Chloroplasts require glutathione reductase to balance reactive oxygen species and maintain efficient photosynthesis

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SUMMARY

Thiol-based redox-regulation is vital for coordinating chloroplast functions depending on illumination and has been thoroughly investigated for thioredoxin-dependent processes. In parallel, glutathione reductase (GR) maintains a highly reduced glutathione pool, enabling glutathione-mediated redox buffering. Yet, how the redox cascades of the thioredoxin and glutathione redox machineries integrate metabolic regulation and detoxification of reactive oxygen species remains largely unresolved because null mutants of plastid/mitochondrial GR are embryo-lethal in Arabidopsis thaliana. To investigate whether maintaining a highly reducing stromal glutathione redox potential (\(E_{GSH}\)) via GR is necessary for functional photosynthesis and plant growth, we created knockout lines of the homologous enzyme in the model moss Physcomitrella patens. In these viable mutant lines, we found decreasing photosynthetic performance and plant growth with increasing light intensities, whereas ascorbate and zeaxanthin/antheraxanthin levels were elevated. By in vivo monitoring stromal \(E_{GSH}\) dynamics, we show that stromal \(E_{GSH}\) is highly reducing in wild-type and clearly responsive to light, whereas an absence of GR leads to a partial glutathione oxidation, which is not rescued by light. By metabolic labelling, we reveal changing protein abundances in the GR knockout plants, pinpointing the adjustment of chloroplast protein degradation and the induction of plastid protein repair and degradation machineries. Our results indicate that the plastid thioredoxin system is not a functional backup for the plastid glutathione redox systems, whereas GR plays a critical role in maintaining efficient photosynthesis.

Keywords: chloroplast, glutathione redox potential, photosynthesis, glutathione reductase, redox-sensitive GFP, reactive oxygen species, non-photochemical quenching, Physcomitrella patens, moss.

INTRODUCTION

In photosynthetic eukaryotes, changes in environmental conditions, such as light intensity or temperature, provoke changes in electron flow both in the chloroplasts and in the mitochondria. Several mechanisms rapidly modulate or redirect electron flow to minimize over-reduction of the two electron transport chains (ETCs), which can otherwise give rise to excessive formation of reactive oxygen species (ROS) (Schwarzländer and Finkemeier, 2013; Schöttler and Toth, 2014). Moreover, ROS serve as important signaling molecules in stress acclimation (Suzuki et al., 2012; Dietz et al., 2016). This implies that the rates of ROS generation and scavenging must be precisely balanced in these organelles. The maintenance of cellular redox pools for...
metabolism, antioxidant defense, and thiol-based redox switching requires the constant influx of electrons via light- and NADPH-powered redox cascades, involving the oxidation and reduction of cysteines in thioredoxins (Trx) and glutathione (Meyer et al., 2012; Yoshida and Hisabori, 2016; Geigenberger et al., 2017; Gütle et al., 2017).

Reduced glutathione (GSH) is present in cells at low millimolar concentrations (Meyer et al., 2001). The functions of glutathione include ascorbate regeneration via the ascorbate-glutathione-cycle, detoxification of potentially toxic organic electrophiles and heavy metals, and the coordination of iron-sulfur clusters by serving as cofactor of monothiol glutaredoxins (Grx) (Foyer and Noctor, 2011; Moseler et al., 2015). In addition, glutathione is also a substrate of dithiol Grx-catalysed protein (de)glutathionylation (Meyer et al., 2012; Zaffagnini et al., 2019). For the latter functions it is essential that glutathione can reversibly switch between its reduced form, GSH, and the oxidized form glutathione disulfide (GSSG), which involves the transfer of two electrons. The glutathione redox potential \( E_{GSH} \) is dependent on the GSH concentration, as well as on the balance between GSH and GSSG. The \( E_{GSH} \) can vary drastically between subcellular compartments (Meyer, 2008; Kojet al., 2012). In unstressed plant cells, the \( E_{GSH} \) of cytosol, peroxisomes, mitochondrial matrix and plastid stroma is highly reducing between \(-310\) and \(-360\) mV (Meyer et al., 2007; Schwarzländer et al., 2008). In these compartments, GSSG is efficiently regenerated to GSH by the action of glutathione reductases (GR) using NADPH as an electron donor. In plants, dual targeting of one GR isoform to plastids and mitochondria is evolutionary conserved (Xu et al., 2013; Marty et al., 2019). In the cytosol and the mitochondria of Arabidopsis thaliana, the loss of GR is partially compensated for by the presence of the NADPH-dependent Trx reductases A and B (NTRA,B) (Marty et al., 2009, 2019). Nevertheless, decreased GR activity of the plastid/mitochondrion-targeted isoform leads to reduced root growth in seedlings (Yu et al., 2013). However, a complete loss of GR in plastids causes early embryo-lethality before greening in Arabidopsis (Ding et al., 2016; Marty et al., 2019).

Because this limits the usability of Arabidopsis as a model for investigating the function of GR in green plastids, the significance of GR in stromal \( E_{GSH} \) maintenance, for photosynthesis and other plastid functions, has remained unclear. Furthermore, to what extent the Trx system can serve as a backup for the glutathione redox system in plastids remains unknown.

In the present study, we investigated the effects of the complete loss of plastid/mitochondrion-localized GR in photosynthetic cells, utilizing the moss Physcomitrella patens as a model (Reski, 2018). We assessed growth and photosynthetic parameters, monitored the dynamics of plastid \( E_{GSH} \) by redox-sensitive GFP (roGFP)-based in vivo imaging and compared protein abundances between wild-type (WT) and GR mutants in response to a shift from low light to high light by quantitative proteomics.

**RESULTS**

Physcomitrella patens lacking GR1 (PpGR1) is viable and displays reduced growth

Loss of plastidic GR in Arabidopsis leads to embryo-lethality (Ding et al., 2016; Marty et al., 2019), not only indicating an essential role in the non-photosynthetic tissues of early sporophyte development, but also preventing further studies of GR function in green tissues. We hence chose *P. patens* as a model because knockout mutants can be generated using protoplastation and regeneration of photosynthetically active vegetative cells in the haploid phase of the life cycle, circumventing embryogenesis and non-photosynthetic tissue (Schween et al., 2005). Furthermore, the high rate of homologous recombination in *P. patens* allows for the targeted removal of the gene sequence, resulting in genuine knockouts (Figure S1). We hypothesized that null mutants of organellar GR might be viable in plants that maintain green plastids throughout their life cycle. We therefore generated knockout constructs replacing exons 2-5 containing the translation start site and the active site in the gene encoding the previously identified dual-targeted mitochondria- and plastid-localized glutathione reductase Pp1s13_127V6.1 (named GR1 in *P. patens*; Xu et al., 2013) with a hygromycin resistance cassette via homologous recombination (Figure S1a). As a genetic background, we generated a *P. patens* line expressing the plastid-targeted \( E_{GSH} \) biosensor Grx1-roGFP2 under the control of the *P. patens* Actin 5 promoter (Weise et al., 2006; Mueller and Reski, 2015). We isolated plants surviving hygromycin selection under constant light. They were genotyped for integration of the knockout construct at the target locus (Figure S1b) and the absence of *PpGR1* transcript was confirmed (Figure S1c). All plants lacking GR1 transcript showed a dwarf phenotype (Figure 1a) compared to the WT line and the line expressing plastid-targeted Grx1-roGFP2 (cpGrx1GFP2 #40, Figure 1a). Two independent lines, \( \Delta gr1 \#48 \) and \( \Delta gr1 \#88 \), were chosen for further analysis. Because the *Arabidopsis thaliana* gr2-1 null mutant is embryo-lethal, we investigated whether \( \Delta gr1 \) knockout mutants were able to complete the moss life cycle. Under inducing conditions, \( \Delta gr1 \#48 \) and \( \Delta gr1 \#88 \) formed sporophytes that underwent complete development and opened to release mature spores (Figure 1b). Thus, GR1 is not necessary for embryo development in *P. patens*. However, spore germination of \( \Delta gr1 \#48 \) and \( \Delta gr1 \#88 \) was delayed by several days, with spores being able to germinate eventually (Figure 1b). Apart from the dwarfed appearance, the \( \Delta gr1 \) mutant lines
grew fewer caulonema filaments and rhizoids than the WT (Figure S2).

The ultrastructure of chloroplasts lacking GR1 was investigated using transmission electron microscopy, which revealed normally packed grana stacks and stroma lamellae, undistinguishable from WT (Figure S3).

**Plastid redox state is dynamic in WT, being shifted to less reducing values in Δgr1 plants and not rescued via Trx reduction under light**

Taking advantage of the stromal-targeted Grx1-roGFP2 sensor, the steady-state of the chloroplast E$_{GSH}$ was determined by confocal in vivo imaging of roGFP2 redox state (Figure 2a). The fluorescence excitation ratio 405/488 nm increases with sensor oxidation. It was 0.91 ± 0.17 in WT, 1.61 ± 0.25 in Δgr1 #48 and 1.81 ± 0.28 in Δgr1 #88 (Figure 2a,b), indicating that the stromal E$_{GSH}$ is less reducing in Δgr1 lines. Total glutathione content (GSH + 2GSSG) was not significantly different from WT (Figure 2b). To test for stability of stromal E$_{GSH}$ after exogenous reduction, plants were incubated with 2 mM dithiothreitol and then exposed to continuous laser scanning under a confocal microscope. As a result, plastid Grx1-roGFP2 was rapidly re-oxidized in the Δgr1 plants but not rescued via Trx reduction (Figure 2c). This result prompted us to investigate the dynamics of E$_{GSH}$ in dark-to-light and light-to-dark transitions (Figure 2d). Dark-adapted plants were exposed to a dark-to-light transition (100 μmol photons m$^{-2}$ sec$^{-1}$ for 10 min) during confocal imaging, resulting in a transient oxidation, followed by a reduction of the Grx1-roGFP2 redox state. The following light-to-dark transition (after 10 min of light exposure) resulted in an oxidation. Pre-incubation of plants with the electron transport inhibitor 10 μM DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea] blocked the E$_{GSH}$ dynamics, indicating a dependence on the photosynthetic ETC. No reduction of Grx1-roGFP2 in the light was observed in Δgr1 plants. Because Trx systems constitute a functional backup for cytosolic and mitochondrial GRs (Marty *et al*., 2009; Marty *et al*., 2019), we next investigated whether the survival of Δgr1 plants was dependent on light exposure, activating the ferredoxin-thioredoxin reductase (FTR) system. Because FTR is supplied with electrons from the chloroplast ETC, we investigated the survival of the Δgr1 mutant lines after transfer from constant light to short day conditions (Figure S4a) and under extended darkness (44 days) (Figure S4b). These experiments revealed that Δgr1 #48 and Δgr1 #88 were not sensitive to incubation in darkness, suggesting that an influx of electrons to Trxs via the chloroplast ETC is not required for the survival of the *P. patens* Δgr1 mutants.

**Δgr1 plants show altered responses of reactive oxygen species dynamics and photosynthetic function**

Because Δgr1 knockout plants were not sensitive to dark-incubation (Figure S4) and they showed an oxidative response to the laser light used for microscopic imaging (Figure 2c), their growth habit under different light intensities was investigated (Figure S5a).

With increasing light fluences (30 to 130 μmol photons m$^{-2}$ sec$^{-1}$), growth of WT plants was increased resulting in higher fresh weights (Figure S5b). By contrast, Δgr1 plants showed no increase in light-dependent biomass accumulation, resulting in an increased difference in biomass gain to WT at higher light fluences. To investigate the impact of different light intensities on ROS scavenging, the levels of ascorbate and dehydroascorbate were determined. The measurements revealed a higher total level of ascorbate in Δgr1 #48 and Δgr1 #88 that reached 500% to 700% of WT levels at different light intensities (Figure 3a and Table S1). Interestingly, dehydroascorbate levels were increased as well, although they were present at a similar ratio to reduced ascorbate as in WT (WT background: 9.9 ± 2.1%; Δgr1 background: 6.6 ± 2.8%; raw data provided in Table S1). We incubated gametophores with nitro blue tetrazolium (NBT) in the dark or in light as a
means to detect superoxide (O$_2^{-}$) and found increased staining intensity in light-incubated Δgr1 plants (Figure 3b). To induce additional ROS formation by photosystem I (PSI), single gametophores were transferred to medium containing 1 μM Paraquat. Although WT plants were still able to grow, Δgr1 #48 and Δgr1 #88 plants bleached and died (Figure 3c, upper). To stop photosynthetic electron flow, moss colonies were transferred to photolithrotrophic growth conditions and their survival on DCMU was tested according to Bricker et al. (2014). WT plants survived when sucrose (1%, w/v) was present in the medium, whereas Δgr1 #48 and Δgr1 #88 plants bleached and died (Figure 3c, middle).

Under exposure to high light (HL) fluencies (450 μmol photons m$^{-2}$ sec$^{-1}$), white sectors appeared in leaflets of Δgr1 plants (Figure 4a). When HL was additionally combined with elevated temperature, Δgr1 #48 and Δgr1 #88 plants died, whereas WT and Δgr1 mutants were able to recover from temperature stress only (Figure 4a). We found that shifting WT plants from control light (CL) into HL for 4 h lead to a 20.1 ± 4.7% increase in total GR activity, whereas the Δgr1 lines showed only 64.7% increase in total GR activity in CL and 70.5 ± 6.6% of WT GR activity after 4 h HL (Figure 4b; for absolute activities, see Table S2), revealing a 35-50% deficit in GR activity, depending on light intensity.

Non-photochemical quenching (NPQ) was functional in Δgr1 protonema and gametophores, whereas NPQ dark relaxation was slower under all light conditions tested (Figure 5a and Figure S6). In Δgr1 gametophores, the quantum yield of PSII (YIIII) recovered more slowly than in WT background and decreased substantially after 4 h.
of HL treatment (Figure S6). Closer examination of photosynthetic parameters revealed a light intensity-dependent decrease of NPQ, linear and cyclic photosynthetic electron flow (LEF + CEF) and an increase in the photosystem I to photosystem II ratio (PSI/PSII) (Figure 5a). CEF was not increased under different light intensities, although it showed a slight increase after 6 h of induction via anoxia (Figure S7a,b). The \( F_v/F_m \) ratio decreased in \( \Delta gr1 \) with increasing light, indicating decreased photosynthetic efficiency (Figure S7a). At the same time, total chlorophyll (\( a + b \)) levels were decreased (Figure S7c).

To investigate the proton motive force (pmf) in \( \Delta gr1 \) chloroplasts compared to WT, electrochromic shift assays (Kramer and Crofts, 1989) were conducted, revealing a decreased pH gradient and increased membrane potential \( \Delta \psi \) in \( \Delta gr1 \), resulting in a similar pmf. The proton conductivity of the ATP synthase was not significantly altered (Figure 5b).
Figure 5. Photosynthetic parameters and xanthophyll cycle pigment analysis.
(a) Photosynthetic parameters measured under low light (LL, 15 \(\mu\)mol photons m\(^{-2}\) sec\(^{-1}\)), control light (CL, 50 \(\mu\)mol photons m\(^{-2}\) sec\(^{-1}\)) and high light (HL, 450 \(\mu\)mol photons m\(^{-2}\) sec\(^{-1}\)). Non-photochemical quenching (NPQ, left). Error bars indicate the SD (\(n = 6\)); the white and black box on top indicates the light and dark phase of the measurements. Actinic light (1076 \(\mu\)mol photons m\(^{-2}\) sec\(^{-1}\)) was switched off after 5 min of illumination. Photosynthetic linear and cyclic electron flow (LEF + CEF; middle; \(n = 6\) LL, CL; \(n = 12\) HL; error bars indicate the SD). Box plots of photosystem (PSI)/PSII ratio (right; \(n = 6\) LL, CL; \(n = 12\) HL; **Significant difference \(P < 0.01\), paired \(t\)-test). WT, wild-type.
(b) Measurement of the proton motive force components pH gradient (\(\Delta p\)) and membrane potential (\(\Delta \Psi\)), and the proton conductivity of the ATP synthase (n = 3). Left: dark relaxation of the carotenoid electrochromic shift signal (ECS) after illumination (white box) with 300 \(\mu\)mol photons m\(^{-2}\) sec\(^{-1}\) \((P < 0.0001\), paired \(t\)-test). The white and black box on top indicates the light and dark phases of the measurements. Right: proton conductivity of the ATP synthase \(g_H\).
(c) High-performance liquid chromatography measurements of pigment levels in plants grown under low light (LL, 15 \(\mu\)mol photons m\(^{-2}\) sec\(^{-1}\)) and plants grown under control light (CL, 100 \(\mu\)mol photons m\(^{-2}\) sec\(^{-1}\)) and shifted for 4 h to high light (HL, 450 \(\mu\)mol photons m\(^{-2}\) sec\(^{-1}\)). Lowercase letters depict significant differences, \(P < 0.05\) (\(n = 3\), two-way ANOVA with interactions, Tukey’s honestly significant difference post-hoc test). Full data available are provided in Table S3. Left: levels of de-epoxidated xanthophyll cycle pigments antheraxanthin (A) and zeaxanthin (Z) (% corrected peak area of all pigments). Right: De-epoxidation state \((\text{antheraxanthin} + \text{zeaxanthin})/ (\text{violaxanthin} + \text{antheraxanthin} + \text{zeaxanthin})\) of xanthophyll cycle pigments under the different light conditions.

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Because NPQ relaxation was slower under all light conditions tested, similar to Arabidopsis npq2 (zeaxanthin epoxidase) mutants (Niyogi et al., 1998), we measured levels of photosynthesis pigments and found that zeaxanthin + antheraxanthin (Z + A) levels were significantly increased under low light (LL) in Agrp1 #88, as well as under control light (CL) and high light (HL) in both Agrp1 lines compared to WT, whereas the zeaxanthin + antheraxanthin to violaxanthin + zeaxanthin + antheraxanthin ratio (Z + A/VAZ) was higher in LL (Figure 5c and Table S3).

Quantitative proteomics reveals light intensity-dependent protein level changes in Agrp1 plants

To assess the significance of GR function for proteostasis under changing light intensities, we used metabolic labelling with the stable isotope 15N in combination with quantitative proteomics. Changes in protein abundances in WT and Agrp1 plants upon a shift from LL to HL were investigated using quantitative proteomics (Figure S8). The sensor expressing line cpGrx1roGFP2 #40 (WT) and Agrp1#48 were labelled in vivo by growth on medium containing Ca(15NO3)2 as an exclusive nitrogen source or normal 14N-containing medium, respectively. Labelled and unlabelled samples of both lines were exposed to HL and samples taken after 1 h, whereas control samples were kept at LL. For the same experimental condition, protein extracts from labelled and unlabelled samples of Agrp1 and cpGrx1roGFP2 #40 (WT), respectively, were mixed in a 1:1 ratio, tryptically digested and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). To exclude any bias introduced by the labelling, one label swap experiment was performed per light intensity, resulting in a total of four experiments (2 x Agrp1 versus WT HL, 2 x Agrp1 versus WT LL) (Figure S8). In total, 1657 proteins with Agrp1 versus WT ratios were quantified, of which 125 were differentially abundant (P < 0.05) in Agrp1 and WT in LL or HL (Figure 6a, blue dots). Of these 125 proteins, 70 were down-regulated and 59 up-regulated (four were either up- or down-regulated, depending on the light conditions). The overlap between differentially regulated proteins in LL compared to HL was 8.6% for the down-regulated proteins and 20.3% for the up-regulated proteins (Figure 6b). Following a manual annotation of subcellular localization (Data S1), based on available organelle proteomics data sets for P. patens (Mueller et al., 2014) and SUBAcon (Hooper et al., 2014) annotation of Arabidopsis homologs, the largest fraction of regulated proteins was attributed to plastids (38), followed by the cytosol (20) and proteins with unknown subcellular localization (20).
unclear localization (13) (Figure 6c). In addition, differentially regulated proteins were sorted into 29 functional categories (Data S1) and categories containing more than two proteins plotted to visualize category-specific down- or up-regulation in the different light conditions (Figure 6d). Here, more proteins with unknown function were down-regulated specifically in LL or HL, whereas several proteins with unknown function were up-regulated in both light conditions. In the categories ‘protein homeostasis’ and ‘photosynthesis light reactions’, more proteins were down-regulated in LL, although more proteins were up-regulated in HL, indicating a strong influence of the light intensity on protein levels in these categories. PSI subunit E (Pp1s101_2V6.1, 2−0.33) was less abundant in LL, whereas the plastid-encoded PSII subunits D1 and D2 were less abundant after the shift to HL (PsBA PhpapaCp046, 2−0.35, PsDB PhpapaCp044, 2−0.2). In the functional category ‘protein homeostasis’, under HL, the increase of one isoform of the plastid proteasome proteolytic subunit ClpP (Pp1s161_14V6.1, 2+6.64) and of chaperonin 60 (Chp 60) alpha and beta subunits (Pp1s16_322V6.1 2+0.48, Pp1s14_298V6.1, 2+0.47, Pp1s15_485V6.1, 2+0.55) indicated an increased demand for protein stabilization and degradation. Proteins of cytosolic translation were down-regulated under LL, whereas proteins of respiratory complex I and photosynthetic dark reactions were only affected in HL. The increase of one isoform of the plastid-encoded glucose-6-phosphate dehydrogenase (Pp1s338_65V6.1, 2+0.87, HL) to be down-regulated. Interestingly, *P. patens* possesses a putative oxidoreductase using GSSG with similarity to bacterial YfcG (Pp1s339_37V6.1).

Proteins of plastid translation were up-regulated in LL, whereas proteins of respiratory complex I and photosynthetic dark reactions were only affected in HL. photosynthetic dark reactions were only affected in HL.

Prominent changes in protein abundances include a plastid ribosome release factor (Pp1s130_293V6.1, 2+6.64, HL) and a KEA (K+-efflux antiporter) homolog (Pp1s2_217V6.1, 2+6.64, HL) with high similarity to AtKEA1 and AtKEA2 (Kunz et al., 2014). Notably some enzyme isoforms were regulated differentially, such as two enolate isoforms (Pp1s15_527V6.1, c. 2+1.2, LL + HL; Pp1s37_237V6.1, 2−6.64, LL + HL).

Because PpGR1 (Pp1s13_127V6.1) was also identified in the proteomics analysis as differentially abundant (Data S1), we confirmed the absence of PpGR1 in Δgr1 lines additionally by a targeted proteomics approach (Figure S9).

Furthermore, we screened the dataset for known redox-regulated proteins and found one of the three isoforms of gamma subunit of chloroplast ATP synthase (Pp1s35_234V6.1, 2−1.35, LL), a putative plastid glucose-6-phosphate dehydrogenase (Pp1s338_65V6.1, 2−0.36, HL), as well as one of two FBPase isoforms (FBPase 2 Pp1s20_373V6.1 2−0.87, HL), to be down-regulated. Interestingly, *P. patens* possesses a putative oxidoreductase using GSSG with similarity to bacterial YfcG (Pp1s339_37V6.1) that was down-regulated under both light conditions.

**DISCUSSION**

**Stromal E<sub>GSH</sub> responds to photosynthetic status**

We generated viable null mutants of PpGR1 and found that an absence of GR1 leads to a shift in the stromal E<sub>GSH</sub>-After Grx1-roGFP2 calibration, sensor 405/488 nm excitation ratio measurements can be translated into the degree of sensor oxidation. Because the redox potential of roGFP2 equilibrates with the redox potential of glutathione, E<sub>GSH</sub> can be calculated, with the limitation that compartment pH has to be estimated (Meyer et al., 2007; Schwarzländer et al., 2008). In Δgr1 plants, the degree of oxidation of the plastid-targeted E<sub>GSH</sub> sensor Grx1-roGFP2 was severely shifted. Based on the change of the 405/488 nm ratio and the *in vivo* sensor calibration (Figure 2b), we calculated a shift from approximately 48% oxidation in the WT background to approximately 92% oxidation in the Δgr1 lines. This would correspond to a 33 mV shift in the redox potential (calculated for pH8: −311 mV in WT versus −278 mV in Δgr1) (Figure 7). In comparison, the redox potential in Arabidopsis epidermal plastids was determined as approximately −361 mV at pH 8 (Schwarzländer et al., 2008).

A shift of 30 mV is equivalent to an increase of the relative amount of GSSG from 0.01% to 0.1%, as calculated for a total concentration of 2.5 mM GSH (Meyer et al., 2007).

Because glutathione- and Trx-dependent thiol switching fuelled by distinct reductases co-exists in the same compartments (Buchanan and Balmer, 2005), the interdependence of these systems has been dissected for the cytosol and the mitochondria. Thus, cytosolic Trx redox state can be rescued via the glutathione system (Reichheld et al., 2007). Vice versa, the NTR/A/B system present in the cytosol and the mitochondria constitutes a functional backup of the glutathione system (Marty et al., 2009). However, a reduction of many plastid Trxs is light-dependent and E<sub>GSH</sub> in other cellular compartments is stable. It was hypothesized that the Trx-dependent redox cascades in plastids cannot provide sufficient backup capacity to reduce the plastid E<sub>GSH</sub> (Marty et al., 2019). Our data indicate that a shift of E<sub>GSH</sub> in plastids occurs in the absence of GR, although such a shift is limited. The resulting steady-state level of E<sub>GSH</sub> may be a consequence of either electron flux to GSSG from Trxs, export of GSSG (Morgan et al., 2013; Noctor et al., 2013) from plastids or increased GSH biosynthesis (Choudhury et al., 2018). However, we did not find increased glutathione levels in the absence of GR1 (Figure 2b).

In dynamic measurements in ectopically reduced Δgr1 plants, the stromal E<sub>GSH</sub> was rapidly re-oxidised by exposure to laser light, suggesting that GSSG rapidly accumulates in mutants upon illumination. Because the regeneration of GSSG to GSH via GR is lacking in the mutant plastids, this likely represents GSSG formed by enzymatic reactions in ROS scavenging and damage repair (Figure 7).

A likely candidate is dehydroascorbate reductase (DHAR) of the ascorbate-GSH cycle. While the relative contributions of monodehydroascorbate reductase (MDHAR) and DHAR to the plastid ascorbate regeneration were debated (Asada, 1999; Polle, 2001), plastid-targeted AtDHAR3 was shown to contribute to ascorbate recycling with mutants.
Mal glutathione is still present in the reduced state (99.9%).

Dynamically, showing that ROS, reactive oxygen species, are generated via glutathione reductase (GR). LL, low light; CL, control light; HL, high light; NPQ, non-photochemical quenching; PET, photosynthetic electron transport; RÖS, reactive oxygen species.

(a) In the wild-type (WT), the stromal glutathione (GSH) redox potential (ΔE_{GSH}) shows light-dependent dynamics; all protective mechanisms against photoinhibition are functional and adjusted to light intensity. Glutathione disulfide (GSSG) generated via enzymatic scavenging and repair pathways is regenerated continuously via glutathione reductase (GR). LL, low light; CL, control light; HL, high light; NPQ, non-photochemical quenching; PET, photosynthetic electron transport; RÖS, reactive oxygen species.

(b) An absence of GR in the stroma leads to a shift of ΔE_{GSH} that is not rescued by light and, as a direct or indirect consequence, triggers changes in NPQ dynamics, pmf partitioning, and ROS and ascorbate levels. PET is reduced compared to WT, compromising photosynthetic efficiency.

(c) After a shift to HL, a further decrease in PET, a further increase in ascorbate, low NPQ and changes in protein levels indicate light-induced damage and triggered stress responses. Differences in font size indicate qualitative changes of parameters.

Figure 7. Schematic summary.

(a) (b) (c)

In the green haploid moss gametophyte, lack of GR1 caused slow growth, as well as defects in photosynthetic parameters. Photosynthetic electron flow was not affected in LL, whereas increasing light fluencies resulted in decreased electron flow in photosynthetic light reactions. On the one hand, increasing light fluencies also caused an almost complete loss of NPQ. On the other hand, relaxation of NPQ kinetics was slower under all tested light conditions, similar to the Arabidopsis npq2 mutant lacking zeaxanthin epoxidase (Niyogi et al., 1998). Under HL, violaxanthin is converted via antheraxanthin into zeaxanthin, which is involved in scavenging ROS, as inferred from the analysis of Arabidopsis vte1 mutant deficient in the...
synthesis of tocopherol, one of the lipid antioxidants in chloroplasts (Havaux et al., 2005). In combination with increased levels of the de-epoxidized xanthophyll cycle pigments zeaxanthin and anteraxanthin and an overall functional xanthophyll cycle, as confirmed by A + Z/VAZ values under HL (Figure 5c), our data suggest an influence of $E_{\text{GR}}$ in the regulation of zeaxanthin epoxidase. Additionally, violaxanthin de-epoxidase, the enzyme involved in zeaxanthin production, is activated by acidification of the lumen pH and uses ascorbate as co-substrate (Arnoux et al., 2009).

Furthermore, an increasing PSII/PSI ratio under higher light fluencies, as well as slow or no recovery of Y(II) after HL, indicated sustained decreases in PSII efficiency. Concomitantly, we found decreased levels of photosystem II subunits D1/D2 and increased protein levels of plastid chaperones and protein degradation, confirming an increased demand for protein repair and degradation in HL (Figure 7). PSII efficiency was already linked to GR activity in a study using a tobacco line with 30% of plastid/mitochondrial GR activity (Ding et al., 2009). Here, the diminished GR activity resulted in decreased chlorophyll, ascorbate, DHA levels and PSII efficiency, as well as increased H$_2$O$_2$ levels under chilling stress (Ding et al., 2012). Concomitantly, overexpression of plastid/mitochondrial GR was beneficial under photoinhibitory conditions in poplar and cotton (Foyer et al., 1995; Kornyeyev et al., 2003).

In Δgr1 plants, we found an elevated ΔΨ and decreased ΔpH, resulting in a similar pmf and H$^+$ conductivity ($g_\text{th}$) compared to WT. An elevated electric field component can increase PSII photodamage (Davis et al., 2016). In addition, we found increased sensitivity to ROS, as well as increased superoxide tissue staining in the Δgr1 mutant. The chloroplasts possess a very efficient removal system for ROS, with several ascorbate peroxidases that detoxify hydrogen peroxide using ascorbate as an electron donor. On the other hand, ascorbate peroxidases themselves represent prominent targets for ROS-induced damage (Dietz, 2016). Therefore, it is possible that the higher ROS fluxes reached in the Δgr1 plants, even under non-stress conditions, leads to decreased enzymatic ascorbate peroxidation and increased ROS-induced damage. Furthermore, ROS inhibit plastid translation and thereby PSII repair (Nishiyama et al., 2011). In tobacco, 30% of plastid GR activity was sufficient to avoid ROS formation under non-stress conditions (Ding et al., 2009). Our data clearly indicate that the presence of functional PSII in increasing light intensities is linked to a highly reducing stromal $E_{\text{GR}}$.

Only a low percentage of quantified proteins differed in abundance between Δgr1 and WT plants in LL and after a shift from LL to HL (7.5%; 125 of 1657). The affected proteins are distributed across several compartments, with the plastid being the most prominent localization (30%), confirming the important role of dual-targeted GR for plastid processes. The impact on cytosolic proteins also shows that mutant cells adjust their protein content to the altered situation in the chloroplasts, possibly suggesting active retrograde signaling between chloroplast and nucleus. Notably, after the shift to HL, mitochondrial proteins (3 complex I subunits) also became affected, suggesting a role of mitochondrial GR under HL conditions.

Following manual annotation of proteins and allocation to process categories, several patterns became apparent. Proteins involved in cytosolic translation were mostly less abundant in Δgr1 plants, whereas several proteins from plastid translation were more abundant. In addition, proteins of plastid glycolysis and fatty acid biosynthesis, as well as proteolysis- and protein folding-related proteins, were more abundant in Δgr1 plants. The adjustment of several photosynthesis-related proteins was already apparent in LL, confirming that GR function is not simply relevant under stress conditions.

In HL, altered levels of transport proteins such as the inner envelope H$^+$/K$^+$ antiporter (KEA) isoform may contribute to phenotypes such as decreased ΔΨ within the pmf component in Δgr1 mutants (Kunz et al., 2014). Interestingly, plant KEA isoforms possess sequence similarity to the glutathione-regulated potassium-efflux system KefC of Escherichia coli (Roosild et al., 2010). This system is important for protection against toxic electrophiles via acidification (Ferguson, 1999) and it is negatively regulated by GSH but activated by glutathione conjugates (Roosild et al., 2010). It is tempting to speculate that activity or an abundance of plant KEA isoforms in the inner plastid envelope is linked to changes in the stromal glutathione redox state. In the absence of AtKEA1/2, NPQ decreased and PSII was compromised (Kunz et al., 2014), which is line with the Δgr1 data. By contrast, deletion of the thylakoid-localized H$^+$/K$^+$ antiporter AtKEA3 resulted in high NPQ in Arabidopsis (Armbuster et al., 2014; Wang et al., 2017). As noted above, analyses of pmf partitioning between ΔΨ and ΔpH revealed a decrease in the putative ΔpH component. Notably, the ΔpH component could be also modulated by ATP hydrolysis/formation in the dark until an equilibrium between the pmf and the phosphorylating potential is reached (Cruz et al., 2001; Aloiiret et al., 2018). Considering the less reducing stromal $E_{\text{GSH}}$ in Δgr1, a difference in phosphorylating potential between WT and Δgr1 is possible, which could also explain differences in the putative ΔpH component.

Notably, the abundance of several known redox-regulated proteins was decreased in the absence of GR1, such as one FBPase isoform and the chloroplastic ATP synthase gamma subunit. It is tempting to speculate that the redox-status of specific cysteines is altered in Δgr1 plants, leading to changes in protein activity or to protein degradation (De Smet et al., 2019; Zaffagnini et al., 2019).
Conclusions

Investigating the role of dual-targeted GR1 in *P. patens* knockout mutants, we found that GR1 is relevant for maintaining plant growth and safeguarding photosynthesis at medium and higher light intensities. Focusing on the effects of a shifted stromal $E_{\text{GSH}}$, we found light intensity-dependent altered NPQ dynamics and zeaxanthin/antixeraxanthin levels, altered pmf partitioning between $\Delta \Psi$ and $\Delta F$, and increased ascorbate levels, as well as changes in electron transport and plastid protein abundance under light stress, indicating damage to PSII and increased demand for repair mechanisms. Notably, in vivo imaging of stromal $E_{\text{GSH}}$ revealed yet unknown GR1-dependent dynamics in dark/light transitions. These findings will stimulate photosynthesis redox research because the phenotype of $\Delta gr1$ mutants is likely a mixture of different effects: direct consequences of the shifted $E_{\text{GSH}}$ and the resulting mis-balance of downstream redox cascades and indirect consequences via ROS-induced protein damage (Figure 7). To understand the revealed link between shifted $E_{\text{GSH}}$ and photosynthetic efficiency, investigations of GSH metabolites and the redox state of thiol switches (Nietzel et al., 2020), as well as Grx function and glutathionylation of proteins, provides scope for future research.

EXPERIMENTAL PROCEDURES

The following procedures are described in Methods S1–S3: Measurement of anoxia-induced cyclic electron flow, glutathione and pigment analyses by high-performance LC, and parallel reaction monitoring.

Plant materials and growth conditions

*Physcomitrella patens* (Hedw.) Bruch & Schimp ecotype ‘Gransden 2004’ (International Moss Stock Centre (IMSC, http://www.moss-stock-center.org), accession number 40001) was grown axenically in agitated liquid Knop medium (250 mg L$^{-1}$ KH$_2$PO$_4$, 250 mg L$^{-1}$ KCl, 250 mg L$^{-1}$ MgSO$_4$, $\times$2H$_2$O, 1 g L$^{-1}$ Ca(NO$_3$)$_2$ $\times$ 4H$_2$O and 12.5 mg L$^{-1}$ FeSO$_4$ $\times$ 7H$_2$O, pH 5.8) (Reski and Abel, 1985) with micro-elements (ME) $\text{H}_{2} \text{BO}_3$, MnSO$_4$, ZnSO$_4$, KL, Na$_2$MoO$_4$ $\times$ 2H$_2$O, CuSO$_4$, Co(NO$_3$)$_2$, (Egener et al., 2002) in a growth cabinet under long day conditions (16:8 h, light/dark photodark; $2^\circ\text{C}$) at 100 $\mu$mol photons m$^{-2}$ sec$^{-1}$. For phenotypic and pigment analyses, *P. patens* was grown on KNOP ME agar plates (12 g L$^{-1}$ purified agar; Oxoid, Thermo Scientific, Waltham, MA, USA) at the indicated light intensity.

For measurements of photosynthetic parameters and preparation of proteins samples for MS/MS, *P. patens* protonema tissue was propagated under axenic conditions either on 9-cm or 4.5-cm grown axenically in agitated liquid Knop medium (250 mg L$^{-1}$ KH$_2$PO$_4$, 250 mg L$^{-1}$ KCl, 250 mg L$^{-1}$ MgSO$_4$, $\times$2H$_2$O, 1 g L$^{-1}$ Ca(NO$_3$)$_2$ $\times$ 4H$_2$O and 12.5 mg L$^{-1}$ FeSO$_4$ $\times$ 7H$_2$O, pH 5.8) (Reski and Abel, 1985) with micro-elements (ME) $\text{H}_{2} \text{BO}_3$, MnSO$_4$, ZnSO$_4$, KL, Na$_2$MoO$_4$ $\times$ 2H$_2$O, CuSO$_4$, Co(NO$_3$)$_2$, (Egener et al., 2002) in a growth cabinet under long day conditions (16:8 h, light/dark photodark; $2^\circ\text{C}$) at 100 $\mu$mol photons m$^{-2}$ sec$^{-1}$. For phenotypic and pigment analyses, *P. patens* was grown on KNOP ME agar plates (12 g L$^{-1}$ purified agar; Oxoid, Thermo Scientific, Waltham, MA, USA) at the indicated light intensity.

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Generation of PpGR1 knockout lines

A knockout construct for Pp1s13_127V6.1 was designed by amplifying homologous regions from genomic DNA with primer pairs PpGR1ko_5PHR_F/PpGR1ko_5PHR_R and PpGR1ko_3PHR_F/PpGR1ko_3PHR_R (Table S4), introducing BspQ1 restriction sites to the homologous ends. DNA fragments containing homologous regions were joined with an expression cassette (nopaline synthase promoter and terminator) for hygromycin phosphotransferase via a triple-template polymerase chain reaction (PCR). This construct eliminates a large part of the Pp1s13_127V6.1 coding sequence including the active site (Figure S1a). The knockout construct was subsequently ligated into the pJetT1.2 (Thermo Scientific, Waltham, MA, USA) vector, digested with BspQ1 and introduced via polyethylene glycol-mediated protoplast transformation (Hohe et al., 2004) into a newly generated line expressing TKTP-Grx1-roGFP2 (Schwarzländer et al., 2008; Speiser et al., 2018) stably integrated at the PTA2 locus under the control of the PpActin5 promoter (Kubo et al., 2013; Mueller and Reski, 2015). In regenerated plants that survived hygromycin selection, integration of the construct into the target locus was verified using primer pairs spanning the 5’ integration site (5P_F and H3b_R, Figure S1b) and the 3’ integration site (NosT_F and 3P_R) (Table S4). An absence of PpGR1 transcript for independent knockout lines was confirmed using the primer pair PpGR1_RT_F and PpGR1_RT_R in a reverse transcription PCR (Figure S1c, Table S4). Moss lines are available from the IMSC under the accession numbers: TKTP-Grx1-roGFP2#40 IMSC 40836, Ppgr#48 IMSC 40834 and Ppgr#488 IMSC 40835.

Microscopy

Microscopy was carried out using a LSM780 (attached to an Axio Observer.Z1) (Carl Zeiss, Oberkochen, Germany) using a 25× (Plan-Apochromat 25×/0.8 Imm Korr NA0.8) or 40× (C-Apochromat 40×/1.2W Korr NA1.2) objective. Bright-field images were taken with an AxioCam MRc (Carl Zeiss). Confocal laser scanning microscopy of roGFP2 redox state was achieved by consecutively exciting the roGFP2 with a 405 nm diode laser (at 2% power output) and a 488 nm argon laser (at 1% power output) in line switching mode, using constant detector gain and emission from 508 to 535 nm. Autofluorescence was recorded after excitation at 405 nm and emission from 430 to 470 nm. Chlorophyll autofluorescence was monitored after 488 nm excitation at an emission of 680 to 735 nm. Image intensities and 405/488 nm ratios were calculated per pixel using a custom MATLAB (MathWorks, Natick, MA, USA)-based software using background subtraction and autofluorescence correction (Fricker, 2016).

Transmission electron microscopy

Transmission electron microscopy was performed as described by Schuessele et al. (2016).

NBT staining

Gametophores were stained in a 0.1 mg ml$^{-1}$ NBT (Duchefa, Haarlem, The Netherlands) solution in 75 mM potassium phosphate buffer (pH 7.0) for 1.5 h in the dark or in the light (120 $\mu$mol photons m$^{-2}$ sec$^{-1}$). Chlorophyll was subsequently removed by incubation in 80% ethanol at 70°C (Lee et al., 2002).

Ascorbate assay

Total and reduced ascorbate in *P. patens* samples was quantified as described by Gillespie and Ainsworth (2007) with the
modification that 2,2'-bipyridyl was dissolved in 95% ethanol. Five to 100 mg of material was flash frozen in liquid nitrogen, homogenized in a bead mill (TissueLyser II; Qiagen, Valencia, CA, USA; 30 Hz for 2 × 1.5 min) and processed immediately.

GR activity assay

GR activity in plant extracts was assessed by following the reduction of DTNB ([5,5'-dithiobis-(2-nitrobenzonic acid)] in the presence of GSSG as described by Marty et al. (2009). Protonema grown in liquid culture was split 4 days after subculture, with one-half remaining under control conditions, whereas the other half was exposed to 4 h of 450 μmol photons m⁻² s⁻¹ (HL).

Metabolic labelling and MS/MS analysis

For isotopic labelling of P. patens, Ca(NO₃)₂ × 4H₂O in solid and liquid PpNO₃ media was replaced by Ca¹⁵NO₃₂ × 4H₂O (Cambridge Isotope Laboratories, Tewksbury, MA, USA). To obtain fully labelled protonema tissue, the protonema cultures were weekly sub-cultivated on fresh¹⁵N labelled solid PpNO₃ media for at least 4 months. To quantify differences in protein abundance between WT and agr1 background, labelled and unlabelled protonema of cpGrx1toGFP2 #40 and agr1 #48 were shifted from LL to HL for 1 h, including one label swap for each light intensity.

For protein extraction, approximately 500 mg of P. patens protonema tissue was harvested and the surface water removed. The samples were frozen in liquid nitrogen and 3 × homogenized using a MM300 mill (Retsch, Haan, Germany) for 30 s with a frequency of 30 Hz. Subsequently, 200–300 μl of protein extraction buffer (25 mM Trizma base, 1% (w/v) sodium dodecyl sulphate, 5 mM ethylenediamine tetraacetic acid, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM benzamidine) was added, followed by centrifugation at 2000 g for 2 min, and the protein concentration was determined in the supernatant using the bicinchoninic acid assay. Equal protein amounts from ¹⁴N and ¹⁵N-labelled samples were mixed and further processed in a filter-aided sample preparation protocol for MS-based analysis as described in Wisniewski et al. (2009). All LC-MS/MS analyses were carried out on a system composed of an Ultimate 3000 RSLcNano UPLC coupled via a nanospray interface to a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA).

Shotgun quantification

Peptides were pre-concentrated and desalted for 3 min on a trap column (Acclaim PepMap 100, 300 μm × 5 mm, 5 μm particle size, 100 A pore size; Thermo Fisher Scientific) using 2% (v/v) acetonitrile/0.05% (v/v) trifluoroacetic acid in ultrapure water at a flow rate of 10 μl min⁻¹. Gradient separation of peptides was performed on a reversed phase column (C18, Acclaim Pepmap, 75 μm × 50 cm, 2 μm particle size, 100 A pore size; Thermo Fisher Scientific) at a flow rate of 300 nl/min using the eluents 0.1% (v/v) formic acid in ultrapure water at a flow rate of 300 nl/min using the eluents 0.1% (v/v) trifluoroacetic acid in ultrapure water (A) and 80% (v/v) acetonitrile/0.1% (v/v) trifluoroacetic acid in ultrapure water (B). The gradient applied was: 25–80% B (v/v) over 105 min, 18–32% B (v/v) over 55 min, 32–99% B (v/v) over 5 min and 99% B (v/v) for 20 min.

MS full scans (MS1, m/z 300–1600) were acquired in positive ion mode at a resolution of 70 000 (FWHM, at m/z 200) with internal lock mass calibration on m/z 445.120025. For MS2, the 12 most intense ions were fragmented by higher-energy c-trap dissociation at 27% normalized collision energy (isolation window size: m/z 1.5). Resolution for MS2 scans: 17 500 (FWHM, at m/z 200), target values for automatic gain control: 1 × 10⁶ and 5 × 10⁴ for MS full scans and MS2, respectively. The intensity threshold for MS2 was set to 1 × 10⁵. Maximum fill times were 50 msec (MS1) and 55 msec (MS2). Unassigned charge states, charged state 1 and ions with charge state 5 and higher were rejected.

Bioinformatic analyses

LC-MS/MS data was processed with Proteome Discoverer, version 2.2 (Thermo Fisher Scientific). Raw files were searched using the SequestHT algorithm against a P. patens protein database based on the V1.6 gene models (Zimmer et al., 2013) supplemented with common contaminant proteins (cRAP; https://www.thegpm.org/crap) with the settings: precursor and fragment mass tolerances 10 ppm and 0.02 Da, respectively; minimum peptide length: 6; maximum of missed cleavages: 2; variable modifications: oxidation of methionine, N-acetylation of protein N-termini. For the identification of ¹⁵N-labelled peptides, a second database search was performed with ¹⁴N to ¹⁵N substitution(s) set as static modification for all amino acids. Peptide-spectrum-matches (PSMs) were filtered using the Percolator node to satisfy a false discovery rate of 0.01 (based on q-values). Identities were filtered to achieve a peptide and protein level false discovery rate of 0.01. LC-MS/MS/MS runs were chromatographically aligned with a maximum retention time drift of 10 min. Precursor ion quantification was performed using unique and razor peptides. Abundances were normalized to the maximum total peptide abundance in all files. Protein ratios (Δagr1 versus WT) were calculated using the ‘pairwise ratio based’ approach with subsequent hypothesis testing (background based t-test) for the calculation of P-values.

NPO measurements

In vivo fluorescence in P. patens was measured with a Maxi-Imaging PAM chlorophyll fluorometer (Heinz Walz, Effeltrich, Germany). Before measurements, 10-day-old LL grown protonema plates were exposed for 1 h to LL, CL or HL and dark-adapted for 40 min. NPO (non-photochemical quenching) was calculated as (Fm - Fo)/Fm‘. Fv (the variable fluorescence) was calculated as Fv'/Fo. The Fv/Fm ratio was used to evaluate the maximum PSII fluorescence in the fully dark-adapted state. Fm‘ represents the maximum PSII fluorescence in the dark-adapted state and in any light-adapted state, respectively; Fv/Fm‘ represents the minimum PSII fluorescence in the dark-adapted state (Kukuczka et al., 2014).

Spectroscopic measurements of photosynthetic parameters

Protonema from LL, CL and HL treated plates was measured with cellophane in buffer (Hepes 20 mM, pH 7.5, KCl 10 mM). LEF + CEF and CEF were measured by monitoring the relaxation kinetics of the carotenoid electrochromic band shift at 520 nm (corrected by subtracting the band shift at 546 nm) in the presence or absence of 10 μM DCMU and hydroxylamine, respectively. CEF and LEF + CEF were calculated as e⁻ sec⁻¹ PSI⁻¹ upon normalization to the PSI amount. The electrochromic shift signal upon excitation with a single saturating turnover flash (5 ns laser flash) in the presence or absence of 10 μM DCMU and 1 mM hydroxylamine was used to estimate the PSI and PSI + PSII amount. DCMU and hydroxylamine in this measurement were used to fully block PSII photochemistry to facilitate determination of the PSI amount (Terashima et al., 2012; Gerotto et al., 2016).

To measure the proton motive force (pHm), which consists of ΔpH (trans-thylakoid proton gradient) and membrane potential (Δψ), 5-day-old protonema tissue from liquid cultures was harvested, dark-adapted for 15 min before analysis and exposed for

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CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

SJMS, PD, MSchw, RR, MH and AJM planned and designed the research. SJMS, RW, DDG, JR, SK, MR, VL and FB performed experiments. MSchw analyzed data. SJMS and AJM wrote the manuscript. All authors discussed the data and approved the final version of the manuscript submitted for publication.

DATA AVAILABILITY STATEMENT

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (Vizcaíno et al., 2014) with the dataset identifier PXD012843. Proteomics data, accession numbers and annotation are provided in Data S1.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Identification of PpGR1 knockout mutants.
Figure S2. Δgr1 mutant phenotype details.
Figure S3. The ultrastructure of chloroplasts is not disrupted in Δgr1 mutants.
Figure S4. Δgr1 plants are viable in extended periods of darkness.
Figure S5. Δgr1 plants cannot profit from higher light fluencies.
Figure S6. NPO and Ylll in in WT and Δgr1 gametophores.
Figure S7. Δgr1 plants are light-sensitive. Measurements of CEF and Fv/Fm.
Figure S8. Experimental set-up for protein quantification via metabolic labelling.
Figure S9. Verification of GR1 protein absence in Δgr1 by targeted LC-MS/MS.
Table S1. Ascorbate measurements.
Table S2. Glutathione reductase activity.
Table S3. Pigment levels.
Table S4. Primer list.
Data S1. Proteomics data and functional annotation.
Methods S1. Anoxia-induced CEF.
Methods S2. HPLC analyses.
Methods S3. Parallel reaction monitoring.

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