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REVIEW

Matrix Redox Physiology Governs the Regulation of Plant Mitochondrial Metabolism through Posttranslational Protein Modifications

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Mitochondria function as hubs of plant metabolism. Oxidative phosphorylation produces ATP, but it is also a central high-capacity electron sink required by many metabolic pathways that must be flexibly coordinated and integrated. Here, we review the crucial roles of redox-associated posttranslational protein modifications (PTMs) in mitochondrial metabolic regulation. We discuss several major concepts. First, the major redox couples in the mitochondrial matrix (NAD, NADP, thioredoxin, glutathione, and ascorbate) are in kinetic steady state rather than thermodynamic equilibrium. Second, targeted proteomics have produced long lists of proteins potentially regulated by Cys oxidation/thioredoxin, Met-SO formation, phosphorylation, or Lys acetylation, but we currently only understand the functional importance of a few of these PTMs. Some site modifications may represent molecular noise caused by spurious reactions. Third, different PTMs on the same protein or on different proteins in the same metabolic pathway can interact to fine-tune metabolic regulation. Fourth, PTMs take part in the repair of stress-induced damage (e.g., by reducing Met and Cys oxidation products) as well as adjusting metabolic functions in response to environmental variation, such as changes in light irradiance or oxygen availability. Finally, PTMs form a multidimensional regulatory system that provides the speed and flexibility needed for mitochondrial coordination far beyond that provided by changes in nuclear gene expression alone.

INTRODUCTION

The mitochondria act as the power stations of aerobic eukaryotic cells by producing and exporting ATP, which is needed to drive life processes such as biosynthesis and transmembrane transport and to energize cellular signaling and selective degradation processes. The requirements of the cell for energy and building blocks change dynamically in response to developmental programs or environmental conditions as well as fluctuations due to diurnal oscillations (Rasmusson and Escobar, 2007). Environmental conditions can change rapidly, over minutes or even seconds. For example, fluctuations in light incidence on a leaf (e.g., due to clouds or sunflecks) alter photosynthesis rates, which in turn affect cellular metabolism on the same time scale (Eberhard et al., 2008). Another example is reoxygenation, which can occur after hypoxia in roots when water retreats after flooding or during seed imbibition and germination (Taiz et al., 2015; Wagner et al., 2018). These rapid changes in substrate or oxygen supply require that the mitochondria modulate their metabolism quickly, for instance by shutting down parts of the tricarboxylic acid cycle (TCA cycle) and operating it in noncyclic mode (Sweetlove et al., 2010). A particularly efficient way of achieving rapid and direct functional modulation is via posttranslational modification (PTM) of existing proteins. Local short-term regulation can bridge the critical time span before long-term reprogramming of mitochondrial function via nuclear gene expression, which can be achieved through retrograde signaling (Ng et al., 2014; Van Aken and Pogson, 2017).

Recent progress in functional proteomics-based investigations has shown that most enzymes involved in mitochondrial metabolism can be modified by PTMs and that their properties are therefore potentially regulated by PTMs (Bykova et al., 2003a; Balmer et al., 2004; Salvato et al., 2014; Nietzel et al., 2020; König et al. 2014a). We are also beginning to understand the effects of specific PTMs on specific enzymes, making it possible to appreciate their regulatory significance. Most PTMs are directly or indirectly associated with redox metabolism and regulation. Rapid progress has recently been made in understanding subcellular

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redox dynamics, not only through proteomic and analytical methods but also through live imaging and biosensing. For instance, fluorescent protein-based biosensors that are genetically targeted to the mitochondria, such as variants of the redox-sensitive green fluorescent protein (roGFP) family, make it possible to continuously monitor the glutathione redox state in the matrix of plant mitochondria inside living cells, tissues, or whole plants aided by confocal laser scanning microscopy or fluorimetric methods (Albrecht et al., 2014; Schwarzländer et al., 2016). This approach and related in vivo analyses can complement proteomic and analytical analyses and provide new insights into the regulation of cellular metabolism (Nietzel et al., 2020).

The role of PTMs as a metabolic regulatory mechanism was recently reviewed by Millar et al. (2019) at the cellular level and by Sandalio et al. (2019) at the peroxisomal level. In this review, we outline an integrated picture of how mitochondrial metabolism can adjust to changes in cellular/environmental conditions to serve the rapidly changing cellular requirements of plants via redox-associated changes that lead to changes in PTMs, which in turn alter protein properties and metabolic flux.

REDOX COUPLES AND REDOX-ASSOCIATED REGULATION OF MITOCHONDRIAL PROTEINS

All the PTMs discussed in this review affect the side chains of protein amino acids (Figure 1; Table 1). Many of the PTMs are oxidations, such as Met and Cys oxidation and carbonylation of Arg, Lys, Pro, Thr, and Trp. Some oxidative modifications are caused by direct interaction with reactive oxygen species (ROS) or reactive nitrogen species, which may be relatively random, and many modifications are irreversible. This does not exclude a regulatory role per se, since protein turnover could act as an off-switch. Disulfide bridge formation is reversible through thioredoxin (Trx)- or glutaredoxin (Grx)-catalyzed reduction, which can constitute an important regulatory mechanism (Nietzel et al., 2017). Likewise, the first step in Met and Cys oxidation (e.g., to Met sulfoxide) is reversible through the action of dedicated reductases (Gustavsson et al., 2002; Biteau et al., 2003; Vieira Dos Santos et al., 2007). Other common PTMs include phosphorylation of Ser, Thr, or Tyr and acetylation of Lys, which occur on well-defined sites and are recognized by dedicated enzymes during both PTM formation and removal. PTMs typically affect protein structure and function by changing the charge of the amino acid side chain (e.g., phosphorylation and acetylation) or by introducing structural constraints, such as disulfide bridges (Figure 1; Table 1).

More than 60 different PTMs caused by oxidation-reduction reactions have been identified (Møller et al. 2011). Relatively few of these have been reported for plant mitochondrial proteins to date: Cys, Met and Trp oxidation, Cys nitrosylation, and carbonylation of Arg, Lys, Pro, Thr, and Trp (Figure 1; Balmer et al., 2004; Kristensen et al., 2004; Møller and Kristensen, 2006; Salvato et al., 2014; Nietzel et al., 2017). These PTMs are formed as a result of the highly reducing conditions for key redox couples in the mitochondrial matrix fueled by the turnover of the TCA cycle and other metabolic pathways. The NADH and succinate (and NADPH) produced by the TCA cycle reduce the electron transport chain (ETC). When electron transport through the ETC is restricted and slowed down, the redox centers of the ETC components become highly reduced, resulting in an increased reduction rate of molecular oxygen, leading to superoxide formation. In turn, superoxide can give rise to several different reactive oxygen and nitrogen species (including H_2O_2, the hydroxyl radical, and peroxynitrite; Møller, 2001; Murphy, 2009; Brand, 2010; Halliwell and Gutteridge, 2015). These compounds show differential reactivity with the different amino acid side chains of proteins, which can lead to changes in PTM patterning.

Redox Conditions in the Plant Mitochondrial Matrix

The redox PTMs all depend on the redox potential of the dominant redox couples in the matrix and, as we shall see later, so do the other PTMs, albeit indirectly. The main soluble redox couples in the matrix are NAD (NADH and NAD^+; $E^{o'} = -320$ mV), NADP (NADPH and $NADP^+$; $E^{o'} = -320$ mV), Trx *o* (Trx-SH and Trx-SS; E = -305 mV at pH 7.5), glutathione (GSH and GSSG; $E^{o'} = -240$ mV), and ascorbate (ascorbate and dehydroascorbate; $E^{o'} = +90$ mV; Noctor, 2006; Queval and Noctor, 2007; Yoshida and Hisabori, 2016). Since a key principle driving redox dynamics in vivo is that thermodynamic differences in the potential of different redox couples are actively maintained to avoid equilibration and to enable regulation, any statement about a redox state or potential of a cell compartment or even the cell without clear reference to a specific redox couple will be inaccurate. We will therefore not use the expression "the redox potential in the matrix." Instead, the redox potential of each redox couple in the matrix needs to be considered separately. We plotted the midpoint potentials for the main redox couples in the matrix-relevant pH 6.8 to 8.0 range in Figure 2. However, although often overlooked, the most abundant redox couples in the mitochondrial matrix (or in any other subcellular compartment) are the thiols in the side chains of Cys and Met residues in proteins (Requejo et al., 2010; Bruhn et al., 2012; Nietzel et al., 2017). Their redox potentials vary widely depending on the molecular environment of each thiol group, and therefore they cannot be included with defined values in Figure 2A.

However, the midpoint potentials of redox couples are of limited use when deciding which redox reactions are likely to occur in the mitochondrion. This would require more detailed information about the actual redox potentials (depending on the relative abundance of the reduced and oxidized forms and temperature and [depending on the specific reaction] the total pool size and pH) and, importantly, the kinetic properties of the reaction, which depend on the available catalysts. This information is typically not easy to obtain for a small cell compartment like the mitochondrial matrix, but important experimental and conceptual progress has been made in recent years.

Here, we will briefly consider the concentration and redox state of each of these redox couples and whether they are in thermodynamic equilibrium with their interacting redox couples. We will also consider the kinetic characteristics of their interaction with other redox couples, which are of central importance for measuring and understanding redox potentials and redox regulation. When the estimated redox potentials for different redox couples in the same cells or the same cell compartment under the same external conditions are different, the redox couples are not in thermodynamic equilibrium. We will also discuss the biosynthesis and import as well as the degradation and export of these redox

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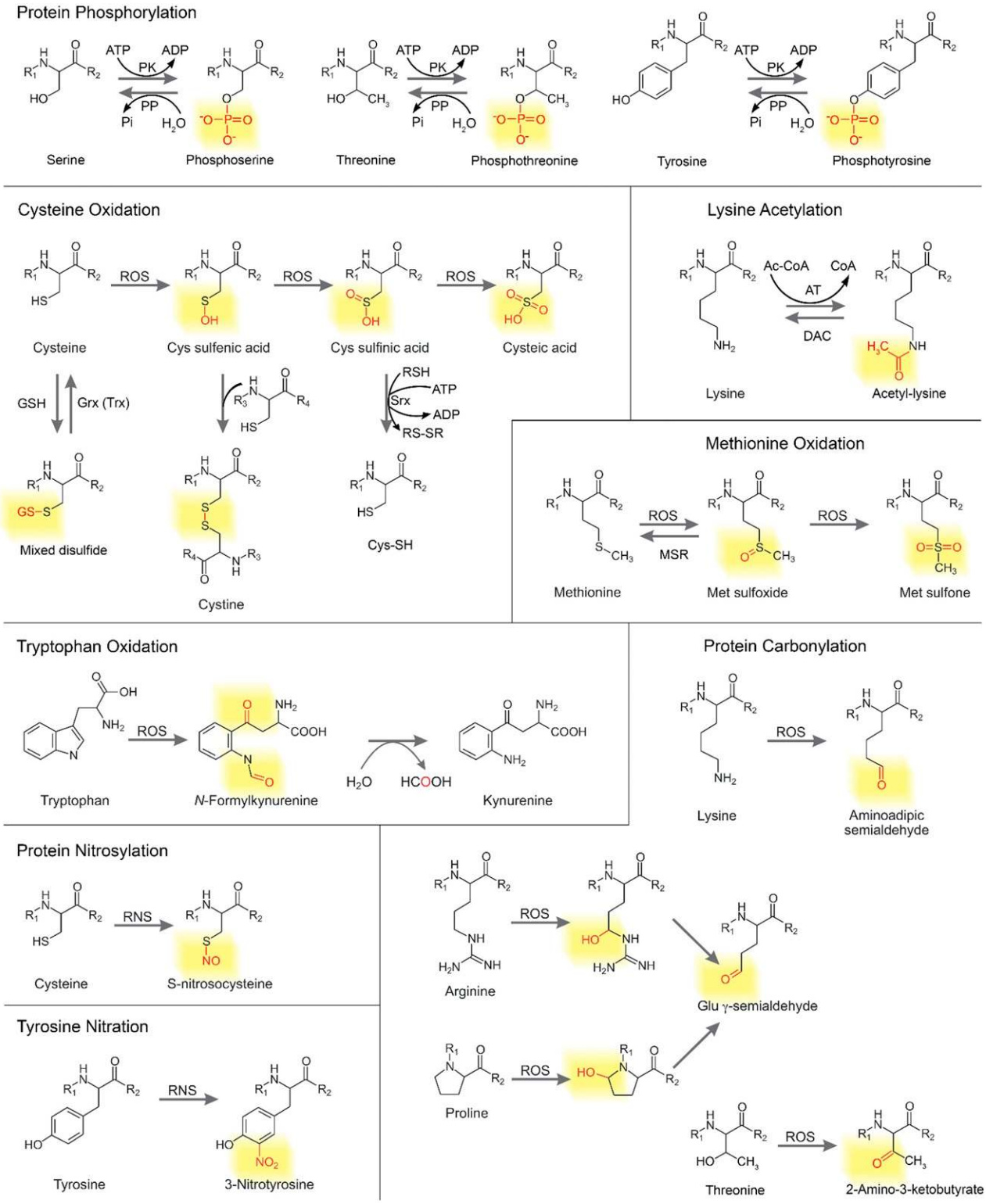

Figure 1. PTMs of Protein Side Chains Discussed in This Review.

Protein phosphorylation of Ser, Thr, and Tyr is catalyzed by protein kinases (PK) and is reversible via a reaction catalyzed by protein phosphatases (PP). Oxidation of Cys can occur via the action of ROS. These nonenzymatic redox modifications form mixed disulfides (e.g., glutathionylation) and Cys derivatives with sulfenic, sulfinic, and cysteic acid groups. The reduction of mixed disulfides, like glutathionylation products, occurs via the action of Trx,

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couples, since the combination of these processes determines their concentration. The concentration of a redox couple is important for two reasons. (1) The rate of enzymatic activity usually depends on the substrate concentration. For example, in plant mitochondria, complex I and NDA have highly different affinities for NADH (K_m = 8 and 80 μM, respectively; Møller and Palmer, 1982). This means that complex I is active even when the NAD pool is relatively oxidized (state 3), while NDA becomes engaged when the NAD pool becomes more reduced (state 4), but also when the NAD pool size increases through NAD^+ uptake (Palmer et al., 1982). (2) In any redox reaction where two molecules dimerize (e.g., 2GSH →GSSG), the monomer concentration will be squared in the Nernst equation used to calculate the redox potential for the reaction ox + z e^- → red:

$$E = E^{o} - \frac{2.3RT}{zF}\left(\log\frac{C_{red}}{C_{ox}}\right)$$

Where E is the actual redox potential, E^o is the standard midpoint potential, R is the gas constant, T is the absolute temperature in Kelvin, z is the number of electrons involved in the reaction, F is the Faraday constant, and C_{red} and C_{ox} are the concentrations of the reduced and oxidized forms, respectively. Thus, at an unchanged redox potential, a decrease in the glutathione pool size will lead to a larger decrease in the GSSG concentration (C_{ox}) versus the GSH concentration (C_{red}; Nicholls and Ferguson, 2014).

NAD

NAD^+ is actively transported across the inner mitochondrial membrane (IMM) into the matrix, where the concentration is 1 to 2 mM (Neuburger et al., 1985). Based on homology to yeast NAD transporters, two mitochondrial carrier family proteins were identified in Arabidopsis (*Arabidopsis thaliana*): NAD^+ TRANSPORTER1 (NDT1) and NDT2 (Todisco et al., 2006; Palmieri et al., 2009). Both proteins can complement NAD^+ transport in a yeast mutant and preferentially transport NAD^+ in vitro. While only NDT2 localized to the mitochondria when transiently expressed in tobacco (*Nicotiana tabacum*) protoplasts as a GFP fusion protein, more recent work suggests mitochondrial localisation also for NDT1. NAD^+ can also be synthesized in the matrix from nicotinamide mononucleotide (Palmieri, 2009; Di Martino and Pallotta, 2011; Noctor et al., 2011; de Souza Chaves, 2019).

Matrix NAD^+ is consumed by (at least) two processes. (1) The enzyme poly[ADP-ribose] polymerase (PARP) adds the ADP-ribose part of NAD^+ to poly[ADP-ribose] tails on proteins, releasing nicotinamide. Poly[ADP-ribose] tails are important for regulating DNA-repair processes (Briggs and Bent, 2011). In mammals, many PARPs are nuclear, but at least one is thought to be mitochondrial, where it participates in the same DNA-repair processes as in the nucleus (Cantó et al., 2015; Xiao et al., 2018). In Arabidopsis, most PARPs are nuclear, but PARP2 has been found in chloroplasts and mitochondria (Pham et al., 2015), although the evidence is unclear. (2) The sirtuin family of proteins are protein deacetylases that can interact with PARP (Bai et al., 2011, 2015) and use NAD^+ as a substrate and release *O*-acetyl-ribose and nicotinamide as by-products of deacetylation (Tanner et al., 2000; Dölle et al., 2013). These reactions are important for protein acetylation, as discussed below.

The redox couple NADH/NAD^+ (and NADPH/$NADP^+$) has the lowest (most negative, most reducing) midpoint potential of all redox couples discussed in this review. A span in NAD reduction level between 10 and 90% is equivalent to a span in redox potential between −290 and −350 mV at pH 7.0 (Figure 2). The reduction level of NAD has been measured in isolated mitochondria by extraction followed by HPLC analysis, providing NAD^+ and NADH concentrations at single time points. Alternatively, NADH fluorescence measurements can be used to continuously monitor changes in the concentration of NADH (and NADPH, which has the same fluorescence spectrum; Neuburger and Douce, 2003; Kasimova et al., 2006). It is now possible to monitor NAD concentration and redox state continuously without interference from NADP using engineered fluorescent protein biosensors (Hung et al., 2011). However, such biosensors have not yet been used in studies of plant mitochondria. Similarly, the separate in vivo measurement of NADH and NADPH concentrations using fluorescence lifetime imaging of autofluorescence has not yet been established in plants (Blacker et al., 2014).

Many highly active enzymes in the matrix, including the TCA cycle dehydrogenases, complex I, and rotenone-insensitive NADH dehydrogenases, require NAD^+ or NADH for activity and therefore contribute to and interact with the NAD reduction level in the mitochondria and, in turn, in the rest of the cell (Møller and Rasmusson, 1998; Møller, 2001; Rasmusson and Møller, 2011; Bykova et al., 2014; Wallström et al., 2014; Bykova and Igamberdiev, 2016; Pétriacq et al., 2017). Due to the presence of several abundant enzymes that use NAD, a major portion of the matrix NAD is bound to proteins, which lowers the free concentration of the coenzyme (Kasimova et al., 2006). However, in isolated mitochondria, direct measurements of metabolites and modeling have shown that the reduction level of matrix NAD is governed solely by the equilibrium constant of NAD-linked malate dehydrogenase, which is present at very high capacities (Figure 3; Palmer et al., 1982; Hagedorn et al., 2004; Fuchs et al., 2020). The NAD reduction level changes dramatically with the respiratory state of the mitochondria and with pH. NAD is largely reduced (>60%) in state 4 respiration of isolated mitochondria, where

Figure 1. (continued).

while at least in peroxiredoxins, Cys sulfinic acid groups can be reduced by sulfiredoxin (Srx) in the presence of ATP (Biteau et al., 2003). Lys acetylation is catalyzed by acetyltransferases (AT) and can be reversed by deacetylases (DAC). Met oxidation to Met-sulfoxide and Met-sulfone is nonenzymatic, and the first step can be reversed by Met sulfoxide reductase (MSR). Trp oxidation is also nonenzymatic and occurs due to the interaction with ROS. Carbonylation of Arg, Lys, Pro, and Thr occurs via interaction with ROS nonenzymatically and irreversibly. Protein *S*-nitrosylation of Cys and nitration of Tyr are nonenzymatic and take place due to the interaction with reactive nitrogen species (RNS). The dominant charged state of the side chains at pH 7.0 is shown only for the phosphorylated Ser, Thr, and Tyr.

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Table 1. PTMs and Their Effects on Protein Structure and Function Presented in the Same Order as in the Text

PTM	Amino Acid	Effect of PTM
Disulfide formation	Cys	Introduces a structural constraint on the protein; blocks the thiol group and interferes with metal ion binding, especially Fe
Met oxidation	Met	Introduces a polarized $-S^+-O^-$ side group; makes the side chain more hydrophilic
Carbonylation	Arg	Removes the positively charged guanidino group
	Pro	Opens the pyrrolidine ring, which removes a kink in the amino acid chain
	Lys	Removes the positive charge and makes the side chain more hydrophobic
Nitrosylation	Cys	Blocks the thiol group and interferes with metal ion binding, especially Fe
Phosphorylation	Ser	Introduces a strong negative charge; acts as a recognition site for reader proteins (e.g. 14-3-3 proteins)
	Thr	
	Tyr	
Acetylation	Lys	Neutralizes the positive charge and adds hydrophobicity; acts as a recognition site for reader proteins, such as bromodomains

See Figure 1 for the structural formulae.

proton backflow through the ATP synthase (to produce ATP) is restricted due to a lack of ADP. This creates a highly negative transmembrane potential across the IMM (around −200 mV, inside negative), which restricts electron flow through the ETC. NAD is <10% reduced in state 3 respiration, when the membrane potential is less negative due to ATP synthase activity (Agius et al., 2001).

In vivo, the reduction level of NAD in the matrix appears to be relatively low (Figure 2), corresponding to a redox potential of approximately −300 mV, indicating that the mitochondria are closer to state 3 than to state 4 in actively respiring cells (Igamberdiev et al., 2001; Igamberdiev and Gardeström, 2003). The reduction level of NADP (which has the same midpoint potential as NAD; Figure 2A) measured in the same mitochondria under the same conditions is clearly higher (more negative redox potential; Figure 2B), demonstrating that these two redox couples are not in thermodynamic equilibrium. The half-reactions for all the main redox couples are shown in the Supplemental Figure.

NADP

$NADP^+$ uptake across the IMM has been reported (Bykova and Møller, 2001), but this has never been validated. There is no known carrier for $NADP^+$, so matrix $NADP^+$ is likely to be formed by an NAD kinase in the mitochondrial matrix, as observed in yeast (Outten and Culotta, 2003) and mammals (Ohashi et al., 2012). However, none of the three NAD kinases encoded in the Arabidopsis genome have been localized to the mitochondria (Waller et al., 2010; Li et al., 2014b; Gakière et al., 2018), leaving open the question of the origin of NADP in the plant mitochondrial matrix. The concentration of NADP in the matrix is much lower than that of NAD (Agius et al., 2001; Igamberdiev and Gardeström, 2003), so the measurements of NAD(P)H autofluorescence mentioned above are clearly dominated by NADH and mask NADPH. In vivo NADPH measurements in the mitochondria may be possible using the fluorescence lifetime imaging approach. In addition, two fluorescent protein sensor families for NADPH have recently been engineered, offering a novel handle on NADP redox measurements for plant mitochondria (Cameron et al., 2016; Tao et al., 2017).

Mammalian mitochondria contain a transhydrogenase in their IMM, which catalyzes the reduction of $NADP^+$:

$$NADH + NADP^+ + H^+(out) \rightarrow NAD^+ + NADPH + H^+(in)$$

This reaction is driven by the IMM proton gradient, which prevents the redox potentials of the two redox couples from reaching an equilibrium and keeps NADP more reduced than NAD (Rydström, 2006). Plant genomes lack transhydrogenase genes. Nevertheless, transhydrogenase-like activity has been observed in plant mitochondria (Bykova et al., 1999), which may conceivably arise from different NAD- and NADP-dependent dehydrogenases under in vitro conditions (albeit associated with redox equilibration and not coupled to the proton gradient).

The concentrations of $NADP^+$ and NADPH in the matrix regulate and are regulated by matrix-localized enzymes, including an NADP-isocitrate dehydrogenase and the rotenone-insensitive NADPH dehydrogenase in the ETC. After oxidation, NADPH can be regenerated by several enzymes, most likely NADP-dependent isocitrate dehydrogenase (NADP-ICDH; Figure 3; Rasmusson and Møller, 1990, 2011; Møller and Rasmusson, 1998; Møller, 2001; Igamberdiev and Gardeström, 2003; Rasmusson et al., 2004), but also by folate metabolism (Goreleva, 2017). There is further evidence for a degree of NADP reduction by matrix enzymes that are typically considered NAD-dependent, such as malate dehydrogenase/malic enzyme and glutamate dehydrogenase in situ, as detected through disulfide reduction of the alternative oxidase (AOX) dimer through the matrix Trx system, which is strictly reliant on electrons from NADPH (Vanlerberghe et al., 1995). It is difficult to estimate the relative contributions of the different enzymes to the reduction of the NADP pool. These contributions are linked to the relative fluxes through the respective dehydrogenases. For instance, it remains unclear if and exactly how isocitrate is allocated to NAD-ICDH and NADP-ICDH, respectively, for oxidation to 2-oxoglutarate. Such a modulation mechanism may have a profound impact on reductant allocation between NAD and NADP and in turn their downstream electron

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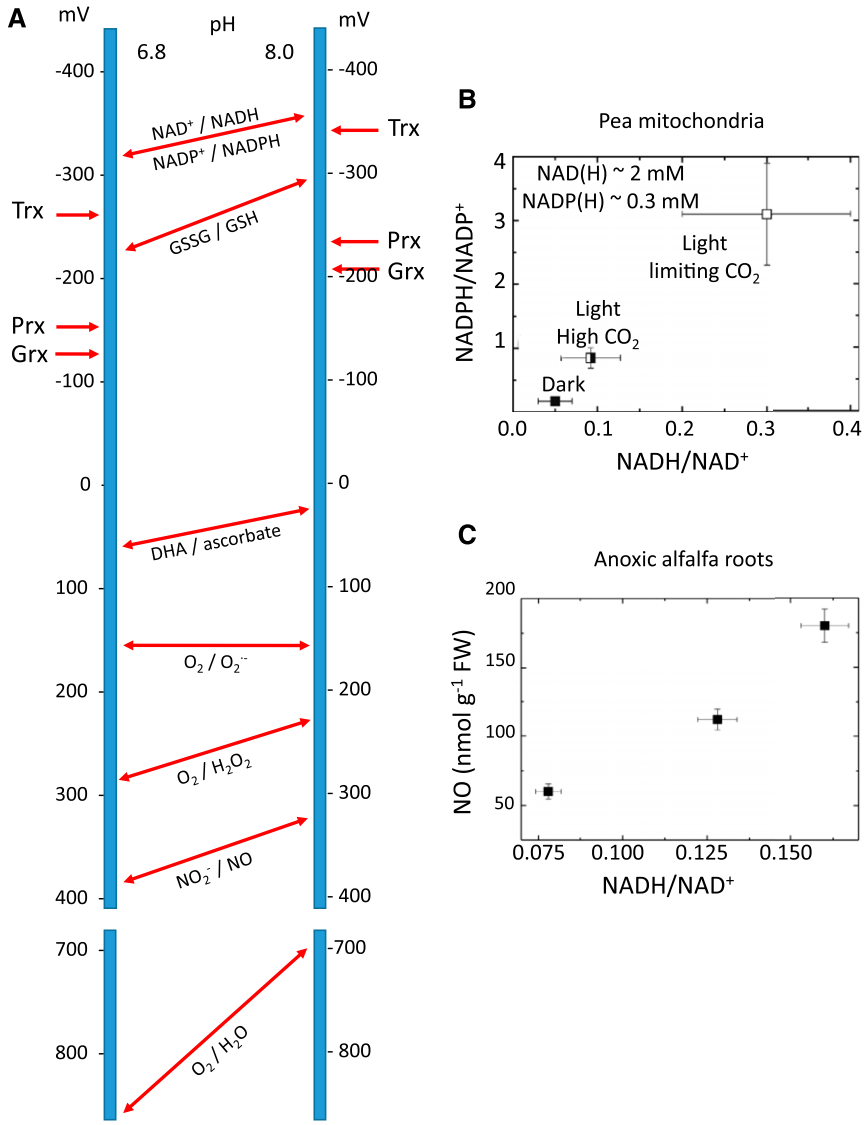

Figure 2. Regulation of the Redox Characteristics in the Matrix of Plant Mitochondria.

(A) The redox potentials of the major redox pairs at pH 6.8 and 8.0. The further the redox potentials are from each other, the more different their reduction levels will be at thermodynamic equilibrium. The values of redox potentials at pH 6.8 and 8.0 for the pairs of oxidized/reduced Trx, peroxiredoxin (Prx), and Grx are shown by arrows pointing to the graph from the left and right to distinguish them from the low-molecular-weight redox pairs. Note that redox potentials of Trx, Prx and Grx proteins can differ strongly within the respective protein families. Specific half reactions under consideration are shown in Supplemental Figure 1.
(B) The pool sizes of NAD and NADP and their redox levels in pea leaf mitochondria depending on light and CO_2 supply (Igamberdiev and Gardeström, 2003).
(C) The accumulation of nitric oxide (NO), depending on the NADH/NAD^+ ratios in anoxic alfalfa (*Medicago sativa*) roots (Dordas et al., 2003; Igamberdiev et al., 2004). FW, fresh weight.

sinks (e.g., thiol redox systems and antioxidant defense versus ATP generation). Substrate channeling via the formation of metabolons has been proposed as a mechanism to direct metabolic fluxes, but the evidence for the dynamic regulation of metabolite channeling in plant mitochondria is currently incomplete and an active field of study (Zhang et al., 2017, 2018).

The reduction level of NADP in the mitochondrial matrix was much higher than that of NAD (−340 mV NADP versus −300 mV NAD), as measured by rapid fractionation of pea (*Pisum sativum*) leaf protoplasts, clearly demonstrating that the two redox couples are not in thermodynamic equilibrium (Igamberdiev and Gardeström, 2003). This suggests that direct $NADP^+$ reduction by matrix dehydrogenases is the dominant mechanism of NADP redox maintenance. By contrast, NADH phosphorylation by the NADH kinase POS5p was proposed to be the major pathway for NADPH regeneration in yeast (Outten and Culotta, 2003). In this system, the maintenance of a more reducing redox potential for NADP than for NAD can be accounted for by ATP hydrolysis. The

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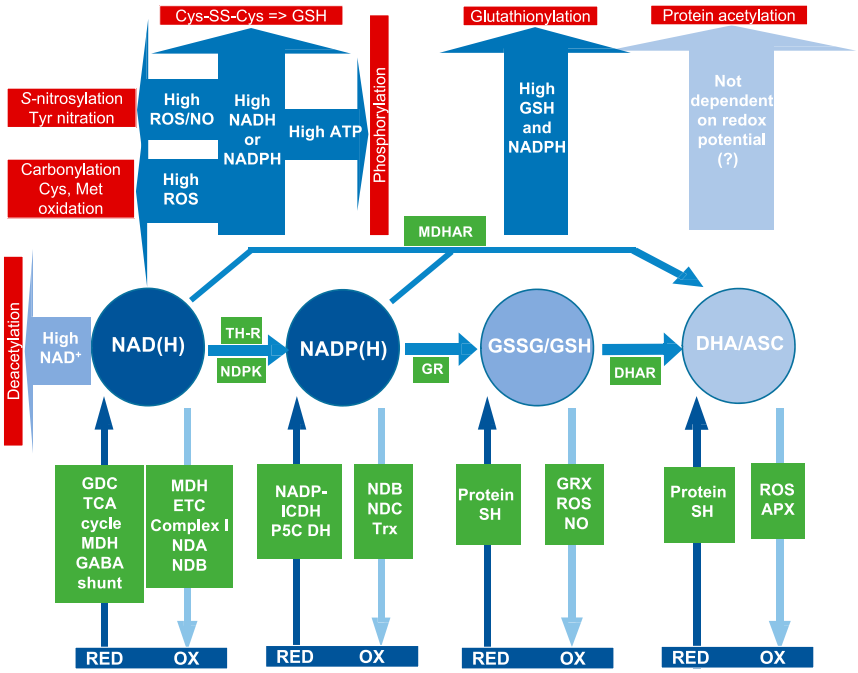

Figure 3. The Link between the Potentials of Matrix Redox Couples and PTMs.

Interactions between pools of the soluble redox couples Cys-SH/Cys-S-S-Cys, NAD(P)H, Trx, glutathione, nitrite, NO, O_2, superoxide, and H_2O_2 are shown. Cys-S-S-Cys to Cys-SH conversions are promoted when the redox potential is very negative [high NAD(P)H/NAD(P)$^+$]. Cys and Met oxidations are promoted when the redox potential is very negative and ROS production is high. Protein nitrosylation occurs when the redox potential is very negative with the concomitant NO and ROS accumulation (e.g., during hypoxia). The formation of highly negative redox potential can also result in protein carbonylations (upon high ROS production). Protein phosphorylation is promoted in state 4 (high ATP level), accompanied by very negative redox potential and ROS accumulation. Protein acetylation is observed upon moderately negative redox potential (allowing PDH to resume acetyl-CoA production). Protein deacetylation by sirtuins takes place when the concentration of NAD$^+$ is high (irrespective of the reduction level). The figure layout was adopted and significantly modified from Noctor (2006). Abbreviations not defined in the text are as follows: APX, ascorbate peroxidase; DHAR, dehydroascorbate reductase; GR, glutathione reductase; MDH, malate dehydrogenase; MDHAR, monodehydroascorbate reductase; NDA, internal rotenone-insensitive NADH dehydrogenase; NDB, external rotenone-insensitive NAD(P)H dehydrogenase; NDC, internal rotenone-insensitive NADPH dehydrogenase; NDPK, nucleoside diphosphate kinase; OX, oxidation; P5C DH, pyrroline-5-carboxylate dehydrogenase; RED, reduction; TH-R, transhydrogenase-like reaction.

Arabidopsis homolog of POS5, the NADH kinase NADK3, was not found in the mitochondria and is likely to reside in the peroxisomes (Turner et al., 2005). More recently, the NADH kinase reaction was found to actually be dispensable in yeast mitochondria, suggesting that yeast also relies mainly on mitochondrial NADP$^+$-dependent dehydrogenases, which reduce NADP$^+$ synthesized from NAD$^+$ by an NAD kinase (Miyagi et al., 2009).

Ascorbate

The last step in ascorbate biosynthesis is catalyzed by the enzyme L-galactono-1,4-lactone dehydrogenase (Noctor and Foyer, 1998), which is attached to the outer surface of the IMM and donates electrons to cytochrome *c* (Bartoli et al., 2000). The IMM presumably contains an as yet unidentified ascorbate transporter similar to the one found in the inner envelope membrane of chloroplasts (Fernie and Tóth, 2015; Miyaji et al., 2015). However, uptake of dehydroascorbate by plant mitochondria has also been reported (Szarka et al., 2004). The concentration of ascorbate in the matrix is much higher than that of NAD (Jimenez et al., 1997), and ascorbate is one of two essential cofactors in the ascorbate/glutathione cycle (see below). The fate of ascorbate in the matrix is currently not resolved, since there are no obvious matrix processes in which ascorbate is degraded/consumed.

It appears that the main function of ascorbate in the mitochondrial matrix is to act as a reductant in the first reaction of the ascorbate-glutathione cycle, where H_2O_2 is removed by the enzyme ascorbate peroxidase. The monodehydroascorbate (MDHA) formed is recycled into ascorbate by a series of reactions involving NADH, NADPH, and GSH. All reducing equivalents ultimately come from NADH and NADPH, while the relative contribution of the two pools may be tuned depending on the relative engagement of the recycling mechanisms via MDHA reductase and dehydroascorbate reductase (Figure 3; Noctor and Foyer, 1998; Møller, 2001, 2007).

Glutathione

Glutathione is a Glu-Cys-Gly tripeptide with a γ-peptide bond between the Glu and Cys, providing protection from degradation by peptidases. Glutathione biosynthesis takes place in two steps catalyzed by GSH1 and GSH2. Both enzymes appear to be

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present in the chloroplast stroma and cytosol in many species, although GSH1 is absent from the cytosol in Arabidopsis (Noctor and Foyer, 1998; Meyer, 2008). The concentration of glutathione in the matrix is very high, much higher than that of NAD (Jimenez et al., 1997). The glutathione concentration is also much higher in the matrix than in plastids and the cytosol, as suggested by immunochemical detection (Zechmann et al., 2008). The enrichment of glutathione anion in the matrix against a negative membrane potential requires the active transport of glutathione across the IMM, but the transporter involved has not yet been identified. Glutathione can be lost from the matrix via export of glutathione polysulfide, which is used in the cytosol for the biosynthesis of iron-sulfur centers (Schaedler et al., 2014).

Genetically encoded fluorescent redox biosensors have been developed containing a CysSH/Cys-SS-Cys redox switch between two juxtaposed β-strands of the barrel structure of GFP. The expression of this roGFP2 (midpoint potential of −272 mV), by itself or fused to Grx1 to improve glutathione specificity and equilibration kinetics, makes it possible to continuously monitor the glutathione redox state in the mitochondrial matrix in living cells and tissues (Meyer et al., 2007; Schwarzländer et al., 2008, 2009, 2016; Albrecht et al., 2014). Interpretation of the data relies on the plausible assumption that the probe is in thermodynamic equilibrium with the glutathione pool, as catalyzed by the Grx activity included in the sensor, and that this equilibrium kinetically dominates over potential interactions with other major redox couples in the matrix.

The roGFP probe was found to be ~90% reduced in the mitochondria of Arabidopsis epidermal cells, corresponding to a redox potential of −358 mV (Schwarzländer et al., 2008). Such a reducing redox potential was unexpected, as it appeared to contradict analytical measurements. However, the biochemical analyses are typically performed on whole-tissue extracts where the glutathione pools from different cell compartments are mixed (discussed in Schwarzländer et al., 2016). As the redox potential of glutathione in the matrix is very similar to the redox potential of NADP, the two redox couples are likely to be in thermodynamic equilibrium. This is consistent with the finding that the recycling of oxidized GSSG is catalyzed by the organellar NADPH-specific glutathione reductase 2 (Figure 3), which is dually targeted to the plastid stroma and mitochondrial matrix (Chew et al., 2003; Marty et al., 2019). An open, intriguing question is whether there are physiological conditions under which glutathione reductase runs backward in vivo to reduce $NADP^+$ using electrons from GSH, which would provide a new role for GSH in NADP redox maintenance (as opposed to the reverse, as generally accepted). The GSH concentration in the matrix is >10 times higher than that of NADP, suggesting that glutathione might act as an effective buffer for the redox potential of NADP.

The roGFP probe responds specifically to changes in the glutathione redox potential. Starting from a largely reduced probe in the mitochondrial matrix, oxidation events can be monitored quite effectively. Since the sensor detects the steady state of the glutathione pool rather than the oxidizing and reducing fluxes, changes may arise from any imbalance between electron influx and efflux. For instance, an increased rate of H_2O_2 reduction via the ascorbate-GSH cycle will cause glutathione oxidation and in turn sensor oxidation. Conversely, decreased $NADP^+$ reduction via metabolism in the matrix could also oxidize the glutathione pool in the absence of any increased rate of H_2O_2 reduction. In addition, the redox potential of the glutathione pool depends on the absolute concentration of the pool, which implies that glutathione import or export/degradation will also affect the sensor response (Schwarzländer et al., 2016).

Trx

Trxs are a family of small proteins (~12 kD) containing a thiol-disulfide active site. Two o-type Trxs, Trx o1 and Trx o2, are present in the mitochondrial matrix, and some plant species might also contain one h-type Trx (Trx h2; Gelhaye et al., 2004, 2005). Grxs contain Trx motifs, and most Grxs and Trxs can react interchangeably with many Grx and Trx targets from a thermodynamic perspective (Vieira Dos Santos et al., 2007). Yet, the decisive factor for an interaction to actually occur in vivo is their reaction kinetics, since their respective specificities are determined by their structural and electrostatic complementarity with their substrates (Berndt et al., 2015; Gellert et al., 2019).

Both Trx and NADPH-Trx reductase (NTR), the enzyme that recycles Trx(ox) to Trx(red) using NADPH as a reductant, are found in moderate abundance in the mitochondrial matrix of potato (*Solanum tuberosum*) tuber cells. This translates into a concentration in the micromolar range (Salvato et al. 2014) or at least an order of magnitude below that of the other redox couples discussed here. The Arabidopsis proteins NTRA and NTRB are dually localized to the cytosol and mitochondrial matrix, where they are likely to serve distinct sets of Trx proteins (Reichheld et al., 2005, 2007). Trx showed a median degradation rate in mitochondria from cultured Arabidopsis cells, with a half-life of 8.6 d (i.e., relatively stable; Nelson et al., 2013).

Protein Cysteine and Methionine

Protein Cys and Met are by far the most abundant (but heterogeneous) sulfur-based redox couples in mitochondria. These amino acids are present at an estimated concentration of 50 to 100 mM, which is likely to be as high or higher than the combined concentrations of all the other regulatory redox couples discussed here (Requejo et al., 2010; Bruhn et al., 2012; Nietzel et al., 2017). The redox potentials of these thiols and S-methyl thioethers vary widely depending on the neighboring amino acid side chains in the 3D protein structure. As such, their thermodynamic buffering capacity covers a broad range, even though their kinetic reactivity is likely to be diverse and specific.

ROS

ROS are involved in the formation of most of the oxidative PTMs (Figure 3). Mitochondria are major sites of intracellular ROS formation in plant cells, even though chloroplasts and peroxisomes display very high ROS production in green cells in the light (Møller, 2001; Foyer and Noctor, 2003; Huang et al., 2016). The mitochondrial ETC in both animals and plants primarily produces superoxide via the single electron reduction of molecular oxygen, and more superoxide is produced when the redox centers of the ETC are reduced (e.g., when substrate supply exceeds the ETC

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electron-output capacity). In mammalian mitochondria, complexes I and III are prime sites of superoxide formation, but several other enzymes can also generate ROS in the matrix (Brand, 2010). For plant mitochondria, complexes I and III are also the main sources of ROS (summarized in Møller, 2001). Superoxide is readily converted to H_2O_2 by mitochondrial superoxide dismutase, and the highly reactive hydroxyl radical can in turn be produced from H_2O_2 in the presence of metal ions (Møller et al., 2011; Halliwell and Gutteridge, 2015). ROS can modify proteins, lipids, DNA, and carbohydrates and in many cases cause damage and oxidative stress, but the oxidation products may also be involved in retrograde signaling (Møller et al., 2007; Møller and Sweetlove, 2010; Schwarzländer et al., 2012; Umbach et al., 2012).

Due to the potential damage caused by ROS, mitochondria have developed an array of mechanisms to remove H_2O_2 before its concentration builds up. At least three different peroxidase systems—ascorbate peroxidase, glutathione peroxidase-like, and peroxiredoxin—are found in the matrix, where they are supplied with electrons by the Trx and glutathione/Grx systems to reduce H_2O_2 to water and to detoxify other peroxides (and reversible Met oxidation may also serve to remove H_2O_2; see below). The reducing power is ultimately derived from NADPH in all cases (Møller et al., 2007; Navrot et al., 2007; Riemer et al., 2015), except for MDHA, which can also use NADH (see above); therefore, all redox couples described above are also involved in this process.

MAJOR PTMs THAT REGULATE MITOCHONDRIAL METABOLISM

Trx: Regulation by Reducing Disulfide Bridges

Photosynthetic carbon fixation was the founding example of Trx-mediated redox regulation. When photosynthesis is active, Trx is kept in the reduced state by reduced ferredoxin, and disulfide bridges are broken in several enzymes in the Calvin-Benson cycle, thereby stimulating activity. This enables the chloroplast to accelerate carbon fixation when there is plenty of NADPH and ATP available. The reverse process occurs in the dark (Taiz et al., 2015; Buchanan, 2016).

Mitochondrial metabolism appears to be regulated in a similar manner, only here Trx is reduced by NADPH-dependent NTRs (Dreyer and Dietz, 2018). More than 100 mitochondrial proteins have been reported to bind to and/or be reduced by Trx in vitro and are therefore potential targets for Trx regulation in pea and Arabidopsis shoots, spinach (*Spinacia oleracea*) leaves and potato tubers (Balmer et al., 2004; Yoshida et al., 2013; Buchanan, 2017). These proteins participate in a wide variety of processes, including photorespiration, Krebs cycle and associated reactions, lipid metabolism, electron transport, ATP synthesis, membrane transport, and stress-related reactions (Figure 4; Supplemental Table; Balmer et al., 2004; Yoshida et al., 2013).

The effect of Trx-mediated disulfide reduction on the properties of target proteins has been determined for a few mitochondrial enzymes. Two TCA cycle enzymes, succinate dehydrogenase (flavoprotein) and fumarase, show decreased activity in response to Trx-mediated reduction in Arabidopsis leaves, which may induce the TCA cycle to operate in a noncyclic mode, taking in malate and exporting citrate or 2-oxoglutarate (Daloso et al., 2015). Citrate synthase is activated by Trx-mediated reduction in vitro (Schmidtmann et al., 2014), but whether this process also operates in green leaves is unclear (Daloso et al., 2015). The most well-studied case is AOX, a small family of diiron ubiquinol oxidases (Moore et al., 2013) that creates a bypass of complexes III and IV in the ETC. AOX acts as a dimer and several plant AOX isoforms contain a conserved Cys residue that can form a disulfide bridge between the two subunits. In isolated mitochondria, the reduction of this disulfide bridge is catalyzed by the Trx/NTR system, which activates AOX and allows the further stimulation of AOX activity through the binding of 2-oxo acids, foremost pyruvate, to one of the free thiol groups (Figure 5; Gelhaye, 2004; Gelhaye et al., 2005; Selinski et al., 2018a, 2018b). However, neither the activation of AOX by Trx nor its further stimulation by organic acids has been observed in vivo (Del-Saz, 2018). Furthermore, the absence of the dominating Trx-o1 had no effect on the reduction of AOX in vivo, and the faster metabolic rates could be linked to the dysregulation of upstream carbon metabolism rather than redox regulation of AOX (Florez-Sarasa et al., 2019; Sanchez-Guerrero et al., 2019). Indeed, the oxidation of AOX would require the oxidation of the TRX system, which in turn would require the oxidation of the NADP pool (and likely the glutathione pool to avoid backup). While the oxidation of these redox couples indeed occurs during the mitochondrial isolation process, there is justified doubt that any physiologically relevant stress would lead to such a harsh oxidation. An exception may be orthodox seeds during storage, when metabolic activity and NADPH delivery cease and oxidation of the present thiols can dominate. In a recent study, Trx-mediated rereduction of mitochondrial Cys residues was found to be important for coordinating the earliest metabolic events in seed germination (Nietzel et al., 2020). Impairment of the matrix Trx system, as well as the glutathione system, led to higher rates of respiration during seed imbibition (similar to the observations of Florez-Sarasa et al. [2019] in leaves) and a decrease in germination vigor.

Glutathionylation of Cys (a mixed disulfide; Figure 1), a PTM that remains largely unexplored in plant mitochondria, will likely also turn out to be as important for the regulation of plant mitochondria as it is for mammalian mitochondria, where it regulates the activity of a number of enzymes in the ETC and the TCA cycle (Mailloux et al., 2013). In plant mitochondria, the only known example is the glycine decarboxylase complex (GDC), whose activity is inhibited by glutathionylation of the P-protein subunit on a Cys residue (Palmieri et al., 2010). Grx treatment relieved the inhibition of the GDC, confirming the notion that glutathionylation plays a role in this process (Hoffmann et al., 2013). Yet, it is currently unclear to what extent glutathionylation of matrix proteins can actually occur in vivo under nonstressed conditions given the highly reduced state of the glutathione pool, which makes protein disulfides thermodynamically unstable. Kinetic control may therefore be an important determinant of glutathionylation, but the required regulatory GRX enzymes have not yet been identified in the mitochondrial matrix (the only confirmed matrix Grx, GrxS15/Grx4, lacks oxidoreductase activity; Moseler et al., 2015).

Cys Nitrosylation

The mitochondrial ETC is a major site of NO production in plant cells under highly reducing conditions (Figure 2C) and especially

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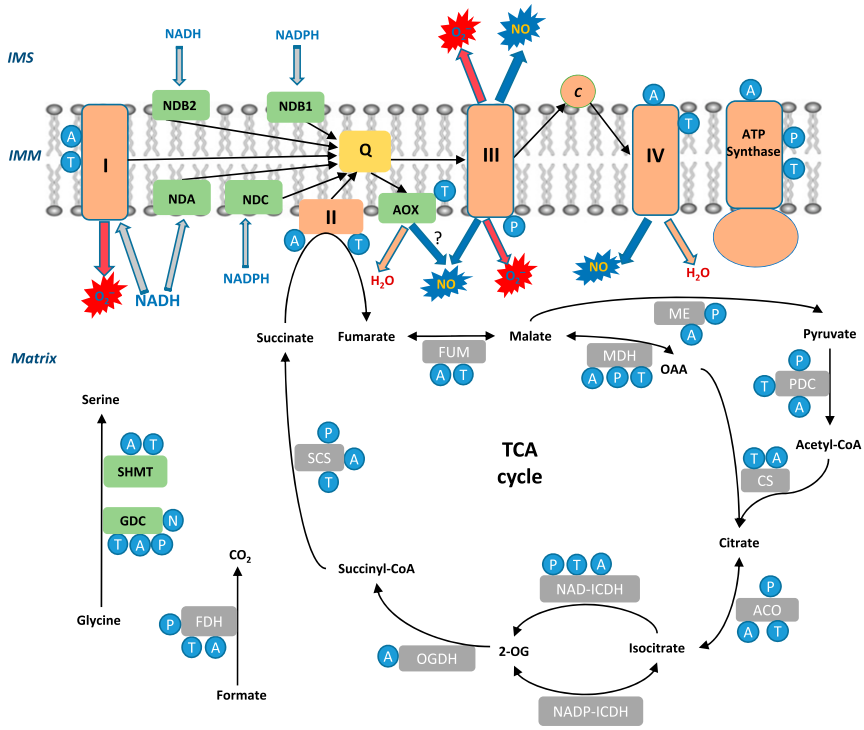

Figure 4. Posttranslational Modifications of TCA Enzymes and ETC Components.

Modifications are denoted as follows: A, acetylation; N, *S*-nitrosylation; P, phosphorylation; T, Trx-dependent reduction/oxidation. The mitochondrial matrix enzymes and ETC components shown are as follows: ACO, aconitase; AOX; bc_1, complex III; c, cytochrome *c*; COX, complex IV (cytochrome *c* oxidase); CS, citrate synthase; FDH, formate dehydrogenase; FUM, fumarase; GDC; I, complex I; II, complex II (succinate dehydrogenase); ICDH; IMM; IMS, intermembrane space; MDH, malate dehydrogenase; NDA, internal rotenone-insensitive NADH dehydrogenase; NDB_1 and NDB_2, external rotenone-insensitive NADPH and NADH dehydrogenases; NDC, internal rotenone-insensitive NADPH dehydrogenase; OGDH, 2-oxoglutarate dehydrogenase complex; PDC; SCS, succinyl-CoA synthetase; SHMT, Ser hydroxymethyltransferase. The figure is based on data from (Bykova et al. (2003a), Balmer et al. (2004), Gelhaye et al. (2004), Kristensen et al. (2004), Umbach et al. (2006), Havelund et al. (2013), Yoshida et al. (2013), König et al. (2014a), Salvato et al. (2014), Nietzel et al. (2017), and Millar et al. (2019). The TCA cycle is fed in plant mitochondria not only by acetyl-CoA formed from glycolytic pyruvate but also from malate that can be converted to pyruvate by NAD-malic enzyme (ME) and oxaloacetate (OAA) that can be converted to pyruvate by MDH and ME. Plant-specific proteins of the mitochondrial ETC and the enzymes that participate in photorespiration are shown in green. A more complete overview of mitochondrial proteins with multiple PTMs is provided in Supplemental Table 1.

under hypoxia (Gupta and Igamberdiev, 2011; Hebelstrup and Møller, 2015; Gupta et al., 2018). Cys nitrosylation is likely to be caused by peroxynitrite produced by a reaction between NO and superoxide. Therefore, nitrosylation is expected to be at maximum levels during the early stages of seed germination, when NO formed during the initial hypoxia reacts with superoxide formed by the ETC during reoxygenation (Møller, 2001; Møller et al., 2007; Hebelstrup and Møller, 2015). Cys nitrosylation is important for the activation or inhibition of key respiratory and antioxidant enzymes. This provides a feedback mechanism for regulating ROS levels (reviewed in Lindermayr and Durner, 2015; Igamberdiev and Bykova, 2018a, 2018b).

Tyr Nitration

Tyr nitration has been demonstrated for several mitochondrial enzymes. Tyr nitration inhibits MnSOD activity (Holzmeister et al., 2015). Tyr nitration has been reported for Ser hydroxymethyltransferase, formate dehydrogenase (FDH), Cys synthase, and subunit T of the GDC (Lozano-Juste et al., 2011), but no information was provided on the effect of Tyr nitration on the activity of these enzymes. Tyr nitration inhibits cytosolic NADP-ICDH (Begara-Morales et al., 2013), which may provide metabolic feedback to mitochondrial NADP-ICDH and NAD-ICDH via isocitrate accumulation.

Met Oxidation

Met oxidation is a very commonly detected PTM, certainly the most common in proteomic profiling studies. For example, Salvato et al. (2014) found that, of the 1060 proteins identified in the potato tuber mitochondrial proteome, 556 proteins (52%) contained one or more PTM. Of the 3066 PTMs identified, Met-SO was by far the most common, with 1373 Met-SO found on 447 proteins (42% of the identified proteins). Most of these proteins contained 1 to 3 Met-SO, but one protein, glycine decarboxylase, contained 24 Met-SO.

The physiological significance of Met-SO formation is still a matter of debate, because it can occur during sample extraction

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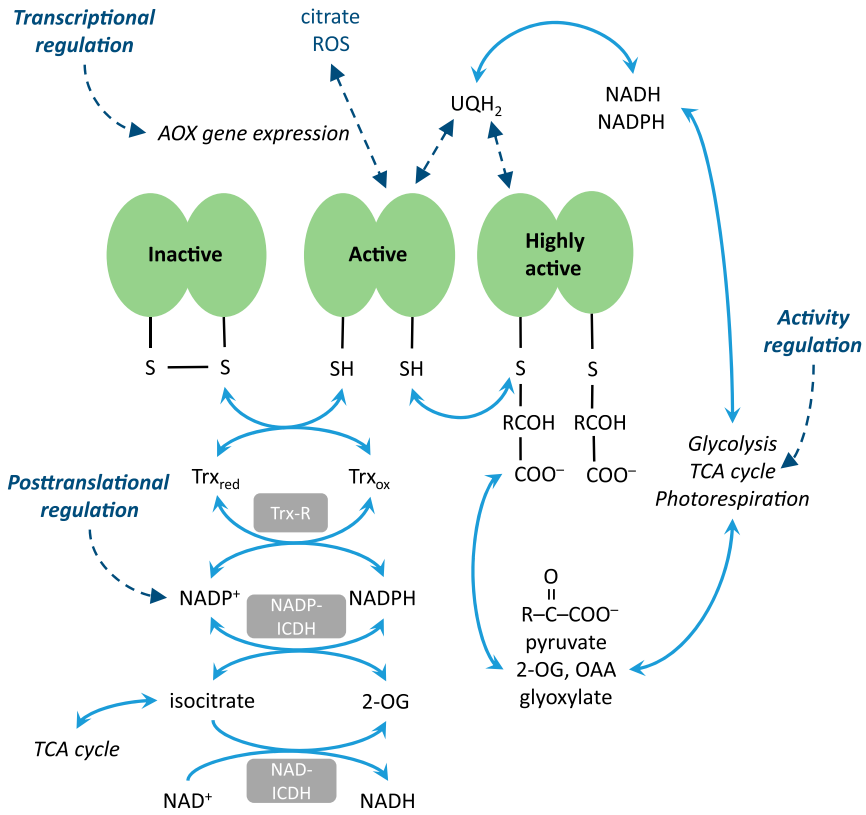

Figure 5. Regulation of AOX by Reduction and Metabolites.

AOX biosynthesis is regulated transcriptionally by citrate and by elevated levels of ROS. AOX is modified posttranslationally by the mitochondrial Trx o, which mediates the conversion between an inactive form with a disulfide bridge between the monomers and an active form with free thiol groups (Gelhaye et al., 2004; Umbach et al., 2006). Thioredoxin reductase (Trx-R) regenerates Trx o to the reduced form; NADPH for this reaction is supplied by NADP-ICDH or other dehydrogenases. AOX is activated by oxo-acids (R-CO-COO-) including pyruvate (coming from glycolysis and malic enzyme reactions), 2-oxoglutarate (2-OG) and oxaloacetate (OAA; formed in the TCA cycle), and glyoxylate (from photorespiration). The posttranslational binding of pyruvate and other oxo-acids to the reduced form further activates the enzyme. The increase in the concentration of the AOX substrate ubiquinol (UQH_2) inside the inner membrane stimulates AOX activity. The figure was significantly modified from Web Figure 12.3C of Taiz et al. (2015): http://6e.plantphys.net/topic12.03.html. The regulation of AOX at different levels is reviewed by Selinski et al. (2018b).

and processing, making it difficult to identify the sites originally oxidized in situ (Liu et al. 2013). However, the first step in Met oxidation to Met-SO is enzymatically reversible, making it a prime candidate for regulation (Figure 1). Plant cells contain Met sulfoxide reductases A and B, each specific for one of the stereoisomers of Met-SO; these dedicated enzymes are able to reduce Met-SO using Trx-SH (or Grx-SH) as a reductant (Gustavsson et al., 2002; Vieira Dos Santos et al., 2007). This implies that both stereoisomers of Met-SO are formed, which is consistent with a nonenzymatic mechanism of formation. Furthermore, Met-SO formation interacts with phosphorylation in the regulation of enzyme activity (see below). It is therefore likely that Met-SO formation is an important regulatory PTM in some cases. However, it is also likely that most Met-SO formation is not specific, occurs in only a small percentage of protein molecules, and may have little or no effect on activity. Therefore, Met-SO is likely to represent "molecular noise" that is now becoming "visible" due to the increasingly sensitive proteomic methods available. The molecular noise is the result of ROS interactions where the proximity to sites of ROS generation is more important than reactivity of the side chain.

Interestingly, in mammals, Met oxidation might occur at sites not important for protein function to protect vital amino acids by absorbing ROS-mediated oxidation, especially in the mitochondria (Levine et al., 1996; Bender et al., 2008). In this case, Met oxidation serves as a ROS-scavenging mechanism rather than a means of regulating metabolic activity.

Protein Carbonylation

One of the main ROS oxidation products is protein carbonylation, which is irreversible (Møller et al., 2011, 2017). This is likely to occur via a direct interaction between ROS and the side chains of the amino acids Arg, Lys, Pro, and Thr (Figure 1). Trp is also oxidized to produce a carbonyl group (Figure 1), but because it does not appear to react with dinitrophenylhydrazine, the standard tag used to identify carbonylated proteins, Trp oxidation is usually not

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included in studies of protein carbonylation (Møller and Kristensen, 2006). Although carbonylation has been observed in many mitochondrial proteins (Kristensen et al., 2004; Salvato et al., 2014), we are not aware of any one case where the effect of in vivo carbonylation on the properties of a protein has been identified. Not surprisingly, very harsh in vitro oxidative treatment of enzymes leads to a loss of activity (Nguyen and Donaldson, 2005). However, protein carbonylation can also occur indirectly via the oxidation of polyunsaturated fatty acids (abundant in the IMM), forming products such as 2-hydroxynonenal, which in turn can form adducts with Lys side chains. Externally added 2-hydroxynonenal modified a range of respiratory enzymes in isolated Arabidopsis mitochondria, and the activity of some of these enzymes was inhibited (Winger et al., 2005, 2007).

Mitochondrial redox changes can also affect cellular protein carbonylation. In Arabidopsis grown on ammonium as an N source, both the *frostbite* mutation of complex I and knockdown of the external NADPH dehydrogenase gene *NDB1* led to lower levels of ammonium-induced cellular protein carbonylation compared with the wild type, where ammonium enhanced protein carbonylation compared with nitrate-grown plants. However, on nitrate nutrition, the *frostbite* mutant instead had more protein carbonylation than the wild type, indicating that the mitochondrial NADH oxidation system affects protein carbonylation (Podgórska et al., 2015, 2018).

It is likely that carbonylation does not regulate the activities of the wide range of enzymes it affects (Kristensen et al., 2004; Salvato et al., 2014). However, carbonylation appears to label protein molecules for degradation, as mitochondria contain an FTSH4 protease that degrades carbonylated proteins (Opalińska et al., 2017). In Arabidopsis plants lacking this protease, carbonylated proteins accumulate in the mitochondria (Smakowska et al., 2016). Thus, carbonylation could be seen as regulatory, because it leads to a decrease in the amount of the affected protein. However, protein degradation, whether induced by carbonylation or not, is likely to be a slow process unsuited for metabolic regulation. The mitochondrial proteins with the fastest turnover in Arabidopsis cell cultures have a half-life of 31 to 72 h (Nelson et al., 2013), which indicates that protein degradation is slow compared with the short-term adjustments in metabolic fluxes we are discussing here.

Protein Phosphorylation

The reversible phosphorylation of Ser, Thr, and Tyr residues in proteins has been recognized as a dynamic protein regulatory mechanism for more than 50 years. Progress in mass spectrometry-based proteomics has led to the identification of >100,000 phosphorylation sites on >20,000 eukaryotic proteins (Rao and Møller, 2012). In mammalian mitochondria, >1000 phosphorylation sites have been identified on several hundred proteins, including several subunits in each of the five respiratory complexes (Kruse and Højlund, 2017). In most cases, the effects of these phosphorylation sites are unknown. In fact, the physiological relevance of many of the phosphorylation sites discovered by increasingly sensitive targeted proteomic methods has been questioned, and they might represent molecular noise (Covian and Balaban, 2012). Another interesting conclusion from studies of protein phosphorylation in mammalian mitochondria is that many phosphorylation events may take place in the cytosol before the proteins are imported into the mitochondria (Kruse and Højlund, 2017). Even the import machinery (TOM22) is phosphorylated by cytosolic kinases that may bind to the outer surface of the IMM. To the extent that phosphorylation modifies the properties of proteins, this places mitochondrial metabolism under cytosolic control via PTMs (Kruse and Højlund, 2017).

Using [^{32}P]ATP labeling or mass spectrometry-based proteomics, ~50 phosphorylated proteins and a number of protein kinases and protein phosphatases have been identified in isolated plant mitochondria. These phosphoproteins include TCA cycle enzymes, ETC components, and components of most other major mitochondrial pathways (Figure 4; Supplemental Table; Bykova et al., 2003a; Heazlewood et al., 2004; Havelund et al., 2013; Salvato et al., 2014). Similar to the situation in mammalian mitochondria (Covian and Balaban, 2012; Kruse and Højlund, 2017), in most cases, we do not know the effect of phosphorylation on these enzymes.

Despite the plethora of proteins potentially regulated by phosphorylation in plant mitochondria, the regulation of only one enzyme complex, the pyruvate dehydrogenase complex (PDC), is understood in detail (reviewed in Tovar-Méndez et al., 2003). The PDC catalyzes the entry reaction into the Krebs cycle:

$$\mathrm{Pyr} + \mathrm{NAD^+} + \mathrm{CoA} \rightarrow \mathrm{Acetyl\text{-}CoA} + \mathrm{NADH} + \mathrm{CO_2}$$

The PDC contains three enzymes (pyruvate dehydrogenase [E1], dihydrolipoyl acetyltransferase [E2], and dihydrolipoyl dehydrogenase [E3]), which act sequentially to catalyze the reaction.

The PDC also contains a protein kinase, which inhibits PDH activity by phosphorylating Ser residues on the E1 subunit as well as a protein phosphatase that removes the phosphate group. High concentrations of the reaction products NADH and acetyl-CoA inhibit PDC activity and stimulate kinase activity so that the enzyme complex is inactive when there is plenty of substrate for the TCA cycle or during photorespiration (Noctor et al., 2007). On the other hand, high concentrations of pyruvate and ADP inhibit the kinase activity so that the enzyme is kept in the active state when pyruvate needs to be processed and ATP is in short supply (Tovar-Méndez et al., 2003). Thus, the PDC is seen as the gatekeeper of the TCA cycle and oxidative phosphorylation, where reversible phosphorylation is used to adjust TCA activity to meet the cellular demands for ATP and metabolic intermediates. However, we now know that the TCA cycle can also function in several noncyclic modes (Sweetlove et al., 2010). Although these dynamic changes in metabolic fluxes can be modeled based on overall constraints without the need for active regulation, there is a large body of experimental evidence that active regulation supports different metabolic modes. The shift between different modes requires the ability to switch other TCA cycle enzymes off and on, which could occur via phosphorylation or Trx-mediated oxidation/reduction (see above).

One phosphoprotein identified in plants is FDH (Bykova et al., 2003b; Roitinger et al., 2015). FDH is found in great abundance in potato tuber mitochondria as well as other plant mitochondria, and it appears to play a role in hypoxic metabolism and other types of stress responses (Colas des Francs-Small et al., 1993; Suzuki

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et al., 1998; Choi et al., 2014; Lou et al., 2016). Although the source of formate in plants is still uncertain, a formate pool is maintained that could originate as a by-product of photorespiration from the nonenzymatic decarboxylation of glyoxylate or as a by-product of methanol metabolism (Igamberdiev et al., 1999). Unlike PDC, FDH is phosphorylated on two Thr residues, but the degree of phosphorylation is regulated by substrates and products in a manner similar to that observed for PDC (Bykova et al., 2003b). The effect of phosphorylation on FDH enzyme properties is unknown, but presumably it inhibits enzyme activity in a manner analogous to that of the PDC. FDH was recently identified as a potential substrate for the RING-type ubiquitin ligase Keep on Going (KEG; McNeilly et al., 2018). The turnover of FDH increased when functional KEG was present in planta, while dephosphorylation of the enzyme increased its stability. FDH phosphorylation is thought to be involved in KEG-mediated protein ubiquitination followed by subsequent degradation (McNeilly et al., 2018). It is, however, presently unclear how the ubiquitin/proteasome system can mediate the degradation of proteins in the mitochondrial matrix (Bragoszewski et al., 2017). Ubiquitination is also involved in autophagy signaling, and the phosphorylation of proteins in different membranes including the outer mitochondrial membrane is involved in general autophagy and induced mitophagy (Li et al., 2014a).

Lys Acetylation

The side chain amino group in the Lys residues of proteins can be modified by acylation due to a reaction with different types of acyl-CoA metabolites, such as acetyl-, propionyl-, butyryl-, crotonyl-, malonyl-, glutaryl-, or succinyl-CoA. This reaction is either enzymatically catalyzed by Lys ac(et)yltransferases (KATs), some of which can accept different types of acyl-CoAs (Kaczmarska et al., 2017), or it proceeds nonenzymatically at pH 7.5, which is a pH value that can be expected in the mitochondrial matrix (König et al., 2014b). Among the different Lys acylations, Lys acetylation (Figure 1) is the most common, and so far the best characterized, of these modifications (Figures 4 and 6).

Histone Lys acetylation was shown to regulate gene expression more than 50 years ago (Gershey et al., 1968), but due to technical bottlenecks, the recognition that it is one of the most common PTMs was delayed until fairly recently. However, these problems have mostly been solved and, over the past 20 years, many thousands of Lys acetylation sites have been identified by mass spectrometry-based proteomics using acetyl-Lys binding antibody matrices for enrichment of the acetylated peptides combined with peptide fractionation techniques (Choudhary et al., 2009). In mammalian cells, a large proportion of Lys-acetylated proteins reside in mitochondria (Lombard et al., 2007). König et al. (2014a) identified 120 acetylated proteins with a total of 243 acetylated Lys residues in Arabidopsis mitochondria, providing a list of potential targets for regulation by Lys acetylation. These proteins participate in a range of important metabolic pathways including the TCA cycle, the ETC, photorespiration, amino acid and protein metabolism, and redox regulation. It is now recognized that Lys acetylation plays an important role in regulating cellular (particularly mitochondrial) metabolism in mammals and perhaps also in plants (reviewed in Hosp et al., 2017).

In mammalian mitochondria, acetylation sites have been found on 63% of the identified proteins. Therefore, similar to Met-SO formation and protein phosphorylation (as discussed above), it is likely that the majority of the observed modifications are molecular noise. However, if this "noise" cannot be removed efficiently from mitochondrial proteins, the hyperacetylation results in strong mitochondrial defects (Carrico et al., 2018). In mitochondria, unlike other subcellular compartments (Narita et al., 2019), Lys acetylation is mainly a negative regulator of protein functions, especially for TCA cycle enzymes (Baeza et al., 2016). One exception to this rule is aconitase, which is stimulated by Lys acetylation (Fernandes et al., 2015).

Mammalian mitochondria contain two noncanonical KATs, while none have thus far been identified in plants (Hosp et al., 2017). All classical histone Lys deacetylases (KDACs) reside in the nucleus and/or cytosol in mammalian cells. In contrast, an organellar histone KDAC, HDA14, which is dual-targeted to both mitochondria and plastids, was recently identified in Arabidopsis (Hartl et al., 2015). However, Lys acetylome profiling analysis of *hda14*-knockout plants revealed increased acetylation mainly on plastidial, but not mitochondrial, proteins (Hartl et al., 2015). Hence, the role of HDA14 in Arabidopsis mitochondria is still unclear. In mammals, all characterized mitochondrial KDACs belong to the sirtuin (silent information regulation2 homolog) family, which use NAD^+ as a substrate and release *O*-acetyl-ribose and nicotinamide as by-products of the deacetylation reaction (Figure 6). Three sirtuins (SIRT3–SIRT5) have been identified in mammalian mitochondria, whereas only one (SRT2) has been found in mitochondria from Arabidopsis (König et al., 2014b). The sirtuin protein family comprises highly conserved NAD^+-dependent protein deac(et)ylases. The loss of any of these enzymes in mammalian cells results in the development of metabolic disorders, diseases such as cancer, or early aging (Imai and Guarente, 2014; Hosp et al., 2017). In mammalian mitochondria, SIRT3 is the major protein KDAC, which positively regulates respiration as well as PDC activity. SIRT4 deacylates several types of acyl-Lys side chains, such as methylglutaryl-Lys, hydroxymethylglutaryl-Lys, 3-methylglutaconyl-Lys (Anderson et al., 2017), and biotinyl- and lipoyl-Lys of the PDC, thereby deactivating its target enzymes (Mathias et al., 2014). The mammalian SIRT5 mainly catalyzes Lys desuccinylation, demalonylation, and deglutarylation (Du et al., 2011; Tan et al., 2014). The Arabidopsis genome, like all angiosperm genomes, only encodes two sirtuins, and only SRT2 resides in the mitochondria. The recombinant Arabidopsis SRT2 exhibited deacetylase activity, and the ATP/ADP carrier protein AAC1-3 had higher acetylation levels in Arabidopsis *srt2*-knockout plants compared with the wild type, which were correlated with higher transporter activities (König et al., 2014b). Since plant SRT2 shares high homology with mammalian SIRT4, it is possible that it possesses all the deacylase activities of SIRT4, but this hypothesis needs to be tested. This concept is attractive, as several other Lys acylations, such as succinylation, butyrylation, crotonylation, and malonylation, have recently been reported for proteins (including mitochondrial proteins) from rice (*Oryza sativa*) and wheat (*Triticum aestivum*; Liu et al., 2018; Lu et al., 2018; Zhou et al., 2018). Since none of the plant sirtuins contain the catalytic active site required for SIRT5-type deacetylase activity (Du et al., 2011), it will be important to establish which enzymes catalyze the removal of Lys succinylation, glutarylation, and malonylation in plants. In addition, the impact of these modifications on mitochondrial metabolism and function deserves exploration.

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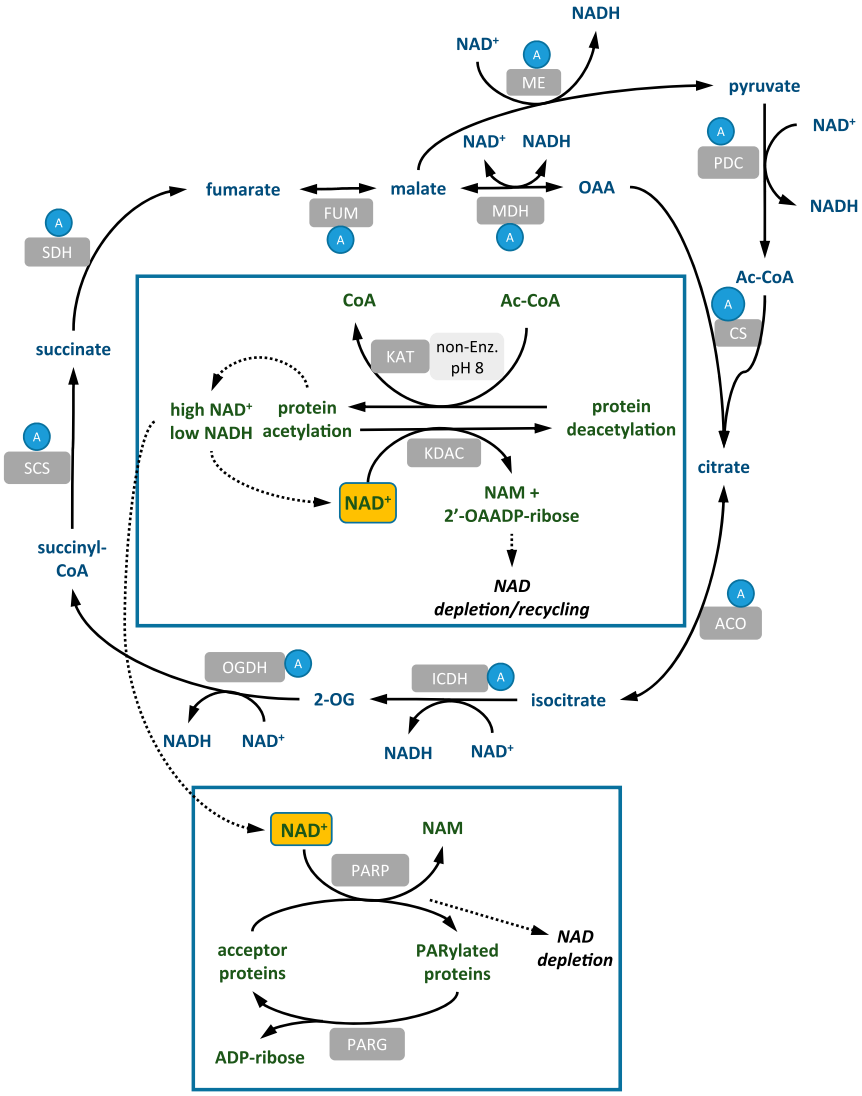

Figure 6. Acetylation of TCA Cycle Proteins and Proposed Redox Regulation via Depletion of the NAD Pool during Sirtuin-Dependent Deacetylation and Poly-ADP-Ribosylation.

The figure is based on data from König et al. (2014a). The proposed joint operation of the TCA cycle and reversible acetylation-deacetylation results in the regulation of NAD pool size and reduction level (top box). Protein Lys acetylation of TCA cycle enzymes can take place via KAT activity or nonenzymatically (non-Enz.) at pH 8.0 and higher in the presence of acetyl-CoA, while NAD^+-dependent KDAC of the sirtuin family consumes NAD^+ during the process of deacetylation. Another process stimulated by high NAD^+ and depleting the NAD pool is poly-ADP-ribosylation of proteins (bottom box). PARP, whose presence in plant mitochondria is still controversial, cleaves NAD^+ and attaches the ADP-ribose moiety to acceptor proteins, whereas poly(ADP-ribose) glycohydrolase (PARG) cleaves the ribose-ribose backbone bond of poly(ADP-ribose), releasing free ADP-ribose (Briggs and Bent, 2011). The inhibition of TCA cycle enzymes by Lys acetylation that affects the metabolic flux, so that only parts of it are used (Sweetlove et al., 2010), is still hypothetical. Pyruvate comes from glycolysis, and malate and oxaloacetate (OAA) can come from glycolysis or be formed in the cycle. Acetyl-CoA is abbreviated as Ac-CoA, and acetylation is denoted by the letter A in a circle. Other abbreviations are as follows: ACO, aconitase; CS, citrate synthase; FUM, fumarase; MDH, malate dehydrogenase; ME, NAD-malic enzyme; NAM, nicotinamide; 2′-OAADP-ribose, 2′-*O*-acetyl-ADP-ribose; OGDH, 2-oxoglutarate dehydrogenase complex; PAR, poly(ADP-ribose); SCS, succinyl-CoA synthetase; SDH, succinate dehydrogenase; SHMT, Ser hydroxymethyltransferase.

REGULATION IN RESPONSE TO DEVELOPMENTAL AND ENVIRONMENTAL STIMULI INVOLVES PTM CROSSTALK AND DISTINCT TIMING

In the previous sections, we provided several examples of how mitochondrial PTMs are involved in modulating enzymes involved in mitochondrial metabolism in response to physiological stimuli. The plant cell constantly needs to adjust its metabolism to changes in the environment and during development. This involves the combined interactions of enzymes with PTM mechanisms derived in response to different cues as well as the combinatorial effects of distinct PTMs on multiple enzymes in a pathway. Here, we present examples that illustrate PTM

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crosstalk and the speed with which PTM modifications modulate mitochondrial metabolism in vivo.

Crosstalk between Different PTM-Linked Mechanisms

Many mitochondrial proteins are modified by several different PTMs (Figures 4, 7, and 8; Supplemental Table). Thus, it is possible that the properties of these proteins are regulated by several different mechanisms, either simultaneously or under different conditions, and that the mechanisms interact to produce a joint outcome. Several examples of such crosstalk between regulatory PTMs include the following. (1) The interaction between protein phosphorylation and Lys acetylation regulates PDC in human H1299 cells (Fan et al., 2014). Acetylation of Lys-321 on the PDHA1 catalytic subunit is required for the recruitment of PDK1, which inactivates the complex by phosphorylation (see the section Protein Phosphorylation above). (2) Met-SO formation can inhibit protein phosphorylation on adjacent Ser/Thr residues by preventing site recognition by the protein kinase involved, possibly by forming a steric/charge barrier (reviewed by Rao et al., 2017).

A complex example of the potential cross-regulation of an enzyme system by several different PTMs involves the parallel enzymes NAD- and NADP-ICDH in the TCA cycle. Isocitrate produced by aconitase must be distributed between these two enzymes to provide the optimal balance between NADH and NADPH production, where NADPH is specifically required for Trx reduction (essential for Trx regulation), GSSG reduction, and ROS removal, as discussed above. The two enzymes regulate each other kinetically, as their products, NADH and NADPH, inhibit the other enzyme (Figure 7; Igamberdiev and Gardeström, 2003). NAD-ICDH is potentially regulated by phosphorylation (Bykova et al., 2003a), acetylation (König et al., 2014a), and Trx (Salvato et al., 2014), while NADP-ICDH activity is dependent on thiol-based redox potential (Nietzel et al., 2020) and may be regulated by glutathionylation in a manner analogous to cytosolic NADP-ICDH (Niazi et al., 2019). The latter enzyme is also regulated by Tyr nitration (Begara-Morales et al., 2013), but the nitrated Tyr residue is not conserved in the mitochondrial isozyme, indicating that the major regulatory mechanism of the system as a whole is the direct regulation of NAD-ICDH, which indirectly affects NADP-ICDH activity and direction. These PTM interactions with NAD-ICDH and NADP-ICDH could provide a mechanism for modulating the NADPH/$NADP^+$ and NADH/NAD^+ ratios, which play a central role in redox homeostasis in the mitochondrial matrix (Figure 7).

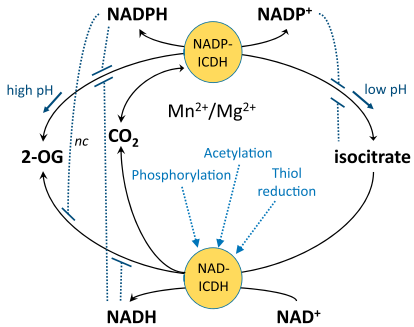

Figure 7. Regulation of NAD- and NADP-Dependent ICDH in Plant Mitochondria.

The NAD-ICDH reaction is essentially irreversible, yet the forward reaction is regulated by phosphorylation (Bykova et al., 2003a) and Trx-mediated thiol reduction (Salvato et al., 2014). The NADP-ICDH reaction is reversible, and the protein is not known to be regulated by PTMs. However, the activity of this reaction is stimulated by PTM-induced inhibition of NAD-ICDH, which functions as the master regulator of this step in the TCA cycle and induces isocitrate accumulation. Likewise, the forward reaction of mitochondrial NADP-ICDH could be stimulated at the substrate level by glutathionylation, as observed for cytosolic NADP-ICDH (Niazi et al., 2019). The regulation of this reaction by metabolites and pH is shown as described by Igamberdiev and Gardeström (2003). nc, noncompetitive inhibition; in all other cases, the inhibition is competitive.

Regulation of Light-Dependent Mitochondrial Metabolism

The GDC system of photorespiratory Gly-to-Ser conversion is strongly regulated at the gene expression level by light (Rasmusson and Escobar, 2007) but is also affected at the posttranslational level by multiple interacting PTMs, which in turn can be affected by light-induced changes in photosynthetic redox metabolism (Noctor, 2006). For example, GDC activity is inhibited by both *S*-nitrosylation and *S*-glutathionylation (Palmieri et al., 2010; Figure 8). In addition, phosphorylation, acetylation, and interactions of Trx with several GDC subunits have been observed (Supplemental Table). Thus, crosstalk between PTMs regulates photorespiratory flux (e.g., by combining light signaling with other environmental cues), although we do not yet have the full picture. In Arabidopsis leaf slices, NO and ROS production induced by the addition of the bacterial elicitor harpin resulted in a 60% inhibition of GDC activity and a strongly increased Gly-to-Ser ratio in the light. This limited the supply of NADH to the ETC, resulting in changes in redox status and promoting cell death (Palmieri et al., 2010).

Upon the transition from light to darkness, leaves display a postillumination respiratory burst (caused by Gly degradation) during the first 1 min after light off. This is followed by a separate phase of light-enhanced dark respiration, which peaks after 3 to 4 min (Atkin et al., 1998; Bathellier et al., 2017). As shown by isotope discrimination, the second phase is caused by malic enzyme-mediated degradation of the malate that accumulated in the light (Werner and Gessler, 2011). However, in tree leaves, light-enhanced dark respiration is preceded by a transient volatile release of acetaldehyde and acetate, which are formed from pyruvate that accumulates in the cytosol before the mitochondrial system has become activated (Karl et al., 2002; Jardine et al., 2012). Together, these findings indicate that it takes several minutes for the pyruvate transporter and/or PDH to become sufficiently reactivated after the light/dark transition. This fits nicely with the observation that the PDC in leaf mitochondria is inactivated by phosphorylation in the light and activated by dephosphorylation within 5 min of darkness (Gemel and Randall, 1992; Tovar-Méndez et al., 2003). To improve our understanding of mitochondrial PTM events in green leaves during the light/dark transition, it will be necessary to obtain a finer temporal resolution using online measurements with fluorescent biosensors for products or substrates.

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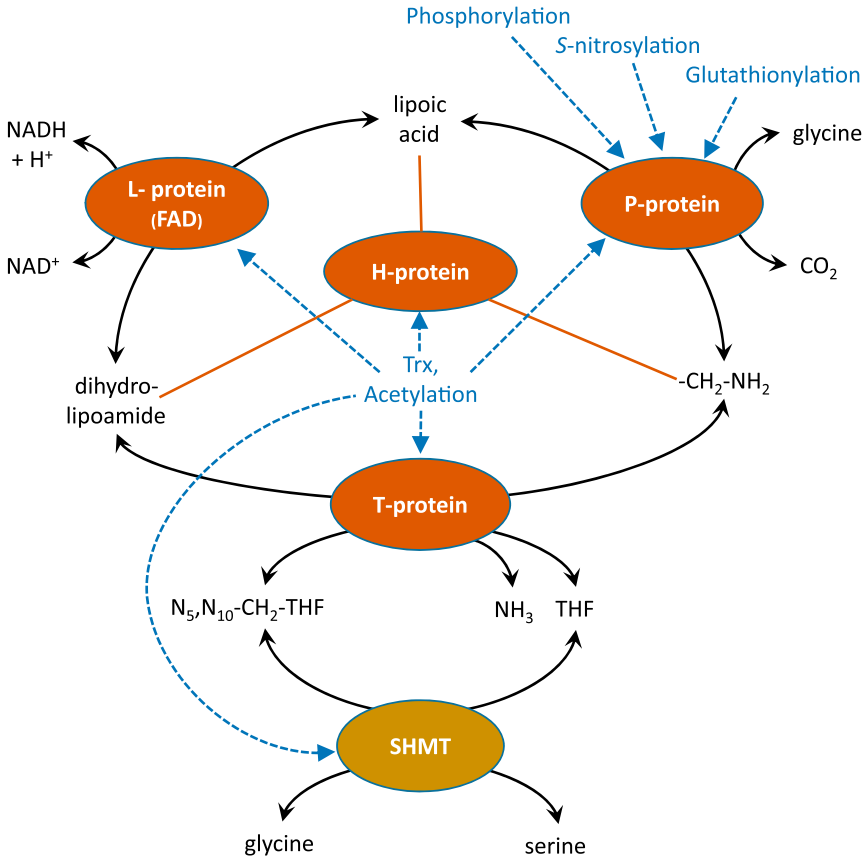

Figure 8. Regulation of Individual GDC Proteins and Ser hydroxymethyltransferase by Trx and Other Posttranslational Modifications.

The P-protein of the GDC is phosphorylated, *S*-nitrosylated, and *S*-glutathionylated, and the latter two processes inhibit GDC activity (Palmieri et al., 2010). All four GDC subunits (P, L, H, and T) as well as Ser hydroxymethyltransferase (SHMT) are potentially regulated by Trx (Balmer et al., 2004) and Lys acetylation (Supplemental Table 1). The figure is based on the data of Balmer et al. (2004), Palmieri et al. (2010), Salvato et al. (2014), and Millar et al. (2019).

Mitochondrial Metabolism and Seed Germination

Increased oxygen consumption and ATP concentration, as detected using ATeam, a biosensor for $MgATP^{2-}$ (De Col et al., 2017), was observed in intact dry Arabidopsis seeds within minutes of the addition of water (i.e., the start of imbibition; Nietzel et al., 2020). In parallel, analysis using roGFP2, a biosensor for mitochondrial glutathione reduction level, indicated that glutathione was becoming reduced in the mitochondrial matrix in intact seeds. In vitro experiments with isolated seedling mitochondria showed that hundreds of Cys-containing peptides became more reduced during substrate oxidation with an NADPH-producing substrate (citrate, via aconitase and NADP-ICDH) but not with 2-oxoglutarate, which only produces NADH via the 2-oxoglutarate dehydrogenase complex (Møller and Rasmusson, 1998; Nietzel et al., 2020). Many of these Cys-containing peptides belong to TCA cycle enzymes, but the most markedly redox-switched peptides belong to glutathione reductase 2, NTRA/B, and Trx-o1. The germination of seeds lacking these redox proteins was associated with markedly enhanced respiration rates and deregulated TCA cycle dynamics, suggesting that the resource efficiency of energy metabolism was reduced during germination of these seeds (Nietzel et al., 2020).

FUTURE PERSPECTIVES

The rapid progress in mass spectrometry-based proteomics has enabled us to shed new light on the roles of PTMs in plant mitochondria in recent years. However, a more comprehensive picture is now required, where the interactions of these PTMs are investigated in more detail to fill in the missing pieces of the puzzle and the dynamics of the fine regulation of mitochondrial functions and metabolic pathways. The biggest hurdle to closing this gap in knowledge is still the requirement for the enrichment of mitochondria from specific tissues and specific cell types, which often have different physiological functions. In addition, some PTMs might be lost, gained, or altered during the long purification procedures due to tissue rupture. Hence, technical advances to obtain better resolution and lower detection levels in imaging mass spectrometry, as well as methods for the rapid purification of plant mitochondria from specific cell types, as recently initiated by proof-of-concept of affinity-based approaches (Kuhnert et al., 2020; Niehaus et al., 2020), are required for further progress.

Supplemental Data

Supplemental Figure. Half reactions and metabolic redox potentials for the reactions described in Figure 2.

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Supplemental Table. Mitochondrial proteins with several post-translational modifications. Supporting Figure 4.

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