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REVIEW PAPER

Photosynthesis and chloroplast redox signaling in the age of global warming: stress tolerance, acclimation, and developmental plasticity

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Abstract

Contemporary climate change is characterized by the increased intensity and frequency of environmental stress events such as floods, droughts, and heatwaves, which have a debilitating impact on photosynthesis and growth, compromising the production of food, feed, and biofuels for an expanding population. The need to increase crop productivity in the context of global warming has fueled attempts to improve several key plant features such as photosynthetic performance, assimilate partitioning, and tolerance to environmental stresses. Chloroplast redox metabolism, including photosynthetic electron transport and CO_2 reductive assimilation, are primary targets of most stress conditions, leading to excessive excitation pressure, photodamage, and propagation of reactive oxygen species. Alterations in chloroplast redox poise, in turn, provide signals that exit the plastid and modulate plant responses to the environmental conditions. Understanding the molecular mechanisms involved in these processes could provide novel tools to increase crop yield in suboptimal environments. We describe herein various interventions into chloroplast redox networks that resulted in increased tolerance to multiple sources of environmental stress. They included manipulation of endogenous components and introduction of electron carriers from other organisms, which affected not only stress endurance but also leaf size and longevity. The resulting scenario indicates that chloroplast redox pathways have an important impact on plant growth, development, and defense that goes beyond their roles in primary metabolism. Manipulation of these processes provides additional strategies for the design of crops with improved performance under destabilized climate conditions as foreseen for the future.

Keywords: Alternative electron transport, chloroplast redox metabolism, climate change, environmental stress, extreme environments, leaf development, photosynthesis, reactive oxygen species, retrograde signaling

Introduction

The Green Revolution of the mid-20th century doubled crop production over a 50 year period, achieved through a combination of improved breeding, more rational agricultural practices, and an extended suite of agrochemicals (Gutteridge, 2018). A similar duplication should be accomplished by 2050 to cope with population growth and demands, requiring novel crops

with major yield improvements and better resilience to environmental hardships. Within this context, enhancing photosynthesis is an obvious goal, and a feasible one too, in principle, because overall photosynthetic efficiency is relatively low due to physical limitations in the use of solar energy and other thermodynamic constraints imposed by the photochemical processes (Ort *et al.*, 2015; Cardona *et al.*, 2018; Baslam *et al.*, 2020). The efficiency of photosynthesis is also limited by the energy invested on protecting the photosynthetic machinery from photodamage (Kromdijk *et al.*, 2016; Alboresi *et al.*, 2019; Fernández-Marín *et al.*, 2020). The theoretical maximum efficiency of light energy conversion into biomass is estimated to be 4–6% depending on the type of photosynthesis (C_3 or C_4), with much lower values normally observed under field conditions (Dahal *et al.*, 2019). The existence of such ample room for improvement prompted many attempts to increase the performance of both the light and dark reactions using a variety of experimental strategies (López-Calcagno *et al.*, 2020; Martin-Avila *et al.*, 2020; Matsumura *et al.*, 2020). The many promising results obtained in this very active field of research are beyond the scope of this article, and the reader is referred to various recently published reviews on the subject for more comprehensive accounts of these advances (see, for instance, Cardona *et al.*, 2018; Baslam *et al.*, 2020; Nölke and Schillberg, 2020; Ort *et al.*, 2015).

In addition to the function of the chloroplast systems responsible for light harvesting, electron transfer, and carbon assimilation, photosynthesis depends strongly on the photosynthetically active leaf area, which is increased by leaf emergence (number) and growth (surface), and decreased by senescence and canopy architecture (Blösch *et al.*, 2015; Feller, 2016). Leaf features that contribute to photosynthetic capacity and photoassimilate transport thus have a huge potential for crop improvement. In this context, the connection between chloroplast function and signaling, on the one hand, and organ development on the other, has been recognized only recently, affecting both leaf growth (Andriankaja *et al.*, 2012; van Dingenen *et al.*, 2016a, b; Mayta *et al.*, 2019a), and senescence progression (Zapata *et al.*, 2005; Mayta *et al.*, 2018, 2019*b*).

In quantitative terms, however, the main limitation to crop productivity is imposed by environmental challenges and suboptimal growth conditions, whose negative impact can only worsen if current global warming predictions prove true. Indeed, evaluation of weather changes in recent decades has indicated an unequivocal escalation of temperatures in the troposphere associated with increased concentrations of greenhouse gasses (Intergovernmental Panel on Climate Change, 2014). These compounds absorb sunlight and convert their energy into infrared radiation that is unable to escape the Earth (Gutteridge, 2018). Most of the greenhouse effects are caused by CO_2, whose levels climbed from <300 μmol mol^{-1} to >400 μmol mol^{-1} in the last two centuries, although methane might also become a major concern in the near future (Intergovernmental Panel on Climate Change, 2014; Cassia *et al.*, 2018; Gutteridge, 2018). Interestingly, tropospheric warming caused by greenhouse gasses allowed life on Earth to develop as we know it. Without them, average world temperatures would be ~30 °C lower (Le Treut *et al.*, 2007).

Available climate models predict more intense and frequent episodes of environmental hardships such as drought, flooding, and heatwaves, with potentially devastating effects on agriculture (Knutti *et al.*, 2016). Abiotic stresses inactivate photosynthesis and other chloroplast oxido-reductive pathways, resulting in growth impairment and yield losses (Baslam *et al.*, 2020, and references therein). In addition, weather changes are expected to affect the distribution, abundance, population dynamics, and virulence of many relevant phytopathogens (Velásquez *et al.*, 2018). Thus, the adequate provision of food, feed, and biofuels in the near future requires the identification of novel traits which could help plants to cope with adverse environments, and their introduction and optimization in crops to increase yield. While plant responses to both biotic and abiotic challenges are complex and operate at various levels, the contribution of chloroplast metabolism and signaling to stress tolerance is far from minor (Gollan and Aro, 2020; Lamers *et al.*, 2020), offering multiple possibilities of intervention to accomplish this goal.

In this review, we summarize the available evidence linking chloroplast redox biochemistry with organ development and stress tolerance, and assess the possibilities of using this knowledge to increase yield and functional adaptation of plants growing under deteriorating environmental conditions by rational manipulation of chloroplast oxido-reductive metabolism.

Environmental hardships knock down photosynthesis and increase propagation of reactive oxygen species

As indicated before, photosynthesis is an early target of environmental stresses, and photosynthetic capacity generally declines before other cellular functions (Feller, 2016; Cardona *et al.*, 2018). Carbon assimilation via the Calvin–Benson cycle (CBC) is particularly sensitive to both drought and high temperatures, even under conditions in which photosynthetic electron transport remains functional (Sharkey, 2005). Paradoxically, atmospheric CO_2 enrichment could improve carbon fixation in those crops that operate a C_3-type photosynthesis such as wheat, rice, and soybean, by outcompeting the oxygenase activity of Rubisco and decreasing photorespiration. However, the environmental hardships accompanying the change in atmospheric composition, such as drought and heatwaves, largely offset this advantage (Gutteridge, 2018). Water limitation, for instance, leads to immediate down-regulation of CO_2 accessibility through stomatal closure, whereas high temperatures increase Rubisco decarbamylation and inactivate Rubisco

activase, a thermosensitive enzyme (Crafts-Brandner and Salvucci, 2004; Kim and Portis, 2006).

Besides providing the energy and reducing power required for growth and development, chloroplasts also act as sensors of environmental status, most conspicuously light intensity, and export signals that modulate plant responses to the external conditions (Crawford *et al.*, 2018; Dietz *et al.*, 2019; Gollan and Aro, 2020; Mielecki *et al.*, 2020). A major share of these signals originates in the photosynthetic electron transport chain (PETC) and other oxido-reductive processes of the organelle (Chan *et al.*, 2016; Dietz *et al.*, 2019; Mielecki *et al.*, 2020; Wang *et al.*, 2020; Jiang and Dehesh, 2021). With its combination of low potential redox intermediates and high oxygen levels, chloroplasts are particularly well suited to this signaling role.

Light-driven reducing equivalents of the PETC are normally delivered, via reduced ferredoxin (Fd) and NADPH, to a plethora of metabolic, dissipative, and regulatory pathways, including the regenerative step of the CBC, but also N and S assimilation, cyclic electron transport (CET), and thioredoxin reduction (Zurbriggen *et al.*, 2008). In addition to their negative effect on the CBC (Baslam *et al.*, 2020), most environmental stresses down-regulate Fd levels (Tognetti *et al.*, 2006; Pierella Karlusich *et al.*, 2017, 2020), leading to acceptor side limitation and over-reduction of the PETC. Under such conditions, excited chlorophylls in PSII cannot relax rapidly enough and may interact via energy transfer with triplet-state oxygen ($^3O_2$). The resulting energy transfer reaction causes electron spin inversion and synthesis of extremely reactive singlet oxygen ($^1O_2$). Electron transfer to oxygen may also occur through interaction with components of the PETC. Kozuleva and Ivanov (2016) have shown that the PSI reaction center is the primary electron donor in a univalent oxygen reduction that results in the formation of the superoxide anion radical ($O_2^{\cdot -}$) in the so-called Mehler reaction (Fig. 1). Superoxide then disproportionates to form H_2O_2 and regenerate O_2, either spontaneously or through the activity of superoxide dismutases (SODs). In turn, H_2O_2 can react with free Fe^{2+} via Fenton-type chemistry to synthesize yet another highly reactive species—the HO· radical (Fig. 1). Singlet oxygen, $O_2^{\cdot -}$, H_2O_2, and HO·, collectively termed reactive oxygen species (ROS), accumulate in photosynthetic tissues under stress conditions and can react, to various extents, with many different types of biomolecules.

Whenever the balance between light harvesting and photochemistry is altered by stress situations, a stream of molecular information exits the plastid to modify nuclear gene expression in a process termed retrograde signaling (Crawford *et al.*, 2018; Dietz *et al.*, 2019; Gollan and Aro, 2020; Mielecki *et al.*, 2020). This regulatory crosstalk originating in mature chloroplasts in response to environmental stimuli is defined as operational control, to distinguish it from the biogenic control signals elicited by developing plastids during chloroplast biogenesis (Chan *et al.*, 2016; Crawford *et al.*, 2018; Mielecki *et al.*, 2020; Jiang and Dehesh, 2021). ROS, and in particular H_2O_2, which is comparatively stable and can travel through biological membranes,

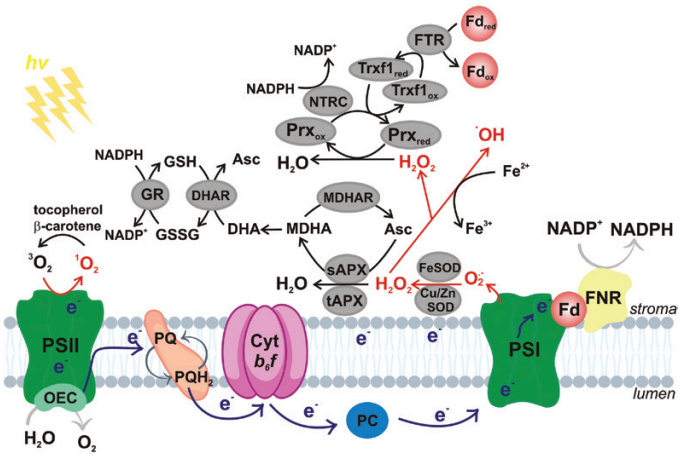

Fig 1. Summary of chloroplast ROS detoxification systems. The Mehler–Asada cycle detoxifies $O_2^{\cdot -}$ and H_2O_2, using both stromal and thylakoid-bound APX isoforms and SOD variants harboring Fe or CuZn. Asc regeneration from MDHA and DHA involves various enzymes and reductants. The electron donor for MDHAR, not shown in the figure, is NADPH. In a different pathway, Prx reduces H_2O_2 to water and is regenerated by the NADPH-dependent activity of NTRC or the Fd-dependent activity of Trxf1 and FTR. If not scavenged, H_2O_2 can be converted into extremely reactive HO· radicals by reduction with Fe^{2+}. Singlet oxygen ($^1O_2$) is detoxified by reaction with tocopherols and carotenoids. ROS are shown in red. Other chemical scavengers are not shown. Further details are given in the text. Asc, ascorbate; DHA, dehydroascorbate; DHAR, DHA reductase; Fd, ferredoxin; FNR, ferredoxin-NADP$^+$ reductase; FTR, Fd-dependent Trx reductase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; MDHA, monodehydroascorbate; MDHAR, MDHA reductase; NTRC, NADPH-dependent Trx reductase C; OEC, oxygen-evolving complex; Prx, 2-Cys peroxiredoxin; ; sAPX, stromal Asc peroxidase; SOD, superoxide dismutase;, tAPX, thylakoid-bound Asc peroxidase; Trxf1, thioredoxin f1.

have been invoked as major players in operational control, either alone or in combination with other intracellular messengers such as nitric oxide, calcium currents, and phytohormones (Chan *et al.*, 2016; Dietz *et al.*, 2019; Mielecki *et al.*, 2020; Wang *et al.*, 2020; Jiang and Dehesh, 2021). It should be emphasized, however, that increased ROS propagation is not the only consequence of exposure to adverse environmental conditions, which affect the whole dynamics of chloroplast redox balance. Accordingly, several other potential signals have been proposed, including the redox status of components of the PETC and the stroma (Exposito-Rodriguez *et al.*, 2017; Gollan and Aro, 2020), suggesting that the network of oxido-reductive pathways of the organelle, rather than any individual process, might be acting in sensing, integration, and export of meaningful information requested to cope with a changing environment.

Improving tolerance to abiotic stresses by manipulation of chloroplast ROS levels

Increased ROS production is thus a universal feature of virtually all environmental stresses, biotic and abiotic. They are generated in chloroplasts, mitochondria, and peroxisomes

as by-products of photosynthesis, respiration, and photorespiration, respectively, and in the apoplast by the activity of NADPH-dependent oxidases (Choudhury *et al.*, 2017; Foyer *et al.*, 2017). Chloroplasts, in particular, wage a continuous war of attrition against their accumulation that might otherwise result in oxidative damage, using a suite of dissipative and scavenging mechanisms, as illustrated in Fig. 1. They operate on different aspects of ROS metabolism, including synthesis, degradation, mobilization, and reaction with susceptible biomolecules. The first line of defense is avoidance of their synthesis via dissipation of the surplus of energy or reducing equivalents to alternative electron transport (AET) pathways. Dissipative systems are particularly important in chloroplasts and will be discussed in some detail in the next sections.

Once ROS are generated, they can be eliminated by direct reaction with a number of reductants present in the cytosol and organelles. The most abundant among them are ascorbate (Asc) and glutathione (GSH in its reduced form), which operate in the soluble phase (Noctor *et al.*, 2012; Zhang, 2012), and tocopherols and carotenoids, which are membrane associated and scavenge mostly organic ROS such as lipid peroxides (Abbasi *et al.*, 2007). These compounds are oxidized during ROS detoxification, and their reduced forms are regenerated by committed pathways (Fig. 1). Compatible osmolytes such as proline, glycinebetaine, and sugar alcohols can also act as antioxidants and are up-regulated under stress conditions (Kaur and Asthir, 2015). Metabolic engineering increasing the chloroplast levels of Asc, GSH, glycinebetaine, proline, α-tocopherol, lycopene, and other carotenoids led to improved stress tolerance in a number of species (Table 1; Gómez *et al.*, 2019). The less reactive ROS ($O_2^{\cdot -}$, H_2O_2) can also be neutralized enzymatically in reactions mediated by SOD activities utilizing various metal cofactors (Cu/Zn, Fe, or Mn), as well as by catalases and peroxidases that detoxify H_2O_2. They are encoded by small gene families, with isoforms directed to different cell compartments including chloroplasts (Mhamdi *et al.*, 2010).

Superoxide radicals generated at the acceptor end of the PETC can be reduced to water by the Mehler–Asada cycle (Fig. 1), which involves sequential activities of SOD and ascorbate peroxidase (APX). Asc, oxidized to monodehydroascorbate (MDHA) during APX reaction and to dehydroascorbate (DHA) after dismutation, is regenerated via MDHA and DHA reductases (MDHAR and DHAR). Electron donors for these reactions are NADPH and GSH, respectively. Oxidized glutathione, in turn, is reduced back to GSH via NADPH-dependent glutathione reductase (GR). Then, the cycle involves five different enzymes and the two major chemical scavengers, and utilizes the reducing power of the PETC to maintain Asc and GSH homeostasis (Fig. 1). All components of the Mehler–Asada pathway have been overexpressed in chloroplasts, either individually or in combination (Table 1). Transformation of model and crop plants with genes encoding APX or Cu/ZnSOD directed to plastids increased tolerance to oxidative stress and various environmental hardships such as heat, high light, and salinity, as reflected by differential preservation of photosynthetic pigments and activities, membrane integrity, and growth rates (Table 1). Introduction of plastid-targeted FeSOD in maize and alfalfa also improved photosynthetic performance under low temperatures and oxidative stress (van Breusegem *et al.*, 1999; McKersie *et al.*, 2000). Stacking of APX and SOD led to even higher levels of stress tolerance in several species, and the incorporation of DHAR in this couple resulted in tobacco plants that grew better under salinity (Table 1).

Peroxiredoxins (Prxs) are ancient enzymes which display a very high affinity for peroxide substrates, presumably inherited from the times when oxygen was still low (Liebthal *et al.*, 2018). The catalytic center of Prxs is a peroxidatic cysteine with low p*K* that reduces peroxides to water employing a simple nucleophilic attack (Rhee, 2016). In chloroplast 2-Cys Prxs, the resulting sulfenic acid is converted into a disulfide bond by reaction with a 'resolving' cysteinyl thiol from a different subunit (Liebthal *et al.*, 2018). The reduced active form of 2-Cys Prxs is regenerated by chloroplast NADPH-dependent thioredoxin reductase C, a committed enzyme with a built-in thioredoxin domain (Pérez-Ruiz *et al.*, 2017), or less efficiently by thioredoxin f1 (Liebthal *et al.*, 2018), thus linking 2-Cys Prx metabolism with NADPH, reduced Fd, and the PETC (Fig. 1). Besides its role in ROS homeostasis, 2-Cys Prx may also participate in various regulatory networks depending on its oxidation and aggregation state (Liebthal *et al.*, 2018). Accordingly, introduction of 2-Cys Prx conferred higher ability to withstand adverse environments when expressed in chloroplasts of potato and tall fescue (Table 1). In addition to APX and 2-Cys Prx, lipid peroxides can be scavenged by plastidic aldehyde dehydrogenase, which employs NADPH as electron donor and is induced by oxidative and abiotic stresses. Overexpression of this dehydrogenase increased drought and salt tolerance in transgenic Arabidopsis (Table 1).

Most environmental hardships involve an associated oxidative stress which was initially assumed to significantly contribute to the damage suffered by the stressed plant through indiscriminate destruction of membrane lipids, proteins, and photosystems, eventually leading to cell death. Accordingly, the protective effects of ROS scavengers were traditionally attributed to damage control. More recent observations, however, indicate that the main role played by these reactive species during plant responses to both developmental and environmental stimuli is to act as signaling molecules (Foyer *et al.*, 2017). Indeed, well-characterized examples of cell death during stress responses show that they are genetically controlled processes in which ROS play a signaling role (Mielecki *et al.*, 2020). Increases in ROS levels might trigger retrograde responses via direct or indirect mechanisms. As the most stable ROS, H_2O_2 can exit the chloroplast through envelope aquaporins or reach the nucleus directly via stromules, bypassing the

Table 1. Manipulation of stress tolerance using ROS scavengers located in chloroplasts

ROS scavenger	Origin	Crop/plant	Stress assayed	Phenotype	References
Enzymatic scavengers					
APX	*Cyanidioschyzon merolae*	Arabidopsis	Heat	Higher pigment contents, lower membrane oxidative damage	Hirooka *et al.* (2009)
	Suaeda salsa	Arabidopsis	High light	Higher photosynthetic activity and pigment contents, lower membrane oxidative damage	Pang *et al.* (2011)
	Pea	Tobacco	UVC radiation	Lower membrane oxidative damage, better growth (fresh weight), higher germination rates	Saxena *et al.* (2011)
Cu/ZnSOD	Rice	Rice	Salt	Better growth (height), higher germination rates	Guan *et al.* (2017)
FeSOD	Arabidopsis	Maize	Oxidative (MV), chilling	Higher photosynthetic activity, lower membrane oxidative damage, better growth (fresh weight)	van Breusegem *et al.* (1999)
	Arabidopsis	Alfalfa	Winter survival	Higher photosynthetic activity	McKersie *et al.* (2000)
APX, Cu/ZnSOD	Pea, cassava	Sweet potato	Chilling	Higher photosynthetic activity	Lim *et al.* (2007)
	Pea, cassava	Sweet potato	Salt	Higher photosynthetic activity and pigment contents, better growth (total fresh weight, root length)	Yan *et al.* (2016)
APX, Cu/ZnSOD, DHAR	Pea, pea, human	Tobacco	Salt	Better growth (shoot and root dry weight)	Lee *et al.* (2007)
APX, Cu/ZnSOD, *codA*	Pea, cassava, *Arthrobacter globiformis*	Potato	Drought, salt	Higher photosynthetic activity and pigment contents, better growth (total dry weight)	Ahmad *et al.* (2010)
APX, Cu/ZnSOD, NDPK2	Pea, cassava, Arabidopsis	Potato	Heat	Higher photosynthetic activity, de-creased wilting	M.D. Kim *et al.* (2010)
MnSOD, GR	Tobacco, *Escherichia coli*	Tobacco	Photo-oxidation, UVB radiation	Higher photosynthetic activity and pigment contents, lower membrane oxidative damage	Poage *et al.* (2011)
DHAR, GR	Rice, *E. coli*	Tobacco	Chilling, salt	Higher photosynthetic activity and pigment contents, better growth rates	Le Martret *et al.* (2011)
GR	*E. coli*	*Populus tremula*×*Populus alba*	Photoinhibition, oxidative (MV)	Higher photosynthetic activity	Foyer *et al.* (1995)
GST, GR	*E. coli*	Tobacco	Chilling, salt	Higher photosynthetic activity and pigment contents, better growth rates	Le Martret *et al.* (2011)
Prx	Arabidopsis	Tall fescue	Heat	Higher photosynthetic activity, lower membrane oxidative damage	K.H. Kim *et al.* (2010)
	Arabidopsis	Potato	Heat	Higher photosynthetic activity	Kim *et al.* (2011)

Table 1. Continued

ROS scavenger	Origin	Crop/plant	Stress assayed	Phenotype	References
ALDH	Arabidopsis	Arabidopsis	Drought, salt	Lower membrane oxidative damage	Sunkar *et al.* (2003)
Non-enzymatic scavengers					
Glutathione	Arabidopsis	Arabidopsis	Drought, salt	Better growth (root length), higher germination rates, increased survival rates	Cheng *et al.* (2015)
γGCL					
γGCL, GS	*Streptococcus thermophilus*	Tobacco	Oxidative (MV)	Lower membrane oxidative damage, better growth rates	Liedschulte *et al.* (2010)
α-*Tocopherol*	Arabidopsis	*Brassica juncea*	Osmotic, salt	Reduced membrane oxidative damage	Kumar *et al.* (2013)
γ-Tocopherol methyltransferase					
Tocotrienol	Yeast - Arabidopsis	Tobacco	Chilling, high light	Higher photosynthetic activity, reduced membrane oxidative damage	Matringe *et al.* (2008)
Prephenate dehydrogenase, hydroxyphenyl pyruvate dioxygenase					
Ketocarotenoids	*Haematococcus pluvialis*	Carrot	UVB radiation	Higher pigment contents, better growth (total fresh weight), decreased wilting	Jayaraj *et al.* (2008)
β-Carotene, ketolase					
Carotenoids	Mulberry	Mulberry	Drought, heat, osmotic, salt	Higher photosynthetic activity and pigment contents, reduced membrane oxidative damage	Saeed *et al.* (2014)
β-Carotene hydroxylase 1					
Lycopene	*Salicornia europea*	Arabidopsis, *Nicotiana benthamiana*	Salt	Higher photosynthetic activity and pigment contents, increased survival rates	Chen *et al.* (2011)
Lycopene cyclase					
Flavonoids	Rice	Rice	Oxidative (MV)	Higher photosynthetic activity, reduced chlorosis	S.G. Kim *et al.* (2010)
Isoflavone reductase-like gene					
Isoprene	White poplar	Tobacco	Heat, oxidative (ozone)	Higher photosynthetic activity	Vickers *et al.* (2009)
Isoprene synthase					
Glycinebetaine	*Arthrobacter globiformis*	Potato	Drought, salt	Higher photosynthetic activity, better growth (total fresh weight)	Ahmad *et al.* (2008)
codA					
	A. globiformis	Potato	Drought	Higher photosynthetic activity and pigment contents, lower membrane oxidative damage	Cheng *et al.* (2013)
	A. globiformis	Alfalfa	Drought, salt	Higher pigment contents, lower membrane oxidative damage, increased survival rates	Li *et al.* (2014)
	A. globiformis	Sweet potato	Drought	Lower membrane oxidative damage	Park *et al.* (2015)
	A. globiformis	Poplar	Chilling, drought, salt	Higher photosynthetic activity, lower membrane oxidative damage	Ke *et al.* (2016)

Abbreviations: ALDH, aldehyde dehydrogenase; APX, ascorbate peroxidase; *codA*, gene encoding choline oxidase; DHAR, dehydroascorbate reductase; γGCL, γ-glutamylcysteinyl ligase; GPX, glutathione peroxidase; GR, glutathione reductase; GS, glutathione synthase; GST, glutathione *S*-transferase; MDHAR, monodehydroascorbate reductase; MV, methyl viologen; NDPK2, nucleoside diphosphate kinase; Prx, 2-Cys peroxiredoxin; SOD, superoxide dismutase. Entries below each non-enzymatic scavenger indicate the transgene product expressed.

cytosol (Exposito-Rodriguez *et al.*, 2017). Identified intermediates of the H_2O_2 signaling network include *oxidative signal inducible 1* (*OXI1*), which encodes a serine/threonine kinase and positively modulates biosynthesis of jasmonic acid and redox-sensitive mitogen-activated protein kinases MPK11 and MPK13 (Mielecki *et al.*, 2020). Among the known targets of the H_2O_2-specific pathway, there are genes involved in central metabolism and stress responses such as heat shock proteins and MPK kinases, as well as transcriptional regulators of the *DREB*, zinc finger, WRKY, and basic helix–loop–helix families (Crawford *et al.*, 2018). The available evidence suggests that transcription factors are activated first, followed by the induction of many downstream target genes.

Singlet oxygen has too short a life span to migrate from plastids, but at least two different signaling pathways are initiated by this reactive species. One of them is mediated by chloroplast proteins EXECUTER (EX) 1 and EX2, and depends on proteolysis of EX1 by the zinc-metalloprotease FtsH. To become a substrate for proteolysis, EX1 must be previously oxidized at a specific tryptophan residue by $^1O_2$ (Mielecki *et al.*, 2020). A distinct pathway is started by reaction of $^1O_2$ with thylakoid β-carotene to render the volatile compound β-cyclocitral which, through the downstream METHYLENE BLUE SENSITIVITY 1 (MBS1) protein, can influence the expression of a set of nuclear genes. Although the two separate pathways originate in $^1O_2$, they share only few common target genes, suggesting that they represent different signaling pathways (Crawford *et al.*, 2018). Interestingly, both lead to up-regulation of genes encoding AAA-ATPases involved in programmed cell death (Chan *et al.*, 2016).

Chloroplast ROS may also promote retrograde responses by modulating the synthesis of other signaling molecules such as 3′-phosphoadenosine 5′-phosphate (PAP), methylerythritol cyclodiphosphate (MEcPP), and Mg-protoporphyrin IX, respectively (Crawford *et al.*, 2018; Mielecki *et al.*, 2020). These three intermediates are exported to the cytosol, where they initiate as many retrograde signaling cascades whose components and targets are beginning to be identified (reviewed in Crawford *et al.*, 2018; Dietz *et al.*, 2019; Mielecki *et al.*, 2020).

In spite of intense research on ROS metabolism and signaling, many mechanistic aspects of their function in development and stress remain unknown. The specific cellular response to ROS-mediated signaling depends on several factors: the type, dose, timing, and duration of the signal and the site of ROS generation (Crawford *et al.*, 2018). As indicated, they can be produced in several cell locations, and growing evidence indicates that signals coming from different sources can integrate to elicit a coordinated operational response (Wang *et al.*, 2020). On the other hand, cellular responses to H_2O_2 are reported to differ depending on whether the signal was generated in chloroplasts or peroxisomes (Sewelam *et al.*, 2014), underscoring the need for renewed research to better understand the mechanisms by which these reactive species provide customized responses to diverse and yet specific environmental situations. The interaction of chloroplast ROS with signaling pathways originating in other cellular compartments is extensively addressed in a number of recent reviews (Crawford *et al.*, 2018; Dietz *et al.*, 2019; Mielecki *et al.*, 2020; Wang *et al.*, 2020), and the reader is directed to them for further details.

Given that ROS play such critical roles in the execution of plant operational responses to adverse conditions, it follows as a logical consequence that preventing their propagation during stress situations could be detrimental to tolerance. The empirical observations summarized in Table 1 indicate otherwise: strengthening of ROS-scavenging systems improves stress tolerance, concurring with yet another widespread observation, namely the induction of antioxidant activities during virtually all stress situations (Choudhury *et al.*, 2017). The question then remains unsolved regarding the nature of the molecular mechanisms responsible for the improved stress performance, demanding further research to solve this apparent contradiction.

Alternative electron transport: roads to multiple stress tolerance

The major electron sinks that consume the redox equivalents originating in the PETC are the carboxylation and oxygenation reactions of Rubisco (photosynthesis and photorespiration, respectively), but AET pathways become important under adverse environmental conditions (Fernández-Marín *et al.*, 2020). These alternative pathways are particularly efficient to relieve the excess reducing power and excitation energy of the PETC and to direct both electrons and energy into productive or dissipative routes (Fig. 2). Since these mechanisms limit the synthesis of all chloroplast ROS, they are useful to control species that are not accessible to enzymatic scavenging. Attempts to increase stress tolerance by genetic engineering of AET pathways and other alternative electron sinks are summarized in Table 2.

Two different routes of CET have been identified, mediated either by the proton gradient regulation 5 (PGR5)–PGR5-like photosynthetic phenotype 1 (PGRL1) complex or by chloroplast NADPH dehydrogenase (NDH) (Fig. 2A). In addition to its role in matching the photosynthetic requirements of ATP and reducing equivalents, CET modulates the release of the excess energy not used for photochemistry in the form of heat, a process monitored by the non-photochemical quenching (NPQ) of chlorophyll fluorescence (Ruban, 2015). Dissipative mechanisms, in general, decrease photosynthetic efficiency under stress, and NPQ is a good example of that. Moreover, some time is required by electron transfer to recover after the environmental adversities have subsided and the whole process has a negative effect on plant growth and yield (Goss and Lepetit, 2015). It is assumed, however, that the benefits conferred by this protective mechanism in the fluctuating environmental conditions found in nature largely compensate its disadvantages, a feature that presumably granted its preservation

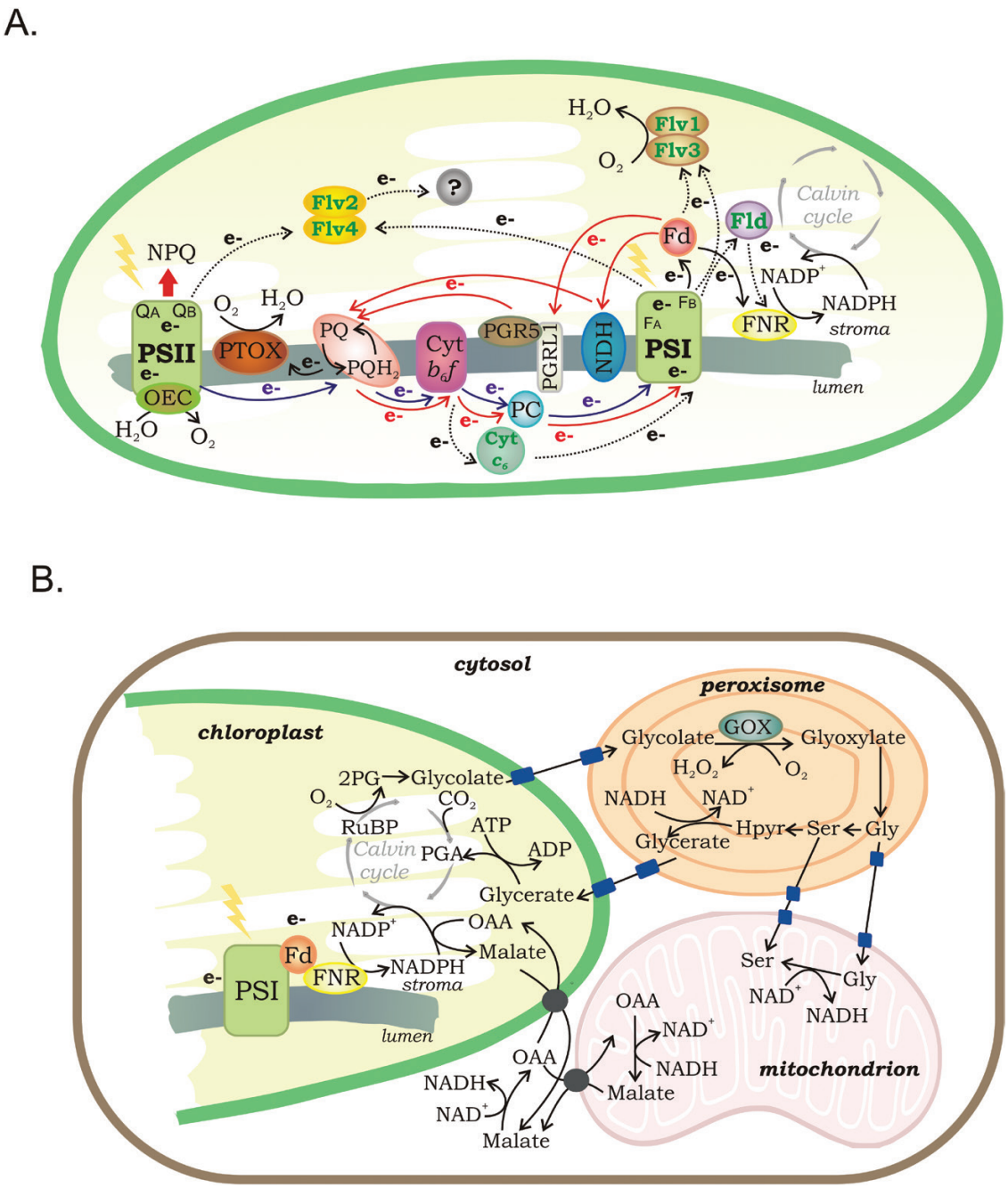

Fig. 2. Alternative electron transport pathways operating in wild-type or genetically modified leaf cells. (A) Schematic representation of the photosynthetic electron transport chain (PETC) showing the linear electron transport from H_2O to $NADP^+$ (blue arrows) and the two pathways of cyclic electron transport (CET) around PSI, depending on either PGR5/PGRL1 or NDH (red arrows). CET and chlororespiration mediated by PTOX relieve the excess reducing power, whereas NPQ is involved in thermal dissipation. Fld and Cyt c_6 are only present in transgenic plants, and Flv1–Flv3 and Flv2–Flv4 are only found in transformed angiosperms. Electron transfer to these alternative electron sinks is represented by dotted arrows. The PSII electron donor to Flv2–Flv4 is Q_B (Bersanini *et al.*, 2017), whereas that from PSI is unknown (Santana-Sánchez *et al.*, 2019). Flv1–Flv3 accept reducing equivalents from either Fd or the PSI final acceptor F_B (Sétif *et al.*, 2020). Electron carriers not found in angiosperms are labeled in green. Other details of their mode of interaction with the PETC and their protective roles are given in the text. (B) In the photorespiratory pathway, toxic 2PG synthesized by the oxygenase activity of Rubisco is recycled via a series of enzymatic steps dispersed between chloroplasts, peroxisomes, and mitochondria. Excess NADPH in chloroplasts is exported through the malate valve. Cyt b_6f, cytochrome b_6f; Cyt c_6, cytochrome c_6; Fd, ferredoxin; Fld, flavodoxin; Flv, flavin-diiron protein; FNR, ferredoxin-$NADP^+$ reductase; GOX, glycolate oxidase; Hpyr, hydroxypyruvate; NDH, NADPH dehydrogenase complex; NPQ, non-photochemical quenching; OAA, oxaloacetate; PC, plastocyanin; 2PG, 2-phosphoglycolate; 3PGA, 3-phosphoglycerate; PGR5, proton gradient regulation 5; PGRL1, PGR5-like photosynthetic phenotype 1; PQ, plastoquinone; PQH2, plastoquinol; PTOX, plastid terminal oxidase; RuBP, ribulose 1,5-bisphosphate.

Table 2. Engineering stress tolerance by manipulation of AET routes and electron sinks in chloroplasts

Transgene product	Origin	Crop/plant	Stress assayed	Phenotype	References
Fld	*Anabaena* PCC 7119	Tobacco	Chilling, drought, heat, high light	Higher photosynthetic activity and pigment contents, lower membrane oxidative damage, reduced leaf bleaching	Tognetti *et al.* (2006)
		Tobacco	Infection with *Xanthomonas campestris*	Reduced ROS levels and lesions. Protection of metabolic routes	Zurbriggen *et al.* (2009)
		Medicago truncatula	Salt	Preservation of nitrogen-fixing activity	Coba de la Peña *et al.* (2010)
		Tobacco	Infection with *Botrytis cinerea*	Inhibited tissue damage and fungal growth. Lower ROS accumulation and higher photosynthetic activity	Rossi *et al.* (2017)
		Arabidopsis	Avirulence factors	Lower photosynthetic inhibition and decreased ROS accumulation	Su *et al.* (2018)
		Agrostis stolonifera	Drought, heat, nitrogen starvation	Higher pigment contents, lower membrane oxidative damage, higher nitrogen content	Li *et al.* (2017)
		Potato	Drought	Higher photosynthetic activity and pigment contents, improved growth and tuber yield	Pierella Karlusich *et al.* (2020)
FNR	Pea	Tobacco	Chilling, high light	Higher photosynthetic activity and pigment contents, lower membrane oxidative damage	Rodriguez *et al.* (2007)
FNR, Fld	*Anabaena* PCC 7119	Tobacco	Oxidative stress (MV)	Higher photosynthetic activity, lower membrane oxidative damage, and ROS accumulation	Giró *et al.* (2011)
Flv1–Flv3	*Synechocystis* sp. PCC6803	Arabidopsis	High light	Faster recovery of photosynthetic parameters, enhanced biomass	Tula *et al.* (2020)
		Barley	Drought	Earlier heading, increased biomass, more spikes and grains per plant, higher total grain weight per plant	Shahinnia *et al.* (2021)
Flv2–Flv4	*Synechocystis* sp. PCC6803	Tobacco, Arabidopsis	High light, salt, oxidative stress (MV), drought	Higher photosynthetic activity, lower membrane oxidative damage and ROS accumulation. Enhanced accumulation of soluble sugars and amino acids under drought	Vicino *et al.* (2021)
NADPH-MDH	Maize	Arabidopsis	Salt	Higher photosynthetic activity and pigment contents, lower membrane oxidative damage, better growth (total fresh and dry weight)	Kandoi *et al.* (2018)

Abbreviations: Fld, flavodoxin; FNR, ferredoxin-NADP$^+$ reductase; Flv, flavin-diiron protein; NADPH-MDH, NADPH-dependent malate dehydrogenase; MV, methyl viologen.

along the evolutionary path of land plants. Indeed, mutants deficient in NPQ display increased sensitivity to photodamage (Johnson, 2020).

NPQ is a complex variable resulting from several quenching mechanisms. Among them, the so-called energy-dependent quenching qE requires low luminal pH and the activity of the PSII subunit PsbS, whereas the xanthophyll cycle is associated with multiple NPQ components including qE and the zeaxanthin-dependent quenching mechanism described by the qZ parameter (Goss and Lepetit, 2015). High light treatments promote violaxanthin de-epoxidation into zeaxanthin through the activity of violaxanthin de-epoxidase (VDE), with zeaxanthin epoxidase (ZEP) catalyzing the back reaction. NPQ relaxes as the high light conditions subside (e.g. upon dark or shade transitions) with slower kinetics than those of induction, and qZ relaxation represents the rate-limiting component, one order of magnitude slower than qE. The rate of CO_2 fixation remains depressed until NPQ relaxation is complete, thus lowering photosynthetic efficiency. Based on these premises, Kromdijk *et al.* (2016) prepared tobacco plants overexpressing VDE and ZEP to accelerate the xanthophyll cycle, together with PsbS. The triple transformants displayed faster NPQ induction under high and fluctuating light and, more importantly, higher relaxation rates upon transfer to dark, which in turn led to faster recovery of CO_2 assimilation and improved photosynthetic efficiency. The transgenic plants grew better in the field, with increases in dry weight of ~15% (Kromdijk *et al.*, 2016).

Photorespiration and chlororespiration are two very important dissipative systems in C_3 plants (Sunil *et al.*, 2019). Photorespiration reduces chloroplast O_2 levels by the oxygenase activity of Rubisco (Fig. 2B), especially under conditions in which CO_2 fixation is limited. It is similar to the Mehler–Asada cycle in the sense that it leads to transient ROS propagation and requires scavenging enzymes to ultimately remove them. Genetic manipulation of this pathway has been largely focused in the improvement of carbon assimilation and yield rather than stress tolerance (Hagemann *et al.*, 2016; Sunil *et al.*, 2019). Chlororespiration mediated by the plastid terminal oxidase (PTOX) reduces oxygen to H_2O using redox equivalents from the PETC, thus removing the substrate required for ROS propagation (Fig. 2A; Nawrocki *et al.*, 2015). Its role in photosynthesis, however, is unclear, and conflicting evidence has been reported indicating both antioxidant and pro-oxidant roles (Krieger-Liszkay and Feilke, 2016).

A most interesting alternative found in photosynthetic microorganisms, non-vascular plants, and gymnosperms is represented by a family of flavin-diiron (Flv) proteins that can also mediate direct reduction of O_2 to H_2O using the PETC as electron source (Ilík *et al.*, 2017; Fig. 2A). Plants and algae contain two Flv isoforms and cyanobacteria up to six. They are reported to act as heterodimers (Flv1–Flv3 and Flv2–Flv4 in *Synechocystis*), although many aspects of their function at the molecular level remain to be elucidated (Ilík *et al.*, 2017). Studies in *Synechocystis* cells have shown that spectral changes associated with Flv1–Flv3 photoreduction are consistent with the involvement of an iron–sulfur cluster, but could not distinguish between Fd or the terminal PSI acceptor F_B (Sétif *et al.*, 2020), whereas Flv2–Flv4 can engage in electron transfer with the primary acceptor Q_B of PSII (Bersanini *et al.*, 2014, 2017). Further research, however, revealed that Flv2–Flv4 can also operate as an electron sink at PSI, although the specific redox partner was not identified (Santana-Sánchez *et al.*, 2019). These proteins are not found in angiosperms, but Flv dimers from bryophytes and cyanobacteria have been introduced in chloroplasts of tobacco, rice, barley, and Arabidopsis (Yamamoto *et al.*, 2016; Gómez *et al.*, 2018; Wada *et al.*, 2018; Tula *et al.*, 2020; Shahinnia *et al.*, 2021; Vicino *et al.*, 2021). The presence of Flv1–Flv3 improved recovery of photosynthesis during dark–light transitions and under fluctuating light (Yamamoto *et al.*, 2016; Gómez *et al.*, 2018; Wada *et al.*, 2018), and plants expressing either this complex (Shahinnia *et al.*, 2021) or Flv2–Flv4 (Vicino *et al.*, 2021) displayed enhanced tolerance to various stresses including drought, salinity, and high light (Table 2). Loss of this adaptive trait from angiosperms has been attributed to a major increase in the efficiency of photorespiration (Hanawa *et al.*, 2017) and CET (Yamamoto *et al.*, 2016) in flowering plants. Indeed, Wada *et al.* (2018) have shown that Flv1–Flv3 significantly improved the photosynthetic efficiency of *pgr5* and *ndh* rice mutants deficient in CET.

An analogous example is provided by flavodoxin (Fld), an electron carrier flavoprotein induced by various stresses in cyanobacteria and some algae, but absent from plant genomes (Pierella Karlusich *et al.*, 2014). Fld is isofunctional with stress-sensitive Fd, and can replace the iron–sulfur shuttle in many reactions including photosynthesis (Zurbriggen *et al.*, 2008; Lodeyro *et al.*, 2012; Fig. 2A). Expression of a plastid-targeted cyanobacterial Fld in different plant species improved delivery of reducing equivalents to productive pathways of the chloroplast, which resulted in down-regulation of ROS levels and increased tolerance to a wide range of abiotic, biotic, and xenobiotic stresses (Tognetti *et al.*, 2006; Zurbriggen *et al.*, 2009; Coba de la Peña *et al.*, 2010; Li *et al.*, 2017; Rossi *et al.*, 2017; Pierella Karlusich *et al.*, 2020; Table 2). Loss of Fld in plants seems to be associated with the strategies of iron bioassimilation in the founder lineage of terrestrial plants (Tognetti *et al.*, 2007; Pierella Karlusich *et al.*, 2015).

Finally, the malate valve functions as a pacemaker for the export of reducing equivalents to the cytosol (Fig. 2B). The key component of this pathway is chloroplastic NADP(H)-dependent malate dehydrogenase (NADPH-MDH), a redox-regulated enzyme that catalyzes the reversible reduction of oxaloacetate to malate using NADPH as an electron donor. Under stress conditions, chloroplast NADPH-MDH acts in consort with cytosolic NAD(H)-dependent isoforms and envelope-bound dicarboxylate transporters to export the surplus of redox equivalents from the plastid and regenerate $NADP^+$, thus relieving acceptor side limitations at PSI (Fig. 2B). In C_4 plants, NADPH-MDH is instead involved in CO_2

concentration mechanisms, and the enzyme is one order of magnitude more active than C_3 counterparts. Kandoi *et al.* (2018) took advantage of this property to increase several fold the NADPH-MDH activity of Arabidopsis leaves by expressing a plastid-targeted maize ortholog. The resulting plants displayed increased tolerance to salt toxicity (Table 2).

As indicated previously, by acting as electron sinks, all these dissipative systems not only limit ROS propagation but also affect chloroplast redox poise, most conspicuously that of the PETC. In this context, the redox state of the plastoquinone pool has been shown to regulate the expression of ~750 nuclear genes, many of them encoding photosynthetic components (Chan *et al.*, 2016). The mechanism by which this status is perceived and transmitted remains unknown, although the protein kinase STN7, which is involved in the modulation of state transitions, has been identified as a post-translational sensor of the plastoquinone redox state (Dietz *et al.*, 2019). During episodes of environmental stress that cause acceptor side limitations, the plastoquinone pool becomes over-reduced. It is expected that incorporation of electron sinks downstream of plastoquinone would alleviate this condition, as demonstrated for Fld (Gómez *et al.*, 2020).

Stress situations cause imbalances between carbon fixation and utilization, and plants have developed the ability to sense altered concentrations of sucrose, glucose, and fructose, among other sugars, and to respond accordingly through a complex signaling network to cope with the environmental challenge (Martínez-Noël and Tognetti, 2018). By preserving photosynthetic activity under stress, ROS scavengers and alternative electron sinks are able to maintain the levels of carbohydrates, amino acids, and other metabolites, as documented in several reports (Tognetti *et al.*, 2007; Zurbriggen *et al.*, 2009; Kandoi *et al.*, 2018; Pierella Karlusich *et al.*, 2020; Shahinnia *et al.*, 2021; Vicino *et al.*, 2021). It is therefore conceivable that signaling by sugars can mediate some of the protective effects observed, although experimental evidence for such a role is still lacking. Besides sucrose and hexoses, abiotic stresses also increase export of dihydroxyacetone phosphate (DHAP), which is required for phosphorylation and activation of MPK6 and regulation of associated transcription factors (Chan *et al.*, 2016). While clearly related to the PETC and the CBC, DHAP function appears to be independent of the plastoquinone redox state and of the various ROS-dependent pathways (Chan *et al.*, 2016).

Although not as extensively explored as the ROS-scavenging systems, genetic manipulation of AET pathways has shown considerable success in the generation of stress-tolerant plants.

A lesson from acclimation to extreme environments: nothing new under the merciless sun

As climate change poses an unequivocal threat to plant welfare, it is worth asking which information can be retrieved from the physiology of species that have adapted to survive in extant inhospitable regions of the world. Extreme environments are defined as those in which one or more environmental factors, such as water and nutrient availability, temperature, and light intensity, are beyond the limits allowing plant physiological processes to occur (Fernández-Marín *et al.*, 2020). They comprise a high proportion of our planet landmass, as represented by deserts, polar zones, and mountains. Despite their harsh conditions, these regions are not devoid of life. Indeed, plants have been able to colonize and thrive in some of the most hostile habitats on Earth. Extreme environments are dominated by non-vascular plants, but angiosperms can also be found there. At least two species performing C_3 photosynthesis have been identified in Antarctica, and various flowering plants grow in alpine regions (Peat *et al.*, 2007; Körner, 2011), indicating that organisms inhabiting these places have succeeded in acclimating to a number of unfavorable conditions.

Many acclimation strategies involve morphological adaptations to the adverse environments, such as height decrease to prevent freezing and wind abrasion (Bjorkman *et al.*, 2018), growth in cushions of tightly compact canopies to isolate leaves from very low air temperatures (Anthelme *et al.*, 2014), or leaf size reduction or absence, and substitution by other photosynthetic organs (i.e. stems) in desert plants (Wright *et al.*, 2017; reviewed in Fernández-Marín *et al.*, 2020). However, beyond those morpho-anatomical features, plant growth and survival under extreme environments was also shown to depend on a suite of biochemical and physiological protective mechanisms, raising the possibility that novel traits could be identified in these highly resilient organisms that could be used to improve crop tolerance to environmental stresses.

Analysis of a limited number of available studies suggests otherwise: protective mechanisms displayed by plants to thrive in environmental extremes do not qualitatively differ from those found in the rest of the species, although in some cases they are stronger. For instance, carotenoids of the xanthophyll cycle involved in NPQ modulation tend to accumulate to higher levels in desert and alpine species compared with counterparts growing in milder environments (García-Plazaola *et al.*, 2015; Magney *et al.*, 2017), and antioxidant enzymes such as SOD, APX, and GR display higher activities in plants from Antarctica and the Tibetan plateau (Navarrete-Gallegos *et al.*, 2012; Cui *et al.*, 2019).

Increased electron sink capacity appears to be one of the most critical features for successful adaptation to extreme habitats. Very high PTOX activities have been reported in alpine species (Streb *et al.*, 2005), and in the desert green alga *Chlorella ohadii* (Kedem *et al.*, 2021). Enhanced function of the Mehler–Asada pathway has also been observed in mountain plants (Cui *et al.*, 2019), and CET rates correlated with drought adaptation in *Phacelia secunda* (Hernández-Fuentes *et al.*, 2019). However, the most extreme form of protection via CET is not found in plants but in green algae and diatoms. For example, *C. ohadii* can thrive in very high light with growth rates that surpass

that of any other phototroph and with minimal photodamage (Kedem *et al.*, 2021). Remarkably, under these extreme conditions, 90% of the electron flow is cyclic while only 10% is linear (Kedem *et al.*, 2021). The somehow striking conclusion was that under those harsh environmental conditions, AET pathways rather than photosynthesis sustain the cellular redox poise (Fernández-Marín *et al.*, 2020).

While the search for novel genes or mechanisms in plants from extreme environments so far has proven futile, it did show that there is ample room for improvement in stress tolerance by manipulation of the existing ROS-controlling systems and AET pathways.

Chloroplast oxido-reductive status impacts biotic stress tolerance

Production losses caused by pathogens and pests have been estimated at 20–40% for the major agricultural crops (Savary *et al.*, 2012). Disease development is deeply influenced by the environment, and for every plant–pathogen interaction there are environmental optima not only for microbial virulence but also for plant immunity and, accordingly, disease progression (Velásquez *et al.*, 2018). While changes in temperature, water availability, or CO_2 levels might accelerate, retard, or prevent disease development, evidence indicates that pathogens are advancing towards the poles as global warming progresses (Bebber *et al.*, 2013; Hamann *et al.*, 2021).

Depending on their lifestyles and strategies of infection, plant pathogens are classified as biotrophs or necrotrophs (Fatima and Senthil-Kumar, 2015). As part of the infection process, biotrophic microorganisms establish a long-term feeding relationship with the living cells of their hosts, without killing them. Necrotrophs, in contrast, promote the destruction of host cells to consume their contents. A third group of microbes, known as hemibiotrophs, exhibit both types of nutrient acquisition strategies, shifting from an initial biotrophic phase to necrotrophy at a later stage of disease development (Lorang, 2019). Plant perception of an invading microorganism is initiated by recognition of pathogen-associated molecular patterns (PAMPs) by predominantly membrane-bound receptors, leading to the activation of PAMP-triggered immunity (PTI). Pathogens, in turn, deliver effector molecules to suppress PTI, often targeting PAMP receptors (Littlejohn *et al.*, 2021). This suppression can be successfully overcome by intracellular plant disease resistance (R) proteins which activate effector-triggered immunity (ETI). ETI provides a stronger response than PTI, launching a hypersensitive response (HR) in the affected tissue. The HR involves the accumulation of ROS and the induction of pathogenesis-related (PR) protein expression. It also promotes localized cell death (LCD) at the site of infection. The resulting barrier of dead cells is regarded as a major factor impeding the spread of biotrophic pathogens, but may facilitate infection by necrotrophic pathogens (Lorang, 2019). PTI may also involve a weaker LCD (Delprato *et al.*, 2015), and growing evidence indicates that PTI and ETI are not distinct but rather interdependent processes acting in concert to boost host defense responses (van der Burgh and Joosten, 2019).

In the past few years, the chloroplast has emerged as both a central player and a target in plant immunity, although its importance for defense has been known for a long time (Littlejohn *et al.*, 2021, and references therein). First, plastids are the main site for synthesis of the precursors of three key phytohormones modulating plant immunity: salicylic acid, jasmonic acid, and abscisic acid, making them central to the integration of signals from PTI and ETI, and a prime effector target (Bürger and Chory, 2019). More recently, however, involvement of redox-based retrograde signaling in orchestrating plant immune responses has been increasingly recognized. Light is required for ETI-triggered HR (Nomura *et al.*, 2012), suggesting the existence of a signaling pathway, different from that of photoreceptors, that links plant immunity with light perception through the photosynthetic apparatus, which constitutes a light-sensing system in its own right (Gollan and Aro, 2020). Collapse of the photosynthetic apparatus during pathogen attack results in perturbations of the chloroplast redox status and increased ROS propagation, both of which can be used as signals to instruct defensive responses. Moreover, treatments with inhibitors that block photosynthetic electron transport at various sites of the PETC have shown that the oxidation state of the plastoquinone pool plays a significant role in plant responses to both excess light and pathogen attack, especially in LCD regulation (Roden and Ingle, 2009; Karpiński *et al.*, 2013). Comparison of chloroplast ROS sources during PTI and ETI suggests that the HR associated with ETI is triggered by $^1O_2$ generation at PSII (Havaux, 2014), whereas PTI is suppressed by inhibitors of the PETC, preventing electron transfer to, and ROS formation at, PSI (Exposito-Rodriguez *et al.*, 2017). Evidence also indicates that ROS generated in chloroplasts can provide information to initiate LCD during non-host and avirulent interactions (Delprato *et al.*, 2015).

As anticipated from these observations, introduction of AET pathways alleviating over-reduction of the PETC and ROS formation inhibited the manifestation of LCD caused by challenge with a non-virulent microorganism (Zurbriggen *et al.*, 2009; Pierella Karlusich *et al.*, 2017). Moreover, expression of Fld prevented chloroplast ROS formation and compromised ETI elicited by a *Pseudomonas* mutant strain in Arabidopsis (Su *et al.*, 2018). A similar intervention protected tobacco plants from infection by a necrotrophic pathogen (Rossi *et al.*, 2017).

Engineering the photosynthetically active surface by manipulation of chloroplast redox poise

Leaf size defines the maximal photosynthetic area and is a key factor for plant performance within a species (Ren *et al.*, 2019).

Given its importance for plant growth and crop yield, foliar development has been extensively studied, revealing a complex and plastic process whose final outcome in terms of size and shape depends on genetic background, position, and environmental conditions (Andriankaja *et al.*, 2012). Leaf growth proceeds through two stages: an initial proliferative phase in which cells divide and simultaneously increase their volume, followed by a post-mitotic cell expansion phase which determines final leaf size (Traas and Monéger, 2010; Gonzalez *et al.*, 2010, 2012; Andriankaja *et al.*, 2012; Kalve *et al.*, 2014). Leaf cell proliferation and expansion are controlled by the interplay of a network of endogenous factors such as phytohormones, ROS, sugars, and other signals which in turn affect the expression of a multitude of genes (Werner and Schmülling, 2009; Traas and Monéger, 2010).

Chloroplasts play a central role during leaf growth, not only by providing energy and carbon via photosynthesis but also through the generation and transmission of redox-based signals (Muñoz and Munné-Bosch, 2018). Andriankaja *et al.* (2012) have shown that timely exit from the proliferative phase requires complete chloroplast biogenesis and can be prevented by herbicides acting on the photosynthetic machinery. Moreover, several mutants lacking chloroplast proteins differentially expressed during leaf development display severe growth phenotypes including major reductions in organ size (van Dingenen *et al.*, 2016a, b).

It is noteworthy that intervention of the PETC with alternative electron shuttles has led to increases or decreases in leaf size depending on the carrier assayed. For instance, introduction of Flv1–Flv3 increased leaf and plant size in Arabidopsis and barley (Tula *et al.*, 2020; Shahinnia *et al.*, 2021). Likewise, expression of algal cytochrome c_6, a luminal electron carrier that replaces plastocyanin in intersystem electron transfer in some phototrophic microorganisms (de la Rosa *et al.*, 2002), resulted in larger plants and leaves in both Arabidopsis and tobacco (Chida *et al.*, 2007; Yadav *et al.*, 2018), even under field conditions (López-Calcagno *et al.*, 2020). Growth improvements were associated with increased photosynthetic rates and water use efficiency in the cytochrome c_6 transformants (López-Calcagno *et al.*, 2020). Fld, which shuttles reducing equivalents beyond PSI, displayed the opposite effect in Fld-expressing plants, that had smaller leaves (Li *et al.*, 2017; Su *et al.*, 2018; Mayta *et al.*, 2019a), despite the higher photosynthetic efficiency displayed by the transformants (Tognetti *et al.*, 2006). The contrasting effects of different electron sinks acting on the same PETC is intriguing and deserves further investigation. Flv complexes act as dissipative systems cycling reducing equivalents around water, in a sort of pseudo-CET (Yamamoto *et al.*, 2016). Instead, cytochrome c_6 can boost photosynthetic activities (Yadav *et al.*, 2018; López-Calcagno *et al.*, 2020) and Fld might also contribute to the distribution of reducing equivalents into productive pathways downstream of PSI (Zurbriggen *et al.*, 2008). They actually integrate into the metabolic network of the chloroplast, yet their expression led to opposite outcomes in terms of leaf growth.

One possible explanation is that the size effect is mediated by altered carbohydrate metabolism and contents, since sugars contribute to plant and leaf growth not only as a source of carbon and energy but also as signaling intermediates (van Dingenen *et al.*, 2016a; Sakr *et al.*, 2018). Plants expressing cytochrome c_6 did show increased levels of soluble sugars when grown under normal conditions (Yadav *et al.*, 2018), but the contents of sucrose, glucose, and fructose were not affected, relative to their wild-type counterparts, by expression of Flv1–Flv3 (Tula *et al.*, 2020; Shahinnia *et al.*, 2021), Flv2–Flv4 (Vicino *et al.*, 2021), or Fld (Tognetti *et al.*, 2007; Mayta *et al.*, 2018). Interestingly, genome-wide RNA profiling of Fld-expressing plants revealed generalized induction of components of the 26S proteasome in both tobacco and potato (Pierella Karlusich *et al.*, 2017, 2020). This proteolytic system is involved in regulation of several developmental and environmental plant responses, and its activity has been shown to negatively modulate leaf size in tobacco (Nguyen *et al.*, 2013). The results suggest that the Fld effect could be mediated by enhanced proteasome activity in the transgenic lines, although further research is required to properly substantiate this hypothesis.

Even a reduction of leaf and plant size might be of agronomic relevance if overall photosynthetic efficiency is maintained, because crop production also depends on the partition of photoassimilates into sink organs. Harvest index (HI) is thus defined as the ratio of grain, fruit, or tuber yield to total plant biomass (Gur *et al.*, 2010), and reflects the ability of a sink tissue to capitalize on the availability of photosynthate to increase the yield of a marketable product. As such, HI has been regarded as a reference parameter to evaluate the progress of breeding programs aimed at improving yield potential. Indeed, much of the success of the Green Revolution stemmed from major increases in HI resulting from the development of dwarf varieties of rice and wheat with diminished leaf biomass coupled to similar or higher grain yields (Khush, 2001). While these dwarfing traits were found to result from mutations of genes involved in gibberellin synthesis and signaling (Hedden, 2003), a similar outcome was obtained by Fld expression in tomato chloroplasts (Mayta *et al.*, 2019a). In this case, the higher photosynthetic efficiency per leaf cross-section driven by Fld activity (Tognetti *et al.*, 2006; Gómez *et al.*, 2020), outpaced the reduction in photosynthetically active surface, leading to significant increases in HI (Mayta *et al.*, 2019a). Calculations based on the horizontal expansion diameters of the engineered plants (Li *et al.*, 2016) indicate that up to 3-fold improvement in absolute fruit yield per planted surface could be gained by increasing plant density per square meter in the field.

Extending photoassimilate production through senescence delay

By determining the time span of photosynthetic activity, leaf senescence is also of critical importance for crop yield. Premature senescence, such as that caused by environmental

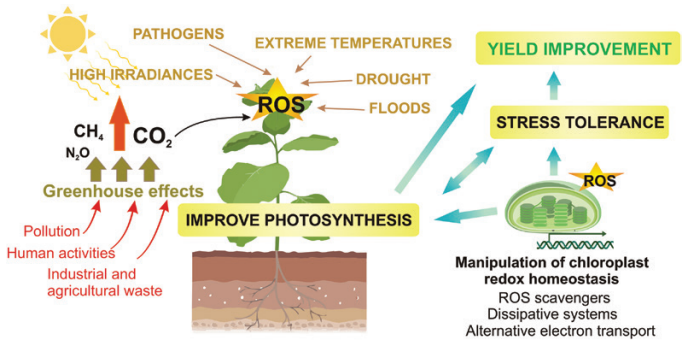

Fig. 3. Manipulation of chloroplast oxido-reductive pathways as a tool to improve plant adaptation to the challenges of global warming. The cartoon shows the consequences of climate change which aggravates biotic and abiotic hardships. Industrial and agricultural pollution and other human activities increase greenhouse gases, most remarkably CO_2, but also methane and nitrous oxide. The resulting global warming affects the intensity and frequency of environmental hardships such as drought, floods, and extreme temperatures, as well as the distribution and virulence of phytopathogens. Most environmental stresses lead to higher rates of ROS propagation, especially in chloroplasts, and affect the redox state of the PETC and the stroma. Manipulation of chloroplast redox homeostasis by overexpression of ROS scavengers, dissipative systems, and additional electron sinks ameliorate the negative effects of these perturbations by preventing over-reduction of the PETC and ROS propagation and accumulation, offering alternative strategies to improve photosynthetic performance, stress tolerance, and ultimately yield.

adversities, is known to negatively affect plant productivity. At the same time, nitrogen recycling requires that some organs senesce and die for others to develop. In this sense, optimization of senescence timing for defined species and growth conditions represents a major goal of crop breeding programs.

While natural leaf senescence is primarily associated with aging, it can also be induced by environmental and nutritional factors including biotic and abiotic stresses, darkness, phytohormones, and ROS. As discussed previously, involvement of chloroplast redox chemistry (including ROS propagation) in modulating cell death is well supported. Chloroplast contribution to senescence has received less attention, but recent findings indicate that changes in the redox balance of these organelles strongly affect senescence timing and progress, operating at an early stage and at a hierarchically high level of developmental decisions (Chen and Gallie, 2006; Abbasi *et al.*, 2009; Mayta *et al.*, 2018).

Accelerated leaf senescence in plants deficient in plastid-borne antioxidant enzymes such as APX, DHAR, and GR supports a role for chloroplast oxido-reductive signaling in this developmental process (Chen and Gallie, 2006; Gou *et al.*, 2015; Ding *et al.*, 2016). The redox poise of the PETC appears to be critical in modulating the onset of leaf senescence. Inactivation of chloroplast NADH dehydrogenase, which is expected to decrease electron input into the chain, leads to a stay-green phenotype in tobacco (Zapata *et al.*, 2005). A similar outcome was obtained by introduction of Fld (Mayta *et al.*, 2018, 2019*b*), suggesting that a more oxidized state of the PETC and/or down-regulation of ROS levels correlate with a protracted induction of senescence and differential preservation of photosynthetic activities. These observations highlight the possibilities offered by manipulation of the PETC to generate crops with extended functional life spans in the fluctuating conditions faced by plants growing in the field (Krieger-Liszkay *et al.*, 2019).

Compared with metabolic engineering, the manipulation of developmental traits for increased and extended photosynthetic capacity and photoassimilate transport is still in its infancy, in part because the genetic and molecular mechanisms controlling leaf shape, size, architectural features, and longevity are very complex, and have only just begun to be understood (Mathan *et al.*, 2016).

Conclusions and perspectives

While our first priority is to prevent further progress of climate deterioration by adopting more rational policies of energy use, we must also increase our knowledge to better understand the potential consequences of this global event, and employ the available genetic resources to better adapt to the challenges of a changing environment. The latter goal involves not only the development of stress-resilient crops to feed a growing population, but also the design of improved tools for ecosystem preservation and restoration.

To date, huge efforts have been made by the scientific community to reach these goals. However, translation of the tolerance observed under defined growth conditions to the field is still complex, and not always predictable. At least some of the difficulties arise from the different responses elicited by plants to natural environmental challenges compared with those occurring in growth chambers or greenhouses. An increasing amount of evidence indicates that plants respond to stress combinations in a non-additive manner and that field trials are thus required to properly characterize these responses (Mittler, 2006; Zandalinas *et al.*, 2020). While several stress-related genetic determinants have been tested in the field, only a few of them were associated with chloroplast redox metabolism (Gómez *et al.*, 2019).

It is widely recognized that the pace of plant growth results from a trade-off between the resources used for growth and development and those invested in protection against environmental challenges (Raven, 2011; Croce and van Amerongen, 2014; Davis *et al.*, 2017). Mounting evidence indicates that within this complex interplay, chloroplasts provide not only energy and reducing power to fuel these processes, but also redox-based signals that modulate the responses to a given situation (Fig. 3). The mechanistic details of these interactions are far from understood. Plants play a dangerous game by employing the levels of hazardous chemicals to modulate stress responses, and it is somehow counterintuitive that genetic interventions that limit the accumulation of such key signaling molecules invariably display beneficial effects on plant survival

and welfare under adverse conditions (Tables 1, 2). It should be noted, however, that stress situations not only increase ROS levels but also induce the expression of antioxidant systems and alternative electron sinks such as PTOX (Choudhury *et al.*, 2017), suggesting that redox-based signaling is kept under tight control during plant stress responses. It is tempting to speculate, therefore, that genetic manipulations such as those illustrated in Tables 1 and 2 display differential effects on the damaging and beneficial consequences of over-reduction of the PETC and ROS propagation, eventually preventing them from reaching deleterious limits without compromising their signaling roles. The use of 'omics' approaches could help to address this question by comparing transcriptional and metabolic profiles generated by stress conditions in wild-type and transformed plants, but their application has been so far quite limited (Pierella Karlusich *et al.*, 2017, 2020). Current efforts to engineer crops with better photosynthetic performance and higher tolerance to environmental stresses are largely based on modifying the expression of a few genes (Tables 1, 2). Even these rather simple approaches have shown considerable promise as exemplified here. The formidable advances in functional genomics will certainly multiply these possibilities, allowing the identification of additional factors that could be targeted in crop improvement, and becoming a way forward to efficiently manipulate plant traits for increasing yield (Mathan *et al.*, 2016; Leisner, 2020).

Crops of the future will require extensive genetic modifications, probably customized for maximum productivity in the particular environment where they will be cultivated. They may incorporate components from multiple sources, including ancestral traits and genes optimized by directed evolution. In this context, manipulation of chloroplast metabolism has shown significant potential to improve not only photosynthetic performance, but also stress tolerance and leaf size and longevity. Little is known about the underlying mechanisms responsible for these effects and therefore a deeper understanding of chloroplast redox biochemistry will be critical to provide more accurate predictions concerning climate change effects on plant growth and reproduction, as well as biotechnological opportunities to improve crop yield and productivity in a warming world.

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