Mechanisms and Functions of Post-translational Enzyme Modifications in the Organization and Control of Plant Respiratory Metabolism

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Summary

The respiratory pathways of glycolysis, the tricarboxylic acid (TCA) cycle, and mitochondrial electron transport chain are central features of carbon metabolism and bioenergetics in eukaryotic cells. As respiration forms the core of intermediary metabolism, it plays a pivotal role in the growth and metabolism of all photosynthetic organisms. The aim of this chapter is to provide an overview of the occurrence and functions of enzyme post-translational modifications (PTMs) in the control of plant respiration including sucrose catabolism. PTMs are covalent alterations of amino acid residues within a particular polypeptide. Diverse PTMs represent pivotal regulatory events that integrate signaling, gene expression, and metabolism with developmental and stress responses in eukaryotic cells. These PTMs are often rapid and reversible and can not only dramatically alter enzyme activity, but may also generate specific PTM-dependent docking sites that influence interactions with other proteins. In yeast, enzyme PTMs exert more control over glycolysis and mitochondrial metabolism than do changes in transcripts or enzyme abundance, and the same situation likely applies to vascular plants. Recent advances in proteomics, particularly the development of novel and specific chemistries along with affiliated mass spectrometry techniques for detection and mapping of diverse PTMs, are rapidly expanding the catalogue of respiratory enzymes whose functions may be controlled by reversible covalent modification. Phosphorylation-dephosphorylation and disulfide-dithiol interconversion appear to be the most prevalent types of reversible covalent modification used in plant enzyme control. Additional PTMs such as monoubiquitination, S-nitrosylation, and acetylation also appear to play important roles. However, the biochemical impact of *in vivo* PTMs on the functional properties of plant respiratory enzymes remains mostly unknown and thus remains an important goal for future research. Rational manipulation of PTM events is expected to make an important contribution to the implementation of effective biotechnological strategies for engineering grain crops for increased yields *via* metabolic engineering.

I. Introduction

A. Multi-faceted Functions of Plant Respiration

Plant respiration is the controlled oxidation of carbon substrates through glycolysis, the tricarboxylic acid (TCA) pathway and mitochondrial electron transport chain (mETC), producing CO_2 and ATP (Plaxton and Podestá 2006). Respiration also has a crucial role to produce low-molecular-weight 'building block' molecules needed as precursors for biosynthesis and nitrogen assimilation, as well as various metabolites required for plant acclimation to unavoidable stresses encountered in their ever-changing environment (Plaxton and Podestá

2006; Tcherkez et al. 2012). Respiration also helps to ensure the efficient operation of other redox pathways, especially photosynthesis. The close connection between respiration and photosynthesis is an intriguing and unique aspect of plant metabolism, and results in respiration behaving quite differently at night and during the day, or otherwise between photosynthetic and non-photosynthetic tissues (Sweetlove et al. 2010; Cheung et al. 2014). Therefore, as occurs for photosynthesis and starch metabolism, various post-translational enzyme controls occur on a diurnal cycle and allow respiration in green tissues to operate in different modes during the day *versus* the night. As discussed below, important insights into the organization and control of

plant respiration have also been gained by studies of heterotrophic tissues such as developing and germinating seeds, ripening fruit, roots and tubers, and suspension cell cultures.

B. The Control of Plant Metabolism Remains Poorly Understood

As we advance the biotechnological application of plants to produce greater and more sustainable yields of food, medicine, fiber, biofuels, and industrial commodities such as biodegradable plastics, a number of important resources have become increasingly available to guide metabolic engineering efforts. Complete genome sequences for a growing number of food, biofuel, and industrial crop and tree species are available and annotations are improving, as are metabolic maps for primary metabolism and increasing amounts of secondary metabolism. One important area that is lagging behind is our understanding of the control of plant metabolism (Stitt and Gibon 2014). Our ability to manipulate the expression of enzymes *via* metabolic engineering continues to outpace our ability to predict the outcome of these manipulations on plant metabolism. Overexpression and knockout lines of metabolic enzymes frequently have unintended, unforeseen and undesirable consequences throughout the metabolic network; often the desired or predicted outcome is not observed (Sweetlove et al. 2008). This is in part due to a lack of consideration and of knowledge of evolved enzyme regulatory mechanisms that are in place to maintain appropriate and optimal rates of metabolic fluxes through the various alternative pathways of plant metabolism.

C. Post-translational Enzyme Regulation Plays a Central Role in the Control of Plant Respiration

As with all organisms, plant metabolic fluxes respond to environmental conditions and developmental processes. Changes in enzyme activities are achieved by either altering the rate of enzyme synthesis or degradation, expressing an alternative isozyme with different properties, involving allosteric effectors and feedback mechanisms, or modifying enzyme activity *via* post-translational modifications (PTMs). Each of these mechanisms is prevalent in plant cells, but the use of PTMs for controlling metabolic fluxes is advantageous because they trigger rapid, appropriate, and highly precise activation or inhibition of key regulatory enzymes of specific metabolic pathways. A recent study investigated the extent to which metabolic flux in the central metabolism of oilseed rape embryos is reflected in the transcriptome (Schwender et al. 2014). With a few exceptions, differential fluxes through the major pathways (*i.e.*, glycolysis, TCA pathway, amino acid and fatty acid synthesis) was not reflected in the abundance of relevant transcripts. A subsequent study concluded that the major discordance between metabolic flux, V_{max}, and enzyme abundance indicated that fluxes in seed central metabolism were not significantly controlled at the level of transcription/translation but rather at the posttranslational level; *i.e., via* the allosteric control and/or PTM of key enzymes (Schwender et al. 2015).

Specific metabolic intermediates often allosterically regulate the activity of upstream and downstream regulatory enzymes, and these feedback inhibition and feed-forward activation mechanisms help metabolism to be a stable, controllable network. In addition, the reversible PTM of key enzymes has long been known to play a dominant role in acute (*i.e.,* short-term) metabolic control, and is the major mechanism whereby cellular metabolism is coordinated and regulated by external signals, particularly in animals and vascular plants. Through the covalent attachment of one or more functional groups to specific amino acid residues within a given polypeptide, PTMs can function as highly versatile molecular switches that control enzyme function. The general model is that the covalently modified enzyme is interconverted between

less active (or inactive) and more active forms owing to conformational-induced effects on the binding of its substrates and/or allosteric effectors. PTMs such as protein-kinase mediated phosphorylation can not only dramatically alter an enzyme's kinetic properties, but may also generate PTM-dependent docking sites that influence its interactions with other proteins, subcellular localization, or susceptibility to turn-over by proteases. PTMs also allows for a very marked sensitivity (amplification) to external signals, much greater than is possible for an enzyme responding to allosteric (metabolite) effectors, and indeed, can regulate some enzymes in an 'on-off' manner.

Some enzyme PTMs may be irreversible as in the case of proteolytic cleavage of a transit peptide, or may include uncatalyzed modifications (e.g. oxidation by reactive oxygen species) which are often indicative of protein damage. However, reversible, catalyzed PTMs represent a crucial mechanism by which plants regulate activities of key flux-controlling enzymes of their metabolic pathways, particularly within the evolutionarily conserved pathways of glycolysis and mitochondrial respiration. Many excellent reviews have appeared concerning different aspect of the study of protein PTMs in plants (Comparot et al. 2003; Huber and Hardin 2004; Buchanan and Balmer 2005; Moorhead et al. 2006; Tran et al. 2012; Rao et al. 2014; Friso and van Wijk 2015; Lamotte et al. 2015; Plaxton and Shane 2015; Nietzel et al. 2016; Wilson et al. 2016).

D. Proteomics Has Revealed Widespread and Diverse PTMs of Plant Respiratory Enzymes

Proteomics is a rapidly evolving field that involves high-throughput identification of proteins and their PTMs in specific tissues, cells, organelles, or membrane fractions during stress acclimation or development. Mass spectrometry (MS) based plant proteomic screens targeting the identification of specific enzyme PTMs have proliferated over the past decade owing to remarkable advances in MS technologies and related bioinformatic databases. These developments have revealed extensive site-specific PTMs of respiratory enzymes in diverse plant tissues under different environmental and developmental situations (Huber and Hardin 2004; Plaxton and Podestá 2006; Finkemeier et al. 2011; Yao et al. 2014; Rao et al. 2014; Plaxton and Shane 2015). Although an important first step, identifying PTMs of individual proteins that occur *in vivo* raises four additional questions, as follows:

1. What is the stoichiometry of the in vivo PTM? By stoichiometry, we mean the proportion of a given protein that carries a specific PTM. Stoichiometry must usually be significant in order for any PTM to have a meaningful impact on the biological function of a target protein in a living cell. Many PTMs of low stoichiometry are likely to result from the non-specificity of catalyzed and uncatalyzed biochemical reactions and are an example of biochemical noise.

2. When is/are the particular amino acid residue(s) post-translationally modified; *i.e.* what is the timing and order of changes in the PTM status of specific respiratory enzymes during development or following exposure to stress?

3. How is/are the particular residue(s) modified? The precise mapping of PTMs to specific amino acid residues of a polypeptide using MS allows the eventual linkage of these PTM sites to specific modifying enzymes (e.g. a protein kinase isozyme that use ATP to phosphorylate a target protein), and ultimately the upstream signal-transduction pathways that control its expression and/or activity (e.g. see Murmu and Plaxton 2007; Hill et al. 2014; Fedosejevs et al. 2016).

4. Why is the enzyme post-translationally modified? This is perhaps the most important problem of all, as we must ultimately understand how a particular PTM influences the activity and biological function(s) of a target enzyme. Although proteomic screens have revealed that most plant respiratory enzymes appear to be subject to multiple PTMs *in vivo*, in

only a handful of cases has a robust characterization of the impact of these PTM events on the target enzyme's biological properties been undertaken (Stitt and Gibon 2014; Friso and van Wijk 2015). In a distinct approach, recent studies have combined flux measurements with metabolomics and phosphoproteomics in leaves to demonstrate correlations between net photosynthetic assimilation and distinct enzyme phosphorylation events (Boex-Fontvieille et al. 2014; Abadie et al. 2016), yet the effect of the majority of these phosphorylation events on enzyme catalysis has not been documented. As a given PTM can affect enzyme properties in different ways (or may have no biochemically meaningful effect), there clearly remains a huge gap in our understanding of many PTM events – we know they exist and are seemingly important, but we don't know what they are doing. Determining how select PTM events influence key enzymes participating in respiratory metabolism has and will continue to provide crucial insights into how plant respiration functions and is controlled *in vivo*.

In this Chapter, we provide a brief description of the allosteric regulation of plant respiration by metabolite intermediates, followed by the description of the known examples of functional characterizations of PTMs of plant respiratory pathway enzymes, including the sucrose catabolizing enzyme sucrose synthase.

II. Phosphoenolpyruvate Branchpoint Is a Primary Site of Glycolytic and Respiratory Control

The metabolic organization of plant respiration, as depicted in Fig. 13.1a, places particular importance on the control of phospho*enol*pyruvate (PEP) metabolism *via* PEP carboxylase (PEPC) and pyruvate kinase (PK). In non-plant systems such as mammalian liver, primary control of glycolytic flux of hexose-phosphates to pyruvate is mediated by the ATP-dependent phospho-fructokinase (PFK), with secondary control at PK. Activation of PFK increases the level of its product, fructose-1,6-bisphosphate, which is a potent feed-forward allosteric activator of the majority of non-plant PKs examined to date. By contrast, *in vitro* and *in vivo* evidence have consistently demonstrated that plant glycolysis is controlled from the 'bottom up' with primary and secondary regulation exerted at the levels of PEP and fructose-6-phosphate utilization, respectively (Fig. 13.1a) (Plaxton and Podestá 2006; Plaxton and O'Leary 2012). These findings are compatible with enzymological studies demonstrating that plant cPK is not activated by fructose-1,6-bisphosphate, whereas plant PFK- and PPi-dependent phosphofructokinase (PFP) demonstrate potent allosteric inhibition by PEP (Plaxton and Podestá 2006). Thus, activation of cPK or PEPC will relieve the feedback inhibition of PFK and PFP exerted by PEP, thereby enhancing glycolytic flux from the hexose-phosphate pool. Reduced cytosolic PEP levels will also elevate Fru-2,6-P_2 levels (and thus activation and inhibition of PFP and cytosolic fructose-1,6-bisphosphatase, respectively) since a drop in PEP results in a fall in 3-phosphoglycerate (these metabolites are at equilibrium *in vivo*), and PEP and 3-phosphoglycerate are potent inhibitors of the kinase activity of plant F2KP (Fig. 13.1a). It follows that the activities of cPK and PEPC must be balanced in a concerted and well integrated fashion to control the overall glycolytic flux of hexose-phosphates as well as the provision of mitochondria with pyruvate and oxaloacetate/malate respectively needed for production of ATP and as biosynthetic precursors. The plastidic pyruvate kinase isozyme (pPK) and the shikimate pathway also require PEP as an initial substrate for their synthesis of pyruvate + ATP, and diverse phenolic compounds, respectively. Therefore, PEP metabolism represents a pivotal branching point in plant carbon metabolism and the various enzymes that utilize PEP must be tightly regulated in a highly coordinated fashion. Thus, it is not
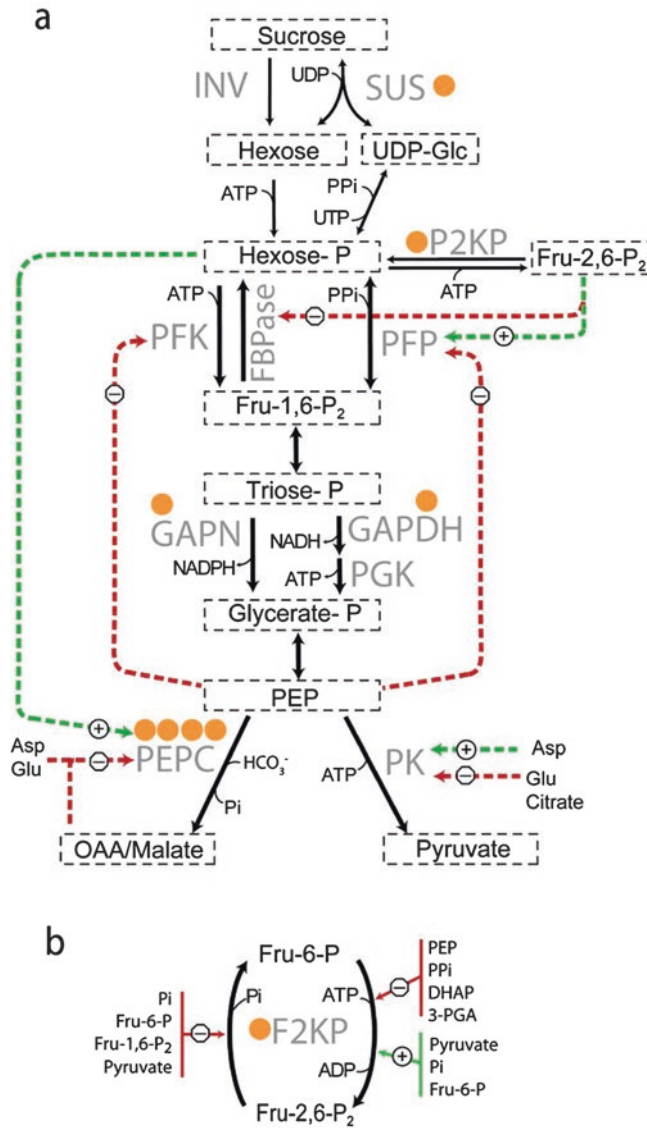
Fig. 13.1. Simplified diagram of plant cytosolic glycolysis showing the location of enzyme PTM's with experimentally described functions, and key features of their control by allosteric effectors. (**a**), the glycolytic pathway including sucrose hydrolysis has been condensed with dashed boxes indicating pools of grouped metabolites; most co-factors and co-substrates have been omitted for simplicity. Selected enzymes have been included to highlight metabolic steps under PTM control and orange circles indicate a described PTM modification on the corresponding enzyme. Central mechanisms of allosteric inhibition (−) and activation (+) are indicated by arrows and illustrate the interrelated importance of PEP and hexose-phosphate metabolism. (**b**), The synthesis and degradation of the potent signal metabolite Fru-2,6-P_2 by the bifunctional enzyme P2KP is sensitive to allosteric control by several glycolytic intermediates, along with Pi and PPi

surprising that PK and PEPC are becoming important targets for metabolic engineering of the PEP branching point to modify levels of agronomically important end products, such as storage proteins and lipids in developing seeds. As discussed below, decades of research into the biochemistry of plant PEP metabolizing enzymes has revealed multiple layers of post-translational control including allosteric effectors, PTMs,

pH, and protein-protein interactions, which may vary considerably between subcellular location and tissue types.

III. Post-translational Modifications of Plant Respiratory Enzymes

A. Phosphorylation

Phosphorylation was the first protein PTM to be discovered and remains the most characterized PTM of eukaryotic proteins (Moorhead et al. 2006). Phosphorylation participates in the control of virtually all aspects of cellular physiology and development including signal transduction, cell differentiation, cytoskeleton organization, active transport (ion pumping), gene expression, disease and stress responses, and metabolic fluxes. Protein phosphorylation is believed to occur with at least 70% of all eukaryotic proteins, with the majority having multiple phosphorylation sites (Moorhead et al. 2006). Protein kinases and phosphatases catalyze the covalent incorporation or hydrolysis, respectively, of Pi groups on target proteins. The central role of protein phosphorylation in plant cell biology is illustrated by the fact that the largest known protein family consists of the protein kinase 'superfamily', accounting for up to 5% of the entire genome. The protein phosphatase catalytic subunits that hydrolyze Pi from phosphoproteins constitute a smaller group of genes compared to kinases. However, protein phosphatase catalytic subunits (such as protein phosphatase type-2A) can associate with a large number of other proteins to form a wide variety of catalytic and regulatory subunit complexes (Moorhead et al. 2006).

As outlined below, there is convincing evidence for the control of at least seven cytosolic or mitochondrial enzymes involved in plant respiration by reversible phosphorylation. This list will undoubtedly expand as recent phosphoproteomic studies indicate that the majority of glycolytic, TCA pathway and mETC enzymes are *in vivo* phosphorylated in diverse plant tissues at some point during their lifetime (Yao et al. 2014). It is notable that protein phosphorylation may not only directly control enzymatic activity, but can also generate specific docking sites for other proteins, control protein shuttling within or between cellular compartments, and regulate proteolytic degradation (Tang et al. 2003; Hardin et al. 2004; Huber and Hardin 2004; Moorhead et al. 2006). In fact, the generation of phosphorylation-dependent docking or interaction sites may be one of the most common functions of protein phosphorylation. Of particular interest in this regard are the 14-3-3s, a family of highly conserved and abundant proteins that play a central regulatory role in all eukaryotic cells (Comparot et al. 2003; Huber and Hardin 2004; Moorhead et al. 2006; Wilson et al. 2016). The 14-3-3s bind to specific phosphorylated sites on diverse target proteins, thereby forcing conformational changes or influencing interactions between their targets and other molecules. In these ways, 14-3-3s 'complete the job' when phosphorylation alone lacks the power to drive changes in enzyme activity. For example, the phosphorylated forms of sucrose-phosphate synthase and nitrate reductase, which are key enzymes in C- and N-metabolism, respectively, are potently inhibited by 14-3-3 binding (Winter and Huber 2000; Moorhead et al. 2006). As discussed below, 14-3-3 proteins appear to participate in the control of the phosphorylated forms of several enzymes involved in plant respiration.

1. Sucrose Synthase

Heterotrophic plant tissues require a large influx of carbon and energy in the form of the disaccharide sucrose, the major form of photosynthetically assimilated carbon translocated from source leaves to sinks *via* phloem. Imported sucrose must initially be

converted into hexose-phosphates needed to fuel respiration and the biosynthesis of structural carbohydrates and storage products, particularly starch, protein, and triacylglycerides. Sucrose synthase (SuSy) is a key player in this process, catalyzing the UDP-dependent cleavage of sucrose into UDP-glucose and fructose (Fig. 13.1a). Sucrose cleavage is vital for vascular plants, not only for the allocation of crucial carbon resources, but also for the initiation of hexose-based sugar signals that alter the expression of diverse genes. Although the SuSy reaction is reversible, it generally catalyzes a net sucrolytic flux *in vivo*. The control of SuSy activity helps to determine sink strength and the partitioning of imported sucrose into various carbon utilizing pathways, including glycolysis and the TCA pathway (Winter and Huber 2000). To fulfill its different functions, SuSy is encoded by a small gene family which displays distinct tissue-specific expression profiles (Bieniawska et al. 2007). Observed differences in SuSy subcellular localization, in particular plasma membrane, cell wall, and cytoskeletal association, also likely contribute to the control of sucrose flux to specific pathways (Winter and Huber 2000; Huber and Hardin 2004; Hardin et al. 2004; Duncan and Huber 2007; Coleman et al. 2009; Brill et al. 2011). SuSy from diverse plant tissues is *in vivo* phosphorylated by a Ca^{2+}-dependent protein kinase (CDPK) at a conserved seryl residue located near its N-terminus. However, the impact of SuSy phosphorylation is somewhat perplexing because contrasting effects of this PTM on the enzyme's properties have been reported. Studies of SuSy from expanding maize leaves, mung bean seedlings, and developing tomato and pear fruits indicated that N-terminal seryl phosphorylation activates the enzyme's sucrolytic activity by lowering its K_m values for sucrose and UDP (Huber et al. 1996; Nakai et al. 1998; Anguenot et al. 1999; Komina et al. 2002; Tanase et al. 2002). By contrast, the same phosphorylation event was reported to

decrease the association of SuSy with membranes (Huber et al. 1996; Winter et al. 1997; Subbaiah and Sachs 2001; Hardin et al. 2004) or to protect the enzyme from proteolytic degradation (Zhang et al. 1999; Asano et al. 2002; Fedosejevs et al. 2014). The differing effects of N-terminal phosphorylation on different SuSy isozymes may reflect the various physiological roles of this enzyme within plant cells.

Observing how a PTM event responds to environmental conditions provides valuable insights into the overarching metabolic regulatory network and ultimately the physiological contribution of a PTM. The extent of SuSy N-terminal phosphorylation initially increased during exposure of maize roots to anoxia (Subbaiah and Sachs 2001), but decreased during nitrogen or salt stresses in soybean root nodules (Komina et al. 2002), or sucrose limitation imposed by depodding of developing castor beans (Fedosejevs et al. 2014). A common connection between these treatments is a change in cellular energy demands. Furthermore, the CDPK responsible for N-terminal SuSy phosphorylation in developing seeds has been identified. In rice, an endosperm-specific CDPK (OsSPK) which phosphorylates SuSy at its N-terminal site was identified as being essential for starch accumulation by the rice grains; mutants lacking *OsSPK* accumulated sucrose in the developing endosperm, resulting in sterility (Asano et al. 2002). This finding strongly implies that phosphorylation maintains SuSy activity necessary for rice grain filling (Asano et al. 2002). In the oil-rich endosperm of developing castor beans, the native CDPK that phosphorylates RcSUS1 (the dominant SuSy isozyme expressed in this tissue) was highly purified and identified by MS as RcCDPK2, the closest castor ortholog to OsSPK (Fedosejevs et al. 2016). RcCDPK2 activity displayed insensitivity to metabolite effectors and paralleled RcSUS1 phosphorylation status during the endosperm filling stage. Both the native and heterologously-expressed RcCDPK2 catalyzed Ca^{2+}-

dependent phosphorylation of RcSUS1 and its corresponding dephosphopeptide at Ser^{11}, while exhibiting an unusually high affinity for free Ca^{2+} ions $[K_{0.5}(Ca^{2+}) < 0.5$ µM] (Fedosejevs et al. 2016). The phosphorylation-dependent protection of SuSy from proteolysis was hypothesized to maintain high levels of SuSy in developing castor oilseeds (which accounts for up to 5% of total soluble protein in this tissue), thereby ensuring appropriate sink strength and levels of hexose-phosphates needed to support fatty acid and storage protein synthesis and affiliated respiratory pathways (Fedosejevs et al. 2014, 2016). It would thus be of interest to determine how RcSUS1 levels, as well as sucrose partitioning *via* glycolysis towards fatty acid synthesis in the developing seeds would be influenced in a castor mutant lacking RcCDPK2.

2. *Fructose-6-Phosphate 2-Kinase/Fructose-2,6-Bisphosphatase*

Fructose-6-phosphate 2-kinase/fructose-2,6-bisphosphatase (F2KP) is a bifunctional enzyme that interconverts fructose-6-phosphate and the cytosolic signal metabolite fructose-2,6-bisphosphate (Fru-2,6-P_2). Low concentrations of Fru-2,6-P_2 potently activate the glycolytic 'bypass' enzyme PFP while inhibiting the oppositely directed gluconeogenic enzyme fructose-1,6-bisphosphatase (Fig. 13.1a), thereby controlling the direction of carbon flux through the upper portion of glycolysis. This mechanism is important for the *in vivo* diurnal control of photosynthetic carbon partitioning in leaves and determining sink strength in heterotrophic tissues (Nielsen et al. 2004). Cytosolic F2KP levels are controlled by the relative rates of F2KP's kinase and phosphatase activities making their reciprocal regulation an important control point in plant respiration. F2KP is subject to complex allosteric activation and inhibition by several intermediates of respiratory metabolism (Fig. 13.1b) (Plaxton and Podestá 2006). Furthermore, F2KP is phosphorylated in seedlings of the model plant *Arabidopsis thaliana* and subsequently binds 14-3-3 proteins (Furumoto et al. 2001; Kulma et al. 2004). In photosynthetic tissues, F2KP phosphorylation appears to vary diurnally and with leaf age (Furumoto et al. 2001). However, additional studies are required to evaluate the impact of phosphorylation and 14-3-3 binding on cytosolic Fru-2,6-P_2 levels and F2KP's *in planta* activity.

3. *Non-phosphorylating Glyceraldehyde-3-Phosphate Dehydrogenase*

Non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPN) is an alternative $NADP^+$-dependent cytosolic bypass of the NAD^+-dependent glyceraldehyde-3-phosphate dehydrogenase (NAD-GAPDH) and 3-phosphoglycerate kinase of classical glycolysis (Fig. 13.1a). GAPN catalyzed conversion of glyceraldehyde-3-phosphate to 3-phosphoglycerate is therefore not coupled to ATP production but represents a source of cytosolic NADPH. Given these differences, the activity of GAPN in heterotrophic tissues is expected to be regulated in accordance with cytosolic energy and reductant demands, and possibly with phosphate availability (Plaxton and Podestá 2006). GAPN is phosphorylated at Ser^{404} in developing wheat endosperm (Bustos and Iglesias 2002, 2003; Piattoni et al. 2011). Phosphorylation triggers its interaction with 14-3-3 proteins which inhibits GAPN activity by lowering V_{max} while increasing sensitivity to inhibition by ATP and pyrophosphate (Bustos and Iglesias 2003). GAPN phosphorylation in wheat endosperm is mediated by a ribose-5-phosphate sensitive SnrK1-like kinase activity, which further ties GAPN regulation to cellular energy and redox status since ribose-5-phosphate levels reflect the activity of the ribose-5-phosphate and NADPH producing oxidative pentose phosphate pathway (Piattoni et al. 2011). Phosphorylation and the consequent 14-3-3 dependent inhibition of GAPN may help plants adjust their glycolytic metabolism to changing energy status. However, details of

physiological situations in which this occurs are lacking.

4. Pyruvate Kinase

Pyruvate kinase is an allosteric enzyme that catalyzes the irreversible conversion of PEP and ADP into pyruvate and ATP. Plants and green algae express cPK and pPK isozymes that show dramatic differences in their physical, immunological, and kinetic/regulatory properties (Plaxton and Podestá 2006). Genetic studies have confirmed that pPK of developing *Arabidopsis* seeds functions in support of fatty acid synthesis (Baud and Lepiniec 2010), whereas cPK exerts control over glycolytic flux to pyruvate in the plant cytosol (Oliver et al. 2008). Plant cPKs characterized to date are sensitive to a variety of allosteric effectors. However, glutamate functions a potent allosteric inhibitor and aspartate as an activator (by relieving glutamate inhibition) of cPK isozymes expressed in tissues active in carbon-nitrogen interactions (e.g. developing seeds, mature leaves) (Plaxton and O'Leary 2012; O'Leary and Plaxton 2015). cPK from developing soybean seeds was proposed to be phosphorylated *in vivo* which was hypothesized to trigger its proteolytic degradation (Tang et al. 2003). Indeed, proteolytic turn-over of cPK may be an important mechanism of control. However, the extent and function of *in vivo* cPK phosphorylation in plants requires confirmation.

5. Class-1 and Class-2 PEP Carboxylase

PEPC is a tightly regulated cytosolic enzyme that catalyzes the irreversible β-carboxylation of PEP in the presence of HCO_3^- to yield oxaloacetate and Pi. In C_4 and crassulacean acid metabolism, photosynthetic PEPCs have been widely studied owing to their central role in atmospheric CO_2 fixation. PEPC also fulfils crucial non-photosynthetic functions, particularly the anaplerotic replenishment of TCA pathway intermediates consumed during biosynthesis and N-assimilation (O'Leary

et al. 2011c). Furthermore, when combined with mitochondrial NAD-malic enzyme and malate dehydrogenase, PEPC also serves as an alternative to cPK for conversion of PEP into pyruvate. Metabolic flux analysis has shown that this "cPK bypass" pathway contributes to pyruvate formation in certain tissues (Schwender et al. 2006).

To fulfill its diverse physiological roles vascular plant PEPC belongs to a small multi-gene family encoding several plant-type PEPCs (PTPCs), as well as a distantly related bacterial-type PEPC (BTPC) (O'Leary et al. 2011c). PTPC genes encode similar 105–110 kDa polypeptides that typically assemble as Class-1 PEPC tetramers that are post-translationally controlled by several allosteric effectors in conjunction with reversible phosphorylation and (as discussed below) monoubiquitination. By contrast, plant *BTPC* genes encode 116–118 kDa polypeptides exhibiting low (<40%) sequence identity with PTPCs and that contain a prokaryotic-like (R/K)NTG C-terminal tetrapeptide (Gennidakis et al. 2007, O'Leary et al. 2011c). Purification of anaplerotic PEPCs from unicellular green algae and then developing castor oil seeds led to the discovery of distinct high molecular mass Class-2 PEPC hetero-octameric complexes composed of a Class-1 PEPC core tightly associated with four BTPC subunits (Fig. 13.2) (Rivoal et al. 2001; Blonde and Plaxton 2003; Gennidakis et al. 2007). *BTPC* genes are a monophyletic group which arose in green algae and native BTPC polypeptides have only been observed tightly associated with PTPC subunits within a Class-2 PEPC complex (O'Leary et al. 2011c). The BTPC subunits of Class-2 PEPC are both catalytic and regulatory as they substantially desensitize the associated PTPC subunits to allosteric inhibition by malate and aspartate (Blonde and Plaxton 2003; O'Leary et al. 2009). BTPC and thus Class-2 PEPC complexes are less common than Class-1 PEPCs and since their discovery in unicellular green algae (Rivoal et al. 2001) have only been observed in actively

expanding sink tissues such as developing castor oil seeds and pollen, and immature leaves (Gennidakis et al. 2007; Igawa et al. 2010; O'Leary et al. 2011b). Key regulatory features of Class-2 PEPCs are their marked insensitivity to allosteric effectors relative to Class-1 PEPC (Rivoal et al. 2001, Blonde and Plaxton 2003, O'Leary et al. 2009), and a dynamic association with the mitochondrial outer membrane (as opposed to the diffuse cytosolic location of Class-1 PEPCs) (Park et al. 2012). Mitochondrial-associated Class-2 PEPC was hypothesized to facilitate rapid refixation of respiratory CO_2 while sustaining a large anaplerotic flux to replenish C-skeletons (of the TCA pathway) withdrawn for biosynthesis (Park et al. 2012). Both Class-1 and Class-2 PEPCs are subjected to multiple regulatory PTMs which have helped to clarify their respective physiological roles *in planta*.

PTPC phosphorylation at its conserved N-terminal serine residue is catalyzed by a dedicated Ca^{2+}-independent PTPC protein kinase (PPCK). This PTM enhances allosteric activation of Class-1 PEPCs by hexose phosphates while diminishing their feedback inhibition by malate and aspartate (Chollet et al. 1996, Law and Plaxton 1997, Nimmo 2003, Izui et al. 2004, Tripodi et al. 2005, Gregory et al. 2009, O'Leary et al. 2011c). Several lines of evidence including transgenic plants expressing a phospho-mimetic version of Class-1 PEPC have verified that phosphorylation increases *in vivo* anaplerotic flux of PEP to oxaloacetate (Rademacher et al. 2002; Nimmo 2003; Figuero et al. 2016). Owing to the importance of phosphorylation as a regulatory mechanism of photosynthetic and non-photosynthetic Class-1 PEPCs, extensive PPCK studies have been performed, including its cloning from crassulacean acid metabolism, C_4 and C_3 plants (Saze et al. 2001; Nimmo 2003; Xu et al. 2003, 2007; Sullivan et al. 2004; Fukayama et al. 2006; Murmu and Plaxton 2007, O'Leary et al. 2011b). Consequently, PPCK-mediated phosphorylation of Class-1

PEPC continues to provide one of the best characterized examples of regulatory enzyme phosphorylation in the plant kingdom.

Although BTPC polypeptides lack the conserved N-terminal phosphorylation site of PTPCs, the BTPC subunits of Class-2 PEPC from developing castor bean endosperm are subject to *in vivo* regulatory phosphorylation on at least two seryl residues (Ser^{425} and Ser^{451}). Phosphorylation at either site inhibits BTPC activity by decreasing the enzyme's affinity for PEP while increasing sensitivity to allosteric inhibition by malate and aspartate (Uhrig et al. 2008a; O'Leary et al. 2011c; Dalziel et al. 2012; Hill et al. 2014; Ying et al. 2017). The pattern of occurrence of these inhibitory BTPC phosphorylation events within castor bean seeds differ markedly from the activation by phosphorylation of PTPC-containing Class-1 PEPC within the same tissue (Fig. 13.2) (Murmu and Plaxton 2007; Tripodi et al. 2005). Phosphorylation of BTPC at Ser^{425} and Ser^{451} increases throughout endosperm development and in response to sucrose limitation by depodding (O'Leary et al. 2011c; Dalziel et al. 2012). These differences imply that castor bean BTPC *versus* PTPC phosphorylation are mediated by different protein kinases. This hypothesis was strongly supported by the purification and characterization of the PPCK that phosphorylates PTPC subunits of castor bean Class-1 PEPC (Murmu and Plaxton 2007), as well as the castor bean CDPK that catalyzes Ca^{2+}-dependent BTPC phosphorylation at Ser^{451} (Hill et al. 2014; Ying et al. 2017). The CDPK was highly purified from developing castor beans and identified by MS as RcCDPK1 (Ying et al. 2017). Both native and heterologously expressed RcCDPK1 displayed high specificity for phosphorylating castor bean BTPC at Ser^{451} as they could not phosphorylate BTPC at any other site, Class-1 PEPC or SuSy, nor various synthetic peptides *in vitro* (Hill et al. 2014; Ying et al. 2017).
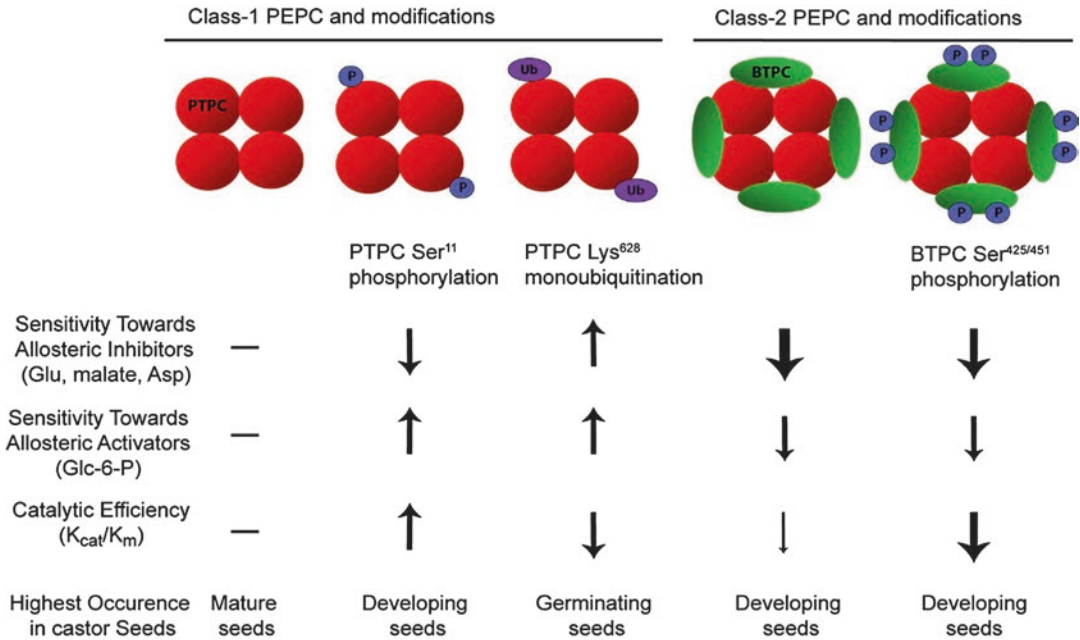
Fig. 13.2. Multiple PTMs of plant PEPC isoforms provide a range of catalytic and regulatory properties. The effects of known regulatory reversible PTMs are compared qualitatively to an unmodified Class-1 PEPC homo-tetramer of PTPC subunits. All PEPC isoforms and modifications occur during development and germination of castor bean seeds. In developing endosperm, Class-1 PEPC is activated *in vivo* by sucrose-dependent phosphory-lation of Ser[11] (Tripodi et al. 2005, Murmu and Plaxton 2007). In the germinating endosperm, approximately half of the PTPC subunits of Class-1 PEPC become monoubiquitinated at Lys[628], inhibiting enzyme activity (Uhrig et al. 2008a). The Class-2 PEPC hetero-octamer that dynamically associates with the mitochondrial outer enve-lope *in vivo* is highly expressed in developing castor endosperm and cotyledons and is markedly desensitized to allosteric feedback inhibitors (Blonde and Plaxton 2003; Gennidakis et al. 2007; O'Leary et al. 2009; Park et al. 2012). The BTPC subunits of Class-2 PEPC are subject to inhibitory *in vivo* phosphorylation at Ser[425] and Ser[451] which increases when sucrose supply is eliminated by removing (depodding) the seeds from the plant (O'Leary et al. 2011c; Dalziel et al. 2012; Hill et al. 2014; Ying et al. 2017)

6. *The Mitochondrial Pyruvate Dehydrogenase Complex*

The mitochondrial pyruvate dehydrogenase complex (mPDH) is an important enzyme complex that connects glycolysis with the TCA pathway by catalyzing the irreversible conversion of pyruvate, coenzyme A, and NAD^+ into acetyl-CoA, NADH, and CO_2. mPDH activity is regulated by feedback inhibition by NADH and acetyl-CoA, and reversibly inactivated following phosphory-lation of its E1α subunits (Tovar-Mendez et al. 2003). Although many other mitochon-drial phosphoproteins have been identified (Yao et al. 2014), mPDH remains the only

well-established example of a regulatory phosphorylation event with plant mitochon-dria. mPDH phosphorylation status is controlled by its tightly associated, dedicated PDH-kinase and PDH-phosphatase (Fig. 13.3). PDH-kinase activity is stimulated by ATP and NH_4^+ and inhibited by pyruvate and ADP. These allosteric features of PDH-kinase are thought to explain why leaf mPDH is rapidly phosphorylated and inhibited in the daytime when high photorespiratory flux produces high ATP and NH_4^+ levels within the matrix (Tovar-Mendez et al. 2003; Tcherkez et al. 2012). A decrease in the expression level of PDH-kinase has also been linked to higher rates of respiration in
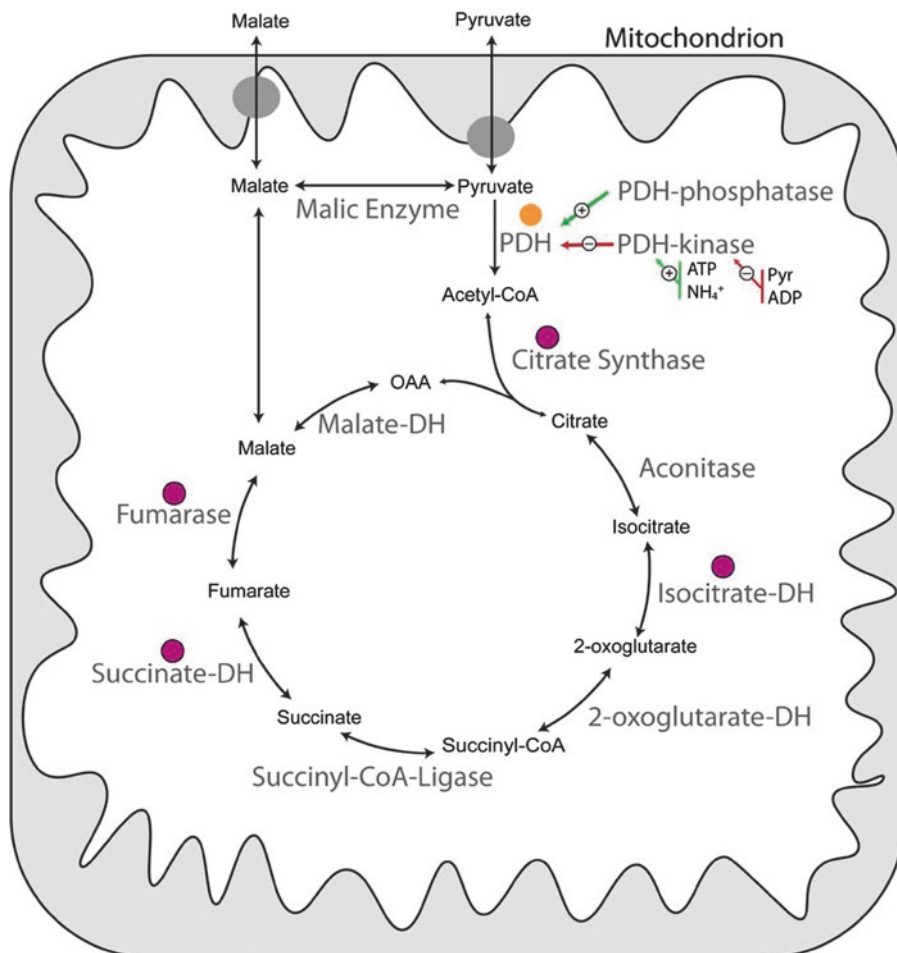
Fig. 13.3.   A simplified diagram showing experimentally observed sites of enzyme PTMs within mitochondrial organic acid metabolism. The pyruvate dehydrogenase complex (PDH) is controlled by regulatory phosphorylation by a PDH-phosphatase and a PDH-kinase which is under allosteric control. Four enzymes within the TCA pathway have been shown by *in vitro* assays to be affected by Trx-mediated thiol reduction, yet because of conflicting reports further evidence is needed to explain the control of the TCA pathway by Trx

leaves and developing seeds of *Arabidopsis* plants (Marillia et al. 2003).

7. ATP Synthase

ATP synthase is an integral protein complex of the mitochondrial inner membrane that catalyzes respiratory ATP production from ADP and Pi using proton motive force established by the mETC. A phosphorylation dependent interaction of 14-3-3 proteins with the F1 β-subunit of ATP synthase was reported to inhibit mitochondrial respiratory activity *in vitro* (Bunney et al. 2001). However, the significance of 14-3-3 interactions within the mitochondria requires further study because 14-3-3 proteins lack a canonical N-terminal mitochondrial target peptide. It is possible, however, that 14-3-3 proteins 'hitchhike' on mitochondrial precursor proteins during their translocation from the cytosol into the mitochondrial matrix (Wilson et al. 2016).

B. Monoubiquitination

Ubiquitination is the covalent attachment of the small protein ubiquitin to lysine residue(s) of a target protein. Ubiquitin is a highly conserved 76 amino acid polypeptide of approximately 8 kDa that is covalently attached through an isopeptide bond between its C-terminal glycine residue and the ε-amino group of a lysine residue on the target protein. A multi-enzyme system consisting of activating (E1), conjugating (E2), and ligating (E3) enzymes attach ubiquitin to cellular proteins. The specificity of ubiquitination is ensured by a huge number of different E3 ligases which recognize particular target proteins; e.g. about 1400 *Arabidopsis* genes encode different E3 ligases. Polyubiquitination is a common PTM that tags many proteins, including Class-1 PEPC (Agetsuma et al. 2005), for degradation by the 26S proteasome. However, protein monoubiquitination has emerged as widespread, non-destructive, and reversible PTM that controls the activity and subcellular location of a wide assortment of eukaryotic proteins. Studies of protein monoubiquitination in yeast and animal cells have established that this PTM functions to recruit 'client proteins' containing a ubiquitin-binding domain, thereby mediating protein:protein interactions that influence diverse cellular processes including gene expression, endocytosis, and signal transduction pathways (Plaxton and Shane 2015).

MS analysis of PTPC subunits of native Class-1 PEPC purified from the endosperm of germinating castor bean seeds led to the serendipitous discovery of what appears to be the first precedent for regulatory monoubiquitination of a metabolic enzyme in nature (Uhrig et al. 2008b). PTPC monoubiquitination at a conserved lysine residue located near the catalytic site was subsequently shown to be a widespread PTM of PTPC subunits of Class-1 PEPC from diverse plant tissues and species (Igawa et al. 2010; O'Leary et al. 2011a; Shane et al. 2013; Ruiz-Ballesta et al. 2014, 2016; Figueroa et al. 2016). The presence of Class-1 PEPC monoubiquitination is easily recognized as it creates an immunoreactive PTPC doublet of ~110 and 107 kDa on PTPC immunoblots, with the higher M_r band being a monoubiquitinated form of the lower band. Monoubiquitination inhibits PEPC activity by increasing enzyme K_m(PEP) and sensitivity to allosteric inhibitors (malate, Asp), while reducing sensitivity to allosteric activators (hexose-phosphates, glycerol-3-phosphate) (Uhrig et al. 2008b; Shane et al. 2013; Ruiz-Ballesta et al. 2014). This PTM therefore clearly opposes the activating effect of PTPC subunit phosphorylation on Class-1 PEPC activity. Progression from PTPC monoubiquitination to phosphorylation and *vice versa* has been observed in specific tissues in response to developmental changes or altered sucrose supply (Uhrig et al. 2008b; O'Leary et al. 2011a; Shane et al. 2013; Ruiz-Ballesta et al. 2014, 2016).

Further evidence for a connection between sucrose supply and the reciprocal control of Class-1 PEPC by phosphorylation *versus* monoubiquitination was provided by a recent study in which trehalose-6-phosphate (Tre-6-P) signaling was shown to operate upstream of the post-translational activation of Class-1 PEPC in *Arabidopsis* leaves by deubiquitination followed by phosphorylation (Figueroa et al. 2016). Tre-6-P levels in plant cells reflect sucrose availability and according to the sucrose-Tre-6-P nexus model, Tre-6-P functions as a signal metabolite that conveys cellular carbon status by modulating flux through the major pathways of carbohydrate catabolism including respiration (O'Hara et al. 2013; Figueroa and Lunn 2016). Inducible expression of the Tre-6-P synthase gene *otsA* in *Arabidopsis* seedlings increased Tre-6-P levels that triggered Class-1 PEPC activation by *in vivo* deubiquitination and subsequent phosphorylation, which coincided with increased anaplerotic flux of PEP into organic acids (Figueroa et al. 2016). Remarkably, nitrate reductase

was also post-translationally activated by the increased Tre-6-P levels, which points to a coordination of nitrate reduction and supply of organic anions (*i.e.*, 2-oxoglutarate and oxaloacetate) needed for N-assimilation and amino acid synthesis. Important areas for future research include assessing: (i) the mechanism by which elevated Tre-6-P mediates deubiquitination and subsequent phosphorylation of the PTPC subunits of Class-1 PEPCs, (ii) potential ubiquitin-binding domain proteins that might interact with monoubiquitinated Class-1 PEPCs, as well as the possible influence of this PTM on the enzyme's subcellular location.

Regulatory monoubiquitination may turn out to be relatively commonplace for enzymes other than PEPC that are involved in plant metabolism. Indeed a recent report demonstrated that monoubiquitination markedly inhibited the activity of a cytosolic NAD-GAPDH isozyme from *Arabidopsis* while triggering its relocation from the cytosol into the nucleus (where NAD-GAPDH may have a 'moonlighting' function as a transcription factor) (Peralta et al. 2016).

C.   Disulfide-Dithiol Interconversion

Thioredoxins (Trxs) are small ubiquitous thiol-oxidoreductases involved in cellular redox regulation in all domains of life. Within chloroplasts a robust system of Trx mediated redox control of carbon assimilatory metabolism has long been established where, in the light, Trx proteins are reduced by the photosynthetic electron transport chain *via* ferredoxin and in turn reduce thiol groups on target enzymes altering their activity (Buchanan and Balmer 2005). This process is critical for the light-dependent activation and inhibition of the stromal-localized reductive and oxidative-pentose phosphate pathways, respectively. A Trx mediated control network also operates in the cytosol and mitochondria *via* cytosolic and mitochondrial targeted NADPH-dependent Trxs and Trx-reductases (Balmer

et al. 2004; Yoshida et al. 2013; Daloso et al. 2015). The existence of such a system is unsurprising because mitochondria reorchestrate carbon metabolism and maintain suitable redox conditions in response to changing cellular and environmental conditions. However, the details of how this system functions *in vivo* to regulate individual mitochondrial enzymes and respiration as a whole are still being uncovered, and this remains an area of high research interest. As with all PTMs, there are currently many putative cytosolic and mitochondrial Trx target enzymes, but only a few well characterized examples of regulatory effects of Trx mediated redox modifications; these are described below. However, *in vitro* studies of the potential effects of redox control are often tested under extremely oxidizing or reducing conditions that may not be prevalent *in vivo,* and the *in vivo* redox status of enzymes is easily lost upon extraction and therefore not easily quantified (Nietzel et al. 2016). Care must therefore be taken to use physiologically relevant redox conditions and reductants like Trx whenever possible to avoid misleading results.

1.   Glycolytic and TCA Pathway Enzymes

The discovery of plant cytosolic (and mitochondrial) specific Trxs suggested that disulfide-dithiol interconversion may also participate in the control of plant respiration (Balmer et al. 2004; Buchanan and Balmer 2005). That plant glycolytic enzymes might be subject to disulfide-dithiol control *in vivo* was indicated by the observations that reduced thiol groups: *(i)* cause activation of plant cytosolic NAD-GAPDH, and *(ii)* elicit maximal activation of plant PFP by Fru-2,6-P_2 (Plaxton and Podestá 2006). Plant PPCK may also be under thioredoxin control as the maize leaf and developing castor bean enzyme was rapidly inactivated by incubation with oxidized glutathione and reactivated by subsequent incubation with thiol reducing agents, particularly in the presence

of thioredoxin (Saze et al. 2001; Murmu and Plaxton 2007). Similar results were obtained with the CDPK that catalyzes inhibitory BTPC phosphorylation at Ser[451] in developing castor beans (Hill et al. 2014).

Proteomic studies have assessed possible *in vivo* targets for thioredoxin action in the mitochondria and cytosol of *Arabidopsis* (Balmer et al. 2004). Cytosolic aldolase and NAD-GAPDH were determined to be likely targets for Trx-mediated disulfide to dithiol interconversion. The same study identified 50 probable Trx-linked proteins in plant mitochondria that are involved in photorespiration, the TCA pathway, mETC, and ATP synthesis, amongst other processes (Balmer et al. 2004). Deduced amino acid sequences for most of these proteins contained a pair of conserved cysteine residues consistent with these residues being Trx targets. Unsurprisingly, the activity of TCA pathway enzymes citrate synthase, isocitrate dehydrogenase, succinate dehydrogenase, and fumarase were reported to respond to Trx-mediated thiol reduction (Fig. 13.3). *Arabidopsis* mitochondrial citrate synthase was activated *in vitro* by Trx thiol reduction, particularly at Cys[365], likely due to reduction of an inter-subunit disulfide bond (Schmidtmann et al. 2014; Daloso et al. 2015). Oxidized *Arabidopsis* mitochondrial NAD^{+} dependent isocitrate dehydrogenase forms inhibitory inter-subunit disulfide bridges that could be reduced by Trx, partially restoring activity (Yoshida and Hisabori 2014). However, a separate study produced the opposite result, observing inhibition of isocitrate dehydrogenase by Trx (Daloso et al. 2015). To identify functional regulation of the TCA pathway by Trx, Daloso et al. (2015) characterized maximal extractable enzyme activities and metabolic fluxes in *Arabidopsis* lines lacking Trx-o, a mitochondrion targeted Trx, or the NADP-Trx-reductases (ntra and ntrb). The results revealed complex effects of the mutations on mitochondrial metabolism and it

was hypothesized that both mitochondrial succinate dehydrogenase and fumarase were inhibited by Trx *in vivo* (Daloso et al. 2015). Clearly, more research is required to determine the extent to which disulfide-dithiol interconversion of plant glycolytic and mitochondrial enzymes function as regulatory switches *in vivo*.

2. Alternative Oxidase of the mETC

Alternative oxidase (AOX) is a prominent feature of plant mETC because it allows increased flexibility in the extent that respiratory electron transport is coupled to the generation of proton motive force across the inner mitochondrial membrane and consequent ATP synthesis by ATP synthase. Depending on its activation state, AOX diverts electrons from ubiquinone directly to O_2, thus bypassing the proton pumping activities of Complex III and IV. This greatly reduces the ATP yield of respiration, but also helps minimize the creation of harmful reactive oxygen species that would otherwise occur if mETC components were overly reduced, especially during any abiotic stress that hinders electron flux to O_2 *via* Complex III and IV (Vanlerberghe 2013). AOX exists in the mitochondrial inner membrane as either a non-covalently or covalently linked homodimer. When the subunits are covalently linked by an intersubunit disulfide bond AOX is inactive. When this disulfide is reduced to dithiol groups by a specific Trx-*h* isozyme, the AOX subunits become non-covalently associated (Gelhaye et al. 2004). AOX activation results from an interaction of these reduced regulatory thiols with α-keto acids (i.e. pyruvate, oxaloacetate) (Umbach et al. 2006; Vanlerberghe 2013). Hence, AOX activity appears to be modulated by both glycolytic flux to pyruvate and the redox state of the mitochondria which controls Trx-*h* activity. This view would be consistent with the increased electron flux to

AOX in complex I mitochondrial mutants that are believed to have higher NADH/NAD^+ ratios in the mitochondrial matrix (see also Chap. 1).

D. S-Nitrosylation

Nitric oxide (NO) has emerged as a key signaling molecule in eukaryotic cells that controls the activity and expression of various enzymes in response to various endogenous and exogenous stimuli (Lamotte et al. 2015). Analyses of NO-dependent events in animal systems have demonstrated that S-nitrosylation of cysteine is an important control mechanism for enzymes. The sensitivity towards S-nitrosylation is determined by the proximity of cysteine residues to specific intracellular sources of NO, by the redox status of the microenvironment, and by the sequence of residues that flank the target cysteine. Leaves and roots of healthy plants produce and emit NO, and at least 100 possible targets of S-nitrosylation in plants have been identified by searching the SwissProt database for the degenerate motif that is characteristic of S-nitrosylated proteins (Huber and Hardin 2004). This *in silico* analysis was corroborated by proteomic studies of *Arabidopsis* that identified numerous proteins as likely candidates for S-nitrosylation (Lindermayr et al. 2005). Interestingly, several glycolytic and TCA pathway enzymes including aldolase, NAD-GAPDH, enolase, PEPC, and malate dehydrogenase were amongst the S-nitrosylated respiratory enzymes that were identified. At the enzymatic level, NO-dependent reversible inhibition of cytosolic NAD-GAPDH activity suggested that this enzyme might be controlled by S-nitrosylation *in vivo* (Lindermayr et al. 2005; Zaffagnini et al. 2013).

The mETC appears to be a major source of NO emissions in plants (see Chap. 10). The disruption of oxidative phosphorylation by NO has been suggested to occur *via* the direct inhibition of cytochrome oxidase (*i.e.,* Complex IV) by NO. Although this disturbance of mitochondrial function by NO may be harmful to the plant cell, a possibly greater deleterious impact of NO is avoided by the presence of AOX. Unlike cytochrome oxidase, electron flow through AOX is insensitive to NO, and AOX expression is induced by NO in *Arabidopsis* (for a review, see Plaxton and Podestá 2006). Far more research is required to fully assess the relationships between NO and plant respiration, including the extent and importance of reversible S-nitrosylation of key enzymes involved in glycolytic and respiratory control.

E. Lysine Acetylation in Plants: The Picture Is Still Unclear

Reversible lysine-acetylation, catalyzed by lysine-acetyltransferases and lysine-deacetylases, is a PTM that has received considerable recent attention from proteomic studies which detected its presence on numerous enzymes throughout metabolism in all domains of life (Choudhary et al. 2009; Wang et al. 2010; Zhao et al. 2010; Finkemeier et al. 2011; Wu et al. 2011; Tran et al. 2012; Wagner and Payne 2013; Konig et al. 2014; Weinert et al. 2014). In animals, yeast, and bacteria there is compelling evidence that reversible Lys-acetylation participates in the control of oxidative carbon metabolism (Wang et al. 2010; Zhao et al. 2010). However, it remains to be determined whether lysine-acetylation plays a similar role in controlling plant respiration. An important feature of lysine-acetylation is that the covalent attachment of an acetyl-group to protein lysine residues can also occur non-enzymatically in a spontaneous chemical reaction that uses acetyl-CoA as the acetyl donor (Wagner and Payne 2013; Konig et al. 2014). As a consequence, the stoichiometry of the vast majority of detected lysine-acetylation events is very low: 86% of detected events have a stoichiometry below 0.1% in the yeast proteome (Weinert et al. 2014). Neither plastids nor mitochondria of plant

cells are thought to contain an acetyltransfer-ase, but both organelles contain relatively high concentrations of acetyl-CoA compared to other compartments that might promote spontaneous protein acetylation (Konig et al. 2014). This indicates that the covalent attachment of acetyl groups to lysine may not be a controlled process in these organelles. However, *Arabidopsis* knockout lines lacking the mitochondrial lysine deacetylase SRT2 displayed modified mitochondrial adenylate transport and respiratory metabolism which correlated with differences in enzyme acetylation status (Konig et al. 2014). Therefore, the mitochondrial deacetylation process is important and controllable *via* SRT2 activity. What remains unclear is whether SRT2 functions simply as a general 'housekeeping' deacetylase that non-specifically reverses lysine-acetylation events to mitigate general protein damage, or whether SRT2 targets specific lysine-acetylation sites to precisely adjust metabolic fluxes. This question cannot currently be addressed in plants because, unlike in animals and yeast, there are no well-established examples of how acetylation controls plant enzyme function.

IV. Conclusions and Perspectives

After having described known examples of regulatory PTMs involved in the control of plant respiration, we can point to some emerging patterns, although we recognize that far more studies of the functional impact of enzyme PTMs are required.

Firstly, PTMs often alter the allosteric regulation of target enzymes by metabolite effectors. This was demonstrated for phos-phorylated forms of GAPN, PTPC subunits of Class-1 PEPC, BTPC subunits of Class-2 PEPC, mPDH, as well as the monoubiquiti-nation of PTPC and NAD-GAPDH, and thiol-reduction of AOX. Modifying the sen-sitivity to allosteric effectors exerts a large

impact on metabolic flux through tightly regulated enzyme such as PEPC and AOX (Gelhaye et al. 2004; Rademacher et al. 2002; Radchuk et al. 2007; Vanlerberghe 2013). This also demonstrates the impor-tance of thoroughly characterizing the influ-ence that PTMs exert on enzyme kinetic properties, beyond simple assays of maximal activity under optimal conditions.

Secondly, phosphorylation-dependent protein-protein interactions with 14-3-3 pro-teins, which are prominent throughout plant primary metabolism, including nitrate reduc-tase, sucrose-phosphate synthase, and gluta-mine synthase, also occur with several respiratory enzymes including GAPN, F2KP, and ATP synthase (Bunney et al. 2001; Bustos and Iglesias 2002, 2003; Kulma et al. 2004; Comparot et al. 2003). The coordina-tion of these 14-3-3 interactions seems likely to facilitate the integration of nitrogen and carbohydrate metabolism especially during light-to-dark transitions (Comparot et al. 2003). Changes in enzyme proteolytic sus-ceptibility is another potential regulatory effect of phosphorylation that has been sug-gested for multiple respiratory enzymes such as SuSy and cPK (Tang et al. 2003; Zhang et al. 1999; Asano et al. 2002; Fedosejevs et al. 2014, 2016). However, more direct experimental methods are needed to prove these connections.

There also appears, so far, to be a distinc-tion between the PTMs that control cyto-solic *versus* mitochondrial metabolisms. Mitochondrial enzymes seem more likely to be regulated by thiol redox status while cytosolic enzymes seem to be more likely regulated by phosphorylation. This pattern might reflect the need for rapid redox-depen-dent metabolic adjustment within mitochon-dria. Furthermore, the established mechanisms controlling reversible mPDH-phosphorylation depends upon mitochon-drial metabolic status rather than signal transduction from the cytosol. Altogether this suggests that the mitochondrion main-

tains some degree of autonomy when it comes to the control of respiratory enzymes by PTMs.

The molecular identification of protein kinases responsible for phosphorylation of SuSy, PTPC, and BTPC along with the finding that Tre-6-P activates Class-1 PEPC by triggering phosphorylation of its PTPC subunits indicates that we are finally starting to piece together a regulatory kinase network that integrates the control of respiratory metabolism with N-assimilation and sucrose supply. Further research must continue to bridge the gap between signal transduction pathways that link Tre-6-P dependent sucrose signaling and crucial target enzymes such as PEPC and nitrate reductase. It is likely that the energy status sensing kinase, SnrK1, which phosphorylates nitrate reductase, sucrose phosphate synthase, F2KP (Kulma et al. 2004), along with major transcription factors (Mair et al. 2015), is involved at some level in controlling respiratory enzyme phosphorylation under changing conditions (O'Hara et al. 2013; Figueroa et al. 2016).

In conclusion, high-throughput proteomic studies have indicated that the majority of plant glycolytic, TCA pathway, and mETC enzymes are subject to *in vivo* PTMs such as phosphorylation in a broad variety of plant tissues and species. For example, more than 50% of the proteins detected in a potato mitochondrial proteome were post-translationally modified on at least one site, with about 100 different proteins having at least ten different PTMs (Rao et al. 2017). However, the functional significance of the vast majority of these PTMs awaits detailed studies of the target enzymes and signaling pathways that control activities of their modifying enzymes. Given the central role that respiration plays in crop productivity, a detailed understanding of how PTMs influence key flux-controlling enzymes of glycolysis, the TCA pathway, and mETC is of significant practical interest. This fundamental knowledge is expected to facilitate the development of rational approaches for crop improvement *via* metabolic engineering, as well as to help predict plant responses to global climate change.

Acknowledgments

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