that leaf senescence was delayed in the *pyl8* RNAi (*ri1-4*) line, as shown by lower membrane ion leakage than that observed in WT and *PYL8*-overexpressing plants (Fig. S2). These results indicate that the physiological processes of leaf senescence began earlier in the *PYL8*-overexpressing line than in the WT and *pyl8* RNAi plants during dark-induced senescence.

3.6. Transcript accumulation of senescence-related genes in PYL8-overexpressing leaves

To evaluate leaf senescence in the *PYL8*-overexpressing line at the molecular level, *SRG1* and *ABI5* transcript levels, which are reported to increase in senescent leaves [21,23], were examined using qPCR (Fig. S3). RNA was extracted from detached rosette leaf numbers 5 and 6 from 3-week-old soil-grown plants that underwent dark-induced senescence. The transcripts of both the senescence-upregulated genes accumulated to a greater extent in the *PYL8*-overexpressing OX1-2 line than in WT and *pyl8* RNAi *ri1-4* plants. Similarly, the transcript levels of *ribulose-1,5-bisphosphate carboxylase small chain 1* (*RBCS1*), which is downregulated during leaf senescence [24], decreased to a greater extent in the *PYL8*-overexpressing line than in WT and *pyl8* RNAi plants (Fig. S3). These results indicate that molecular events associated with leaf senescence begin earlier in *PYL8*-overexpressing lines than in WT and *pyl8* RNAi plants.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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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.bbrc.2015.05.010.

Transparency document

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