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REVIEW PAPER

Reactive oxygen species and redox regulation in mesophyll and bundle sheath cells of C_4 plants

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Abstract

Redox regulation, antioxidant defence, and reactive oxygen species (ROS) signalling are critical in performing and tuning metabolic activities. However, our concepts have mostly been developed for C_3 plants since *Arabidopsis thaliana* has been the major model for research. Efforts to convert C_3 plants to C_4 to increase yield (such as IRRI's C_4 Rice Project) entail a better understanding of these processes in C_4 plants. Various photosynthetic enzymes that take part in light reactions and carbon reactions are regulated via redox components, such as thioredoxins as redox transmitters and peroxiredoxins. Hence, understanding redox regulation in the mesophyll and bundle sheath chloroplasts of C_4 plants is of paramount importance: it appears impossible to utilize efficient C_4 photosynthesis without understanding its exact redox needs and the regulation mechanisms used during light reactions. In this review, we discuss current knowledge on redox regulation in C_3 and C_4 plants, with special emphasis on the mesophyll and bundle sheath differences that are found in C_4. In these two cell types in C_4 plants, linear and cyclic electron transport in the chloroplasts operate differentially when compared to C_3 chloroplasts, changing the redox needs of the cell. Therefore, our focus is on photosynthetic light reactions, ROS production dynamics, antioxidant defence, and thiol-based redox regulation, with the aim of providing an overview of our current knowledge.

Key words: Antioxidant enzyme, bundle sheath cell, C_4 photosynthesis, mesophyll cell, NAD-ME, NADP-ME, reactive oxygen species (ROS), redox regulation, thiol, thioredoxin.

Introduction

Photosynthesis is the unique process by which cyanobacteria, some protists, and plants convert atmospheric CO_2 and water into complex organic molecules. Oxygenic photosynthesis has persisted for at least 2.5 billion years on Earth (Tipple and Pagani, 2007). It is achieved by two distinct stages, namely the light reactions that produce ATP and reducing power, mostly in the form of NADPH, and the Calvin–Benson–Bassham cycle (CBB cycle) that converts inorganic carbon into simple

Abbreviations: $^1O_2$, singlet oxygen; 2PG, 2-phosphoglycolate; 3-PGA, 3-phophoglycerate; AOX, alternative oxidase; APX, ascorbate peroxidase; Asc, ascorbate; BS, bundle-sheath; CAT, catalase; CBB cycle, Calvin–Benson–Bassham cycle; CET, cyclic electron transport; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; Fd, ferredoxin; FNR, ferredoxin-dependent NADPH reductase; GPX, glutathione peroxidases; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulphide; H_2O_2, hydrogen peroxide; LET, linear electron transport; MDHAR, monodehydroascorbate reductase; NAD-ME, NAD malic enzyme; NTRC, NADPH thioredoxin reductase C; NADP-ME, NADP malic enzyme; NDH, NAD(P)H dehydrogenase-like complex; $O_2^{\cdot-}$, superoxide anion radical; PCK, phosphoenolpyruvate carboxykinase; PEP, phosphoenol pyruvate; PEPC, phosphoenolpyruvate carboxylase; PET, photosynthetic electron transport; PGR5, proton gradient regulation 5; PGRL1, PGR5-like photosynthetic phenotype 1; PPDK, pyruvate orthophosphate dikinase; PQ, plastoquinone; PRX, peroxiredoxin; PSI, photosystem I; PSII, photosystem II; PTOX, plastid terminal oxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; TRX, thioredoxin; UCP, uncoupling protein.

organic molecules. The ancestral CBB cycle originated in prehistoric times when the levels of CO_2 were very high, whereas O_2 concentrations were much lower relative to present day values (Bekker *et al.*, 2004). Thus, ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), the first enzyme of the CBB cycle, evolved and initially fixed CO_2 in the absence of O_2, or at least in the presence of very low O_2 levels. Similarly, reduced photosystem reaction centres were able to transfer electrons to their acceptors without the potential intervention of O_2 and production of reactive oxygen species (ROS). The conditions of a high CO_2/O_2 ratio increased the efficacy of Rubisco to fix CO_2 due to low oxygenase activity and maximized the thermodynamic efficiency of the CBB cycle (Raven and Allen, 2003). The competition between Rubisco's oxygenase and carboxylase activity started almost 2.1 billion years ago due to the increase in atmospheric O_2 concentrations that resulted from the cyanobacteria that used oxygenic photosynthesis (Roy and Andrews, 2000). The initial product of the oxygenase reaction with the ribulose-1,5-bisphosphate substrate is one molecule of 2-phosphoglycolate (2PG) and one molecule of 3-phophoglycerate (3-PGA). To counteract the accumulation of 2PG, photosynthetic organisms developed several strategies, such as improvement of the kinetic properties of Rubisco (Tabita, 1999) and the development of the photorespiratory pathway (Bauwe *et al.*, 2010). The C_4 photosynthetic pathway evolved much more recently as an adaptation to high photorespiratory pressure caused by decreasing atmospheric CO_2, high temperatures, aridity, and/or salinity (Sage, 2004). Within the plant kingdom, the photosynthetic trait of C_4 metabolism came about by convergent evolution and it has evolved independently at least 66 times during the past 35 million years (Sage *et al.*, 2012). C_3 and C_4 photosynthesis share fundamental metabolic processes, such as the CBB cycle, light-harvesting complexes, and electron transport components. Hence, C_4 photosynthesis does not change the fundamental machinery of the CBB cycle, but functionalizes structural and biochemical additions around C_3 photosynthesis to improve its efficiency. Most C_4 plants fix the CO_2 in mesophyll cells with phosphoenolpyruvate carboxylase (PEPC), an enzyme that, unlike Rubisco, is insensitive to O_2. Subsequently, the CO_2 is released in the bundle sheath cells where Rubisco is localized and the CBB cycle occurs. This additional step increases the availability of CO_2 around Rubisco and minimizes its chance of catalysing the oxygenation reaction (Gowik and Westhoff, 2011). From a physiological point of view, C_4 photosynthesis has remarkable advantages over plants performing solely C_3 photosynthesis, since in C_4 leaves photorespiration remains very low under a range of environmental conditions and runs at about 3.5–6% of the total CO_2 assimilation rates (Carmo-Silva et al., 2008). In contrast, in C_3 leaves under current atmospheric CO_2 concentrations and at a temperature of 25 °C, photorespiration proceeds at about 20–30% of the rate of CO_2 fixation (Sage, 2004; Carmo-Silva *et al.*, 2008). Establishment of this CO_2-concentration mechanism requires adaptation in other cellular metabolic pathways in order to integrate the CO_2-pumping C_4 cycle.

Recently, high-throughput tools for examining the transcriptome, proteome, and metabolome at the cell or tissue level have greatly enhanced our knowledge about the biochemical and physiological mechanisms of C_4 photosynthesis; however, these tools have not yet revealed how C_4 photosynthesis is integrated into the cellular redox system. Photosynthesis can be considered as a series of redox reactions, and it is mutually linked with the cellular redox status (Dietz *et al.*, 2016). The photosynthesizing chloroplast is the main regulator of the cellular redox state in the light. Our knowledge about the production of ROS, redox regulation of photosynthesis, and antioxidant defence is mostly derived from C_3 plants. This started with the intensive use of, for example, spinach and pea in studies on photosynthesis, and continued with the model plant Arabidopsis, all of which have C_3 photosynthesis. However, efforts to convert C_3 plants to C_4 plants, especially to NADP-ME C_4 plants, (Li *et al.*, 2017), in order to increase yields (see C_4 RiceProject, http://c4rice.irri.org/, and von Caemmerer *et al.*, 2012) and meet our future food demands require us to have a better understanding of the regulatory processes in C_4 plants. Various photosynthetic enzymes that take part in the light reactions and carbon reactions are regulated via redox components. Due to its centrality, understanding redox regulation in chloroplasts is of paramount importance: it appears impossible to efficiently integrate C_4 photosynthesis with the existing regulatory and metabolic systems without understanding its exact redox necessities in metabolism and regulation. Therefore, this review addresses redox regulation in C_3 and C_4 photosynthesis. Special focus is given to the differences between mesophyll and bundle sheath cells based on recent progress in transcriptome, proteome, and metabolome research in C_4 plants.

Advantages of evolution of C_4 photosynthesis from a viewpoint of redox regulation

The development of C_4 photosynthesis from an ancestral C_3 state probably occurred with a smooth evolutionary trajectory (likened to a 'Mount Fuji landscape'; Heckmann *et al.*, 2013), without troughs of fitness along the way. The ancestral C_3 plant was probably highly photosynthetically active with dense venation, since evolution of intermediacy towards C_4 requires abundant, photosynthetically activated bundle sheath cells and sufficient photorespiratory flux via release of CO_2 into the bundle sheath by glycine decarboxylase to act as a CO_2 pump to sustain CO_2 enrichment (Bräutigam and Gowik, 2016). The evolution towards C_4 started with the shift of photorespiratory glycine decarboxylation to the bundle sheath (Sage, 2004; Schulze *et al.*, 2013; Mallmann *et al.*, 2014). Assuming high photosynthetic and therefore high photorespiratory flux, this shift resulted in increased CO_2 concentration in the bundle sheath and hence improved plant fitness under carbon-limiting conditions (Heckmann *et al.*, 2013). Indeed, the CO_2 compensation point is reduced in plants with a photorespiratory CO_2 pump (i.e. Rawsthorne *et al.*, 1988; Schlüter *et al.*, 2017). The distribution of photorespiration between two cell types also results in increased transcript abundances of the uncoupling protein

(UCP) and of mitochondrial alternative oxidase (mAOX), probably as a valve to detoxify NADH produced during glycine decarboxylation in the bundle sheath mitochondria of evolutionary intermediates (Schlüter *et al.*, 2017). Increasing release of NADH feeds into the respiratory electron transport chain and AOX counteracts over-reduction and excessive ROS development. In *Flaveria* species, this increase in mAOX transcripts is no longer detectable once C_4 is fully evolved, indicating that the redox imbalance no longer exists (Schlüter *et al.*, 2017).

The photorespiratory pumping of CO_2 also results in an amino-group imbalance between mesophyll and bundle sheath cells, which may have prompted the evolution of the C_4 cycle (Rawsthorne *et al.*, 1988; Mallmann *et al.*, 2014; Bräutigam and Gowik, 2016). A very low-level C_4 cycle may have immediately sprung up to transfer an amino group back to the mesophyll, and was then selected upon for increased fitness under carbon-limiting conditions (Mallmann *et al.*, 2014). Modelling predicts that during this phase of evolution, flux through the C_4 cycle increased and mesophyll Rubisco activity was reduced (Heckmann *et al.*, 2013; Mallmann *et al.* 2014). The selection of enzymes and transporters for the C_4 cycle was probably based on transcriptional abundance in the C_3 ancestor, since C_4 orthologues correspond to the most abundant isoforms in C_3 sister species (Emms *et al.*, 2016; Moreno-Villena *et al.*, 2017). There is currently no evidence for preferential selection based on regulatory or metabolic context. Once flux through the C_4 cycle is sufficient to supply the carbon needs of the plant, Rubisco can be terminally lost from the mesophyll, and concomitantly also photorespiratory 2PG production. C_4 plant species generally have reduced photorespiratory gene expression (Bräutigam *et al.*, 2011; Gowik *et al.*, 2011; Bräutigam *et al.*, 2014; Covshoff *et al.*, 2016), probably due to drift in the absence of the strong selective pressure that would fix C_4 photosynthesis as a trait (Bräutigam and Gowik, 2016). Loss of photorespiration also means loss of a sink for reducing equivalents and ATP (Bauwe, 2010), which is probably counteracted by the increased availability of CO_2 that acts as a larger sink for reducing equivalents and ATP in the CBB cycle. The optimization of the cycle and its efficient integration into the underlying C_3 metabolism were probably late events during C_4 evolution (Sage, 2004; Heckmann *et al.*, 2013). Comparisons of different C_4 species with C_3 species have not revealed general changes in regulatory or redox-based systems (Bräutigam *et al.*, 2014), probably because different species can use different decarboxylation enzymes, each of which may require its own adaptations.

Comparison of photosynthetic machinery of mesophyll and bundle sheath chloroplasts and dynamics of ROS production in these compartments

Three biochemical subtypes of C_4 photosynthesis have been distinguished based on the steps during concentration of CO_2, on the transported metabolites, and on the subcellular localization of the decarboxylation reactions and the type of enzymes used. Thus, C_4 plants are grouped into NAD malic enzyme-type (NAD-ME), NADP malic enzyme-type (NADP-ME), and phosphoenolpyruvate carboxykinase-type C_4 photosynthesis (PCK) (Hatch, 1987; Rao and Dixon, 2016). The metabolic pathways used by NADP-ME and NAD-ME plants are given in detail in Fig. 1. The two C_4

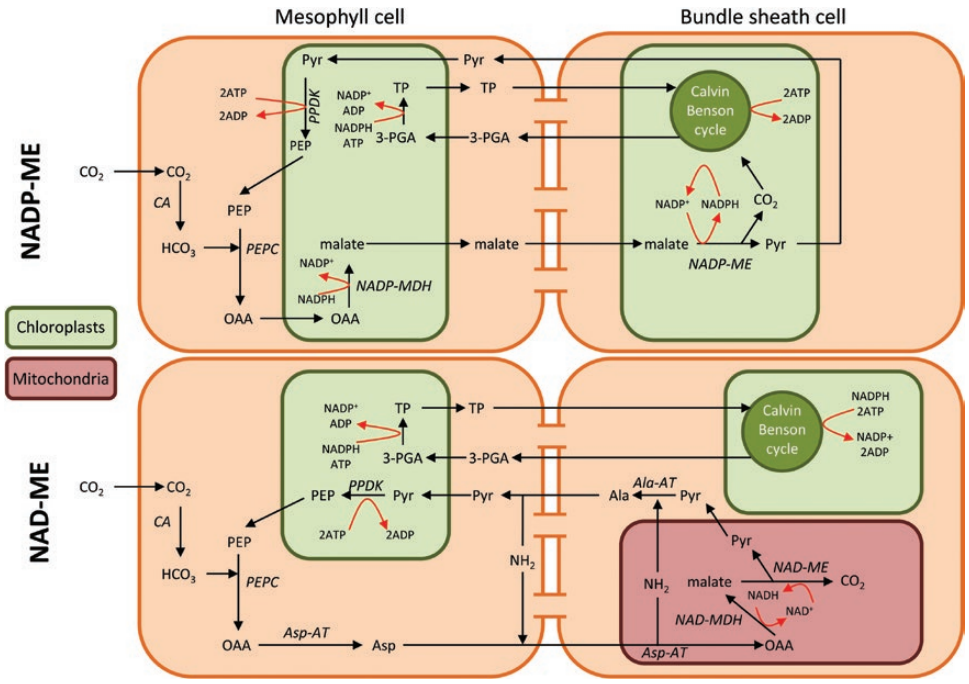

Fig. 1. Reactions involved in the NADP-ME and NAD-ME subtypes of C_4 photosynthesis. The NADP-ME subtype requires two NADPH and three ATP in mesophyll cells, and two ATP in bundle sheath cells since NADPH is metabolically shuttled to this cell type. For the NAD-ME subtype the corresponding values are one NADPH and three ATP for mesophyll cells and one NADPH and two ATP for bundle sheath cells. Reactions involving NADPH/$NADP^+$ and ATP/ADP are indicated with red arrows. 3-PGA, 3-phosphoglycerate; Asp-AT, aspartate aminotransferase; CA, carbonic anhydrase; NAD-MDH, NAD malate dehydrogenase; NAD-ME, NAD malic enzyme; NADP-MDH, NADP malate dehydrogenase; NADP-ME, NADP malic enzyme; OAA, oxaloacetic acid; PEPC, phosphoenolpyruvate (PEP) carboxylase; PPDK, pyruvate orthophosphate dikinase; Pyr, pyruvate; TP, triose phosphate.

cycles probably fundamentally differ in their cellular integration with regard to redox signalling as the decarboxylation enzymes inhabit different organelles: NADP-ME resides in the chloroplast and NAD-ME resides in the mitochondria.

NADP-ME and NAD-ME C_4 plants use at least five ATP and two NADPH for each CO_2 fixed (Laisk and Edwards, 2000), while C_3 species use three ATP and two NADPH for the CBB cycle and additional ATP to fuel photorespiration. In C_4 plants, the two additional ATP molecules are needed for regeneration of phosphoenol pyruvate (PEP) by pyruvate orthophosphate dikinase (PPDK). This ATP demand increases proportionally to the amount CO_2 leakage from the bundle sheath back to the mesophyll before it can be fixed by Rubisco (von Caemmerer and Furbank, 2003). Linear electron transport (LET) from photosystem II (PSII) to NADPH in photosynthetic electron transport (PET) creates a fixed ratio of ATP and NADPH, which is approximately 1.3–1.5 and is insufficient for the energetic needs of C_4 photosynthesis (Kramer and Evans, 2011).

Bundle-sheath (BS) chloroplasts of NADP-ME plants such as maize, sorghum, and sugarcane, are depleted of PSII or display very low PSII activity. They show little or no grana stacking (Laetsch, 1974; Rao and Dixon, 2016). It has been demonstrated that peripheral components of the water-splitting complex are especially depleted in maize and sorghum, indicating that the PSII activity in BS chloroplasts is limited due to a lack of oxidizing sites of the complex (Meierhoff and Westhoff, 1993). In these NADP-ME type plants, NADPH is produced metabolically in the BS during decarboxylation of malate. The reduction of 3-PGA by glyceraldehyde-3-phosphate dehydrogenase, the NADPH-consuming step of the CBB cycle, is divided between the mesophyll and the BS (Majeran *et al.*, 2008) (Fig. 1). Therefore, there is no need for the occurrence of LET, which explains the absence or low PSII activity in the BS chloroplasts. However, chloroplasts of NAD-ME plants do not show chloroplast dimorphism. In these plants, the C_4-acid aspartate is converted to malate and decarboxylated in the BS mitochondria to produce NADH, and therefore there is no mechanism to metabolically deliver NADPH from the mesophyll to the BS chloroplasts. Hence, PSII activity and grana development is enhanced and a higher rate of LET is observed in BS chloroplasts of NAD-ME plants (Takabayashi *et al.*, 2005). At the same time, a triosephosphate/3-PGA shuttle probably assists in distributing the CBB cycle reduction to both the mesophyll and BS (Bräutigam *et al.*, 2011) (Fig. 1).

To compensate for the additional ATP requirement in PEP regeneration, C_4 plants use cyclic electron flow (CET) around photosystem I (PSI), in which electron transport from ferredoxin (Fd) to plastoquinone (PQ) creates a proton gradient across the thylakoid membranes (ΔpH). Consistent with the increased need for CET to feed the energy requirements of PEP synthesis by PPDK, it has been demonstrated that C_4 plants show a higher degree of accumulation of PSI subunits at both the protein and mRNA levels in NAD-ME and NADP-ME C_4 species (Bräutigam et al., 2011; Nakamura *et al.*, 2013), but the cellular localizations of these increases differ. Possible PET pathways for the mesophyll and BS of NADP-ME plants are summarized in Fig. 2. In the NADP-ME species maize, which has no or reduced PSII activity in the

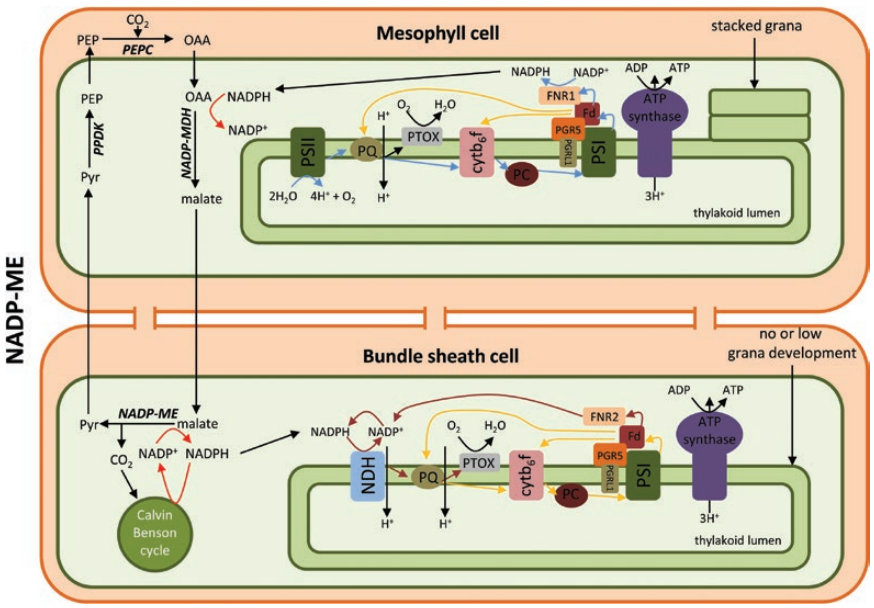

Fig. 2. Simplified overview of the photosynthetic electron transport (PET) reactions in mesophyll and bundle sheath (BS) cells of NADP-ME C_4 plants. Linear electron transport (LET, indicated with blue arrows) and cyclic electron transport (CET, indicated with orange arrows) and components involved in these reactions are shown. Note that mesophyll chloroplasts have stacked grana and higher photosystem II (PSII) activity, while no or little grana development is observed in BS chloroplasts. BS chloroplasts utilize CET around PSI via the NAD(P)H dehydrogenase-like complex (NDH) or the proton gradient regulation 5- PGR5-like photosynthetic phenotype 1 (PGR5-PGRL1) complex. Chlororespiration involving NDH, plastoquinon (PQ), and plastid terminal oxidase (PTOX) is indicated with maroon arrows. Fd, ferredoxin; FNR, ferredoxin NADPH reductase; NADP-MDH, NADP malate dehydrogenase; NADP-ME, NADP malic enzyme; OAA, oxaloacetic acid; PC, plastocyanin; PEPC, phosphoenolpyruvate (PEP) carboxylase; PPDK, pyruvate orthophosphate dikinase; Pyr, pyruvate.

BS, it has been shown that PSI levels are 1.6-fold higher in BS chloroplasts (Majeran *et al.*, 2008). CET can be mediated by feeding electrons into the NAD(P)H dehydrogenase-like complex (NDH) or the proton gradient regulation 5-PGR5-like photosynthetic phenotype 1 (PGR5-PGRL1) complex (Shikanai, 2014; Suorsa *et al.*, 2016). Nakamura *et al.* (2013) demonstrated that accumulation of NdhH, a subunit of the NDH complex, increases with the progression of C_4 evolution in the genus *Flaveria*, where C_3, C_3-C_4, C_4-like, and C_4 species show a linear increase of NdhH levels with a maximum of 10-fold in C_4 species. In terms of cell type, NdhH in NADP-ME C_4 plants accumulates in the BS, with BS/mesophyll ratios of 2.75, 1.6, and 3 for maize, *Portulaca grandifolia*, and *Flaveria*, respectively (Takabayashi *et al.*, 2005; Majeran *et al.*, 2008; Nakamura *et al.*, 2013). In contrast to NADP-ME C_4 plants, BS chloroplasts in NAD-ME C_4 plants retain a functioning PSII. In these species, NDH accumulation is increased in the mesophyll when compared to the BS. Takabayashi *et al.* (2005) showed that the mesophyll cells of the NAD-ME plant *Portulaca oleracea* have 3-fold higher NDH levels than the BS. It has been concluded that the pattern of NDH accumulation is inversely related to PSII accumulation. Thus, NDH accumulates in cells needing high ATP/NADPH ratios (Ishikawa *et al.*, 2016). Other differences in NADP-ME C_4 species concern the accumulation of PGR5 and PGRL1, which are assumed to form a complex with PSI (DalCorso *et al.*, 2008). Within the genus *Flaveria*, it was found that PGR5 and PGRL1 levels were three-fold higher in species with C_4 photosynthesis. Among different species, the respective protein levels only increased in species lacking grana in BS chloroplasts (Nakamura *et al.*, 2013). In maize, PGRL1 accumulated six times more in BS chloroplasts while no differences were detected for PGR5 between the two cell types. However, in *F. bidentis* (C_4), both proteins were equally distributed. These findings indicate that C_4 plants use the PGR5-PGRL1 pathway to differing degrees. To modulate potential excess flux through PET, chloroplasts evolved mechanisms for relaxation. One of these safety valves is the plastid terminal oxidase (PTOX or IMMUTANS), which is an alternative oxidase (AOX)-like protein (Krieger-Liszkay and Feilke, 2016). By oxidizing the PQ pool, PTOX transfers the electrons to O_2 (forming water) to relax electron pressure during LET. In addition, it can also function in chlororespiration in which stromal reducing power (NADPH) is transferred to the PQ pool by NDH and then to O_2 via PTOX (Foudree *et al.*, 2012). Friso *et al.* (2010) demonstrated that PTOX in maize accumulated in BS chloroplasts where CET was predominant. In this case, PTOX might be important for regulating the stromal redox state and CET around PSI in BS chloroplasts of NADP-ME C_4 plants.

In summary, the data show that the distribution of CET between mesophyll and BS cells and the CET/LET ratio change with the carboxylation pathway used in the species, namely C_3, NADP-ME C_4, or NAD-ME C_4. It is, however, unclear, how the redox control of electron partitioning between LET, CET, and the Mehler reaction (also known as the water–water cycle, part of the Asada–Halliwell–Foyer cycle; Asada, 2006; Dietz *et al.*, 2016) is accomplished when the CBB cycle is distributed between two cell types with different energy requirements.

Antioxidant metabolism in C_3 and C_4 plants

One of the inevitable consequences of oxygenic photosynthesis is the production of ROS, such as the superoxide anion radical ($O_2^{\cdot-}$) and H_2O_2 mainly at PSI (Asada *et al.*, 1974) and singlet oxygen ($^1O_2$) mainly at PSII (Telfer *et al.*, 1994), with over-excitation of PET (Dietz *et al.*, 2016). Production of $O_2^{\cdot-}$ is known as the Mehler reaction (Mehler, 1951), which only proceeds at low rates and functions in regulation (Heber, 2005). The dismutation of $O_2^{\cdot-}$, spontaneously or via superoxide dismutase (SOD), produces H_2O_2 that can be further reduced to water by different classes of peroxidases or catalase (CAT) (Mittler *et al.*, 2004). The flux into $O_2^{\cdot-}$ at PSI is modulated by the redox status, and while it is probably present in all chloroplasts in C_4 plants, most likely differs depending on the presence of LET. $^1O_2$ is produced by the reaction of triplet-state chlorophyll (^{3}Chl) with O_2 in its ground state ($^3O_2$), and $^1O_2$ production is especially increased under high light intensities, when the PQ pool is over-reduced (light absorption >PET) (Dietz *et al.*, 2016). Since $^1O_2$ is produced during LET at PSII, it is absent in BS chloroplasts of NADP-ME species. ROS generated during PET act as messengers (Dietz *et al.*, 2016) while at the same time posing a risk to the chloroplasts and requiring detoxification.

Redox homeostasis in the plant cell is defined as the balance between oxidation and reduction reactions (Foyer and Noctor, 2005). Hence, scavenging of ROS produced during photosynthesis is a vital part of this regulatory network, since it acts as a sink for reducing power and protects PET components. Plants have evolved a variety of enzymatic and non-enzymatic components for ROS scavenging; these include SOD, CAT, ascorbate peroxidase (APX), and various thiol-based peroxidases such as glutathione peroxidases (GPX) and peroxiredoxins (PRX). The scavenging enzymes are supported by glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), and dehydroascorbate reductase (DHAR), which are responsible for shifting the redox state of the Asc/DHA and GSH/GSSG redox couples to the reduced form (Mittler *et al.*, 2004). Among these components, SOD, APX, DHAR, MDHAR, and GR in conjugation with Asc and GSH form the ascorbate-dependent water–water cycle (Asada–Halliwell–Foyer cycle) in photosynthesis, which starts with oxidation of water by PSII and ends with reduction of H_2O_2 to water (Asada, 2006). There exists an ascorbate-independent water–water cycle, that relies on Fd/Fd-dependent thioredoxin reductase (FTR)/thioredoxin (TRX) or NADPH/NADPH thioredoxin reductase C (NTRC) and PRX (Dietz *et al.*, 2006). The total activities of these antioxidant enzymes have been compared in sunflower (C_3) and sorghum (C_4 NADP-ME) (Zhang and Kirkham, 1996), wheat (C_3) and maize (C_4 NADP-ME) (Stepien and Klobus, 2005; Nayyar and Gupta, 2006), *Cleome spinosa* (C_3) and *C. gynandra* (C_4 NAD-ME) (Uzilday *et al.*, 2012), *F. robusta* (C_3) and *F. anomala* (C_3-C_4 intermediate), *F. brownii* (C_4-like), and *F. bidentis* (C_4 NADP-ME)

(Uzilday *et al.*, 2014). Meta-analysis of all these data indicates some differences in the total activities of antioxidant defence enzymes that are unrelated to the prevailing metabolism. However, restriction to a comparison of C_4 plants of the NADP-ME type with C_3 plants reveals a significant correlation for some enzyme activities. For example, APX and GR activities are always higher in NADP-ME C_4 plants, while CAT activities are higher in C_3 plants. This relationship is confirmed by the gradual increase in APX and decrease in CAT activity observed in *Flaveria* species belonging to different evolutionary steps of C_4 photosynthesis (Uzilday *et al.*, 2014). As well as the decrease in total activity, a CAT isoform detected in native activity gels and observed in non-C_4 species (*F. robusta*, *F. anomala*, *F. brownii*) was lost in *F. bidentis*, which has evolved a complete C_4 carboxylation mechanism, suggesting a role for this CAT isoform in the context of photorespiration (Uzilday *et al.*, 2014). However, in contrast to NADP-ME C_4 plants, NAD-ME C_4 *C. gynandra* has lower APX activity when compared to C_3 *C. spinosa* (Uzilday *et al.*, 2012). The same phenomenon was also observed in *Bienertia sinuspersici*, which is a single-cell C_4 plant that uses the NAD-ME subtype (Uzilday *et al.*, 2018). Young leaves of *B. sinuspersici* exhibit C_3 photosynthesis while mature leaves develop full NAD-ME-dependent C_4 photosynthesis. In this plant species, the cell is divided into peripheral and central compartments that contain dimorphic chloroplasts and act as analogues of mesophyll (peripheral compartment) and bundle sheath (central compartment) chloroplasts (Lara *et al.*, 2008). During transition from C_3 to single-cell C_4 photosynthesis in *B. sinuspersici*, total APX activity decreases by 4-fold, while immunodetectable PRXQ increases by about 5-fold (Uzilday *et al.*, 2018), suggesting that the contribution of APXs and PRXs to the H_2O_2-scavenging metabolism depends on the type of photosynthesis and may be adjusted in a reciprocal manner in this species. When either one of the chloroplastic APX or PRX activity is inhibited via genetic mutations, the other is induced to compensate for the decrease in H_2O_2-scavenging capacity (Baier *et al.*, 2000; Kangasjärvi *et al.*, 2008), suggesting that the total peroxide-scavenging capacity of chloroplasts operates in a balance between APX and PRX activity depending on the metabolic situation. The switch from APX to PRX may also indicate a shift from a preferential role in detoxification to one in signalling.

The antioxidant capacity of phosphoenolpyruvate carboxykinase (PCK) plants has not been studied. Thus, at present, redox regulation in PCK plants cannot be included in our comparison. The overall decrease in antioxidant capacity in NAD-ME plants may reflect a higher capacity to funnel reducing power into the CBB cycle. The counterintuitive increase in NADP-ME plants may result from the sole occurrence of LET in mesophyll cells, which has to supply reducing power to both cell types and which may incur more regulatory challenges.

Within a single NADP-ME C_4 plant, the abundance of antioxidant enzymes differs between the mesophyll and the BS. In maize, tocopherol synthesis, GR, thylakoid-bound APX, and DHAR as well as thiol peroxidases predominantly accumulate in the mesophyll cells, whereas SOD appears to be nearly evenly distributed (Doulis *et al.*, 1997; Friso *et al.*, 2010). SODs scavenge $O_2^{\cdot-}$, which ultimately stems from PSI, and are therefore required in both the mesophyll and BS. The mesophyll-dominant thiol peroxidases can detoxify reactive lipid species formed by $^1O_2$ (Dietz *et al.*, 2016). The preferred accumulation of tocopherol-synthesizing enzymes in the mesophyll may also reflect the presence of PSII, which may generate singlet oxygen (Dietz *et al.*, 2016). In the case of GR, it has been shown that post-transcriptional regulation prevents accumulation of GR in the BS of maize (Pastori *et al.*, 2000). The distribution of antioxidant enzymes suggests that antioxidants in maize leaves are partitioned between the BS and mesophyll according to the availability of reducing power, and hence of LET. There appears to be the need for transport of oxidized forms of redox couples such as GSSG and DHA to the mesophyll for regenerating the reduced forms of GSH and Asc, respectively. Interestingly, Bilska and Sowinski (2010) have shown that maize plasmodesmata are closed during chilling stress, which might intervene in the transfer of redox couples besides C_4 metabolites. Whether this phenomenon also occurs during other environmental stresses is unknown and is worth investigating. Elucidation of this dynamic transport and metabolism seems to be important because the inability to transfer reducing power via plasmodesmata would expose the BS of NADP-ME-type plants to oxidative stress. This hypothesis is in agreement with the fact that BS proteins are more sensitive to oxidative damage than mesophyll proteins exposed to paraquat or low temperature (Kingston-Smith and Foyer, 2000). If this is the case, then breeding or genetic manipulations that can sustain plasmodesmatal transfer during stress might increase both the stress tolerance and the photosynthetic capacity of C_4 plants.

The thiol network of mesophyll and bundle-sheath chloroplasts

The dual nature of ROS as both signaling molecules and toxic agents necessitates not only particular detoxifying mechanisms but also alterations to the signal processing network. Mesophyll chloroplasts of the NADP-ME C_4 plant maize display a LET where water-splitting in PSII is linked to the reduction of NADP. Fd serves as the electron distribution hub in mesophyll chloroplasts of maize, similar to as it does in chloroplasts of C_3 plants. The maize genome codes for four Fds, namely ZmFdI, FdII, FdV, and FdIX. The predominant ZmFdI donates electrons to ferredoxin-dependent NADPH reductase (FNR) 1, 2, and 3 (Goss and Hanke, 2014). In maize mesophyll chloroplasts, thioredoxins (TRXs) can be reduced by Fd-dependent thioredoxin reductase (FTR). All classes of chloroplast TRXs known from Arabidopsis have been identified in maize at the protein level (Friso *et al.*, 2010). Importantly, mesophyll chloroplasts can also generate $O_2^{\cdot-}$ and subsequently H_2O_2 via the Mehler reaction, and thus they maintain a water–water cycle with ascorbate peroxidase (Ivanov and Edwards, 2000). However, it is questionable whether the water–water cycle reaches appreciable rates and thus whether it may function as a major alternative electron sink if NADPH accumulates. Driever and Baker (2011)

observed little O_2 uptake in maize leaves at the CO_2 compensation point and 3% O_2, and also during induction of photosynthesis. The authors proposed a single role of the Mehler reaction-derived ROS in cell signaling (Mullineaux and Baker, 2010; Driever and Baker, 2011). The physiological observations coincided with histochemical results where, under control conditions, $O_2^{\cdot-}$ and H_2O_2 were undetectable both in the mesophyll and BS chloroplasts (Omoto *et al.*, 2013).

The signaling function of ROS is related to the thiol redox regulatory network of chloroplasts, where redox input elements via redox transmitters reduce redox target proteins (Fig. 3). For reversibility of redox regulation, the network depends on redox sensors that react with ROS with high affinity. ROS act as final electron acceptors, representing the electron sinks in the thiol network. Thiol peroxidases, PRX and GPX, take over the function as redox sensors (Dietz, 2008). Thiol peroxidases have some specificity towards certain peroxides, but in general they display a very high substrate affinity to H_2O_2, and in some cases also to alkylhydroperoxides and peroxynitrite, and thus they efficiently react with ROS even at low concentrations (Dietz, 2016). The simultaneous supply of reductant in the light by the LET to redox transmitters and the generation of ROS allows for balancing the redox state of the thiol redox regulatory network of the mesophyll chloroplast (Fig. 3A). Analysis of the proteomes of mesophyll and BS chloroplasts provides insight into the relative distribution of the components of the thiol-disulfide network between these plastid types (Friso *et al.*, 2010). The lowest relative protein levels in maize BS chloroplasts were observed for NTRC (BS/Mesophyll=0.09), Fd3 (0.10), Fd1 (0.12), and FTRB (0.14). In this group of proteins with high abundance in mesophyll chloroplasts there were also three glutaredoxins and an ascorbate peroxidase-like protein (Table 1). It was obvious from this proteomic study that many redox input elements, redox transmitters, and sensors were enriched in mesophyll chloroplasts. Among the sensors, PRXQ was particularly abundant in the mesophyll chloroplasts (BS/M=0.36), indicating a function in the context of LET. PRXQ is associated with the thylakoids (Lamkemeyer *et al.*, 2006), has been suggested to be localized in the thylakoid lumen (Petersson *et al.*, 2006), and assists in protecting the photosynthetic metabolism in Arabidopsis and cyanobacteria from oxidative stress (Lamkemeyer *et al.*, 2006; Tailor and Ballal, 2017).

In a converse manner, BS chloroplasts essentially lack LET and produce $O_2^{\cdot-}$ and H_2O_2 at much lower rates than mesophyll chloroplasts (Omoto *et al.*, 2013). CET generates a proton motive force to generate ATP. Levels of PGR5 and NDH-subunits that are involved in CET increase with the establishment of C_4 photosynthesis in *Flaveria* genotypes, as discussed above. The increase is particularly strong in the BS, showing the preferential role of CET in this plastid

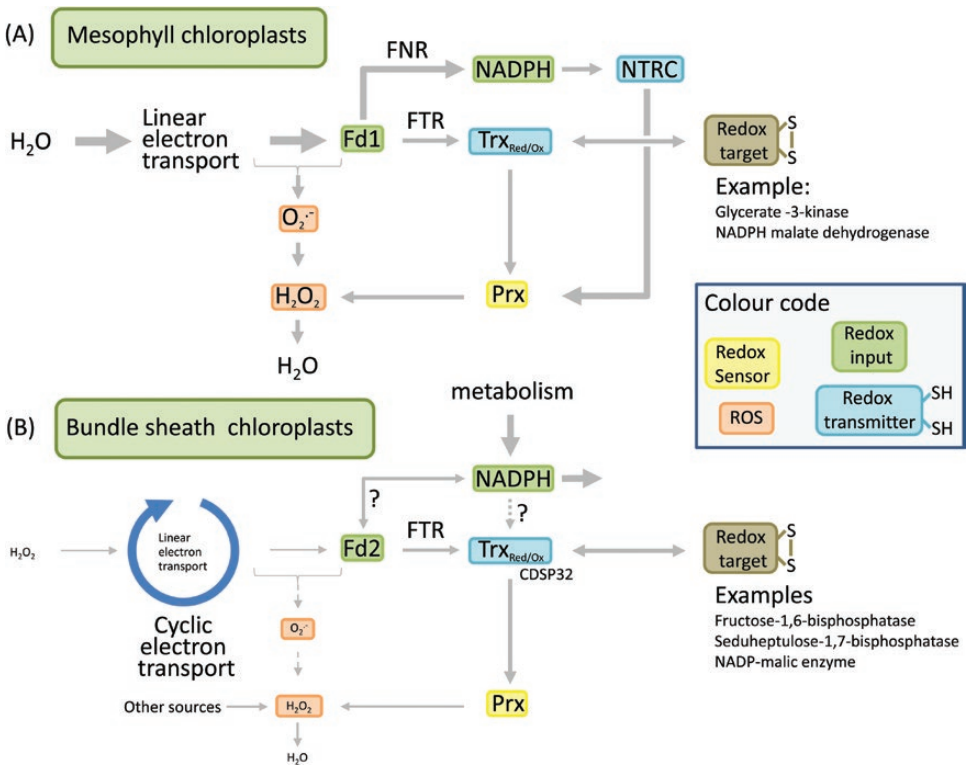

Fig. 3. Depiction of the thiol regulatory network of mesophyll and bundle sheath chloroplasts of maize. (A) In the mesophyll, linear electron transport produces NADPH and thioredoxin (TRX) via ferredoxin (Fd1). TRXs reduce target enzymes such as glycerate-3-kinase, but also peroxiredoxins (PRXs). The predominant reductant of 2-CysPrx is NADPH thioredoxin reductase C (NTRC). Linear electron transport also produces H_2O_2, which oxidizes PRXs. Oxidized PRXs oxidize TRXs. This mechanism balances the redox state of the target proteins and their activity. (B) Cyclic electron transport in the bundle sheath scarcely reduces Fd and little $O_2^{\cdot-}$ is released; ROS come from other sources. NTRC is missing. NADPH is provided by metabolic reactions. Reduction of TRXs such as CDSP32 and TRX-*f* is probably achieved by NADPH-dependent processes. PRXs are oxidized at lower rates than in mesophyll chloroplasts, but they also contribute to redox target oxidation.

Table 1. *Distribution of redox and ROS network elements between mesophyll and bundle sheath chloroplasts: the network elements are sorted from preferential mesophyll to preferential bundle-sheath localization.*

Highly enriched in mesophyll		**BS/M<0.25**
Transmitter	Glutaredoxin, various	0.05–0.10
Transmitter	NTRC	0.09
Input	Fd1	0.10
Antioxidant	Ascorbate peroxidase	0.10
Input	FTR-B	0.14
Antioxidant	MDHAR	0.23
Preferentially in mesophyll		**0.25<BS/M<1**
Input/Metabolism	FNR-1	0.26
Input	Fd2-1	0.33
Input/metabolism	Glutathione reductase	0.35
Antioxidant	DHAR-2	0.35
Sensor	PrxQ	0.38
Transmitter	Trx-f1	0.40
Input	FNR-1	0.40
Antioxidant	tAPX	0.41
Input	FTR-A	0.41
Transmitter	Trx-x	0.41
Transmitter	Trx-y1	0.42
Transmitter	ACHT2-Trx	0.45
Transmitter	Trx-f2	0.46
Sensor	GPX-2	0.46
Metabolism/Input	γ-ECS/GSH1	0.66
Input	FTR-A	0.68
Antioxidant	Cu,Zn-SOD	0.73
Sensor	2-CysPrx	0.74
Transmitter	Trx-m2, m4	0.75
Sensor	PrxIIE	0.76
Transmitter	Trx-m4	0.78
Input	Fd-like	0.78
Preferentially in bundle sheath		**BS/MS>1**
Antioxidant	APX	2.22
Input	Fd2-2	3.15
Transmitter	Trx-h2	4.76
Transmitter	CDSP (Trx)	22.82

The color coding is taken from Fig. 3: input elements are labelled green, transmitters blue, and sensors yellow. The red shading groups proteins according to their BS/MS ratio. The data are taken from Friso *et al.* (2010).

(Munekage *et al.*, 2010). Except for the thioredoxin-like protein CDSP32, which was essentially localized exclusively in the BS, and TRX-h2, which may be a cytosolic contamination of BS chloroplasts, all other TRXs were depleted from the BS chloroplasts (Table 1). It is tempting to speculate that CDSP32 takes over the function of NTRC in BS chloroplasts. CDSP32 donates electrons to 2CysPRX (Broin *et al.*, 2002) and its deletion causes enhanced photooxidative stress and 2CysPRX oxidation in potato (Broin and Rey, 2003).

Considering the uneven distribution of the elements of the thiol regulatory network between the mesophyll and BS chloroplasts, it is hypothesized that a similar state of redox regulation of target proteins can be achieved by distinct metabolic states. In the mesophyll cells, LET generates NADPH at high rates, and $O_2^{\cdot-}$ and H_2O_2 are produced at appreciable rates sufficient for redox regulation, and thus the regulatory thiol network is operated with significant turnover between the reduction-oxidation cycle of the individual redox target proteins. In the BS, NADPH is generated in metabolic reactions. TRXs including CDSP32 donate electrons to PRXs at low rates. Considering the lower rate of $O_2^{\cdot-}/H_2O_2$ generation in the BS, this lower reduction rate is assumed to be sufficient to maintain the PRX system in a balanced redox state, enough to catalyse oxidation of target proteins if the reduction by specific redox-transmitting TRXs ceases, for example upon transfer to darkness or low light. An open question is where the regulatory amounts of ROS are generated in the BS if the rate of the Mehler reaction is insignificant. Other sources of ROS may be: (i) via incomplete suppression of photorespiration, particularly in high light (Kromdijk *et al.*, 2010), and thus leakage of ROS from peroxisomes; (ii) via production in mitochondria; or (iii) via photosynthetic electron flow by other mechanisms, as described for tobacco (Michelet and Krieger-Liszkay, 2012).

Redox regulation in C_4 photosynthesis has been described for several photosynthesis-related enzymes in maize. Maize fructose-1,6-bisphosphatase and sedoheptulose-1, 7-bisphosphatase are activated by TRX-*f*, as are their C_3 counterparts (Nishizawa and Buchanan, 1981). The recently proposed requirement for NTRC and 2CysPrx for proper redox regulation of fructose-1,6-bisphosphatase in *Arabidopsis thaliana* (Pérez-Ruiz *et al.*, 2017) probably must be modified in BS chloroplasts since, as mentioned above, NTRC appears to be absent from the BS. Alternatively, there is no need for NTRC in the BS chloroplasts due to low ROS production in CET. Glycerate-3-kinase produces glycerate-3-phosphate in the mesophyll (Bartsch *et al.*, 2010). It is regulated by Cys-residues in a small C-terminal extension. The oxidized form is poorly active and prevails at night. TRX-*f* activates glycerate-3-kinase in the light and relieves the autoinhibition (Bartsch *et al.*, 2010). It has been proposed that mesophyll-derived triosephosphate is needed to build up CBB-cycle intermediates in the BS chloroplast for rapid activation of CO_2-fixation (Stitt and Zhu, 2014). Another example of redox-regulated target proteins is the BS-localized NADP-ME (Alvarez *et al.*, 2012). NADP-ME exists in two isoforms, one with a housekeeping function and one with a function in C_4 photosynthesis. The latter decarboxylates malate imported from the mesophyll and concomitantly releases NADPH. It is activated upon reduction by TRXs and inactivated upon oxidation (Drincovich and Andreo, 1994; Alvarez *et al.*, 2012). Redox regulation assures that CO_2 release from malate stops when environmental conditions inhibit photosynthesis. In addition, mesophyll-localized NADP-MDH is also redox-regulated by the Fd–TRX system. Similar to NADP-ME, NADP-MDH is also activated upon reduction and inactivated with oxidation (Lemaire *et al.*, 2007). Each of these redox-regulated target proteins undergoes reduction upon illumination and oxidation upon darkening or in low light. The oxidation by the thiol network must be precise and timely. The different scenarios for redox regulation in the mesophyll and BS chloroplasts as outlined in this paragraph are summarized in Fig. 3. The main differences concern the generation of ROS,

the reduction pathway of PRXs, and also the reduction of TRXs. These all await further scrutiny.

What does it take to make a C_4 plant in terms of redox regulation?

ROS production and redox regulation are specific for the type of decarboxylation enzyme a C_4 plant uses and therefore the PET capacities of the chloroplasts in the mesophyll and BS. NAD-ME plants with C_3-type chloroplasts in both the mesophyll and BS have reduced capacities with regards to some antioxidant enzymes. The reduction probably stems from a relaxation of redox stress due to high levels of CO_2 and therefore high demand for reductant in the CBB cycle. NADP-ME plants with two different chloroplast types, one of which lacks PSII, have increased capacity in antioxidant enzymes and a shift of thiol network components to the mesophyll cells. Both the increase and the shift probably stem from the challenges to redox poise imposed by two cell types being dependent on the LET in only one of them. It has previously been suggested that at least the following evolutionary steps need to be achieved in order to convert a C_3 plant to a C_4 (Schuler *et al.*, 2016): increasing vein density and BS to mesophyll ratio; enlargement of the BS; engineering of dimorphic chloroplasts; and, finally, compartmentalization of photosynthetic enzymes between the BS and mesophyll. However, our current knowledge as covered in this review strongly suggests that it is also necessary to integrate attempts at engineering a C_4 metabolic pathway to a C_3 chassis with the redox network of the cell. Acquiring the knowledge to distribute LET and CET between the BS and mesophyll cells seems to be a requirement, which is only possible with an in-depth understanding of how these processes are regulated in C_4 plants during growth and development, and under fluctuating conditions. One of the most challenging endeavours for achieving distribution of PET seems to be the efficient over-expression within a cell type of the NDH complex (which is encoded by more than 30 genes; Takabayashi *et al.*, 2009) integrated with the ATP requirements of the two cell types. Intervening in PET would inevitably change the dynamics of ROS production in the two cell types, which would then further require an adjustment in antioxidant defence. On the other hand, as well as the components of light reactions, C_4 enzymes, and CBB enzymes, there is also a need to engineer the thiol network required to control the light-driven regulation of photosynthesis in both cell types, which would sense and transduce the redox status and regulate target proteins, without losing its specificity.

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