

# Posttranslational Protein Modifications in Plant Metabolism<sup>1</sup>

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Posttranslational modifications (PTMs) of proteins greatly expand proteome diversity, increase functionality, and allow for rapid responses, all at relatively low costs for the cell. PTMs play key roles in plants through their impact on signaling, gene expression, protein stability and interactions, and enzyme kinetics. Following a brief discussion of the experimental and bioinformatics challenges of PTM identification, localization, and quantification (occupancy), a concise overview is provided of the major PTMs and their (potential) functional consequences in plants, with emphasis on plant metabolism. Classic examples that illustrate the regulation of plant metabolic enzymes and pathways by PTMs and their cross talk are summarized. Recent large-scale proteomics studies mapped many PTMs to a wide range of metabolic functions. Unraveling of the PTM code, i.e. a predictive understanding of the (combinatorial) consequences of PTMs, is needed to convert this growing wealth of data into an understanding of plant metabolic regulation.

The primary amino acid sequence of proteins is defined by the translated mRNA, often followed by N- or C-terminal cleavages for preprocessing, maturation, and/or activation. Proteins can undergo further reversible or irreversible posttranslational modifications (PTMs) of specific amino acid residues. Proteins are directly responsible for the production of plant metabolites because they act as enzymes or as regulators of enzymes. Ultimately, most proteins in a plant cell can affect plant metabolism (e.g. through effects on plant gene expression, cell fate and development, structural support, transport, etc.). Many metabolic enzymes and their regulators undergo a variety of PTMs, possibly resulting in changes in oligomeric state, stabilization/degradation, and (de)activation (Huber and Hardin, 2004), and PTMs can facilitate the optimization of metabolic flux. However, the direct *in vivo* consequence of a PTM on a metabolic enzyme or pathway is frequently not very clear, in part because it requires measurements of input and output of the reactions, including flux through the enzyme or pathway. This Update will start out with a short overview on the major PTMs observed for each amino acid residue (Table I), followed by a brief discussion of techniques for the characterization of protein PTMs, including determination of the localization within proteins (i.e. the specific residues) and occupancy. Challenges in dealing with multiple PTMs per protein and cross talk between PTMs will be briefly outlined. We then describe the major physiological PTMs observed in plants as well as PTMs that are nonenzymatically induced during sample preparation (Table II). Finally, this Update is completed with a number of well-studied, classical examples of the

regulation of plant metabolism by PTMs, in particular for enzymes in primary metabolism (Calvin cycle, glycolysis, and respiration) and the C<sub>4</sub> shuttle accommodating photosynthesis in C<sub>4</sub> plants (Table III). As will be evident, most of these classic examples involve regulation by (de)phosphorylation, reversible formation of disulfide bonds involving thioredoxins, and changes in oligomeric state and protein-protein interactions.

There are many recent reviews focusing on specific PTMs in plant biology, many of which are cited in this Update. However, the last general review on plant PTMs is from 2010 (Ytterberg and Jensen, 2010); given the enormous progress in PTM research in plants over the last 5 years, a comprehensive overview is overdue. Finally, this Update does not review allosteric regulation by metabolites or other types of metabolic feedback and flux control, even if this is extremely important in the regulation of metabolism and (de)activation of enzymes. Recent reviews for specific pathways, such as isoprenoid metabolism (Kötting et al., 2010; Banerjee and Sharkey, 2014; Rodríguez-Concepción and Boronat, 2015), tetrapyrrole metabolism (Brzezowski et al., 2015), the Calvin-Benson cycle (Michelet et al., 2013), starch metabolism (Kötting et al., 2010; Geigenberger, 2011; Tetlow and Emes, 2014), and photorespiration (Hodges et al., 2013) provide more in-depth discussions of metabolic regulation through various posttranslational mechanisms. Many of the PTMs that have been discovered in the last decade through large-scale proteomics approaches have not yet been integrated in such pathway-specific reviews, because these data are not always easily accessible and because the biological significance of many PTMs is simply not yet understood. We hope that this Update will increase the general awareness of the existence of these PTM data sets, such that their biological significance can be tested and incorporated in metabolic pathways.

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**Table I.** Reactivity of amino acids and their PTMs observed in plants

Amino Acid Residue	Observed Physiological PTM in Plants	PTMs Caused by Sample Preparation
Ala (A)	Not known	
Arg (R)	Methylation, carbonylation	
Asn (N)	Deamidation, N-linked glycosylation	Deamidation
Asp (D)	Phosphorylation (in two-component system)	
Cys (C)	Glutathionylation (SSG), disulfide bonded (S-S), sulfenylation (-SOH), sulfonylation (-SO <sub>3</sub> H), acylation, lipidation, acetylation, nitrosylation (SNO), methylation, palmitoylation, phosphorylation (rare)	Propionamide
Glu (E)	Carboxylation, methylation	Pyro-Glu
Gln (Q)	Deamidation	Deamidation, pyro-Glu
Gly (G)	N-Myristoylation (N-terminal Gly residue)	
His (H)	Phosphorylation (infrequent)	Oxidation
Ile (I)	Not known	
Leu (L)	Not known	
Lys (K)	N-ε-Acetylation, methylation, hydroxylation, ubiquitination, sumoylation, deamination, O-glycosylation, carbamylation, carbonylation, formylation	
Met (M)	(De)formylation, excision (NME), (reversible) oxidation, sulfonation (-SO <sub>2</sub> ), sulfoxation (-SO)	Oxidation, 2-oxidation, formylation, carbamylation
Phe (F)	Not known	
Pro (P)	Carbonylation	Oxidation
Ser (S)	Phosphorylation, O-linked glycosylation, O-linked GlcNAc (O-GlcNAc)	Formylation
Thr (T)	Phosphorylation, O-linked glycosylation, O-linked GlcNAc (O-GlcNAc), carbonylation	Formylation
Trp (W)	Glycosylation (C-mannosylation)	Oxidation
Tyr (Y)	Phosphorylation, nitration	
Val (V)	Not known	
Free NH <sub>2</sub> of protein N termini	Preprotein processing, Met excision, formylation, pyro-Glu, N-myristoylation, N-acylation (i.e. palmitoylation), N-terminal α-amine acetylation, ubiquitination	Formylation (Met), pyro-Glu (Gln)

## REACTIVITY OF AMINO ACIDS IN PLANTS

PTMs can occur spontaneously (nonenzymatically) due to the physical-chemical properties of reactive amino acids and the cellular environment (e.g. pH, oxygen, reactive oxygen species [ROS], and metabolites) or through the action of specific modifying enzymes (Ryšlavá et al., 2013). PTMs are also determined by neighboring residues and their exposure to the surface. The 20 amino acids differ strongly in their general chemical reactivity and their observed PTMs (Table I). In particular, Cys and Lys can each carry many types of PTMs, whereas the N-terminal residue of proteins is also prone to multiple PTMs, ranging from N-terminal cleavage to N-terminal lipid modifications (acylation), acetylation, and ubiquitination (Table I). In addition to these PTMs that occur in vivo and presumably have physiological significance (but see discussion below), several PTMs are often generated during sample preparation due to exposure to organic solvents (e.g. formic acid leading to the formylation of Ser, Thr, and N termini), (thio)urea (N-terminal or Lys carbamylation), reducing agents and oxygen, unpolymerized acrylamide (Cys propionamide), and low or high pH (cyclization of N-terminal Gln or Glu into pyro-Glu; Table I). A large-scale proteomics study of *Arabidopsis thaliana* leaf extracts

did address the frequency of PTMs that do not require specific affinity enrichment based on a data set of 1.5 million tandem mass spectrometry (MS/MS) spectra acquired at 100,000 resolution on an LTQ-Orbitrap instrument followed by error-tolerant searches and systematic validation by liquid chromatography retention time (Zybailov et al., 2009). This revealed, for example, that modification of Met and N-terminal Gln into oxidized Met and pyro-Glu, respectively, showed by far the highest modification frequencies, followed by N-terminal formylation, most likely induced during sample analysis, as well as deamidation of Asn/Gln (approximately 1.2% of all observed Asn/Gln). Several of these nonenzymatic PTMs (in particular deamidation, oxidation, and formylation) can also occur in vivo and, therefore, cannot be simply dismissed as artifacts but need to be considered as potential regulators.

## IDENTIFICATION, MAPPING, AND QUANTIFICATION OF PTMS

PTMs are typically identified by mass spectrometry (MS; Young et al., 2010; Olsen and Mann, 2013; Chalkley et al., 2014) or, alternatively, by immunoblotting using an antiserum that recognizes the specific PTM (Černý

**Table II.** Most significant and/or frequent PTMs observed in plants

Type of PTM (Reversible, Except if Marked with an Asterisk)	Spontaneous (S; Nonenzymatic) or Enzymatic (E)	Comment on Subcellular Location and Frequency
Phosphorylation (Ser, Thr, Tyr, His, Asp)	E	His and Asp phosphorylation have low frequency
S-Nitrosylation (Cys) and nitration* (Tyr)	S (RNS), but reversal is enzymatic for Cys by thioredoxins	Throughout the cell
Acetylation (N-terminal $\alpha$ -amine, Lys $\epsilon$ -amine)	E	In mitochondria, very little N-terminal acetylation, but high Lys acetylation; Lys acetylation correlates to [acetyl-CoA]
Deamidation (Gln, Asn)	S, but reversal of isoAsp is enzymatic by isoAsp methyltransferase	Throughout the cell
Lipidation (S-acetylation, N-meristoylation*, prenylation*; Cys, Gly, Lys, Trp, N terminal)	E	Not (or rarely) within plastids, mitochondria, peroxisomes
N-Linked glycosylation (Asp); O linked (Lys, Ser, Thr, Trp)	E	Only proteins passing through the secretory system; O linked in the cell wall
Ubiquitination (Lys, N terminal)	E	Not within plastids, mitochondria, peroxisomes
Sumoylation (Lys)	E	Not within plastids, mitochondria, peroxisomes
Carbonylation* (Pro, Lys, Arg, Thr)	S (ROS)	High levels in mitochondria and chloroplast
Methylation (Arg, Lys, N terminal)	E	Histones (nucleus) and chloroplasts; still underexplored
Glutathionylation (Cys)	E	High levels in chloroplasts
Oxidation (Met, Cys)	S (ROS) and E (by PCOs; see Fig. 1B), but reversal is enzymatic by Met sulfoxide reductases, glutaredoxins, and thioredoxins, except if double oxidized	High levels in mitochondria and chloroplast
Peptidase* (cleavage peptidyl bond)	E	Throughout the cell
S-Guanylation (Cys)	S (RNS)	Rare; 8-nitro-cGMP is signaling molecule in guard cells
Formylation (Met)	S, but deformylation is enzymatic by peptide deformylase	All chloroplasts and mitochondria-encoded proteins are synthesized with initiating formylated Met

et al., 2013). MS is the most versatile and precise technique, since it can identify multiple different PTMs in the same sample and, importantly, determine which residue(s) carries the PTM. The latter is important for understanding how the PTM may affect protein function; however, such localization can be challenging, since it requires a high-quality MS/MS sequence and diagnostic fragment ions around the site of PTMs. MS-based PTM studies often involve enrichment for specific PTMs using an affinity matrix, followed by an optimized liquid chromatography-MS/MS acquisition. Whereas collision-induced dissociation is the most common peptide fragmentation technique used for protein identifications, electron-transfer dissociation and electron-capture dissociation are valuable complementary techniques to identify PTMs and/or improve the localization of PTMs (e.g. phosphorylation [Jünger and Aebersold, 2014] and ADP ribosylation [Haag and Buck, 2015]). Major improvements of modern mass spectrometers, in combination with novel enrichment strategies, have allowed for large-scale identification studies for numerous PTMs. Quite often, following a technological breakthrough, multiple independent studies for a specific PTM are published within a short time span, thus temporarily piquing the interest in this PTM. Recent examples for plants are Lys acetylation (Finkemeier et al., 2011; Wu et al., 2011; König et al.,

2014a), Cys acylation (Hemsley et al., 2013a, 2013b), Cys nitrosylation, and sumoylation (Elrouby and Coupland, 2010; Miller et al., 2010). MS is inherently nonquantitative due to variable peptide ionization efficiencies; therefore, the quantification of occupancy can be challenging, in particular if the PTM results in a change in ionization and/or fragmentation efficiency (both are required in MS-based analysis) and/or a change in hydrophobicity (Cox and Mann, 2011; Altelaar et al., 2013; Olsen and Mann, 2013).

#### FALSE DISCOVERY OF PTMS

As with all measurements, but in particular large-scale omics-type studies, false discoveries are made. The false-positive rate of PTM assignment can be broken down in three questions of confidence, and ideally, for a given modified spectrum, a *P* value should be determined for each confidence question. (1) Assignment of the amino acid sequence, typically solved using a target-decoy database approach (Nesvizhskii, 2010; Muth et al., 2015; Savitski et al., 2015). (2) Assignment of a particular PTM to a peptide precursor. Using high-resolution instruments to determine the precursor ion mass, assignment of the PTM to the peptide is generally reliable, in

particular when the mass shift due to the PTM is greater than 10 ppm (Zybailov et al., 2009). For example, the difference between monoisotopic peaks of peptides with deamidation of Asn/Gln (+0.984-D shift) and the  $^{13}\text{C}$  peak (+1.003-D shift) of the corresponding non-modified peptides is 0.019 D, which for a precursor mass of 1,500 D corresponds to 12 ppm. Similarly, the difference between Lys trimethylation (delta mass = 42.046950) and acetylation (delta mass = 42.010565) for a precursor mass of 1,500 D corresponds to a 24-ppm difference. The expected change in hydrophobicity due to simple PTMs generally correlates with the change in the reverse-phase liquid chromatography elution time; therefore, the incorporation of hydrophobicity prediction tools can improve PTM analysis dramatically (Zybailov et al., 2009). (3) Assignment of the position of the PTM within the amino acid sequence, search programs, and search options and issues are discussed by Chalkley and Clauser (2012).

#### PROTEINS WITH MULTIPLE PTMS, COMBINATORIAL PTMS, AND CROSS TALK BETWEEN PTMS

Individual proteins often carry multiple PTMs, which pose specific experimental and bioinformatics challenges, as illustrated nicely in a community-wide study (Chalkley et al., 2014). Since many PTMs are reversible, specific residues can also alternate between PTMs (e.g. dependent on cellular conditions, protein configuration [folding], or protein-protein interactions), and one PTM can influence the generation of other PTMs. This can result in an explosion of possible proteoforms and in cross talk between PTMs occurring on the same protein. Cross talk between PTMs on the same protein can coordinately determine the activity, function, and/or interactions of a protein. Finally, cross talk also exists between PTMs located on interacting proteins. Time-resolved and quantitative determination of combinatorial PTMs is challenging, and understanding of the biological outcomes is only in its infancy. For recent reviews on combinatorial PTMs and cross talk, see Nussinov et al. (2012) and Lothrop et al. (2013). Prominent examples of PTM cross talk are Lys ubiquitination and acetylation or Lys ubiquitination and phosphorylation (Shane et al., 2013). Phosphorylation can also directly promote substrate proteolysis by caspase (peptidase) during apoptosis (Dix et al., 2012). Recent biochemical and proteomics studies suggested that most if not all enzymes of the Calvin-Benson cycle undergo redox regulation through selective redox PTMs, including reversible disulfide bond formation, glutathionylation, and nitrosylation, with an interplay between these PTMs dependent on (sub)cellular conditions (Michelet et al., 2013). Moreover, the regulators carrying out these PTMs (e.g. thioredoxins, glutaredoxins, etc.) themselves can also undergo some of these PTMs, making for a complex network of PTMs (Michelet et al., 2013).

The identification, localization, and quantification of different combinations of PTMs on the same protein can

sometimes be better solved by so-called top-down or middle-down proteomics, as opposed to the more common bottom-up proteomics (Lanucara and Evers, 2013; Catherman et al., 2014). Bottom-up proteomics involves the cleavage of proteins into smaller fragments (by proteolysis or chemical cleavage) prior to MS analysis. In contrast, in top-down proteomics, proteins are not digested into smaller fragments but rather injected and analyzed by a specialized mass spectrometer in its entirety. In middle-down proteomics, the intact proteins are cleaved into just a few fragments by limited proteolysis prior to injection into the mass spectrometer. Top-down and middle-down proteomics are not high throughput and are best carried out on either purified proteins or protein mixtures of low complexity. Classic examples of studies using top-down, middle-down, but also bottom-up proteomics on proteins with different PTMs involve histones (Karch et al., 2013) and the p53 tumor suppression protein (DeHart et al., 2014).

#### PROTEIN PREPROCESSING, MATURATION, AND DIFFERENT PROTEOFORMS

Most proteins undergo different forms of N- or C-terminal processing by peptidases prior to their full activation. The majority of proteins undergo (cotranslational) N-terminal methionine excision (NME) by the action of methionine aminopeptidases in cytosol, plastids, and mitochondria. NME occurs if the Met is followed by a small residue (Gly, Ala, Thr, Pro, Ser, Val, and to a lesser degree Cys), which is referred to as the NME rule (Bonissone et al., 2013; Giglione et al., 2015). The NME rule can be explained in terms of steric hindrance of the methionine aminopeptidases action by the size of the side chain of the residue in the penultimate position. The initiating N-terminal Met in plastid- and mitochondria-encoded proteins is incorporated in formylated form through the action of a specialized tRNA-formylated Met. NME of these proteins only occurs after deformylation of Met by a peptide deformylase (Giglione et al., 2015). Methionine aminopeptidases and peptide deformylases are monomeric metalloproteases.

Many plant proteins are synthesized as precursor proteins and have cleavable N- or C-terminal signal peptides for sorting or secretion to the relevant subcellular compartment (Jarvis and López-Juez, 2013; Krause et al., 2013; Murcha et al., 2015). For instance, chloroplast/plastid and mitochondrial proteins have N-terminal signal peptides, whereas proteins sorting into the endoplasmic reticulum (ER) have a relatively short and cleavable N-terminal signal peptide. A subset of peroxisomal proteins have cleavable N-terminal signal peptides (assigned Peroxisomal Targeting Signal2 [PTS2]; Hu et al., 2012). These organellar sorted proteins can have additional, frequently cleavable, downstream sequence information for additional sorting steps (e.g. into the thylakoid lumen of chloroplast or to the plasma membrane and apoplast). The cleavages of these signal peptides and downstream signals are carried out by

dedicated and specialized processing peptidases (van Wijk, 2015).

In addition to the removal of sorting information, a subset of proteins undergo further N- or C-terminal trimming events (for some peptidases autocatalytic) by specific peptidase activity to fully activate and/or stabilize the proteins or release the protein from a membrane for full activity (Johnson et al., 2008; Watanabe and Lam, 2011; Cavazzini et al., 2013). In some cases, the newly formed termini then undergo specific PTMs, such as N-terminal acetylation, phosphorylation, or acylation.

### **ACTION MECHANISMS AND CONSEQUENCES OF PTMS**

Enzymes can act alone as monomers or as homo-oligomers, or they interact with different proteins (either catalytic subunits or regulatory proteins) to form stable or transient heteromeric complexes. It is hard to generalize where PTMs locate in proteins, but many PTMs are surface exposed, in particular those that are reversible through enzymatic activity. PTMs can be found on residues that are evolutionarily conserved, whereas others are enriched on nonconserved residues; for discussion, see Christian et al. (2012) and Ryšlavá et al. (2013). Conceptually, direct control of metabolic enzymes and their regulators by PTMs can occur through five mechanisms. (1) Protein processing and activation. This was discussed in the previous section. (2) Protein localization. Several PTMs can directly affect subcellular localization. This is the case in particular for proteins that move between the nucleus and cytosol through the action of ubiquitination, sumoylation, and acetylation, but also through lipid modifications that anchor proteins to membranes, or through glycosylation, in particular for proteins passing through the ER-Golgi network. (3) Protein stability. Several PTMs contribute to protein stability or mark proteins for degradation. Examples include Lys polyubiquitination marking for proteasomal degradation. (4) Protein-protein interactions. Many PTMs influence protein-protein interactions through changed protein charge or hydrophobicity (e.g. phosphorylation) or through change in protein conformation (e.g. oxidation/reduction of Cys affecting disulfide bonds). (5) Enzyme activity and kinetics. PTMs can directly influence enzyme activity through allosteric regulation or by affecting access to the active site.

### **PROTEOLYTIC GENERATION OF SMALL BIOACTIVE PEPTIDES AND THEIR PTMS IN PLANTS**

Plants produce hundreds of biologically active small peptides and peptide hormones (typically less than 20 amino acids) through posttranslational cleavage of proteins by a variety of endopeptidases and exopeptidases (Czyzewicz et al., 2013; Krause et al., 2013; Matsubayashi, 2014). These peptides can act systemically through long-distance transport or from cell to cell or by secretion into

the apoplast, where they interact with receptors and transcription factors, thus fulfilling roles in plant cell differentiation and development, growth, and defense, indirectly influencing plant metabolism. Three different PTMs have been observed for a subset of these peptides, namely Tyr sulfation (Tyr-SO<sub>3</sub>H), Pro hydroxylation (Hyp), and Hyp arabinosylation, a type of glycosylation. They are generated through the action of transferases, hydroxylases, and glycosyltransferases located in the ER or Golgi (Matsubayashi, 2014). A well-studied example of such bioactive peptides is in the control of stomatal density and its response to environmental CO<sub>2</sub> concentrations, directly impacting leaf photosynthesis through the availability of CO<sub>2</sub> (Schlüter et al., 2003). In summary, peptides EPIDERMAL PATTERNING FACTOR1 (EPF1), EPF2, and STOMAGEN (EPF-LIKE9) are secreted into the apoplast by the activity of apoplastic/cell wall subtilisin peptidases CO<sub>2</sub> RESPONSE SECRETED PROTEASE and STOMATAL DENSITY AND DISTRIBUTION1. These peptides then interact with membrane receptors, thus activating signaling pathways (Richardson and Torii, 2013; Engineer et al., 2014; Lee et al., 2015).

### **ARE ALL IN VIVO PTMS BIOLOGICALLY SIGNIFICANT?**

Small- and large-scale proteomics studies have identified many PTMs, yet typically they do not provide evidence that all these PTMs have biological function and significance. For instance, in the case of phosphorylation, it has been argued that perhaps many of these PTMs have no biological function but are simply phosphorylated due to the lack of kinase specificity and/or weak consensus sequence for the phospho site. A community-wide effort to collect and assemble all PTM data and map these systematically to the predicted proteome for individual plant species would be a good starting point to systematically determine the functional implications for plant metabolism and other aspects of plant biology.

### **PTMS IN PLANTS AND THEIR INFLUENCE ON PLANT METABOLISM**

In the following sections, a comprehensive but concise overview of the major PTMs observed in plants is provided. Where possible, we will emphasize specific direct impacts on plant metabolism and cite original (experimental) studies.

#### **Phosphorylation**

Phosphorylation is one of the most widespread reversible PTMs and plays a major role in plant signal transduction and metabolism by altering protein activities, protein interactions, or subcellular location (Mithoe and Menke, 2011; Schönberg and Baginsky, 2012; van Wijk et al., 2014; Silva-Sanchez et al., 2015). Phosphorylation is catalyzed by kinases, which transfer a phosphoryl group typically from ATP, but also from ADP (e.g.

chloroplast pyruvate orthophosphate dikinase during the night), to the hydroxyl group of specific Ser, Thr, or Tyr residues within their target proteins (on average, 75%–80%, 15%–20%, and 1%–5% for Ser, Thr, and Tyr, respectively; Champion et al., 2004). However, also His (e.g. for histones; Bigeard et al., 2014) and both His and Asp in plant two-component signaling (Heyl et al., 2013; Osakabe et al., 2013) can be phosphorylated (Cieřla et al., 2011). Phosphatases are responsible for removing phosphorylated residues from the modified proteins. Plant genomes encode about twice as many kinases compared with mammalian genomes (Zulawski et al., 2013); 1,052 protein kinases and 162 phosphatases are encoded by the Arabidopsis genome (Wang et al., 2014), indicative of the important role of protein phosphorylation in plants.

Several dozen large-scale phosphorylation studies have been published for Arabidopsis, *Medicago* spp., maize (*Zea mays*), rice (*Oryza sativa*), and other plant species; most of these data can be mined using various plant phosphorylation databases (Silva-Sanchez et al., 2015). A recent review summarized the phosphorylation of enzymes in the photorespiratory pathway located in chloroplasts, peroxisomes, and mitochondria and discussed each of these enzymes in fair detail (Hodges et al., 2013; Table III). A recent meta-analysis of available phosphoproteome data for Arabidopsis covering a range of processes, (non)photosynthetic tissue types, and cell cultures resulted in 60,366 phosphopeptides matching 8,141 nonredundant proteins and provides insight into the challenges/pitfalls of using large-scale phosphoproteomic data sets to nonexperts (van Wijk et al., 2014). The distribution of phosphorylated proteins across cellular functions and subcellular compartments was determined, and subcellular compartmentalization of phosphorylation motifs, likely reflecting localized kinase activity, was observed. Careful verification of phospho-site assignments by systematic evaluation of original spectral will be needed, in particular to evaluate the large differences in Tyr phosphorylation between studies (see also Lu et al., 2015). In addition to these large-scale studies, there are hundreds of articles studying the role of plant protein phosphorylation for specific proteins and pathways, including those involved in plant primary (e.g. light and dark reactions of photosynthesis and nitrogen metabolism) and secondary metabolism. We will provide a number of examples in the last section of this Update.

### N-Terminal Acetylation

N-terminal  $\alpha$ -amine acetylation typically occurs following cotranslational NME in cytosol. In the case of chloroplasts, it occurs either posttranslationally after removal of the plastid sorting sequence or cotranslationally in the case of plastid-encoded proteins (Rowland et al., 2015). In contrast, there is little evidence for enzymatic N-terminal acetylation within plant mitochondria (Huang et al., 2009). N-terminal acetylation results in the loss of a positive charge and is carried out by N-terminal acetyltransferases (the NAT family). It appears

that these NATs can also acetylate the  $\epsilon$ -amine of Lys (Starheim et al., 2012). Similar to Lys acetylation (next section), N-terminal acetylation is positively correlated to levels of acetyl-CoA, the source of the acetyl moiety for NATs, thereby linking cellular metabolic state to this PTM. The majority of proteins in plants and other eukaryotes are N-acetylated, and a NAT-A loss-of-function Arabidopsis mutant showed pleiotropic developmental and growth defects in Arabidopsis (Ferrández-Ayela et al., 2013), whereas loss of function of NAT-C showed reduced plant growth and photosynthetic capacity (Pesaresi et al., 2003). A chloroplast NAT enzyme (AtNAA70) was recently identified in Arabidopsis and showed N- $\alpha$ -acetylation when expressed in *Escherichia coli*, in particular for Met, Ala, Thr, and Ser (Dinh et al., 2015). Comparing this specificity with in vivo Arabidopsis chloroplast N-terminome data (Zybailov et al., 2008) suggests that additional NATs operate in chloroplasts (Dinh et al., 2015). Because the majority of proteins undergo N-terminal acetylation, it is unclear to what extent this PTM is really regulated (Starheim et al., 2012). Yet, for specific proteins, this PTM has been shown to impact protein sorting and location, protein-protein interactions, as well as protein stability (Starheim et al., 2012). A recent and fascinating study of the N-terminal before amino transferases (NAT)-A and NAT-B loss-of-function mutants in Arabidopsis showed that the stability of a nod-like receptor (Suppressor of NPR1, Constitutive1 [SNC1]) is controlled by alternative translation initiation and N-terminal acetylation: NatA-mediated acetylation of one isoform served as a degradation signal, while NatB-mediated acetylation of the alternative isoform stabilizes the SNC1 protein, thus revealing antagonistic N-terminal acetylation of a single protein substrate (Xu et al., 2015). Surprisingly, transgenic or abscisic acid-induced NatA down-regulation induced adaptation to drought stress (Linster et al., 2015). We anticipate that N-acetylation will impact many other plant proteins and plant metabolism in various ways.

### Lys Acetylation

Lys  $\epsilon$ -amine acetylation has been studied in detail as a prominent histone PTM in plants and nonplant species; until recently, Lys acetylation was mostly considered an epigenetic modification (Berr et al., 2011). However, improvements in MS-based proteomics identified hundreds of nonhistone targets in plants (Finkemeier et al., 2011; Wu et al., 2011; Nallamilli et al., 2014) and other eukaryotes (Mischerikow and Heck, 2011; Weinert et al., 2014) as well as in prokaryotes (van Noort et al., 2012; Choudhary et al., 2014). Nonhistone acetylation appears widespread in chloroplasts and mitochondria. In chloroplasts, several Calvin cycle enzymes, including Rubisco, and a few chloroplast membrane proteins were found acetylated (Finkemeier et al., 2011; for more on Rubisco, see below). In mitochondria, Lys acetylation occurs on tricarboxylic acid cycle proteins as well as on matrix-exposed proteins of

the mitochondrial electron transport chain (Konig et al., 2014a; Papanicolaou et al., 2014). Lys acetylation is a reversible PTM carried out enzymatically by Lys acetyltransferases and deacetylases, including sirtuins. Recently, an Arabidopsis mitochondrial sirtuin (SRT2) was shown to be a Lys acetylase in vitro and in vivo, and the SRT2 loss-of-function mutant showed a metabolic phenotype with altered levels of sugars, amino acids, and ADP and reduced efficiency of oxidative phosphorylation (Konig et al., 2014b). Furthermore, the cellular concentration of acetyl-CoA positively correlates with protein Lys acetylation, likely because acetyl-CoA is the substrate for the Lys acetyltransferases but perhaps also because nonenzymatic Lys acetylation does occur at the basic pH value, such as found in the mitochondrial matrix and chloroplast stroma. Lys acetylation in Arabidopsis mitochondria seems to occur preferentially immediately downstream of Glu and to a lesser extent Phe or Leu (Konig et al., 2014b).

### Methylation of Lys and Arg

One of the first discovered Lys methylated proteins was Rubisco (Houtz et al., 1989). However, similar to acetylation, Lys methylation is particularly recognized and studied as a histone modification in nonphotosynthetic eukaryotes and plants (Liu et al., 2010; Biggar and Li, 2015). Recent studies with nonplant material (e.g. mammalian cells and yeast [*Saccharomyces cerevisiae*]) using various novel enrichment techniques followed by MS/MS showed that high numbers of Arg (monomethylation and demethylation) and Lys (monodimethylation and trimethylation) are methylated (for an excellent overview on innovative methodologies, see Carlson and Gozani [2014]). These methylations are carried out by Arg or Lys methyltransferases (PRMTs and PKMTs, respectively) with S-adenosylmethionine (AdoMet) as methyl donor, thereby releasing S-adenosyl-homo-Cys (Biggar and Li, 2015) and coupling the cellular metabolic state to this PTM. Lys methylation can be reversed by the action of multiple Lys demethylases, but Arg demethylases have yet to be identified (Biggar and Li, 2015). Ribosomal proteins are enriched among methylated proteins, and methylation appears important for ribosome biogenesis (ribosomal RNA maturation) and perhaps function, as also demonstrated by an Arabidopsis mutant for a cytosolic Arg methyltransferase (PRMT3; Hang et al., 2014).

The first systematic MS-based Lys and Arg methylation study in plants (Arabidopsis) focused on chloroplasts; however, no affinity enrichment was used, but rather existing global protein profiling data were reinvestigated for possible methylated peptides (Alban et al., 2014). This resulted in the identification of 24 Lys and seven Arg methylation sites from 23 stromal and membrane chloroplast proteins, enriched for functions in photosynthesis (Calvin cycle and light reaction) and biogenesis. Several of these methylated proteins were validated by methylation isotope-labeling experiments. A follow-up study showed that Arabidopsis methyltransferase (PrmA) was dual

targeted to chloroplasts and mitochondria, and is responsible for methylation ribosomal protein L11 in both organelles (Mazzoleni et al., 2015). Other plastid methyltransferases and (putative) substrates have been identified, namely plastid transcriptionally active chromosome complex14 (PTAC14) associated with plastid nucleoids (Gao et al., 2012) and Lys methyltransferase-like targeting Fru-1,6-bisP aldolase (Mininno et al., 2012). Loss-of-function PTAC14 mutants have an albino phenotype due to blocked chloroplast development, probably related to the loss of plastid gene expression rather than metabolism (Gao et al., 2012). Trimethylated Lys was observed for an Arabidopsis mitochondrial elongation factor and NAD-malate dehydrogenase (Zybailov et al., 2009). Large-scale methyl-proteomics studies in plants using the novel affinity or other enrichment techniques are overdue and would provide a strong basis for studying the regulatory role of Arg and Lys methylation in plant metabolism, including possible coupling to the AdoMet subcellular concentrations.

### Carbonylation

Protein carbonylation is an irreversible PTM associated with protein inactivation due to oxidative stress and aging in prokaryotes and eukaryotes alike; carbonyl levels often increase gradually with age (Nyström, 2005; Lounifi et al., 2013). Carbonylation typically results from metal-catalyzed ROS-induced oxidation of the side chains from Lys, Pro, Arg, and Thr residues but can also be indirectly ROS induced through lipid peroxides (for Cys, His, and Lys) and even by glycation/glycoxidation (for Lys). A wide range of carbonylation proteins in plants and nonplants have been detected, and quantification techniques have been developed in recent years and reviewed extensively (Fedorova et al., 2014; Rogowska-Wrzesinska et al., 2014). Carbonylated proteins are considered marked for proteolysis, but in particular when proteins are heavily carbonylated, they often accumulate as cytotoxic aggregates due to their increased hydrophobicity and are associated with age-related disorders in humans (Nyström, 2005). Surprisingly, it was suggested that the pattern of (chloroplast) protein carbonylation in Arabidopsis (and presumably other plants) is distinct from that in nonphotosynthetic eukaryotes, in that carbonylation first increases with age (similar to other species) but drops abruptly prior to the vegetative-to-reproductive transition and does not coincide with senescence (Johansson et al., 2004). This suggests that old leaves rid themselves of oxidized proteins prior to bolting. We speculate that this is due to the targeted removal of organellar content through a combination of chlorophagy, Rubisco-containing bodies, and senescence-associated vacuoles (Wang and Blumwald, 2014; van Wijk, 2015; Xie et al., 2015). There are multiple protein carbonylation studies with various plant species focusing on chloroplasts and mitochondria in leaves as well as seed biology and fruit ripening; we refer for details and citations to an excellent review,

including a list with carbonylated (and S-nitrosylated) Arabidopsis proteins (Lounifi et al., 2013). Carbonylated proteins have been detected especially in cytosol, mitochondria (Smakowska et al., 2014), and chloroplasts and are involved in various metabolic pathways, protein folding, and other processes. However, within these pathways, carbonylation is not evenly distributed, and specific proteins appear preferentially targeted for this PTM. It was also proposed that there is cross talk between ROS-induced carbonylation and reactive nitrogen species (RNS)-induced protein nitrosylation (see below), particularly under biotic and abiotic stress (Lounifi et al., 2013). The impact of carbonylation on metabolic functions in relation to plant life cycle, mechanistic aspects of selective carbonylation, and the reduction of carbonylation load by proteolysis (proteasome and organellar proteolytic systems) should be addressed in future studies using state-of-the-art quantitative technologies.

### Deamidation

Spontaneous deamination of Asn to Asp and isoAsp, or of Gln to Glu observed *in vivo*, is a relatively slow process (time scale of days to years), and has been correlated to protein stability, aging, and degradation (Wright, 1991a, 1991b). The process is irreversible; however, (abnormal) isoAsp can be converted into Asp *in vivo* through the activity of isoAsp methyltransferase (PIMT) through an AdoMet-dependent reaction. This reversion is of physiological importance for longevity and stress repair in plants (Mudgett et al., 1997; Ogé et al., 2008), bacteria, and animals (Reissner and Aswad, 2003). Deamidation can occur rapidly *in vitro*, in particular when exposed to basic pH and at increased temperatures. Therefore, it frequently occurs during sample preparation for MS/MS analysis (e.g. during tryptic digests, typically approximately 12 h at 37°C at pH 8.2) and thereby complicates the assessment of physiological deamidation. The rate of deamidation *in vivo* and *in vitro* is also greatly dependent on sequence context (i.e. deamidation is faster if Asp residues are followed by a small amino acid residue, in particular Gly; Krokhin et al., 2006), but deamidation is also influenced by folding and proximal residues (Wright, 1991a, 1991b).

Two independent studies in Arabidopsis identified isoAsp repair by PIMT1 as critical for seed quality (Ogé et al., 2008; Nayak et al., 2013). PIMT overexpression lines showed reduced levels of proteins with isoAsp residues, whereas reduced PIMT1 lines showed increased levels. Consistently, PIMT1 levels corresponded with prolonging seed lifetime and germination vigor (Ogé et al., 2008). Nayak et al. (2013) focused specifically on the function of a DEAD box RNA helicase (plant RNA helicase75 [PRH75]) that is important in translation upon imbibition in seeds. PRH75 becomes inactivated upon the formation of isoAsp, and the study showed that PIMT1 is essential to convert isoAsp to Asp, thereby restoring PRH75 function. Finally, a study of aquaporins (plasma membrane intrinsic proteins [PIPs]) in Arabidopsis roots showed

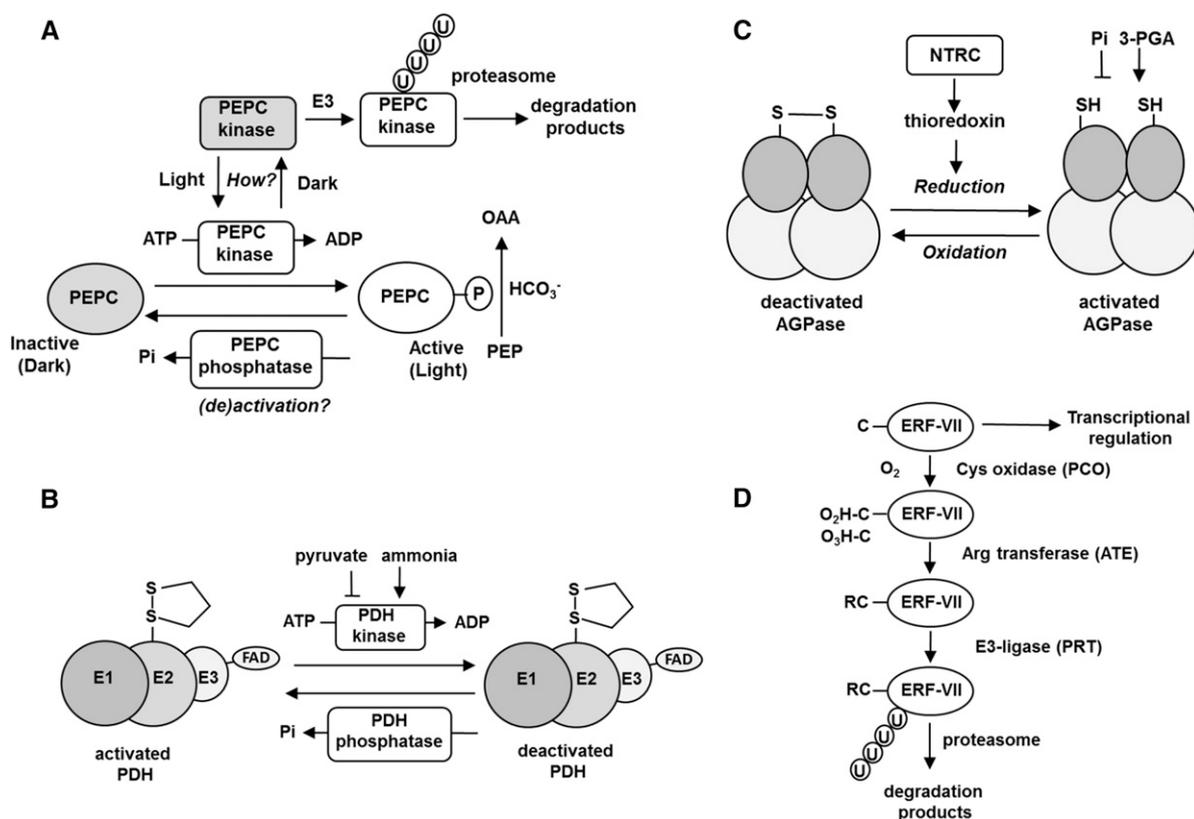
increased deamidation (by MS/MS analysis and spectral counting) in response to (a)biotic stress conditions. Moreover deamidation levels of PIP2 appeared coupled to its phosphorylation status, suggesting cross talk between these PTMs (di Pietro et al., 2013). It was suggested that deamidation could be involved in regulating aquaporin activity. Despite the challenges to determine physiologically induced protein deamidation, the impact of this PTM on plant metabolism is worth exploring in more detail.

### Sulhydryl Oxidations of Met and Cys Induced by ROS

The wide range of oxidation states of sulfur make Cys and Met residues the major sites of oxidation within proteins. However, the rate of oxidation of these residues is inversely correlated to their pKa (which is determined by neighboring residues), accessibility, and the local pH (Akter et al., 2015; Waszczak et al., 2015). Cys oxidation and its important role in redox homeostasis in plants have been studied extensively through mechanistic studies (e.g. within the Calvin cycle and glycolysis in the chloroplast; Balsera et al., 2014; Dietz and Hell, 2015) as well as through a variety of proteomics approaches, as summarized in excellent reviews (Akter et al., 2015; Waszczak et al., 2015). The initial oxidation of Cys by hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) typically results in sulfenic acid, which can then form a mixed disulfide bond with glutathione (GSH; glutathionylation; see next section) or a disulfide bond with other thiols. Further oxidation of sulfenic acid leads to sulfinic acid and irreversible sulfonic acid. In most cases, also sulfinic acid is irreversible; however, sulfinic acid could be rereduced by a sulfiredoxin enzyme in the case of specific mitochondrial and chloroplast peroxiredoxins (Rey et al., 2007). In addition to the nonenzymatic oxidation of Cys, a small set of plant Cys oxidases (PCOs) is capable of enzymatically oxidizing N-terminal Cys in the case of several Ethylene Response Factor (ERF)-VII transcription factors, generating a sulfenic acid PTM (Weits et al., 2014). The PTM turns these transcription factors into a substrate for arginyltransferases, ultimately leading to polyubiquitination and proteasomal degradation (for details, see final section and Fig. 1; for review, see van Dongen and Licausi, 2015). In contrast to Cys oxidation, relatively little is known about the extent of Met oxidation and its impact on plants. However, an elegant study in Arabidopsis identified and quantified hundreds of Met oxidation sites induced by H<sub>2</sub>O<sub>2</sub> generated *in situ* in the peroxisomes through enhanced photorespiration (by shifting from high to low [CO<sub>2</sub>]) and in a peroxisomal catalase (which detoxifies H<sub>2</sub>O<sub>2</sub>) mutant (Jacques et al., 2015). Furthermore, oxidation of Met can be reduced by a family of Met sulfoxide reductases (Rouhier et al., 2006; Tarrago et al., 2009), thus repairing these oxidized (damaged) proteins.

### Sulfur Glutathionylation

GSH is a tripeptide (Glu-Cys-Gly) and is present at millimolar concentrations in plant cells, thereby acting as



**Figure 1.** Examples of PTMs regulating metabolic enzymes and pathways. **A**, Regulation of cytosolic phosphoenolpyruvate carboxylase (PEPC) in C4 plants such as maize. The regulation of PEPC kinases and phosphatase is still unknown, but it may involve additional PTMs. The cellular concentration of PEPC kinase is kept very low through proteasomal degradation. PEP, Phosphoenolpyruvate. **B**, Regulation of mitochondrial pyruvate dehydrogenase (PDH) by (de)phosphorylation through a PDH kinase and phosphatase. The PDH kinase is allosterically inhibited by pyruvate and stimulated by ammonia (produced through photorespiration), ensuring cross talk between PDH activity and the cellular metabolic state. **C**, Regulation of heterotetrameric ADP-Glc pyrophosphorylase (AGPase) by NADP-dependent thioredoxin reductase (NTRC) and thioredoxin. AGPase has two large and two small subunits; thioredoxin can reduce the disulfide bond (S-S) between the two small subunits, resulting in activation of the enzyme. In this activated state, AGPase is allosterically inhibited by inorganic phosphate (Pi) and stimulated by 3-phosphoglycerdehyde (3-PGA), ensuring cross-talk between AGPase activity and the cellular metabolic state. **D**, Sequential PTMs control the adaptive response of plants to hypoxia through enzymatic oxygen-dependent Cys PTMs (sulfinic acid and sulfonic acid) of the transcriptional factor ERF-VII. Nitric oxide (NO) can also contribute to the Cys oxidation. The modified Cys is the N terminus of ERF-VII. RC, Arg-Cys.

a buffer against oxidative stress and maintaining thiol homeostasis (Zaffagnini et al., 2012b; Rouhier et al., 2015). GSH becomes oxidized (GSSG) by acting as an electron donor to reduce oxidized Cys or Met residues, and GSSG can be reduced back to GSH by a family of glutaredoxins. The GSH-GSSG ratio in plant cells is typically well over 90%. GSH can form a mixed disulfide bridge with accessible free thiol on a protein, a reaction termed protein S-glutathionylation. Several plant enzymes have been shown to undergo glutathionylation, in particular proteins in mitochondria and chloroplasts but also in the cytosol (Zaffagnini et al., 2012b). The most extensive glutathionylation proteomics study in photosynthetic organisms was recently done for *Chlamydomonas reinhardtii*, showing that many metabolic pathways (e.g. nucleotides, thiamine, chlorophyll, fatty acid, and respiration) are targets for this PTM (Zaffagnini et al., 2012a).

### Nitrosylation and Nitration of Cys and Tyr by RNS

NO is a lipophilic gas produced in particular during (a)botic stress and recognized as an important regulator and signaling molecule in plants (and animals; Yu et al., 2014). However, unlike in animals, which possess a specific NO synthase, there is no well-established route for NO production in plants, and it appears that NO is produced by multiple sources in plants (Yu et al., 2014). NO reacts rapidly with superoxide and GSH, generating ONOO and S-nitrosylated GSH, which are considered RNS. RNS react with reactive Cys thiols to form reversible S-nitrosothiols or with Tyr residues to form 3-nitrotyrosine, a mostly irreversible PTM in plants. Several proteomics studies identified targets of RNS in various plant species (Navrot et al., 2011), and RNS-driven PTMs are implicated in nearly every aspect of plant biology, including direct impacts on metabolic

enzymes (Wang et al., 2013; Yu et al., 2014; Sanz et al., 2015; Trapet et al., 2015). Recent reports also identified S-guanylation as a stable NO-related PTM in Arabidopsis caused by 8-nitro-cyclic GMP (cGMP), which is generated by the reaction of NO with cGMP (Sawa et al., 2011, 2013). 8-Nitro-cGMP is essential for guard cell signaling in plants (Joudoi et al., 2013).

### Ubiquitination

Protein ubiquitination consists of the reversible, covalent binding of a single ubiquitin molecule (monoubiquitination) or a chain of ubiquitin molecules (polyubiquitination) to Lys residues of the substrate protein (Vierstra, 2012). Ubiquitin is a small (76 amino acids) protein and is added to the substrate by the sequential participation of three proteins: the ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2), and ubiquitin protein ligase (E3). Approximately 7% of all Arabidopsis coding genes are predicted to be involved in protein ubiquitination, most of which encode for E3 ubiquitin ligase enzymes or associated factors (i.e. adaptors and substrate recruiters) that facilitate substrate selection. Indeed, this massive investment of plants (and to a lesser degree other eukaryotes) into this PTM reflects its enormous impact on plant growth and development, photomorphogenesis, hormone signaling, (abiotic responses, and metabolism (Vierstra, 2009; Guerra and Callis, 2012; Stone, 2014; see also the special issue in *Plant Physiology* [Bartel and Citovsky, 2012]). In this Update, we just highlight a few key aspects and a few cases where this PTM directly affects metabolism. The unprecedented impact of this PTM lies in (1) the large number of E3 enzymes and associated factors facilitating substrate selection, (2) the different configurations that polyubiquitin can have, (3) the reversibility of ubiquitination through the action of deubiquitinating enzymes (approximately 50 in Arabidopsis), (4) the 26S proteasome degradation system, through which polyubiquitinated molecules can be selectively and rapidly degraded, and (5) cross talk with other PTMs such as phosphorylation (Ser, Thr, and Tyr) and Lys sumoylation and acetylation. Essentially, polyubiquitination targets proteins for degradation to the proteasome, whereas monoubiquitination affects protein-protein interactions, protein function, signaling, and trafficking, including endocytosis. Many of the reported effects of polyubiquitination concern the targeted degradation of transcription factors and components of signaling pathways (e.g. receptors and kinases), resulting in many downstream effects. Fewer cases have been described so far where metabolic functions are directly targeted, but examples include ALDEHYDE OXIDASE3, which is directly involved in abscisic acid synthesis and is polyubiquitinated by E3 ligase (SENESCENCE-ASSOCIATED UBIQUITIN LIGASE1), resulting in proteasomal degradation to prevent premature senescence (Raab et al., 2009). Plasma membrane-localized boron and iron transporters can be

monoubiquitinated on cytosol-exposed Lys residues, resulting into endocytosis and degradation in the vacuole (Barberon et al., 2011; Kasai et al., 2011). Plant cytosolic PEPC involved in glycolysis is monoubiquitinated to reversibly inhibit its activity (Shane et al., 2013). The ubiquitin ligase ATL31 regulates plant growth in response to nutrient balance between carbon and nitrogen by 14-3-3 proteins for ubiquitination dependent on the phosphorylation state of the 14-3-3 protein (Yasuda et al., 2014).

### Sumoylation

Similar to ubiquitin, SUMO (for small ubiquitin-like modifier) proteins are small (approximately 110 amino acids) proteins reversibly regulating protein fate in the cell, but not by targeting proteins to degradation (like ubiquitin; Miura and Hasegawa, 2010; Park and Yun, 2013). SUMO proteins have little sequence overlap with ubiquitin but have comparable three-dimensional structures. Similar to ubiquitin, SUMO goes through a C-terminal maturation step phase, but for SUMO exposing a di-Gly motif. The C-terminal Gly reversibly binds to Lys residues in target proteins through the sequential action of E1, E2, and E3 enzymes. Proteomics and yeast two-hybrid screens, combined with various follow-up analyses, identified more than 200 SUMO targets in Arabidopsis (Elrouby and Coupland, 2010; Miller et al., 2010); this showed that SUMO targets a wide range of functions, including abiotic stress responses, transcriptional regulation, hormonal signaling (abscisic acid) and cell division/development, and protein trafficking (in particular in/out of the nucleus). So far, most functional studies involving sumoylation are only indirectly related to plant metabolism, with the exception of regulation of the cytosolic nitrate reductases (Park et al., 2011, 2012). The Arabidopsis nitrate reductases, NIA1 and NIA2, are sumoylated by the E3 SUMO ligase SIZ1, which dramatically increases their activity, thereby promoting nitrate assimilation. The Arabidopsis *siz1* mutant growth phenotype is suppressed by exogenous ammonium but not by nitrate (Park et al., 2011). The low nitrate reductase activity also correlated with an overall decrease in alternative mitochondrial respiration [involving type II NAD(P)H dehydrogenases and alternative oxidases] and with a low carbohydrate content to maintain the carbon-to-nitrogen ratio in the mutants (Park et al., 2012). Finally, considerable antagonistic and cooperative cross talk between sumoylation and the PTMs ubiquitination and phosphorylation has been observed, but this cross talk is still vastly underexplored in plants (Park and Yun, 2013).

### Lipid Protein Modifications

Proteins can be cotranslationally or posttranslationally modified by covalent lipid attachments, which

increase the affinity of proteins to cellular membranes. Protein lipid modifications allow otherwise soluble proteins to be membrane anchored but also directly affect protein function (e.g. ion channels; Shipston, 2014). Dynamic microdomains or nanodomains of plant plasma membranes play important functional roles, as they allow high local concentrations of specific proteins; lipid modifications are intimately involved in these events (Konrad and Ott, 2015; Tapken and Murphy, 2015). Hundreds of plant proteins with (reversible) lipid anchors have been identified over the last decade through proteomics and MS/MS analyses, and more are predicted based on potential binding motifs (for review, see Running, 2014; Hemsley, 2015; Hurst and Hemsley, 2015). These proteins impact many functions in plant biology, including plant metabolism. There are essentially four types of lipid anchors known in plants, and they have mostly been studied in *Arabidopsis*. (1) N-terminal myristoylation irreversibly binds to N-terminal Gly, catalyzed by the enzyme *N*-myristoyltransferase. More than 400 proteins have been predicted to be myristoylated in *Arabidopsis*, but experimental evidence is still lacking for many of them. Some of the identified myristoylated proteins are kinases, phosphatases, thioredoxins, GTP-binding proteins, transcription factors, and proteins with unknown functions (Running, 2014). (2) Cys-linked prenylation, in particular farnesyl and geranylgeranyl, C15 and C20 isoprenoids, respectively. Based on C-terminal binding motifs (e.g. CxC, CCx, CCxx, and CCxxx), more than 700 *Arabidopsis* proteins are predicted to be potentially prenylated. These have various biological functions, such as transcription, regulation of cell cycle, alteration of cell wall, homeostasis, and defense mechanisms. (3) Reversible Cys-linked fatty acids (*S*-acylation), mostly involving C16 palmitate. *S*-acylated proteins often also carry other lipid modifications, such as prenylation or myristoylation. More than 600 proteins involved in different biological processes are predicted to undergo palmitoylation in *Arabidopsis* and cover a wide range of biological functions, including many types of kinases (e.g. mitogen-activated protein kinases and Leu-rich repeat receptor-like kinases), membrane transporters, and ATPases (Hemsley et al., 2013a, 2013b), and pathogen defense (Boyle and Martin, 2015). Some 24 *Arabidopsis* putative protein *S*-acyltransferases are predicted to be localized in the plasma membrane or ER and Golgi, and not much is known about them. We also note that (typically ER-localized) CAAX proteases (e.g. EST24) will cleave isoprenylated proteins, such as RAS proteins, immediately downstream of the Cys, frequently followed by methylation of the now C-terminal Cys (Bracha-Drori et al., 2008). (4) The glycolipids glycosylphosphatidylinositol and viglycosylinositol-phosphoryl-ceramide are bound to the C termini of proteins in the outer leaflet of the plasma membrane. Prediction and proteomics studies in *Arabidopsis* indicate the presence of more than 200 putative glycosylphosphatidylinositol- and viglycosylinositol-phosphoryl-ceramide-linked proteins (Borner et al., 2003; Elortza et al., 2006). Some of these proteins are

involved in cell wall formation, accumulation of cuticular wax, suberin, cellular signaling, and proteolysis, and many have unknown functions.

Despite earlier reports on lipid PTMs in chloroplasts (i.e. palmitoylation of the D1 reaction center protein [Mattoo and Edelman, 1987] and prenylation within chloroplasts [Parmryd et al., 1999]), there is very little new support for protein lipid modifications within either chloroplasts or mitochondria. Indeed, most lipid PTMs are found on proteins within or passing through the secretory system, in particular on either side of the plasma membrane. However, there are also transcription factors known with lipid PTMs (Hemsley, 2014, 2015). It is worth mentioning that N-terminal protein acylation can change the intercellular sorting; for example, inhibition of the *N*-myristoylation of a calcium-dependent protein kinase was sufficient to alter its localization from the plasma membrane to chloroplasts, and chloroplast localization of ferredoxin-NADP<sup>+</sup> reductase and Rubisco activase could be efficiently suppressed by the artificial introduction of myristoylation and palmitoylation sites (Stael et al., 2011).

#### ***N*-Linked and *O*-Linked Glycosylation**

Glycosylation occurs when a carbohydrate is attached to a protein via a covalent bond. Most eukaryotic proteins that are secreted into the ER become cotranslationally *N*-glycosylated via an Asn that is part of Asn-X-Ser/Thr and/or Asn-X-Cys motifs. The *N*-linked glycan subsequently undergoes varying degrees of processing and modifications by enzymes that are spatially distributed over the ER, Golgi, and post-Golgi apparatus. In particular, the initial (cotranslational) glycosylation steps in the ER play an important role in protein folding and quality control, including recognition by the ER-associated degradation pathway. The biology, significance, and detection of *N*-glycoproteins (the glycoproteome) in plants were recently extensively described and referenced (Song et al., 2011, 2013; Strasser, 2014). Since the vast majority of secretory proteins are glycosylated, many aspects of plant growth, development, metabolism, and (a)biotic responses are affected by *N*-glycosylation, including direct effects on enzyme functions. Much less understood is *O*-glycosylation, which involves linkages via hydroxylated Pro often together with an *O*-linked glycan of a neighboring Ser (via its hydroxyl side chain; for review, see Nguema-Ona et al., 2014). *O*-glycosylated proteins are mostly found in the cell wall, where they make up a significant percentage (approximately 10%) of cell wall proteins. However, reversible linkage of the carbohydrate *O*-linked  $\beta$ -GlcNAc (*O*-GlcNAc) to the hydroxyl groups of Ser and Thr residue is observed in the nucleus and cytoplasm (e.g. on transcription factors; Olszewski et al., 2010; Steiner et al., 2012). In plants, this PTM is regulated by two *O*-GlcNAc transferases, named SPINDLY and SECRET AGENT; loss-of-function double mutants for these two proteins results in embryo lethality.

## PTM DISTRIBUTION ACROSS SUBCELLULAR LOCALIZATIONS

Several PTMs show uneven distribution across the different subcellular compartments (Table II). This relates to the specific biological function of the PTM (e.g. *O*-glycosylation in the cell wall) and/or the absence of enzymes in a compartment that can carry out the PTM. PTMs induced by ROS or RNS are typically of higher frequency where the ROS and/or RNS species are produced. In the case of ROS, the electron transport activities in chloroplasts (from photosynthesis) and mitochondria (oxidative phosphorylation) are a major source for the production of H<sub>2</sub>O<sub>2</sub> and superoxide, whereas peroxisomes in leaves can produce large amounts of H<sub>2</sub>O<sub>2</sub> during photorespiration, which is typically detoxified by catalase. Phosphorylation is perhaps the PTM for which most in-depth, large-scale studies have been carried out. A meta-analysis for *Arabidopsis* showed that the highest number of phosphoproteins were detected in nuclei (35%), followed by the secretory system (23%) and cytosol (23%), non-photosynthetic plastids and chloroplasts (6%–7%; nearly 300 proteins), mitochondria (4%; approximately 160 proteins), and just 1% in peroxisomes (van Wijk et al., 2014). A recent article use such meta-phosphoproteomics to precisely map the observed phosphorylation sites of enzymes in the photorespiratory pathway located in chloroplasts, peroxisomes, and mitochondria; the significance and context of these observed phosphosites have been discussed in detail (Hodges et al., 2013). Finally, a bias to specific subcellular compartments for some of the PTMs may be due simply to the interest of researchers in those specific compartments (e.g. nonhistone methylation).

## (CLASSIC) EXAMPLES OF PLANT METABOLIC ENZYMES AND PATHWAYS REGULATED BY COMBINATORIAL PTMS

Over the last decades, the regulation of a relatively small number of important and abundant enzymes, in particular in primary metabolism and the C<sub>4</sub> shuttle in C<sub>4</sub> plants, have been studied in great detail at the biochemical level. Table III list several of these highly studied plant enzymes, located in the cytosol, chloroplast/nongreen plastids, or mitochondria, including their known PTMs and recent references (we refer to the listed references for hundreds of older citations). As will be discussed below, most of these classic examples involve regulation by (de)phosphorylation, reversible formation of disulfide bonds involving thioredoxins, and changes in oligomeric state and protein-protein interactions. We use a few of these examples to illustrate how combinatorial or sequential PTMs can function in plant metabolic regulation (Fig. 1). Importantly, these examples highlight how rates of photosynthesis, respiration, photorespiration, and glycolysis are coordinated through changes in enzyme oxidation/reduction and (de)phosphorylation state via a network of thioredoxins, kinases, and phosphatases driven by subcellular ATP-ADP and NAD(P)H-NAD(P)<sup>+</sup> ratios as well as allosteric and feedback

regulation by metabolites (Noguchi and Yoshida, 2008; Hodges et al., 2013). However, recent proteomics studies for glutathionylation, nitrosylation, and Lys acetylation suggest that the classic view of redox regulation of primary metabolism is too simple and that additional layers and cross talk of PTMs must be considered and integrated into current models. We also discuss a very recent example of how the environment (in this case, molecular oxygen) can be sensed through a specific PTM, allowing the plant to substantially modify its proteome (Fig. 1D).

## Regulation by (De)phosphorylation and Redox during the Light/Dark Cycle and in Response to the Metabolic State

In particular in chloroplasts, many enzymes are regulated through a combination of oxidation/reduction of Cys residues and (de)phosphorylation involving thioredoxins, kinases, and phosphatases as well as 14-3-3 proteins (Denison et al., 2011; de Boer et al., 2013), frequently combined with allosteric regulation by metabolites (Huber and Hardin, 2004; Hodges et al., 2013). Because photosynthesis and respiration are associated with fluctuating rates of production and the consumption of NAD(P)H (and FADH<sub>2</sub>) directly impacting the redox state of the cell, regulation of the enzyme activities in these pathways through oxidation/reduction (of Cys residues) is an excellent way to coordinate both pathways. This is mediated through an extensive family of the thioredoxins (Schürmann and Buchanan, 2008; Michelet et al., 2013; Nikkanen and Rintamäki, 2014) as well as the redistribution of reducing equivalents through metabolic exchange of the malate/oxaloacetate and triose phosphates/3-PGA between subcellular compartments (Scheibe, 2004; Taniguchi and Miyake, 2012). Surprisingly, compared with the Calvin-Benson cycle, the regulation of the tricarboxylic acid cycle *in vivo* by PTMs is relatively unexplored (note that regulation by mitochondrial metabolites is well explored), but proteomics studies suggested that the abundance of several PTMs (in particular Lys acetylation [Konig et al., 2014a, 2014b] and phosphorylation [Havelund et al., 2013] but also *S*-nitrosylation and *S*-glutathionylation [Lázaro et al., 2013]), oxidation/reduction by thioredoxins (Balmer et al., 2004; Yoshida et al., 2013), or glutaredoxin (Rouhier et al., 2005; Noctor et al., 2007) play a role in the regulation and stress response of mitochondrial respiration (Lázaro et al., 2013). The potential for thioredoxin regulation of respiration was recently probed through complementary *in vitro* and *in vivo* analysis, including metabolite labeling, leading to the conclusion that just two enzymes in the tricarboxylic acid cycle (succinate dehydrogenase and fumarase) are deactivated by thioredoxin-mediated reduction. In contrast, cytosolic ATP-citrate lyase was activated by reduction, thus drawing citrate away from the tricarboxylic acid cycle for the production of acetyl-CoA for fatty acid synthesis (Daloso et al., 2015). The *in vitro* observed thioredoxin-mediated activation of citrate synthase (Schmidtman et al., 2014) was not supported by *in vivo* data (Daloso et al., 2015).

**Table III.** Regulation by PTMs in plant metabolism and classic examples of well-studied enzymes and pathways

Many of these enzymes also undergo allosteric regulation through cellular metabolites. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; PRK, phosphoribulokinase.

Process	Enzymes	PTMs, Protein Modifiers, Localization	References
Calvin-Benson cycle (chloroplasts)	Many enzymes	Oxidoreduction of S-S bonds, reversible nitrosylation, glutathionylation; through ferredoxin/ferredoxin-thioredoxin reductase/thioredoxins (mostly <i>f</i> and <i>m</i> ) and glutaredoxins; proteomics studies in Arabidopsis and <i>C. reinhardtii</i>	Michelet et al. (2013)
	Rubisco	Methylation, carbamylation, acetylation, N-terminal processing, oligomerization; classical studies in pea ( <i>Pisum sativum</i> ), spinach ( <i>Spinacia oleracea</i> ), and Arabidopsis	Houtz and Portis (2003); Houtz et al. (2008)
	GAPDH/CP12/PRK supercomplex	Dynamic heterooligomerization through reversible S-S bond formation controlled by thioredoxins	Graciet et al. (2004); Michelet et al. (2013); López-Calcano et al. (2014)
Glycolysis	Cytosolic PEPC	Phosphorylation (S, T), monoubiquitination	O'Leary et al. (2011)
Photorespiration	Seven enzymes are phosphorylated	Phosphorylation from meta-analysis of public phosphoproteomics data for Arabidopsis; located in chloroplasts, peroxisomes, mitochondria	Hodges et al. (2013)
	Maize glycerate kinase	Redox-regulated S-S bond; thioredoxin <i>f</i> ; studied extensively in chloroplasts of C4 maize	Bartsch et al. (2010)
Respiration (mitochondria)	Potentially many enzymes, but functional/biochemical consequences are relatively unexplored	Recent studies suggested PTMs for many tricarboxylic acid cycle enzymes, including Lys acetylation and thioredoxin-driven S-S formation; in particular, succinate dehydrogenase and fumarase are inactivated by thioredoxins	Lázaro et al. (2013); Schmidtman et al. (2014); Daloso et al. (2015)
	PDH	Ser (de)phosphorylation by intrinsic kinase and phosphatase; ammonia and pyruvate control PDH kinase activity; see Figure 1B	Thelen et al. (2000); Tovar-Méndez et al. (2003)
C4 cycle (C3 and C4 homologs also involved in glycolysis and/or gluconeogenesis)	Pyruvate orthophosphate dikinase	Phosphorylation by pyruvate orthophosphate dikinase-RP, an S/T bifunctional kinase-phosphatase; in chloroplasts	Chastain et al. (2011); Chen et al. (2014)
	PEPC	Phosphorylation; allosteric regulation by malate and Glc-6-P; in cytosol in mesophyll cells in C4 species (e.g. <i>Panicum maximum</i> ); see Figure 1A	Izui et al. (2004); Bailey et al. (2007)
	PEPC kinase	Ubiquitination resulting in degradation (note also diurnal mRNA levels and linkage to activity level; very low protein level); in cytosol in mesophyll cells in C4 species (e.g. <i>Flaveria</i> spp. and maize)	Agetsuma et al. (2005)
	PEPC kinase	Phosphorylation in cytosol in bundle sheath cells	Bailey et al. (2007)
Starch metabolism (chloroplasts)	ADP-Glc pyrophosphorylase	Redox-regulated disulfide bonds and dynamic oligomerization; thioredoxins; see Figure 1C	Geigenberger et al. (2005); Geigenberger (2011)
	Starch-branching enzyme II	Phosphorylation by Ca <sup>2+</sup> -dependent protein kinase; P-driven heterooligomerization	Grimaud et al. (2008); Tetlow and Emes (2014)

(Table continues on following page.)

**Table III.** (Continued from previous page.)

Process	Enzymes	PTMs, Protein Modifiers, Localization	References
Suc metabolism (cytosol)	SPS (synthesis of Suc)	(De)phosphorylation; SPS kinase and SPS phosphatase; 14-3-3 proteins; cytosol (maize and others)	Huber (2007)
	Suc synthase (breakdown of Suc)	Phosphorylation; Ca <sup>2+</sup> -dependent protein kinase; correlations to activity, localization, and turnover	Duncan and Huber (2007); Fedosejevs et al. (2014)
Photosynthetic electron transport (chloroplast thylakoid membranes)	PSII core and light-harvesting complex proteins	(De)phosphorylation by state-transition kinases (STN7/8) and PP2C phosphatases (PBCP and PPH1/TAP38)	Pesaresi et al. (2011); Tikkanen et al. (2012); Rochaix (2014)
Nitrogen assimilation	Nitrate reductase	(De)phosphorylation; 14-3-3 proteins	Lillo et al. (2004); Huber (2007)

A classic case is PEPC in the cytosol of mesophyll cells of maize and sorghum (*Sorghum bicolor*), a key enzyme in C4 photosynthesis (Izui et al., 2004; Fig. 1A). PEPC captures CO<sub>2</sub> from bicarbonate to carboxylate phosphoenolpyruvate into the C4 acid oxaloacetate, which is then reduced to malate, exported to bundle sheath cells, and decarboxylated to release CO<sub>2</sub> for Rubisco (Westhoff and Gowik, 2004). Light activates PEPC kinase by an unknown mechanism, presumably one or more PTMs. In turn, the kinase phosphorylates PEPC on a conserved Ser residue, thereby activating PEPC to produce oxaloacetate. Interestingly, this low-abundance PEPC kinase is degraded by the proteasome after polyubiquitination, but the E3 ligase is not yet known (Agetsuma et al., 2005). An unidentified phosphatase (Protein phosphatase 2A family) dephosphorylates PEPC in darkness, thereby down-regulating PEPC activity (Dong et al., 2001; Izui et al., 2004). The regulation of this phosphatase is still unknown.

A similar type of regulation also occurs for cytosolic Suc phosphate synthase (SPS), which is the main regulator in Suc synthesis (Winter and Huber, 2000). Similar to PEPC, a dedicated kinase and phosphatase change the phosphorylation status, but in the case of SPS, phosphorylation occurs in the dark, rendering the enzyme less active (Huber, 2007). Furthermore, a member of the 14-3-3 family binds to phosphorylated SPS, but the effect remains controversial (de Boer et al., 2013). The kinase, phosphate, and SPS are also each allosterically regulated by Glc-6-P and inorganic phosphate, illustrating the complexity of metabolic regulation. Similarly, Suc synthase, involved in the breakdown of Suc in heterotrophic tissues (e.g. the nonphotosynthetic base of a developing maize leaf and developing seeds) is phosphorylated by a Ca<sup>2+</sup>-dependent protein kinase; the phosphorylation influences both protein activity and membrane association (Duncan and Huber, 2007; Fedosejevs et al., 2014).

PDHs are complex multienzyme complexes catalyzing the oxidative carboxylation of pyruvate to produce acetyl-CoA for the tricarboxylic acid cycle in mitochondria and for fatty acid synthesis in chloroplasts and in nonphotosynthetic plastids (Thelen et al., 2000; Tovar-Méndez et al., 2003). The activity of mitochondrial PDH is regulated by reversible Ser phosphorylation by an associated kinase and type 2C phosphatase;

in contrast plastid-localized PDH does not contain this pair of kinase/phosphatase and consistently is not regulated by phosphorylation but instead is regulated by changes in plastidic pH and [Mg<sup>2+</sup>]. Mitochondrial PDH is active in its dephosphorylated form under conditions of high photosynthesis rates and associated photorespiration and inactive in its phosphorylated form in darkness (Fig. 1B). Key to this on-off switch mechanism is the inhibition of PDH kinase by pyruvate, the substrate for PDH, and the activation by ammonia produced during photorespiration (Fig. 1B).

ADP-Glc pyrophosphorylase is a heterotetrameric complex that catalyzes the first committed step of starch synthesis, and its activity is regulated by redox through the reduction by thioredoxin *f* or *m* (Fig. 1C; Geigenberger, 2011). Reduction of an intermolecular disulfide bond between two large subunits leads to a change in kinetic properties, including a change in substrate affinity, and modified sensitivity to the allosteric metabolic regulators 3-PGA and inorganic phosphate (Geigenberger, 2011).

### Enzymatic Cys Oxidation Triggers Proteasomal Degradation through the N-End Rule Degradation Pathway

A wonderful example of how a series of PTMs can help plants adapt to their environment is the response of plant cells to hypoxia (Fig. 1D). A high rate of respiration, or submergence of tissue under water, can lead to very low cellular oxygen concentration (hypoxia); since oxygen is the ultimate electron acceptor in oxidative phosphorylation, this results in reduced rates of respiration and ATP production (van Dongen and Licausi, 2015). In order to adjust cellular metabolism to hypoxia, plant cells are able to sense low oxygen concentrations through a set of ERF-VII transcription factors. Under aerobic conditions, the concentration of these transcription factors is kept very low through continuous proteasomal degradation through an enzymatic PTM induced by oxygen. During hypoxia, these transcription factors stabilize; therefore, they can act to modify gene expression to adjust to hypoxia (Fig. 1D). A small set of Cys oxidases (PCOs) are capable of enzymatically oxidizing the N-terminal Cys of ERF-VII, generating a

sulfenic acid (Weits et al., 2014). The PTM turns these transcription factors into a substrate for arginyl-transferases, which adds an Arg residue to the N terminus. The new N terminus is now recognized by specific single-subunit E3 ligases that polyubiquitinate the transcription factors, ultimately leading to degradation by the proteasome (van Dongen and Licausi, 2015).

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