A role for GLABRA1 in dark-induced senescence*

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The GLABRA (GL1) gene, belonging to the transcription factor-encoding myb gene family, is responsible for trichome formation in Arabidopsis thaliana (L.) Heynh. The leaves and stems of glabra1 mutant plants are devoid of trichomes. Having an easily observable phenotype, the gl1 mutation was one of the first markers established for genetic mapping of Arabidopsis thaliana. Since then, the GL1 gene has been assigned roles in other processes, also related to leaf structure. In this study we present some previously undescribed effects of the gl1 mutation on dark-induced senescence. This process was induced by covering selected mature leaves of Columbia wild-type and gl1 Arabidopsis with black paper for 4 days, while the plants remained growing in a normal photoperiod. While no visible differences in the external symptoms of senescence could be observed in the darkened leaves, the expression of senescence-associated genes was significantly lower in gl1 plants as compared to the wild type. The darkening of leaves led to a decrease in photosynthetic activity and the expression of photosynthesis-associated genes, in comparison to the control leaves. This effect was much less pronounced in gl1 than in the wild type plants. Therefore, gl1 plants seem to be less susceptible to dark-induced aging, suggesting a possible role for the GL1 gene in controlling the onset and progress of senescence. This result is also of practical importance, since gl1 is the genetic background of many other mutants. It may therefore be advisable to revise some of the results obtained with such mutants in light of findings presented here.

Key words: GLABRA1, senescence, darkness, photosynthesis, photosynthetic pigments, trichomes

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Abbreviations: CAB, CHLOROPHYLL A/B BINDING PROTEIN; gl1, glabra1; F/Fm, maximum quantum yield of photosystem II; PAL1, PHENYLALANINE AMMONIA-LYASE 1; PSI/PSII, photosystem I/II; RbcS1A, RIBULOSE BISPHOSPHATE CARBOXYLASE SMALL CHAIN 1A; SAG, SENESCENCE ASSOCIATED GENE; SEN1, SENESCENCE1; WT, wild type

INTRODUCTION

Arabidopsis thaliana (L.) Heynh trichomes, the so-called leaf hairs, are branched, non-glandular structures originating from single epidermal cells. Their formation is controlled by a complex interplay of several transcription factors, belonging to different families. GLABRA (GL1) was one of the first genes identified as being responsible for trichome development (Herman & Marks, 1989; Marks & Feldmann, 1989; Oppenheimer et al., 1991). It encodes an R2R3 MYB transcription factor, which along with one of the two redundantly acting basic helix-loop-helix (bHLH) transcription factors GLABRA3 (GL3) or ENHANCER OF GLABRA3 (EGL3), and TRANSPARENT TESTA GLABRA1 (TTG1, WD40 family), forms the trichome activator complex (Hauser, 2014; Dai et al., 2016; Matías-Hernández et al., 2016). Activation of this complex (also called MBW: MYB-bHLH-WD40), triggers the trichome formation pathway in a given epidermal cell, whereas it inhibits this process in surrounding cells. This complex induces transcription of GLABRA2 (GL2) and other downstream genes required for trichome development (Dai et al., 2016). At the same time it also initiates expression of several R3 MYBs acting as trichome repressors: CAPRICE (CPC), ENHANCER OF TRIPTYCHON AND CAPRICE1 (ETC1), ETC3 and TRIPTYCHON (TRY) (Matías-Hernández et al., 2016). These negative regulators move into adjacent cells, where they inhibit the formation of the activator complex by competing with GL1 for GL3 or EGL3 binding. Other R3 MYB trichome repressors are controlled by an independent pathway mediated by microRNA156 and 3QUAMOXA PROMOTER BINDING PROTEIN LIKE (3PL), regulating temporal trichome patterning (Yu et al., 2010). Moreover, the phytohormones: gibberellins and cytokinins, act as positive regulators of trichome formation (Matías-Hernández et al., 2016).

Although they may seem inconspicuous, trichomes have a wide array of important physiological functions. Trichomes increase the light reflectance of leaves, protecting them against excessive heat and photoinhibition, as well as UV-B damage. The presence of trichomes affects such leaf properties as wettability, water repellence and boundary layer thickness, thus regulating transpiration, water uptake and gas exchange (Bickford, 2016). Trichomes also provide protection against biotic stresses, especially those caused by herbivorous insects (Hausser, 2014). The leaves and stems of glabra1 (gl1) mutant
plants are devoid of trichomes, while some remain present on the leaf margins. Having an easily observable phenotype and exhibiting no other apparent abnormalities, the natural gl1 mutation was one of the first markers established for the genetic mapping of Arabidopsis in the 1960’s (Redei & Hirono, 1964; Koornneef et al., 1982). Later, the gl1 mutation was introduced into the widely used Landsberg and Columbia ecotypes. Since then, gl1 has been used as a genetic background for generating other mutants. Recently, some other effects of the gl1 mutation, also related to leaf structure, were reported. gl1 leaves exhibit a lower stomatal density and a less ordered pattern of stomatal distribution than wild type (WT) (Tsujii & Coe, 2013). The gl1 mutation has been also found to affect cuticle formation (Xia et al., 2010). The defective structure of gl1 cuticle was caused by a reduced content of several cuticular wax components. This in turn compromised the systemic acquired resistance (SAR) of gl1 plants, as shown by their enhanced susceptibility to fungal pathogens.

In this study, we found a novel function of the GL1 transcription factor. The motivation came from some ambiguous results obtained previously using Arabidopsis mutants with a gl1 background. We found differences in the dark-induced chlorophyll degradation between WT Columbia and mutants with a gl1 background and gl1 plants (unpublished data). To clarify whether the lack of GLABRA1 is involved in the observed effect, we compared the process of dark-induced senescence in Arabidopsis thaliana WT Columbia and the gl1 mutant. We used an experimental protocol involving the darkening of individual leaves, while the plants remained in a normal photoperiod (Weaver & Amasino, 2001; Keech et al., 2007). This model shares many common features with natural, age-dependent senescence, including leaf yellowing, chlorophyll degradation and induction of senescence associated genes (SAGs) (van der Graaff et al., 2006). To assess the progress of senescence we used several well-established markers, such as photosynthetic efficiency and photosynthetic pigment content in the leaves (Weaver & Amasino, 2001; Sztatelman et al., 2015). We also assessed expression of several genes involved in senescence (SeneCence AsSOCIAted GeNeS: SAG13, SAG12 and SeneCenceF:1 – SENF), photosynthesis (RIBULOSE BI-SPHOSPHATE CARBOXYLASE SMALL CHAIN 1A – RsC1A and CHLOROPHYLL A/B BINDING PROTEINS – CABs, including CAB1 and CAB2) and anthocyanin biosynthesis (PHENYLALANINE AMMONIA-LYASE: 1 – PAL1). All genes selected for investigation in this study have been previously used in our work on darkening of detached Arabidopsis leaves (Sztatelman et al., 2015).

Light/dark treatment. Two or three adult, healthy leaves from each plant were darkened by wrapping in black paper. The plants remained in the growth chamber and after 4 days the darkened and non-darkened leaves were collected from each plant. Both darkened and non-darkened samples were chosen from leaves number 5 to 10. Whole plants were dark-adapted for 16 h before leaf collection (starting with the onset of darkness in the growth chamber). The harvested leaves were either directly used for chlorophyll fluorescence measurements or frozen in liquid nitrogen and stored at –80°C for further experiments (pigment extraction, RNA and protein isolation). Frozen samples consisted of 1–3 leaves (gl1) or 3 leaves (WT) pooled from different plants.

Pigment extraction/HPLC measurement. Leaf samples (50–200 mg of fresh mass) were ground in a mortar in 1–2 ml of methanol with a few grains of CaCO3. The homogenate was collected quantitatively and centrifuged, and the extraction of the pellet was repeated. Supernatants from both extractions were pooled and adjusted to known volume with methanol. Pigment separation, detection and quantitative analysis were performed according to (Sztatelman et al., 2015).

Measurement of chlorophyll fluorescence. The maximum quantum yield of photosystem II (PSII) (Fv/Fm) was measured using an Open FluorCam FC 800-0/1010 imaging fluorometer. Detached leaves (darkened and non-darkened) were placed on water-soaked paper and dark-adapted for 30 min. After recording the basal fluorescence (F0) for 2 s, samples were illuminated with saturating white light (2000 μmol m−2 s−1) for 960 ms. The average Fv/Fm value for each leaf was calculated using the FluorCam7 software.

RNA isolation and real-time PCR. Total RNA was extracted using the Spectrum Plant Total Kit (Sigma-Aldrich) and digested with DNaseI (Sigma-Aldrich) during purification on columns. RNA concentration was measured using a NanoDrop Lite (Thermo Fisher Scientific) spectrophotometer and its integrity was checked by electrophoresis in 1% agarose gel. First-strand cDNA synthesis was performed using the ReverTra Aid M-MuLV Reverse Transcriptase Kit (Thermo Fisher Scientific) with 1000 ng of RNA and oligo(dT)18 primers. Real-time PCR was performed using SYBR Green JumpStart Taq ReadyMix (Sigma Aldrich) and a Rotor-Gene 6000 thermal cycler (Corbett Research, Australia) according to (Łabuz et al., 2012), with the following modifications: reactions were run in triplicates, and ÛBC, ÛBq10 and ÛSAND were used as the reference genes. Primer sequences and annealing temperatures were based on (Sztatelman et al., 2015) and are listed in Table S1 (at https://ojs.ptbioch.edu.pl/index.php/abp/). cDNA pooled from six different samples (darkened and non-darkened leaves) was used as a reference for calculating relative gene expression levels. Normalization was performed using normalization factors based on reference gene levels, calculated using geNorm v3.4 (Vandesompele et al., 2002).

Protein extraction and Western blot. Leaf samples were homogenized and adjusted to an equal mass. Proteins were extracted as described by Sakamoto and Briggs (Sakamoto & Briggs, 2002). SDS-PAGE was performed on 12% polyacrylamide gels and was followed by a semi-dry protein transfer (BioRad) to a PVDF membrane. The membrane was blocked overnight with 5% (w/v) non-fat powdered milk in phosphate-buffered saline (PBS), 0.5% Tween-20. Primary antibodies, anti PsbaA (D1 protein, Agrisera AS05084A), were applied at a dilution of 1:10000

MATERIALS AND METHODS

Plant material. Arabidopsis thaliana WT Columbia-0 and gl1 (CS28174) seeds were purchased from the Nottingham Arabidopsis Stock Center (NASC, Nottingham, UK). After sowing in Jiffy-7 pots (Jiffy International AS), the seeds were stratified at 4°C for 2 days. Plants were cultured in a Sanyo MLR 350 H growth chamber, at 25°C, 80% humidity, in a 14 h light/10 h dark photoperiod and illuminated with fluorescent lamps (Oriel 350W/77 and Philips Master TL-D 36/W/840) with an average photosynthetic photon flux density of 70 μmolm−2s−1. Plants used for experiments were 5–6 weeks old.
(Agrisera) at room temperature for 1 h. After washing, secondary antibodies (goat anti-rabbit horseradish peroxidase (HRP) conjugated IgG, Agrisera) were applied at a dilution of 1:10,000, at room temperature for 1 h. The signal was detected with Clarity Western ECL Blotting Substrate (Bio-Rad) and the BioSpectrum Imaging System (UVP Ultra-Violet Products Ltd). Then, the membranes were stripped with Restore Plus Western Blot Stripping Buffer (Thermo Scientific) and probed again with anti-actin antibody (AS132640, Agrisera) at a dilution of 1:2500, at room temperature for 1 h, followed by incubation with secondary antibodies and ECL detection. Densitometric quantification was performed using ImageJ. Intensities of the chemiluminescent signals were normalized to actin levels in each sample.

**Statistical analysis.** The significance of the effects of the plant line (WT, gl1) and light conditions (control, darkened) was calculated with two-way ANOVA using the R software. The significance of the four pairwise differences of means (WT control vs WT darkened, gl1 control vs gl1 darkened, WT control vs gl1 control and WT darkened vs gl1 darkened) was tested using the glht command of the multcomp package. Holm’s correction was used to adjust the p-values for multiple comparisons. In general, variance was not uniform across groups. Thus, the heteroskedasticity-consistent variance – covariance matrix was used in the pairwise comparison procedure, calculated using the sandwich library. The results of the expression experiments (mRNA and protein levels) were log – transformed before statistical analysis.

**RESULTS**

**Photosynthetic efficiency**

Photosynthetic efficiency, measured as $F_v/F_m$ was comparable in WT and gl1 plants grown under the control conditions. In the darkened leaves of both genotypes, the $F_v/F_m$ parameter values were significantly lower. However, the decrease in $F_v/F_m$ was smaller in gl1 and the difference between both genotypes was statistically significant at the level of 0.001 (Fig. 1a). Additionally, we assessed the levels of the photosynthetic D1 protein, a core subunit of PSII (Fig. 1b). In control plants the amount of D1 was visibly higher in gl1 than in WT. After leaf darkening the amount of D1 increased in the WT plants and it slightly decreased in gl1, resulting in equal amounts of protein in both genotypes. However, all observed differences in the D1 protein levels between WT and gl1 plants were statistically non-significant (Fig. 1b).

**Photosynthetic pigments**

After four days of darkening, the leaves of both, the WT and gl1, showed partial yellowing indicating a change in photosynthetic pigment composition (Fig. S1 at https://ojs.ptbioch.edu.pl/index.php/abp/). The contents of all analyzed photosynthetic pigments (chlorophyll a and b, neoxanthin, violaxanthin, β-carotene and lutein) per g of fresh weight were comparable in the WT and gl1 light-grown plants. After leaf darkening, the content of all pigments per fresh weight was visibly reduced, especially in the case of both chlorophylls and β-carotene (by up to 50%) (Fig. 2a–c). This decrease was found to be statistically significant in the case of almost all pigments (apart from violaxanthin) in the WT plants (Fig. 2b). Although in gl1 plants the differences between control and darkened leaves were not statistically significant, the decrease in pigment content was also visible, suggesting the same process occurred as in the WT plants (Fig. 2). The seemingly minor differences between both genotypes were more pronounced when the pigment content was expressed per amount of total chlorophyll (Fig. S2b–c https://ojs.ptbioch.edu.pl/index.php/abp/).
In the light-grown WT and gl1 plants all pigment content ratios were comparable (Fig. S2a–c at https://ojs.ptbioch.edu.pl/index.php/abp/). After darkening, the content of carotenoids (neoxanthin, violaxanthin and lutein) in relation to chlorophyll had increased, while the β-carotene:chlorophyll ratio remained unchanged (Fig. S2 https://ojs.ptbioch.edu.pl/index.php/abp/). The dark-induced increase in carotenoid:chlorophyll ratios was slightly higher in the WT plants than in gl1 (Fig. S2b–c at https://ojs.ptbioch.edu.pl/index.php/abp/). In the case of violaxanthin and lutein, the differences between the control and darkened plants were statistically significant for both, the WT and gl1 (Fig. S2b–c cat at https://ojs.ptbioch.edu.pl/index.php/abp/).

### DISCUSSION

Until recently, physiological and molecular studies of the *A. thaliana glabra1* mutant were restricted to the process of trichome formation and some other effects related to the leaf structure (Xia *et al.*, 2010; Tsuji & Coe, 2013). To our knowledge, other aspects of the mutant’s physiology, including the progress of senescence, have not been investigated in more detail to date.

The experimental scheme of darkening individual leaves on growing plants applied here has been established as a good model of natural senescence (Weaver & Amasino, 2001; van der Graaff *et al.*, 2006; Keech *et al.*, 2007). While whole darkened plants seem to enter a ‘stand-by’ mode, aimed to preserve the activity of crucial metabolic pathways for as long as possible, the darkening of individual leaves triggers the degradation of cellular components and nutrient retrieval (Keech *et al.*, 2007). This is reflected in a rapid decline in photosynthetic activity. Similarities between natural and dark-induced senescence in individual leaves have been confirmed by a transcriptome study, showing at the same time that the former process is much more complex (van der Graaff *et al.*, 2006).

WT and gl1 plants, when grown under control conditions, were comparable by all parameters tested. The only pronounced difference was the lower expression of *RbcS1A* and *CABs* in gl1 (Fig. 3b), which did not affect the photosynthetic efficiency measured as Fv/Fm (Fig. 1a). This allows us to assume that all the differences observed between both genotypes after leaf darkening are truly linked to differences in the process of senescence. The significantly higher value of the Fv/Fm parameter in the darkened gl1 leaves as compared to WT was the first hint to a slower progress of senescence in mutant plants. The decline in photosynthetic activity in darkened leaves was not reflected in the levels of the D1 protein which increased in the WT and only slightly decreased in gl1 (Fig. 1b).

Lower photosynthetic efficiency in darkened leaves was correlated with the downregulation of *RbcS1A* and *CABs* photosynthetic genes (Fig. 3b), which is consistent with results of previous similar studies (Weaver & Amasino, 2001; Sztateczman *et al.*, 2015). This suggests that although the biosynthesis of new components has already been inhibited, the degradation of the photosynthetic ma-
chinery might not be significantly advanced yet. Higher \textit{RhPS1A} and \textit{C4B} transcript levels in the darkened \textit{gl1} leaves as compared with the WT ones further point to a slower progress of senescence in these plants. This effect was statistically significant in the case of \textit{RhPS1A}. Degradation of the D1 protein is also known to be induced by abiotic stresses, such as excessive light, UV-B radiation or high salinity (Kato & Sakamoto, 2009; Sakuraba \textit{et al.}, 2014; Sztatelman \textit{et al.}, 2015). The lack of an evident decrease in D1 levels and the absence of degradation products may therefore mean that the applied dark treatment specifically triggers senescence, without being an additional source of stress. The \textit{PAL1} gene is involved in anthocyanin biosynthesis and induced by light (Jones, 1984; Kimura \textit{et al.}, 2003). Downregulation of this gene in darkness probably reflects the reduced need for the production of photoprotective pigments under these conditions. The small decrease in \textit{PAL1} transcript level, observed in the darkened \textit{gl1} leaves, may therefore be another indicator of slower senescence in these plants (Fig. 3b).

Natural senescence is invariably connected with gradual leaf yellowing. This could be also observed in our experimental model (Fig. S1 at https://ojs.ptbioch.edu.pl/index.php/abp/). This leaf yellowing is due to the degradation of photosynthetic pigments, which contributes to the decline in photosynthetic activity. This was confirmed by the analysis of photosynthetic pigment content. As expected, the levels of all pigments had decreased substantially after dark treatment (Fig. 2). No major, significant differences could be observed in the reaction to darkening between \textit{gl1} and WT. In both genotypes, an increase in the amount of carotenoids (neoxanthin, violaxanthin and lutein) as compared to total chlorophyll content was observed after leaf darkening, which is consistent with the usually faster degradation of chlorophylls (Young \textit{et al.}, 1991; Lu \textit{et al.}, 2001) (Fig. S2b–c at https://ojs.ptbioch.edu.pl/index.php/abp/). This parameter revealed some subtle differences between S2b–c at https://ojs.ptbioch.edu.pl/index.php/abp/). which is consistent with the usually faster degradation of chlorophylls after leaf darkening, oxanthin, violaxanthin and lutein) as compared to total chlorophyll content. As expected, the levels of all pigments was confirmed by the analysis of photosynthetic pigment content. As expected, the levels of all pigments degradation of photosynthetic pigments, which contributes to the decline in photosynthetic activity. This was confirmed by the analysis of photosynthetic pigment content. As expected, the levels of all pigments could explain a higher PSI efficiency and slower progression of senescence in the darkened \textit{gl1} leaves. On the other hand, taking into account the nature of \textit{GLABRA1} as a transcription factor, its direct role in regulating the expression of genes involved in senescence cannot be excluded. It is also possible that both of the proposed (and perhaps also other) mechanisms contribute to the observed delayed senescence in the \textit{gl1} mutant.

The authors declare no conflict of interest.

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The results obtained in this work provide ample and robust evidence that the onset and progress of dark-induced senescence is delayed in the \textit{A. thaliana glabra1} mutant. Taking into account previous reports comparing different models of senescence (Weaver & Amasino, 2001; van der Graaff \textit{et al.}, 2006), it is highly probable that the same holds true for the process of natural senescence in these plants. The lack of trichomes makes the \textit{gl1} mutant more susceptible to numerous environmental stresses, both abiotic and biotic, which may contribute to the induction of senescence in these plants (Hauser, 2014; Bickford, 2016). Nevertheless, trichome production comes at a cost (Sletvold \textit{et al.}, 2010; Hauser, 2014). Although the presence of trichomes may be beneficial to the plant, they are developed at the expense of other traits. As demonstrated in \textit{Arabidopsis lyrata}, trichome density (and the resulting resistance to herbivores) was negatively correlated with plant size and fitness (Sletvold \textit{et al.}, 2010). Perhaps in the case of the \textit{gl1} mutant, the resources which would otherwise be used for trichome production can be allocated to other processes. This could explain a higher PSI efficiency and slower progress of senescence in the darkened \textit{gl1} leaves. On the other hand, taking into account the nature of \textit{GLABRA1} as a transcription factor, its direct role in regulating the expression of genes involved in senescence cannot be excluded. It is also possible that both of the proposed (and perhaps also other) mechanisms contribute to the observed delayed senescence in the \textit{gl1} mutant.

The demonstrated effect of the \textit{gl1} mutation on the process of senescence should be taken into account in any experiments using other mutants with a \textit{gl1} genetic background and the results should be interpreted with caution. The \textit{gl1} example shows that a single mutation can affect very different, seemingly unrelated processes. It is therefore advisable to avoid using such “cryptic” multiple mutants. If this is impossible, an appropriate control, that is the \textit{gl1} mutant, should be used.

Conflict of Interest Disclosures

The authors declare no conflict of interest.
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