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# The Scope, Functions, and Dynamics of Posttranslational Protein Modifications

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## Abstract

Assessing posttranslational modification (PTM) patterns within protein molecules and reading their functional implications present grand challenges for plant biology. We combine four perspectives on PTMs and their roles by considering five classes of PTMs as examples of the broader context of PTMs. These include modifications of the N terminus, glycosylation, phosphorylation, oxidation, and N-terminal and protein modifiers linked to protein degradation. We consider the spatial distribution of PTMs, the subcellular distribution of modifying enzymes, and their targets throughout the cell, and we outline the complexity of compartmentation in understanding of PTM function. We also consider PTMs temporally

in the context of the lifetime of a protein molecule and the need for different PTMs for assembly, localization, function, and degradation. Finally, we consider the combined action of PTMs on the same proteins, their interactions, and the challenge ahead of integrating PTMs into an understanding of protein function in plants.

## Contents

## INTRODUCTION

Proteins are synthesized on ribosomes generating a nascent polypeptide chain. Many proteins then undergo posttranslational modifications (PTMs) to form the mature proteoforms that ultimately accumulate in plant cells to form the observed proteome (157). Different PTMs occur throughout the life cycle of proteins. These PTMs range from cotranslational modifications; to enablers of location, function, and signaling; and finally to markers for stability and degradation. PTMs occur on amino acid side chains or at a protein's C or N termini, and they extend the chemical decoration and properties of the 20 standard amino acids by modifying existing functional groups or introducing new ones. PTM of the N-terminal α-amino group follows a different set of characteristics because of the chemical reactivity of this site. Posttranslational cleavage of a protein reveals a new C or N terminus that can have altered function and stability of a protein. Here we describe five major classes of modifications, each with subclasses, and the evidence of specific targets of their action.

## COTRANSLATIONAL AND PREFOLDING PROTEIN MODIFICATIONS

The N termini of proteins in polypeptides are very reactive sites that are exposed to a highly diverse set of modifications collectively called N-terminal protein modifications (NPMs). Many NPMs are cotranslational and are catalyzed by ribosome-associated enzymes acting as soon as a nascent polypeptide emerges from the ribosomal exit tunnel (22). The earliest and most extensively observed cotranslational NPMs comprise N-terminal methionine excision (NME) and two acylation reactions, *N*-α-acetylation (NTA) and *N*-myristoylation (MYR). A further set of NPMs involves N-terminal transit peptide removal when proteins reach a final location in mitochondria, plastids, and membrane networks or upon secretion from the cell.

### N-Terminal Methionine Excision

NME is the removal of the first amino acid from the nascent chain, the initiating methionine (iMet) for nuclear-encoded open reading frames and *N*-formyl-methionine (fMet) in chloroplasts and mitochondria (**Figure 1**). In these latter organelles, the NME starts with the removal of the formyl group by peptide deformylases (PDF1A, PDF1B), which are enzymes that are dually targeted to plastids and mitochondria. Knockout of both PDFs is lethal (22). Biochemical and genetic studies indicate that PDF1A acts during oxidative stress, whereas PDF1B operates under standard conditions (22). Removal of the formyl group is a prerequisite for iMet excision by methionine aminopeptidases (MetAPs). In plants, six nuclear-encoded MetAPs distributed among cytosol, mitochondria, and plastids ensure the excision of iMet (**Table 1**). Cleavage of iMet occurs when the second amino acid has a small side chain, and such cleavage is not possible or is incomplete when the second amino acid has a large side chain (50). Prediction tools for NME that match well with in vivo identified plant MetAP substrates have been developed (e.g., terminator3) (17) (**Table 1**).

### *N*-α-acetylation

NTA transfers an acetyl moiety from acetyl–coenzyme A (acetyl-CoA) to the α-amine of the N-terminal amino acid of a nascent chain (**Figure 1**). The reaction is catalyzed by *N*-α-acetyltransferases (Nats). In the cytosol, the majority of NTA relies on the heteromeric complexes NatA, NatB, and NatC (3). The existence of plant cytosolic cotranslational NTA machinery was shown from the characterization of N termini from many photosynthetic organisms (9, 108, 115, 209). Homology searches have identified several potential Nats in *Arabidopsis thaliana* (**Table 1**), including homologs of the known metazoan catalytic and auxiliary subunits. Characterization of *Arabidopsis* NatA shows conservation with metazoan Nats, including interaction with the ribosome,

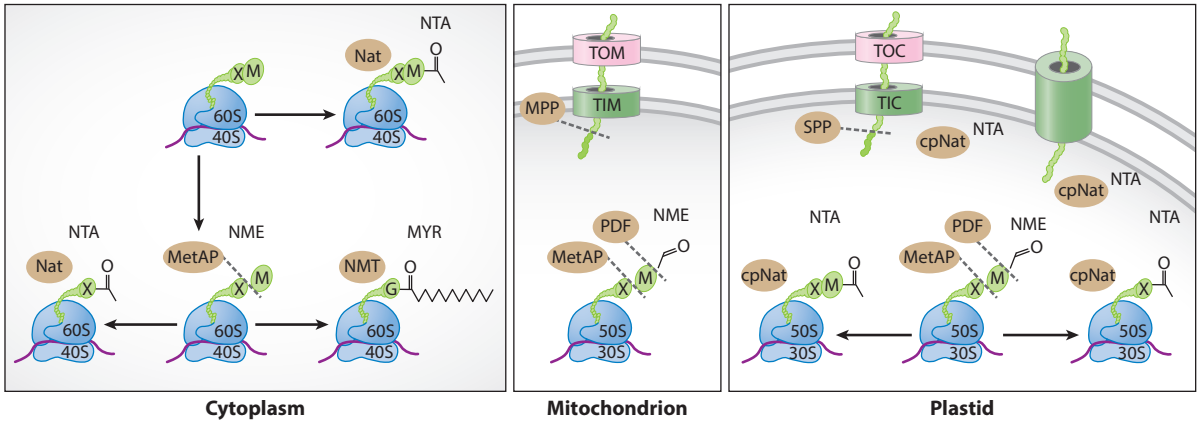


**Figure 1**

N-terminal modifications during translation and after subcellular trafficking. Dashed lines indicate proteolytic action of PDF, MetAP, MPP, or SPP. Abbreviations: 30S, prokaryotic small ribosomal subunit; 40S, eukaryotic small ribosomal subunit; 50S, prokaryotic large ribosomal subunit; 60S, eukaryotic large ribosomal subunit; cp, chloroplastic; G, glycine; M, methionine; MetAP, methionine aminopeptidase; MPP, mitochondrial processing peptidase; MYR, *N*-myristoylation; Nat, N-α-acetyltransferase; NME, N-terminal methionine excision; NMT, N-myristoyltransferase; NTA, *N*-α-acetylation; PDF, peptide deformylases; SPP, stromal processing peptidase; TIC, translocon at the inner chloroplast membrane; TIM, translocon at the inner mitochondria membrane; TOC, translocon at the outer chloroplast membrane; TOM, translocon at the outer mitochondria membrane; X, any amino acid.

and also shows that the catalytic subunit Naa10 contains all the information for the complex specificity in plants (106, 190). A number of putative plastid Nat catalytic subunits, but no corresponding auxiliary subunits, have been identified (22). Characterization of AtNaa70 (NatG or cpNat) revealed broad substrate specificity and clear plastid localization (35). Plastid NTA occurs cotranslationally on plastid-encoded proteins and posttranslationally on imported nuclear-encoded proteins after plastid transit peptide cleavage (14, 17, 18, 81, 209). Although some metazoans carry out NTA in mitochondria, the extent of this modification is uncertain and not confirmed in plant mitochondria to date.

## *N*-Myristoylation

MYR is a major protein fatty-acylation modification of eukaryotes. It occurs exclusively in the cytosol and involves the irreversible transfer of a myristate moiety from myristoyl-CoA to the α-amino group of glycine (Gly) of the target protein unmasked by the NME action (**Figure 1**). MYR is catalyzed by *N*-myristoyltransferases (NMTs). In *Arabidopsis*, NMT1 is the major enzyme responsible for this modification (19, 135). MYR is considered to act mainly cotranslationally in eukaryotes, although presence of NMT in the ribosomal fraction has not yet been shown in plants. Hundreds of cotranslational MYR sites and one example of posttranslational addition of MYR have been reported in plants (113) (**Table 1**).

## Prepeptide Cleavage: Processing and Maturation of Proteins

Precursor proteins can also have substantial cleavable N- or C-terminal signal peptides for selective sorting to different subcellular compartments or for secretion (**Figure 1**) (84, 97, 123). Plastid and mitochondrial proteins have N-terminal signal peptides (123), endoplasmic reticulum (ER)-located proteins have a relatively short and cleavable N-terminal signal peptide (97), and peroxisomal proteins can have cleavable N-terminal signal peptides (PTS2) (91). Sorted proteins can also have additional cleavages for secondary sorting, e.g., to the thylakoid lumen or to the

**Table 1 Posttranslational modifications discussed in this review, known substrate selection components, and experimentally defined *Arabidopsis* substrates**

| Modification | Known substrate selection components | Number of experimentally confirmed substrates[a] | Reference(s) |
|---|---|---|---|
| Ubiquitin | ~1,500 | 3,468 | 92, 181 |
| SUMO | 2 (SIZ1, HPY2/MMS21) | 1,099 | 120, 145 |
| Rub1 | 1 (RBX1) | 5[b] | 119, 206 |
| Met-Ox | ROS | 403 | 83 |
| Cys-Ox | ROS | 311 | 4, 183 |
| Carbonylation | ROS, RNS | 40 | 114 |
| Phosphorylation | 940 kinases<br>150 phosphatases | 7,603 | 71[e] |
| NME | PDF1A, PDF1B<br>6 MetAPs | 1,793 | 24[f] |
| NTA | 10 Nats (NatA-F)<br>12 hypothetical Nats<br>cpNat1 | 1,875 | 24[f] |
| MYR | NMT1, NMT2 | 525 | 24, 113 |
| Nt-cleavage | 8 processing peptidases (SPP, TPP, MPP, PPP) | 349 | 80, 143 |
| *O*-linked sugars | SPY, SEC, HPAT, RRA, XEG113 | 266 | 191, 193, 201 |
| *N*-linked glycans | OST complex (12 proteins)<br>7 GTs<br>7 GHs | 1,667 | 159, 200 |
| GPI anchors | 5 GTs<br>GPI-GlcNAc transferase complex (7 proteins) | 68 | 20, 42–44 |
| *O*-linked glycans | HPAT, RRA, XEG113, ExAD, 14 GTs | 166[c] | 124, 154 |
| Nt-arginylation | ATE1, ATE2 | 5[d] | 57, 58, 199 |
| Gln-specific Nt-amidohydrolase | NTAQ1 | - | 62 |
| Asn-specific Nt-amidohydrolase | NTAN1 | - | 62 |
| Nt-Cys oxidation | PCO1–PCO5 | 5[d] | 186, 187 |

Abbreviations: Asn, asparagine; ATE, arginyl transferase; Cys, cysteine; ExAD, EXTENSIN ARABINOSE DEFICIENT TRANSFERASE; Gln, glutamine; GPI-GlcNAc, glycosylphosphatidylinositol-acetylglucosamine; GT, glycosyltransferase; OST, oligosaccharyltransferase; SPY, SPINDLY; GH, glycosyl hydrolase; HPAT, HYDROXYPROLINE *O*-ARABINOSYLATRANSFERASE; MYR, *N*-myristoylation; Nt, amino-terminal; NME, N-terminal methionine excision; NTA, *N*-α-acetylation; NTAN, Nt-Asn amidohydrolase; NTAQ, Nt-Gln amidohydrolase; PCO, PLANT CYSTEINE OXIDASE; RNS, reactive nitrogen species; Rub1, related to ubiquitin1; ROS, reactive oxygen species; RRA, REDUCED RESIDUAL ARABINOSE; SEC, SECRET AGENT; SUMO, small ubiquitin-related modifier; XEG113, XYLOGLUCANASE 113.

[a]Collated experimental data based on key publications as of June 2018; detailed information is provided in **Supplemental Data 1**. New data are now being collated online at the Plant PTM Viewer (**http://www.psb.ugent.be/PlantPTMViewer**).

[b]Four cullin subunits of approximately 1,000 cullin-based ubiquitin ligases and Rub1 activating enzyme (AXR1).

[c]Majority determined through bioinformatic analysis of the genome.

[d]ERFVII transcription factors.

[e]PhosPhAt database (**http://phosphat.uni-hohenheim.de**).

[f]Energomic database (**https://bioweb.i2bc.paris-saclay.fr/n-terpred/**).

plasma membrane (174). Peptidases define the position of the cleavage with a variety of identified primary sequence motifs. Presequences removed can be as short as a few or up to 150 amino acids in length. Some proteins undergo later N- or C-terminal trimming events to activate them, stabilize them, or release them from a membrane to allow movement and action (e.g., ER-tethered

Supplemental Material >

transcription factors) (26, 185). Several bioinformatic tools now available predict transit-peptide cleavage sites in sequences (e.g., TargetP, ChloroP, SignalP), but they show low accuracy in plants. However, hundreds of experimentally determined N-terminal cleavage sites have been reported in plants (**Table 1**) and can be used to train plant-specific predictors in the future.

## N-Terminal Protein Modification Targets and Associated Modification Roles in Plants

NPMs influence the folding, activity, complex associations, localization, and half-life of proteins (3, 59). One of the major roles of the NME process is associated with protein stability, and by linking thiol status and proteolysis, cytoplasmic NME becomes essential for normal plant growth and development (49). Plastid NME directly contributes to protein stability, and removal or maintenance of iMet is perceived as a stabilizing or destabilizing signal, respectively (60). The plastid proteolytic machinery involved in degradation of NME-destabilizing proteins is still unknown. However, D1 and D2 that are correctly processed by NME are degraded primarily by the FtsH protease complex, whereas inhibition of chloroplastic NME compromises the specific recognition of D1 and D2 by FtsHs (2). Loss of *Arabidopsis* NatA is lethal (106, 190). The cytosolic NatA-NTA is a hormone-controlled dynamic process, and downregulation of NatA results in drought resistance in *Arabidopsis* (106). NTA catalyzed by different Nats can both stabilize proteins and act as a built-in degradation signal (190). Mutants for NatB auxiliary and NatC catalytic subunits are viable, and their loss negatively affects photosynthesis efficiency and induces a pleiotropic growth-retarded phenotype, respectively (107).

It has been generally assumed that MYR ensures proper targeting of the myristoylated proteins to the plasma membrane. Studies on *Arabidopsis* thioredoxins showed that only MYR localized proteins to the endomembrane system and that partitioning between the endomembrane and cytosol correlated with the catalytic efficiency of the NMT enzyme (168). They further demonstrated that an S-acylation adjacent to the MYR site is crucial to readdress proteins to micropatches in the plasma membrane (168). *At*NMT1 inactivation leads to late-embryo abortion due to early developmental defects affecting shoot apical meristem differentiation (135). Shoot apical meristem defects induced by the absence of *At*NMT1 are directly linked to the lack of MYR of the protein kinase SnRK1. Additionally, a low level of *At*NMT1 induces a dwarf phenotype (139) and impairs flower differentiation, fruit maturation, fertility, and innate immunity (135). These defects have been ascribed to postembryonic *At*NMT1 roles of MYR. The majority of plant MYRed proteins have roles in calcium signaling and pathogen responses, but the impact of MYR on these functions has not been characterized (113). Alternative localization of MYRed proteins in non-plasma-membrane compartments has been uncovered in plants and mammals (24, 103, 113), suggesting that MYR also contributes to dedicated functions in other membrane compartments.

## Methods for Characterizing and Predicting N-Terminal Protein Modifications

Edman degradation chemistry was the original method of choice for investigating the N termini of proteins; however, its failure with N-terminal-modified peptides, except when the peptide was formylated, limited its use for N-termini characterization in bacteria and plant organelles such as the characterization of thylakoid lumen proteins (133). In vitro assays with purified enzymes, including PDFs, MetAPs, Nats, or NMTs with short peptides mimicking the natural substrates, have been used to provide information on both substrate specificity and enzyme efficiency. In vitro transcription-translation experiments performed with radiolabeled precursors ($^{3}$H- or $^{125}$I-myristic acid, $^{35}$S-Met, or $^{3}$H-acetyl-CoA) also provided early data (117). Indirect in vivo approaches have also been used to support the presence of specific NPMs. In MYR, site-specific

mutagenesis of implicated N-terminal Gly residues and their effects on protein subcellular localization have been exploited for indirect evidence of MYR (see references in 113). N termini and related modifications are poorly identified in conventional shotgun proteomics experiments. Published data show that N termini are identified much less frequently than the theoretical estimation of one N terminus every ten identifications (117). As a result, the NPMs currently identified represent <10% in the detectable *Arabidopsis* proteome (**Table 1**). Several new approaches based on selective labeling of the α-amino group of the proteins in a complex mixture followed by positive or negative selection of the labeled N-terminal peptides will enable more systematic analysis of NPMs by mass spectrometry. Dedicated protocols for plant N-terminome profiling are available using TAILS (32, 143), COFRADIC (170), ChaFRADIC (177), and SILProNAQ (15). Although many NPM techniques are not amenable for quantitation, the development of some in association with new tools to uncover mature N termini (16) has allowed determination of the N terminus acetylation yield for more than 2,000 proteins in *Arabidopsis* (**Table 1**) (113).

## GLYCOSYLATION IN STRUCTURE, FOLDING, SORTING, AND SIGNALING

Covalent linkage of sugars to proteins represents one of the most complex PTMs in biology. Addition of a simple sugar or an elaborate oligosaccharide can have profound effects on the role, location, and function of plant proteins. This can include affecting the solubility of a protein (*N*-linked glycans), generating transient lectins for protein-protein interactions [*O*-linked acetylglucosamine (*O*-GlcNAc)], turning proteins into large, complex macromolecules (*O*-linked glycans), and enabling the sophisticated delivery of proteins to specific lipid domains [glycosylphosphatidylinositol (GPI) anchors].

### *N*-Linked Glycans: Protein Folding and Sorting

Attachment of an oligosaccharide to asparagine (Asn) to generate an *N*-linked glycoprotein is a highly conserved process across eukaryotes. *N*-linked glycans play an important role in protein folding within the lumen of the ER, whereas more recent evidence suggests a role in endomembrane sorting and trafficking (141). A conserved series of biosynthetic steps within the ER and Golgi operate sequentially to generate various *N*-glycan structures (**Figure 2**). These include the most elaborate *N*-linked glycan structure described in plants, a biantennary oligosaccharide comprising 13 sugar residues of mannose, *N*-GlcNAc, fucose (Fuc), xylose, and galactose (Gal) (48, 159).

Variations in the *N*-glycan structure along with the presence of the oligosaccharide result in various technical issues that have limited initial surveys of plant *N*-glycoproteins. To reduce these analytical issues, initial approaches employed endoglycosidases prior to mass spectrometry. These studies collectively identified approximately 3,000 *N*-glycan sites from more than 1,600 *Arabidopsis* proteins (158, 207); however, the data lacked structural information about the *N*-glycan. Several recent surveys of *Arabidopsis* using high-resolution mass spectrometry have revealed the extent of site-specific variation of *N*-glycan structures in approximately 500 *N*-glycoproteins (112, 192, 200) (**Table 1**).

The most documented function associated with *N*-linked glycosylation in plants is its role in protein folding within the ER (159). Transfer of a hydrophilic *N*-linked glycan to the nascent polypeptide increases protein solubility and prevents aggregation (100). Mutations in the biosynthetic machinery at early stages of *N*-glycan assembly result in severe plant growth phenotypes (23) and are often lethal owing to accumulation of unfolded proteins in the ER. These observations

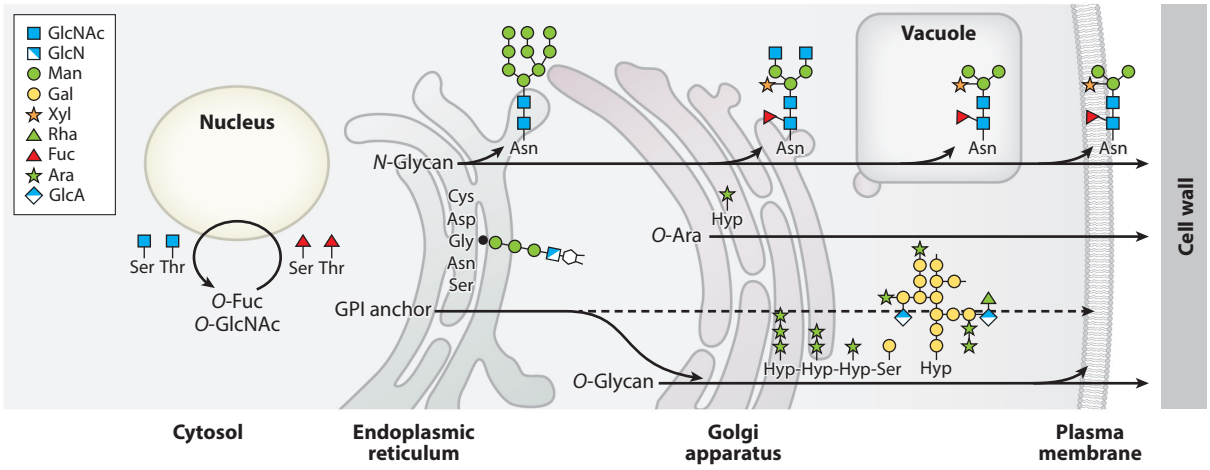


**Figure 2**

Glycosylation pathways in plants. Protein glycosylation is a highly prevalent posttranslational modification within the endomembrane of plants with modified proteins trafficked to the vacuole, plasma membrane, and cell wall. The *N*-linked glycan pathway (*N*-glycan) is well characterized and is associated with protein folding within the endoplasmic reticulum and protein sorting in the endomembrane. The GPI anchor pathway has mainly been elucidated in mammalian and yeast systems but is also present within plants and can direct proteins to specific regions of the plasma membrane (*dashed arrow*) after assembly in the endoplasmic reticulum. The *O*-linked glycan pathway (*O*-glycan) is involved in the generation of elaborate sugar structures on proteins and likely enables structural interactions with polysaccharide of the cell wall. Many proteins carrying *O*-glycans also harbor GPI anchors. *O*-Ara of signal peptides results in highly activated peptide hormones that are secreted for intercellular communication. Protein glycosylation is also a feature of the cytosol with evidence for single *O*-GlcNAc and *O*-Fuc sugars generating novel epitopes for protein-protein interactions. These cyclic modifications appear to enable transient interactions between proteins that can be used in processes such as the control of gene regulation in the nucleus. Solid arrows indicate that further steps occur in the secretory pathway; the dashed arrow indicates that no further steps occur in the secretory pathway. Abbreviations: Ara, arabinose; Asn, asparagine; Fuc, fucose; Gal, galactose; GlcA, glucuronic acid; GlcN, glucosamine; GlcNAc, acetylglucosamine; GPI, glycosylphosphatidylinositol; Hyp, hydroxyPro; Man, mannose; Rha, rhamnose; Ser, serine; Thr, threonine; Xyl, xylose.

are distinct to interruptions in *N*-glycan maturation steps within the Golgi apparatus where only conditional effects (e.g., salt sensitivity) have been observed (90). This salt sensitivity is attributed to the mislocalization of an underglycosylated endo-β1,4-glucanase (KORRIGAN) necessary for cellulose biosynthesis (141). The minimal effect of aberrant mature *N*-glycans initially put into question the biological significance of these PTMs in plants. However, characterization of equivalent mutants in *Oryza sativa* (rice) resulted in a range of growth and developmental defects (46), supporting diverse functions for different *N*-glycan structures in plants beyond protein folding.

## *O*-Linked Glycans: Structure and Signaling

The majority of proteins containing *O*-linked glycans belong to the hydroxyproline-rich glycoprotein (HRGP) superfamily associated with the cell wall (124). *O*-linked glycans are assembled within the Golgi apparatus by glycosyltransferases, many of which are unknown (**Table 1**). Proline (Pro) residues are hydroxylated in the ER to hydroxyPro (Hyp) to which *O*-linked glycans are attached and elaborated (73). The HRGP superfamily is divided into arabinogalactan proteins (AGPs), extensins (EXTs), and Pro-rich proteins (88). AGPs are rich in sugars (>90%) and possess glycan structures comprising Gal backbones with side chains of Ara and terminal rhamnose, Fuc, and glucuronic acid (42). EXTs contain short glycan chains of four to five Ara residues on Hyp,

with a serine (Ser) residue harboring a single *O*-Gal linkage (124). Pro-rich proteins have been described with various *O*-glycans, including Gal/Ara-type motifs and small Ara extensions (72). Thus, *O*-linked glycoproteins contain extensive structural heterogeneity that likely has specific functional roles within the cell wall.

Determining roles for *O*-glycoproteins has been hampered by an inability to easily characterize these structures. Only a handful of studies have purified plant *O*-glycoproteins and analyzed the glycan structure and composition (160). Whereas enrichment approaches have provided structural and compositional insights (132), computational approaches have been relied on to define HRGPs and thus *O*-glycoproteins in plant genomes (88). The collection of glycoproteins containing *O*-linked glycans in plants has generally been defined using motif and feature analysis. Typically, plants encode >100 different proteins with complex *O*-linked glycans (88), with *Arabidopsis* encoding approximately 165 HRGPs (154) (**Table 1**).

HRGPs have been implicated in an array of developmental processes, many associated with cell expansion and elongation reflecting their location in the cell wall. The rich carbohydrate structures on these proteins enable intimate contact with cell wall glycans, for example, ARABINOXYLAN PECTIN ARABINOGALACTAN PROTEIN 1 and cell wall polysaccharides pectin and arabinoxylan (162). Such connections could enable the transmission of signals produced from structural changes during processes such as cellular expansion and elongation. Indeed, a member of the Pro-rich EXT-like receptor kinase family has been implicated in abscisic acid signaling and regulating cell elongation (10).

## *O*-Linked Sugars: Protein Interaction, Signaling, and Crosstalk

Attachment of a single sugar residue to proteins or peptides can influence protein functions including stability, interactions, and activity. Attachment of *N*-GlcNAc to Ser or threonine (Thr) occurs in the cytoplasm and is mediated by the *O*-GlcNAc transferase SECRET AGENT (SEC) (191) (**Figure 2**). This type of glycosylation is akin to phosphorylation and can affect other PTMs (195). Evidence for specific protein-level *O*-GlcNAcylation in plants exists for nearly 1,000 sites on more than 260 proteins and is prominent on proteins involved in transcription, mRNA processing, and chromatin remodeling (191) (**Table 1**). *O*-GlcNAcylation has a significant role in vernalization and gibberellic acid signaling. In wheat, an AP1 clade MADS-box transcription factor (TaVRN1) is responsible for vernalization-induced flowering (189). The RNA-binding protein TaGRP2 binds and inhibits TaVRN1 transcript accumulation. However, vernalization results in *O*-GlcNAc modification of TaGRP2, which promotes protein interactions with VER2, a carbohydrate-binding protein that reduces TaGRP2 interactions with TaVRN1 transcripts.

Presence of *O*-Fuc on Ser and Thr residues has only recently been revealed in plants with a single protein characterized, a DELLA transcription factor from *Arabidopsis* (201). Attachment of *O*-Fuc by the *O*-fucosyltransferase SPINDLY (SPY), a homolog of SECRET AGENT, occurs in the cytosol (201) (**Table 1**). *O*-fucosylation may share similar characteristics to *O*-GlcNAc in plants, namely regulating protein-protein interactions.

*O*-arabinosylation of Hyp residues of *O*-linked glycoproteins in the endomembrane is a feature of EXTs present in cell walls. It appears that this machinery has been co-opted by some plant peptide hormones. The well-characterized CLAVATA3 (CLV3)/EMBRYO SURROUNDING REGION–related (CLE) family of peptide hormones plays a role in intercellular communication. Such signaling roles include root to shoot–mediated regulation of nodulation in legumes (193). The active CLE peptides appear to be *O*-arabinosylated at a Hyp residue in the Golgi apparatus (190). The presence of *O*-Ara modification appears to increase binding of CLE peptides to the CLV receptor, whereas the potency of the signal seems to increase depending on arabinose

numbers. Such activity was recently demonstrated when exogenously fed triarabinosylated CLE peptides were shown to specifically suppress nodulation in pea (70).

## Glycosylphosphatidylinositol Anchors: Flexible Membrane Tethers

Proteins carrying GPI anchors are found at the outer leaflet of the plasma membrane. The GPI anchor is considered an alternative attachment mechanism for proteins and has unique features compared with those of a transmembrane domain, including release of the protein by phospholipases, localization to membrane microdomains, and increased membrane mobility (197). The core structure of the GPI anchor contains a phosphoethanolamine linked to a simple oligosaccharide chain comprised of three mannose residues, glucosamine, and a phosphatidylinositol linked to a long-chain fatty acid (130) (**Figure 2**). The GPI structure is initially assembled at the ER membrane and then covalently attached to the C terminus of the protein by the multisubunit GPI transamidase complex. This attachment process involves the cleavage of 20–30 residues with the GPI anchor most commonly found on cysteine (Cys), aspartic acid (Asp), Gly, Asn, and Ser residues (134).

The importance of GPI anchors to plant growth and development was demonstrated through the analysis of mutants in GPI biosynthesis. These studies found gametophytic or embryo-lethal phenotypes (197). Mistargeting of essential GPI anchor–containing proteins likely causes these phenotypes. GPI anchor proteins from plants have been characterized using phospholipases to release them from membrane preparations and identification by mass spectrometry. Such studies have revealed approximately 70 proteins with experimentally determined GPI modifications from *Arabidopsis* (20, 43, 44) (**Table 1**). These include proteins with extracellular functions such as β-1,3-glucanases, early nodulin-like proteins, and COBRA-like proteins. Surprisingly, only a single plant GPI anchor structure has been elucidated so far, PcAGP1 from a *Pyrus communis* (pear) cell suspension culture (130). This work identified the conserved core structure of plant GPI anchors and also found Gal residue substitutions, showing heterogeneity in the core GPI structure that could enable levels of specificity and functionality. Heterogeneity may explain the specific membrane localizations of GPI-anchored proteins in plants, such as COBRA from *Arabidopsis*, which is necessary for orientated cellulose deposition (149). COBRA localizes to specific regions along the longitudinal surfaces of cells, and this targeting may rely on specific features of the GPI anchor. Indeed, GPI-anchored proteins appear to be highly abundant in lipid/protein-enriched microdomains of the plasma membrane (161).

# PHOSPHORYLATION IN SIGNALING, FUNCTION, AND LOCALIZATION

Protein phosphorylation is the most well-studied reversible protein modification, especially with respect to its functional characterization. It is catalyzed by protein kinases (208) and reversed by protein phosphatases (152). Within plant genomes, more genes encode protein kinases (940 in *Arabidopsis*) than protein phosphatases (150 in *Arabidopsis*), suggesting substrate specificity is higher for kinases than for phosphatases (**Table 1**). Protein phosphorylation occurs as ester bonds mainly with the hydroxyl groups of the amino acids Ser (80–85%), Thr (10–15%), and tyrosine (Tyr) (0–5%) (175). Currently, in *Arabidopsis*, at least 7,603 nonredundant proteins have been experimentally identified as phosphorylated at 42,649 different sites, mainly by large-scale bottom-up mass spectrometry after enrichment over charged metal ions ($Fe^{3+}$, $Ga^{3+}$, $Zr^{3+}$) or by complexing with titanium oxide (**Table 1**). Out of these, 4,653 proteins had only one phosphorylation site, whereas 203 phosphorylation sites were identified for ribosomal RNA biogenesis factor AT1G48920. Most

(74%) phosphorylation sites were identified under only one experimental condition, 14% were identified twice, and 12% were identified more than three times. The most frequently identified phosphorylation site is Ser338 of phosphoenolpyruvate carboxylase AT4G10750 (41). A plant-specific phosphorylation site predictor that reaches 61% precision has been developed (71). Phosphorylation of histidine (His) and Asp residues is found in two-component phosphorelay signaling pathways, for example, in cytokinin signaling (13, 109). More recently, arginine (Arg) phosphorylation has been described occurring in plants (169), but its functional relevance remains poorly understood.

## Phosphorylation as a Modulator of Protein Activity

Protein phosphorylation can function as a molecular switch in regulation of protein activities. A prominent example is the plasma membrane $H^+$-ATPase AHA2 for which a total of eight different phosphorylation sites have been identified in mass spectrometry experiments (144). Two of these phosphorylation sites increase proton pump activity, whereas two other sites inhibit activity (68). Particularly, the C-terminal Thr T947 is well known to activate AHA2 by stabilizing a conformation in which the autoinhibitory C terminus is moved away from the pore (51). A second activating phosphorylation site was discovered in the C terminus at Thr881 (53). In contrast, the proton pump is inhibited by phosphorylation at Ser899 (69) and Ser944 (52). Different proteins such as receptor kinases or membrane-directed soluble kinases catalyze phosphorylation at each activating or inhibiting site (68). It is a widespread principle that membrane channels, transporters, or metabolic enzymes are activated (e.g., aquaporin PIP2A) (116), inhibited (e.g., ammonium transporter AMT1.3) (101), or altered in affinity (e.g., nitrate transporter NRT1.1) (74) through phosphorylation.

## Phosphorylation and Subcellular Localization

Subcellular localization of proteins is also regulated by protein phosphorylation. Prominent examples are transcription factors that are mobile between cytosol and nucleus, including BZR1, which functions in activation of brassinosteroid-regulated genes when localized in the nucleus (93). In the phosphorylated state, BZR1 is localized to the cytosol, and following activation of a phosphatase by phosphorylation, BZR1 is dephosphorylated and moves to the nucleus (93). Phosphorylation can also regulate the targeting of proteins to subcellular compartments as has been shown for the aquaporin PIP2A, which is trafficked to the plasma membrane only when a dedicated Ser residue in the C terminus is phosphorylated (138). Dephosphorylation of that Ser residue leads to removal of the water channel from the plasma membrane through internalization.

## Phosphorylation in Signaling Cascades

Many signaling pathways work through phosphorylation to (*a*) transmit and amplify signals through enzyme activations and second messengers and (*b*) transmit signals through a phosphorelay system. The cascade model implies activation of a receptor through perception of its ligand (89). Activated receptor kinases then activate downstream kinases in a so-called transducer layer of the plant signaling network (38), which ultimately results in phosphorylation of effector proteins, such as transcription factors or metabolic enzymes. A well-studied example of a signaling cascade is the MAP kinase network consisting of a hierarchy in which MAP triple-kinases (ste-like MKKKs, 37 proteins in *Arabidopsis*) phosphorylate and activate MAPK kinases (MKKs, 10 proteins in *Arabidopsis*), which in turn phosphorylate and activate MAPKs at conserved Thr and Tyr residues (MPKs, 20 proteins in *Arabidopsis*) (208). At each phosphorylation and activation

step, signal amplification occurs through one upstream kinase phosphorylating and activating more than one molecule of the downstream kinase. Almost all biotic and abiotic stresses result in activation of such MAPK signaling cascades (121). The specificity within MAPK signaling was established by MKK and MPK interaction pairs on protein arrays (136), through identification of shared and specific substrate proteins of MPKs (140) and the study of protein complexes containing components of MAPK signaling (12).

Phosphorelay signaling involves the transmission of the exact same phosphate molecules from one protein to another (65). This signaling system is most prominent in bacteria but also exists in plants in the form of membrane-bound sensor-His kinases, phosphotransfer proteins, and their response regulators functioning as transcriptional regulators. Phosphorylation is physically transferred between a His residue of the donor domain or protein and an Asp residue of an acceptor domain or protein, until the phosphorylation finally is accepted by nuclear response regulator proteins (65). Cytokinin receptors, ethylene receptors, and the putative osmosensor AHK1 are representatives of such sensor-His kinases. More recently, it was discovered that sensor-His kinases feed into the Ser-Thr-Tyr signaling cascades, possibly through protein-protein interactions (30). Finally, signals can be transferred through the activation of downstream responses by second messengers that activate specific protein kinases. Calcium is a versatile second messenger that directly activates the protein kinases of the CPK (78) and CIPK families (36).

### Phosphorylation as a Signal Integrator

Many proteins have multiple phosphorylation sites that can be targets of different kinases and can thus integrate information from different signaling pathways. A study of the distribution of experimentally identified phosphorylation sites within proteins revealed accumulation of phosphorylation sites in unstructured regions of proteins (29). These phosphorylation hot spots were overrepresented in proteins with functions as effector proteins in signaling cascades, such as transcription or splicing factors. For example, BZIP transcription factors show enhanced DNA-binding properties when phosphorylated at multiple Ser residues (95). Thus, the degree of phosphorylation through the action of one or more kinases can directly modulate the degree of transcriptional activity. PIF3, a light- and auxin-induced transcriptional regulator of the basic helix-loop-helix (bHLH) family, also contains multiple phosphorylation sites. Phosphorylation of a total of three sites results in DNA binding, even though the exact position of the three sites that are phosphorylated is not relevant (125). This provides possibilities for different signals targeting different phosphorylation sites and the enhancement of signal strength by increased phosphorylation. Multiple phosphorylation sites provide an increased negative charge to the phosphorylated protein and induce positional effects within the specific sequence and structural context. Thus, increased effects of multiple phosphorylations (such as increased DNA binding) could also be attributed to accumulation of negative charges. Such charge effects are of primary importance for the activity of the membrane-localized protein OCTOPUS (21), which regulates phloem development.

## OXIDATION IN DAMAGE AND REGULATION

Owing to the presence and production of reactive oxygen species (ROS) and reactive nitrogen species in plant cells, there is a substantial potential for both reversible and irreversible oxidation of amino acid side chains. These oxidations can be involved in signaling, interact with other PTMs, inactivate proteins, and trigger structural changes or N-degron pathways leading to protein degradation. Despite numerous reports of oxidized peptides being identified in shotgun proteomic studies, many are formed during sample processing and do not represent in vivo evidence of oxidation. The focus in recent studies has been evidence of increases in oxidation status arising

from in vivo treatments and coupled mass spectrometry approaches aimed to capture in vivo oxidation events. The two major classes of oxidation are those that modify amino acid side groups to produce a carbonyl group on a wide variety of amino acid residues and sulfhydryl group oxidation states of His and Cys residues.

## Carbonylation

Protein amino acid residues can be oxidized in vivo, leading to the formation of carbonylation sites. This includes irreversible metal-catalyzed ROS-induced oxidation of lysine (Lys) to α-aminoadipic semialdehyde, Pro to 2-pyrrolidone or glutamic semialdehyde, Arg to glutamic semialdehyde, and Thr to 2-amino-3-ketobutyric acid (**Figure 3**). Indirect ROS-induced carbonylation of Cys, His, and Lys occurs via reaction with lipid peroxidation products. Oxidative carbonylation of proteins is not evenly distributed, and specific proteins appear preferentially targeted, probably via metal interaction of surface residues. Carbonylation leads to the loss of protein function and ultimately to degradation of oxidized proteins. Owing to the low abundance of endogenously carbonylated proteins in vivo, unambiguous identification of carbonylated proteins and modified sites via proteomic technologies is rare. The primary technique used to find carbonylations is through their reactions with hydrazines. Biotin-hydrazide or 2,4-dinitrophenylhydrazine reacts with carbonyls to form stable hydrazones that can be visualized on gels using antibodies to the derivatizing agent or via immune affinity that purifies carbonylated proteins by chromatography. There is still a challenge in such enrichments of determining the site at which proteins are carbonylated: In most cases, proteins are simply identified as coming from a protein spot that reacts with the derivatizing agent or through nonoxidized peptide-based tandem mass spectrometry

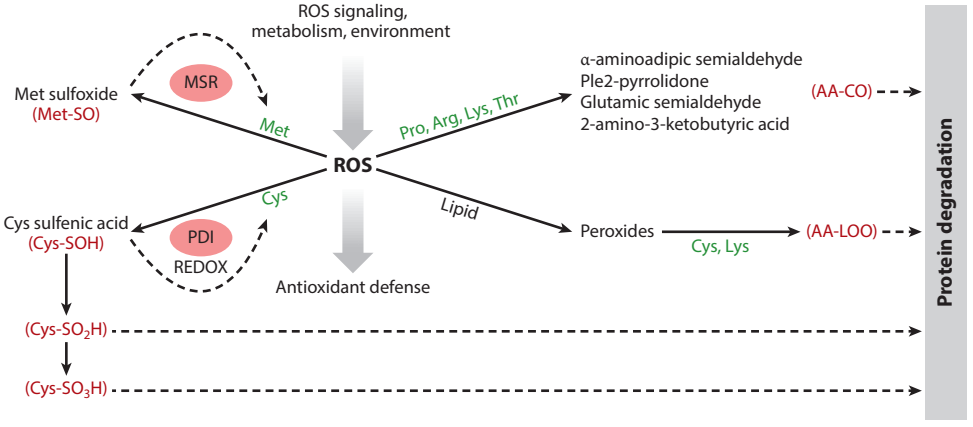


**Figure 3**

Oxidative modification and damage. ROS produced in metabolism, during stress, or in signaling are largely degraded by antioxidant defenses, but a small percentage react with sensitive amino acid residues in proteins (amino acids) to produce a range of oxidative PTMs observed in vivo. Cys and Met oxidation to Met-SO and Cys-SO yield PTMs that are reversible by MSR and PDIs, and these PTMs have increasingly defined roles in signaling and protein function. Further oxidation of Cys and a range of carbonylation and peroxidation reactions on other amino acids yield PTMs with ROS-linked damage that alter protein function and stability in the cell. Dashed lines are enzyme-catalyzed reactions; solid lines are enzyme-independent chemical reactions with ROS or lipid peroxides. Abbreviations: AA-CO, carbonylated amino acid; AA-LOO, lipid peroxide-conjugated amino acid; Arg, arginine; Cys, cysteine; Lys, lysine; Met, methionine; MSR, methionine sulfoxide reductase; PDI, protein disulfide isomerase; Pro, proline; PTM, posttranslational modification; ROS, reactive oxygen species; Thr, threonine.

derived from proteins or peptide populations that are enriched by antibodies to derivatizing agents. Two studies in plants have moved beyond gels to use either quantitative enrichment of carbonylated proteins using affinity chromatography followed by mass spectrometry identification or a more detailed assessment of the presence of oxidation of affinity-enriched peptide populations. Mano et al. (114) used lipid peroxide antibodies and N-(aminooxyacetyl)-N0-biotinyl-hydrazine to identify different populations of carbonylated proteins in salt-stressed *Arabidopsis*. Using lipid peroxides, they found 17 proteins with twice as many modifications in the stressed versus the non-stressed plants and 22 additional proteins with increased hydrazine derivatization in the stressed plants. Zhang et al. (204) combined biotin hydrazide-labeled chromatography with the SWATH method to analyze protein carbonylation in rice embryos during germination. In total, 288 carbonylated peptides corresponding to 144 proteins were determined; 66 had altered carbonylation intensity over four stages of germination. To our knowledge, this is the first study to combine en masse enrichment of carbonylated proteins with oxidized peptide identification and quantitation in plants.

## Sulfhydryl Oxidation

The sulfhydryl group in Met and Cys can be oxidized across the oxidation states of sulfur, leading to reversible and irreversible PTMs. Rates of Cys and Met oxidation are not random within proteins but depend on protein structure and neighboring residues and the pH of their surroundings (5, 184). Despite prolonged interest in detection of disulfide bonds (122), the recent focus of sulfhydryl PTM analysis in plants has been on the reversible oxidative modifications of single Cys and Met, which may be the first oxidative events impacting protein activity (**Figure 3**). In Cys, the oxidation product is sulfenic acid, and not the higher-order sulfinic acid or sulfonic acid forms (**Figure 3**). In Met, the oxidation product is Met sulfoxide. Various proteomics approaches have been used to identify and quantify these species because specific antibodies for affinity enrichment are not available (5, 184). In alkylation-based sulfhydryl proteomics, reversible Cys oxidative modifications are inferred by the properties of different reducing agents. However, given uncertainty about this process and its specificity, direct methods have now been developed. Based on a genetic chimeric construct that traps sulfenic acid into a disulfide and enables enrichment with a TAP tag, one approach has identified 100 *Arabidopsis* proteins containing sulfenic acid (183). Based on sulfenic acid selectively reacting with dimedone in click chemistry functionalized analogs to trap and enrich peptides containing sulfenic acid, another approach enabled identification of 226 sulfenylated proteins after $H_2O_2$ treatment of *Arabidopsis* cells (4). Oxidation of Met to Met sulfoxide can be reduced in plants by Met sulfoxide reductases (MSRs) repairing Met-oxidized proteins and enhancing seed longevity (27). Jacques et al. (83) used diagonal peptide chromatography and the specificity of Met sulfoxide reductases to isolate peptides carrying Met residues oxidized in vivo that were subsequently analyzed by tandem mass spectrometry. This allowed the identification of the 500 sites of Met oxidation on 400 proteins induced by $H_2O_2$ in a catalase mutant in *Arabidopsis*.

## Examples of Oxidation Impacting Protein Function and Regulation

Changes in carbonylation have been observed in transition to flowering; dormancy, germination, and aging of plant seeds; and in plant stress responses (86, 110, 156). Yin et al. (198) studied the transition from the plateau phase to the rapid decreasing phase of seed aging in rice. They also showed the abundance of 112 proteins and how the carbonylation levels of 68 of these proteins markedly changed, indicating oxidative damage, just at the point where the germination rate of

seeds decreased by 20%. Satour et al. (147) studied seed maturation in *Medicago truncatula* and identified several proteins whose extent of carbonylation varied during impairment of seed quality. They focused on a cellulose synthase (PM34), which exhibits high sensitivity to carbonylation and catalyzes a process required for germination and subsequent seedling growth. Met oxidation makes the amino acids more hydrophilic, and this property change alters Ser phosphorylation of nitrate reductase (67) and results in a loss of chaperone-like activity of a chloroplast small heat shock protein upon oxidation of Met (66). Cys oxidation has a clear role in redox homeostasis in plant primary metabolism (e.g., Calvin cycle and glycolysis) (11, 33). At lower $H_2O_2$ levels, glutathione-S transferase Tau23 (GSTU23) forms Met sulfoxides, which interferes with both glutathione binding and catalytic activation of the enzyme; however, at higher $H_2O_2$ levels, a disulfide bond protects Cys and Met residues from oxidation. This provides an example of stress-induced catalytic function changes under oxidizing conditions that are regulated by Met sulfoxide reductases and glutaredoxin (167).

## MECHANISMS AND FUNCTIONS OF N-DEGRON PATHWAYS

The N-degron pathways (formerly named N-end rule pathways) (176) link a range of protein PTMs directly to the ubiquitin proteasome system (UPS). The pathways were originally identified in *Saccharomyces cerevisiae* (8), and versions have subsequently been found in plants. They define a set of processive rules that relate the stability of proteins to the identity of their amino-terminal (Nt) residues, showing that almost all residues can be destabilizing in a sequence context-dependent manner (56). Three pathways were originally proposed in nonplant systems [Arg/N-degron, Acetyl (Ac)/N-degron, and Pro/N-degron]; however, direct evidence for functional Ac/N-degron and Pro/N-degron pathways does not yet exist in plants. Destabilizing residues are exposed following nonprocessive endoprotease action on substrates that reveals a new Nt-residue, and Nt-Met can also be destabilizing depending on the sequence context (56). Amino-terminal destabilizing residues are defined as primary, secondary, or tertiary, depending on the combination of subsequent PTMs that lead to degradation through the ubiquitin proteasome system (**Figure 4**) (176). Primary destabilizing residues are recognized directly by N-recognin E3 ligases of the N-degron pathways that also require correct stereospecific context and a proximal Lys (for ubiquitin addition) to form a functional N-degron. Secondary destabilizing residues [glutamic acid (Glu), Asp, and oxidized Cys] are arginylated by arginyl transferases (ATEs) that produce Nt-Arg, a primary destabilizing residue (**Figure 4**). The tertiary destabilizing residues glutamine (Gln) and Asn are deamidated, respectively, by Nt-Gln (NTAQ1) and Nt-Asn (NTAN1) amidohydrolases to Glu and Asp. The tertiary destabilizing residue Cys is oxidized by molecular oxygen and nitric oxide (NO) that produce a bioisostere of Asp (99). Thus, multiple PTMs are required for ubiquitin proteasome system substrate degradation via N-degron pathways.

### Components of N-Degron Pathways in Plants

Plant components relating to N-degron pathways have been uncovered through forward and reverse genetic approaches (**Figure 4**). Researchers have identified two N-recognin E3 ligases, PROTEOLYSIS6 (PRT6) and PRT1, that respectively recognize basic and hydrophobic (aromatic) Nt-residues. PRT1 was cloned from *Arabidopsis* using a screen to identify genetic components responsible for degradation of phenylalanine (Phe)-DHFR (7, 137), and it encodes a protein unlike other N-recognins with two RING finger domains and one ZZ domain. PRT6 is a RING domain E3 ligase containing a UBR box that is required for interaction with Nt-primary destabilizing residues (54). It was identified via a forward genetic screen for hypersensitivity to seed

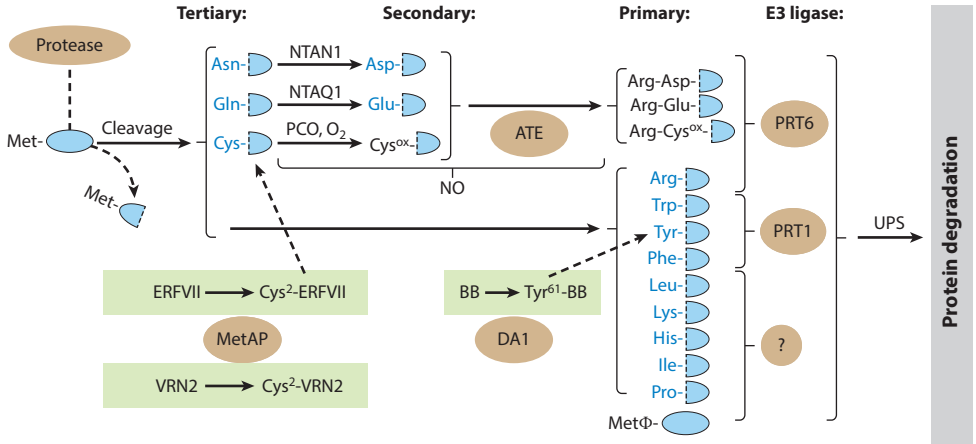


**Figure 4**

Posttranslational modifications are essential components of plant N-degron pathways. The hierarchical structure of the pathways is shown from initial exposure of a novel Nt-residue by protease action to eventual degradation through the UPS following ubiquitylation via E3 ligases. Known physiological protein substrates are shown in green boxes with the destabilizing residue and residue position indicated by dashed arrows and the associated protease shown in the brown ovals. Solid horizontal lines indicate the progressive processes in the degron pathways. Residues shown to be destabilizing in plants using artificial substrates are shown in blue (54, 62). The position of NO in Nt-Cys oxidation is not yet clarified in vivo (187). The identity of N-recognins recognizing a variety of proven destabilizing residues remains to be resolved (*brown circle question mark*). Primary, secondary, and tertiary destabilizing residues are indicated. Blue ovals and semi-ovals represent proteins. Residues are indicated by three-letter codes. Abbreviations: Φ, hydrophobic residue; Arg, arginine; Asn, asparagine; Asp, aspartic acid; ATE, arginyl transferase; BB, BIG BROTHER; Cys, cysteine; ERFVII, ETHYLENE RESPONSE FACTOR VII; Gln, glutamine; Glu, glutamic acid; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; MetAP, methionine aminopeptidase; NO, nitric oxide; Nt, amino-terminal; NTAN, amino-terminal asparagine amidohydrolase; NTAQ, amino-terminal glutamine amidohydrolase; OX, sulfinic acid; PCO, PLANT CYSTEINE OXIDASE; Phe, phenylalanine; Pro, proline; PRT, PROTEOLYSIS; Trp, tryptophan; Tyr, tyrosine; UPS, ubiquitin proteasome system; VRN2, VERNALIZATION2.

germination (76) and via reverse genetic approaches through sequence homology (54, 63, 180). In animal and yeast systems, primary destabilizing residues are all recognized by sequence-similar UBR1-type N-recognins (176). Because plants contain at least two different N-recognins recognizing different primary destabilizing residues, we suggest that the plant pathways should be named based on the N-recognin to describe the separate branches. Thus, primary destabilizing residues recognized by PRT6 would be part of the PRT6 N-degron pathway, and those recognized by PRT1 would be part of the PRT1 N-degron pathway. Arginyl transferase was originally identified in a genetic screen for delayed leaf senescence (199). Proteins that have sequence similarity to NTAQ1 and NTAN1 are encoded by plant genomes; for example, *Arabidopsis* encodes a putative NTAN1 (AT2G44420) and NTAQ1 (AT2G41760) (62). Recently, plant-specific enzyme components [PLANT CYSTEINE OXIDASES (PCOs)] that oxidize Nt-Cys to Cys sulfinic acid were identified (186). Analysis of the function of complete sets of destabilizing residues using artificial substrates in transgenic plants showed that all branches of the pathway operate to control protein stability in *Arabidopsis* (62). In addition to *Arabidopsis*, N-degron pathways were experimentally shown to have a role in barley (*Hordeum vulgare*) (118) and the bryophyte *Physcomitrella patens* (150). Plant proteins with similarity to Ac/N-recognins have also been identified (205). A recent

report showed that acetylation via the NatA complex destabilized SUPPRESSOR OF NPR1, CONSTITUTIVE 1 (SNC1) in *Arabidopsis* (190), suggesting that this pathway exists in plants.

## Physiological Roles in Growth and Development

Many physiological roles have been identified for the PRT6 N-degron pathway in plant growth and development and in response to biotic and abiotic stresses. Genetic analyses showed that in *Arabidopsis* the pathway exerts a major influence on the regulation of seed germination and seedling establishment (57, 76, 203) and also plays a role in the regulation of leaf development and senescence (63), flowering time, and root growth (179). The pathway acts as a sensor for molecular oxygen, thereby enhancing plant response to hypoxia (low oxygen), and also for NO, both through the Cys branch (**Figure 3**) (57, 58, 105). This pathway branch controls responses to drought and salinity in both *Arabidopsis* and barley (179). Different branches of the pathway also play roles in plant responses to biotic stresses (31, 64) that include a role for NTAQ1 as a component of the plant immune system (180).

## Discovery of Physiological Substrates in Plants and Use of N-Degron Pathways

Although identification of components of N-degron pathways through sequence homology is relatively facile, identification of physiological substrates is not, because there is no a priori knowledge of the substrate and protease combination or of the site of protease cleavage and identity of the revealed Nt-residue. This accounts for the fact that very few N-degron pathway substrates have been identified in any system. Proteomics analyses of N-degron pathway mutants in *Arabidopsis* have revealed regulated protein landscapes but not substrates (177, 202). Nevertheless, three substrates have been identified in plants (**Figure 4**). The group VII ETHYLENE RESPONSE FACTOR (ERFVII) transcription factors are physiological substrates of the Cys branch of the PRT6 N-degron pathway, and their stability is directly controlled by oxygen and NO (57, 58, 105). Through this regulation, these factors control a variety of responses to oxygen [including flooding/hypoxia tolerance (58, 105), photomorphogenesis (1), and biotic stress (64)] and to NO [including seed germination and stomatal aperture (57) and response to abiotic stresses such as salinity (179)]. The Polycomb Repressive Complex 2 component VERNALIZATION (VRN)2 was also shown to be a substrate of the Cys branch, and VRN2 was shown to enhance tolerance to water-logging and hypoxia stress (58a). ERFVIIs and VRN2 are exposed as oxygen/NO conditional substrates following cotranslational cleavage of Nt-Met by MetAP activity that reveals a Cys-2 residue. Recently, the E3 ligase BIG BROTHER (BB) was shown to be a substrate of the PRT1 N-degron pathway following cleavage by the ubiquitin-activated peptidase DA1 to reveal Tyr-61 (37). Potential substrates of arginyl transferase were also identified in *P. patens* (75), though formal identification as physiological substrates in vivo is still required. Data from new proteomics approaches that are focused on identification of Nt-residues (as discussed in the section titled Methods for Characterizing and Predicting N-Terminal Protein Modifications) should greatly increase the number of potential substrates and aid chemical validation of Nt-destabilizing residues in vivo, especially when combined with assessment of protease substrates. For example, the published N-terminome associated with targets of *Arabidopsis* METACASPASE9 has revealed many neo-Nt-sequences with destabilizing residues (171).

Key questions remain regarding the function, spatial distribution, and potential use of plant N-degron pathways. The role of NO in oxidation of Nt-Cys has not been resolved, as PLANT CYSTEINE OXIDASE can conduct this reaction in vitro without NO (187). Does a chloroplast N-degron pathway exist, as suggested by analysis of stability of chloroplast-localized artificial

substrates (6)? Is there a functional Ac/N-degron pathway in plants (55, 106)? Finally, the pathways using the associated PTMs show great promise for biotechnological exploitation, including great potential to enhance plant responses to environmental stresses (118, 179); they could also be used with conditional N-degron systems for controlled protein expression (45).

## MODIFICATIONS BY SMALL PROTEINS FOR CHANGES IN FUNCTION AND DEGRADATION

A group of small proteins including ubiquitin, related to ubiquitin1 (Rub1), and small ubiquitin-related modifier (SUMO) can be covalently attached to substrate proteins in plants (**Table 1**). For each of these modifiers, different substrates and specific functions have been documented. The basic biochemistry of protein modifiers requires their C terminus to protrude from the compact body of the protein. The C-terminal Gly (in most cases, part of a di-Gly motif) becomes linked to a Cys residue of the activating enzyme to form a thioester. Transfer to substrates requires an intermediary protein termed conjugating enzyme that carries the modifier in a thioester bond. Substrates are selected by proteins of the ligase class, which bring substrate and conjugating enzyme into an optimal position for modifier transfer. ε-Amino groups of internal substrate Lys residues serve as attachment sites. In exceptional cases, an oxy-ester bond is formed with the OH group of Thr or Ser residues in the substrate protein, thus replacing the normal isopeptide bond to Lys (61). Enzymes of the conjugation cascade are specific for each modifier. Likewise, the removal of modifiers from substrates is accomplished by dedicated, modifier-specific proteases. Pathogen effectors can target modifier conjugation. Effectors can be modifier-specific proteases (e.g., SUMO proteases) (129), ligases (155), or inhibitors of modification enzymes (82, 131).

Modifier and substrate usually do not interact noncovalently—the ligase catalyzes the docking event. In contrast, an attached modifier generates a new interaction surface (or blocks previously accessible interaction surfaces) on the substrate. Correspondingly, ligases can be considered writers of a certain modification code, whereas cellular proteins with affinity to the new interaction surface are readers of the code, enabling cellular responses to modifications. For ubiquitin, researchers have described almost 20 different domains that bind to ubiquitin and can thus be part of reader proteins (34, 188). For SUMO, binding by readers is accomplished via flexible loops containing so-called SUMO interaction motifs (188).

### Detection of Modifier-Linked Substrates

Mass spectrometry approaches are instrumental in detecting modifier-substrate covalent complexes. Proteins modified by ubiquitin generate branched peptides in tryptic digests. The C-terminal end of ubiquitin is Arg-Gly-Gly. A tryptic digest leaves the Gly-Gly dipeptide attached to the ε-amino group of a substrate Lys residue. Because this modified Lys is not a site of tryptic cleavage, the result is a peptide with an internal Lys and a mass increased by the two branching Gly residues. Antibodies specific for the Gly-Gly dipeptide linked to a Lys residue have been successfully used for enrichment of branched peptides (94). Similarly, ubiquitin-binding domains can be used to enrich substrates decorated with ubiquitin chains (87, 92). Extension of the ubiquitin N terminus to include peptide tags for enrichment has also been used in many experimental settings. Most SUMO isoforms also end in a di-Gly motif, which is not preceded by a basic residue. However, the branch generated by trypsin on a substrate peptide is usually so long that it compromises detection based on mass spectrometry. To alleviate this problem, amino acid changes (introduction of Arg or Lys close to the C-terminal end) have been successfully applied (120). In this way, large

lists of experimentally determined SUMO- or ubiquitin-modified proteins have been published (**Table 1**); though by no means complete, they offer an insight into the substrate space.

## Specific Features of Different Modifiers

Ubiquitin conjugation utilizes a complex modifier system. With approximately 1,500 substrate selection components (ligases), the range of substrates is significant. Most pathway substrates obtain a chain of ubiquitin moieties and are targeted for proteolytic destruction by the proteasome. Fewer ubiquitylation substrates follow other routes. In particular, single ubiquitin attachment to histones H2A and H2B mediates epigenetic switches and is part of transcription regulation (47). Mono-ubiquitylation also participates in membrane protein endocytosis (39) and enzyme regulation (172). Ubiquitin chains linked via ubiquitin Lys 63 are more akin to reversible modifier action. Lys 63 chain formation occurs as part of DNA repair pathways and of membrane protein quality control, where it can lead to vacuolar targeting of endocytosed plasma membrane proteins (142, 164). All signal transduction pathways with sufficiently described chains of events contain at least one step of ubiquitin-dependent proteolysis (153). This pertains in particular to hormone action. Auxin acts as a glue to increase affinity of AUX/IAA corepressor proteins to the cullin-based ubiquitin ligase $SCF^{Tir1}$ (102, 146). After breakthrough work elucidating this mechanism, a similar hormone-triggered degradation route was discovered for jasmonic acid (40, 79). Critical degradation steps have also been found for gibberellin (182) and salicylic acid (194) action.

The major, if not the only, function of Rub1 is the regulation of ubiquitin ligases containing the cullin scaffold subunit (cullin1, -3a, -3b, or -4). Conjugation and removal of Rub1 is part of an assembly-disassembly cycle for these multisubunit complexes that ensures dynamic integration of unassembled substrate recognition components into active ligases (111). Some mutants in the Rub1 conjugation pathway display an auxin-resistant phenotype (the *aux1* mutation affects the Rub1 activating enzyme), explained by the central role of $SCF^{Tir1}$ in auxin response.

Sumoylation is accomplished by a surprisingly small number of enzymes (126, 178). Only two SUMO ligases have been described, and the SUMO-conjugating enzyme SCE can act as ligase to link SUMO to ΨKxE peptide motifs (where Ψ is a hydrophobic amino acid, K is the modified Lys residue, x is any amino acid, and E is Glu). A significant number of SUMO-specific proteases may influence the abundance of sumoylated substrates and thereby regulate substrate specificity (196). SUMO modification often occurs in the form of group modification, meaning that protein assemblages are modified by the addition of single SUMO moieties to several subunits or sites (85). Nonetheless, SUMO chains can also be formed (166) and may be attached to a specific subset of sumoylation substrates (165). Another specific feature of sumoylation is the existence of species-specific noncanonical SUMO variants with specialized functionality, next to standard SUMO (28, 173). The vast majority of sumoylated proteins reside in the nucleus, but cytoplasmic substrates have been identified as well.

SUMO conjugation is essential and acts primarily by providing a new interaction surface on a protein. It has been linked to stress responses in multiple ways. Characteristically, mutants in SUMO ligase SIZ1 show changes in response to drought, cold, and salt stress, but they also display developmental phenotypes including reduced growth (25, 178). In addition, there is a strong linkage to pathogen defense. *siz1* mutants have increased salicylic acid content, which significantly contributes to the reduced-growth phenotype. Furthermore, the noncanonical SUMO isoform SUMO3 of *Arabidopsis* is pathogen induced and plays a role in defense induction in plants (173).

## INTERACTIONS BETWEEN POSTTRANSLATIONAL MODIFICATIONS BUILDING NETWORKS OF COMPLEXITY

Most systematic studies of PTMs by mass spectrometry consider the role of single modification types and provide detailed information of the altered sites in peptide sequences (**Table 1**). Comparatively few studies consider either the evidence for multiple PTMs on a given peptide or protein or the functional implications of such interactions. Assembling the available data for *Arabidopsis* (21,527 nonredundant claims of protein modifications for 11,575 nonredundant proteins) (**Table 1**; **Supplemental Data 1**) for the five classes of PTMs discussed in this review provides several insights. First, most PTMs have been reported in all of the ten major compartments of the plant cell (**Figure 5**). SUMO/ubiquitylation and phosphorylation predominate in the cytosol and in the nucleus, oxidation is focused on the cytosol and plastid, and glycosylation predominates in the plasma membrane and secreted protein sets. Second, experimental reports indicate that more than 800 proteins are modified by at least three of the five classes and more than 100 by four of the five classes (**Figure 6**; **Supplemental Data 1**). Central regulators of assimilation

Supplemental Material >

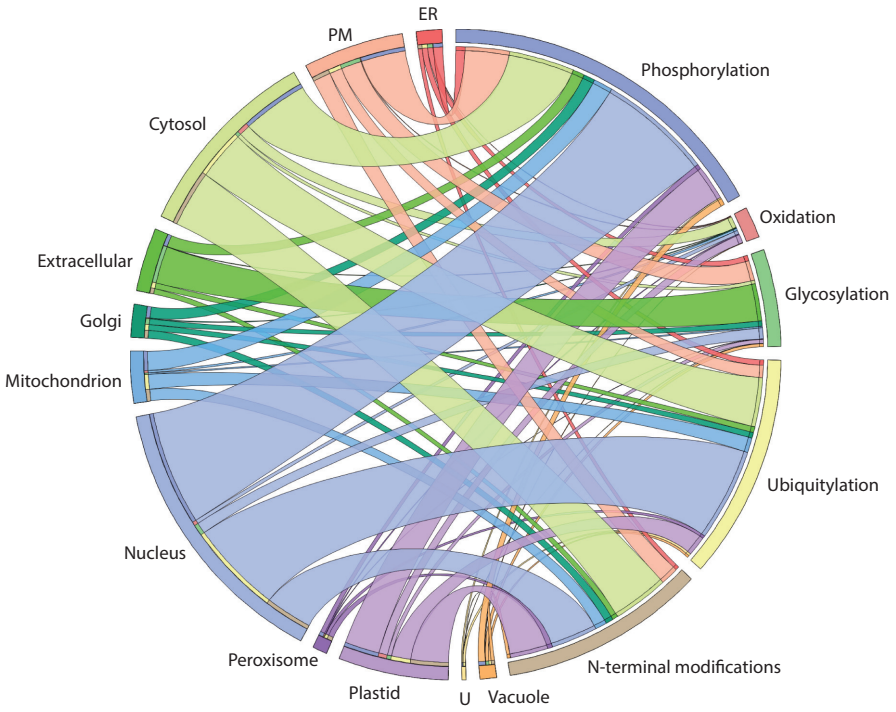


**Figure 5**

Subcellular locations of PTMs based on location of proteins. Experimental reports of PTMs on peptides for proteins in *Arabidopsis* were collated and reported on the basis of a single subcellular location from SUBAcon (77) using Circos (98) for visualization. Phosphorylation contains serine, threonine, and tyrosine phosphorylations; N-terminal modifications contain *N*-myristoylation, *N*-α-acetylation, N-terminal methionine excision, and N-terminal-cleavage sets; glycosylation contains *O*-linked acetylglucosamine, *N*-linked and *O*-linked glycosylation, and GPI anchors; oxidation contains carbonylation as well as $Met^{OX}$ and $Cys^{OX}$ modifications; and ubiquitylation contains ubiquitin and SUMO modifications as shown in **Table 1**. Abbreviations: ER, endoplasmic reticulum; GPI, glycosylphosphatidylinositol; OX, sulfinic acid; PM, plasma membrane; PTM, posttranslational modification; SUMO, small ubiquitin-related modifier; U, unknown location.

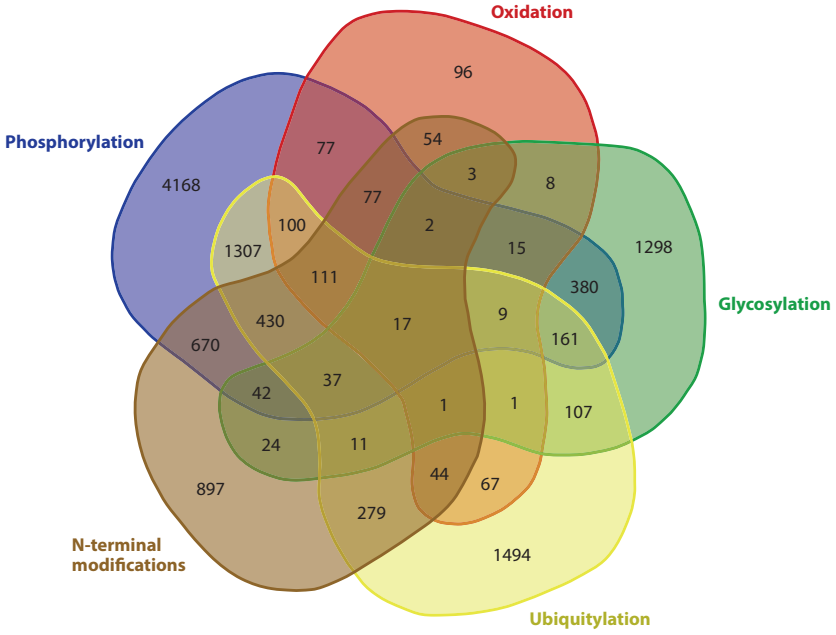


**Figure 6**

Multiple posttranslational modifications (PTMs) on *Arabidopsis* proteins. Venn diagram of experimental reports of PTMs on peptides for proteins in *Arabidopsis* were collated and reported across the five classes discussed in this review. Phosphorylation contains serine, threonine, and tyrosine phosphorylations; N-terminal modifications contain *N*-myristoylation, *N*-α-acetylation, N-terminal methionine excision, and N-terminal-cleavage sets; glycosylation contains *O*-linked acetylglucosamine, *N*-linked and *O*-linked glycosylation, and glycosylphosphatidylinositol (GPI) anchors; oxidation contains carbonylation as well as $Met^{OX}$ and $Cys^{OX}$ modifications; and ubiquitylation contains ubiquitin and small ubiquitin-related modifier (SUMO) modifications as shown in **Table 1**. Generated using **http://bioinformatics.psb.ugent.be/webtools/Venn/**.

are among the highly modified proteins (e.g., Rubisco, nitrate reductase), whereas MAPMAN annotation (163) classes of glycolysis, TCA cycle, photosynthesis, and N-metabolism are significantly overrepresented among the multiply modified proteins (**Supplemental Data 1**). There is emerging evidence that modification at one site can depend on another modification on neighboring residues. For example, $Met^{ox}$ can inhibit neighboring phosphorylation due to oxidative damage (67), ROS-induced carbonylation and reactive nitrogen species–induced protein nitrosylation interact in biotic and abiotic stress (110), and there is crosstalk between SUMOylation and phosphorylation in nutrient acquisition, signaling, and plant growth (127). The wider potential for such interactions between different PTMs is also suggested from evidence for nonrandom distribution of modification sites (96).

Supplemental Material >

## FUTURE PERSPECTIVES ON POSTTRANSLATIONAL MODIFICATION DISCOVERY AND FUNCTION

There will be an exponential expansion of knowledge of PTM-regulated sites in plant proteomes from large-scale discovery studies and quantitation of PTM sites under different external and internal stimuli. Conditional dependence of PTMs is widely documented, and many conditions will need to be assessed to define the PTM landscape in plants. The timing of PTMs during the life

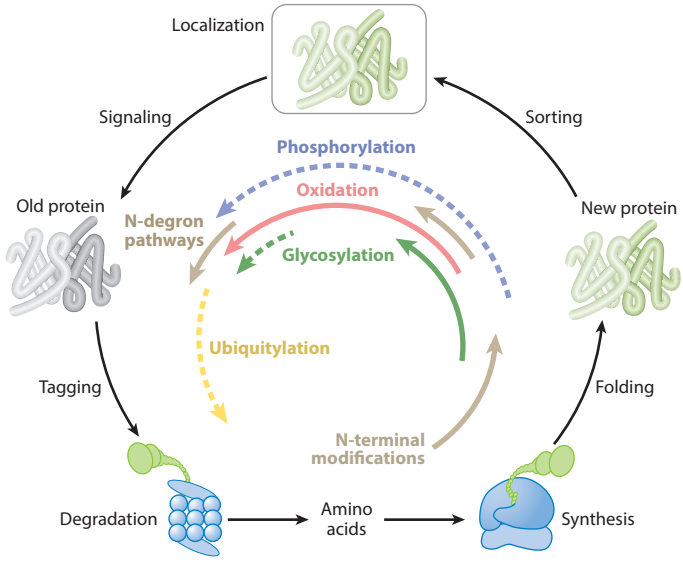


**Figure 7**

Life cycle of protein modification. Posttranslational modifications (PTMs) shown with reference to the time of modification during synthesis, folding, sorting, localization, signaling, tagging, and degradation of proteins over their lifetimes. Phosphorylation contains serine, threonine, and tyrosine phosphorylations; N-terminal modifications contain *N*-myristoylation, *N*-α-acetylation, N-terminal methionine excision, and N-terminal-cleavage (during sorting) sets; glycosylation contains *N*-linked and *O*-linked glycosylation, glycosylphosphatidylinositol (GPI) anchors, and *O*-linked sugars (during signaling); oxidation contains carbonylation as well as $Met^{OX}$ and $Cys^{OX}$ modifications; and ubiquitylation contains ubiquitin and small ubiquitin-related modifier (SUMO) modifications as shown in **Table 1**. Colors are as shown in **Figures 5** and **6** for each PTM class; dotted lines indicate reversible modifications.

cycle of proteins (**Figure 7**) and how all PTMs influence the half-life of proteins also need to be considered (104). Mass spectrometry studies in plants already document large numbers of unidentifiable peptide spectra, which are due, in part, to the presence of unknown PTMs. Grouping and analysis of these spectra will enable new classes of PTMs to be discovered in plants.

PTM discoveries in plants need to be coupled to detailed functional studies through site mutations. Functional validation is experimentally much more difficult and much slower than large-scale detection and quantitation of the PTM sites. Furthermore, PTMs can result in either activation or inactivation of functions and can also have localization effects, so the functional relevance of each event cannot directly be inferred from large-scale quantitative PTM data sets. Divergence of PTM identification rates and their functional analysis is set to widen considerably unless many more researchers begin to study PTMs through both enzymology and genetics.

Understanding both the degree to which a certain protein pool is modified by a PTM and what level of modification is needed to produce a biological effect are key technical challenges requiring systematic and large-scale detection of PTM stoichiometry. Technically, capturing site occupancy on large-scale data is possible, for example, in phosphorylation (128), but it requires comparison between extended sample-analysis times for enriched PTM-containing peptide samples and nonenriched total proteins. In many cases, synthetic modified and unmodified peptides are required as standards to define stoichiometry.

Most current plant PTM data sets identify modified peptides while losing the context of the protein molecules from which they were derived. How multiple PTMs are arranged on a

single protein molecule can be studied via intact protein mass spectrometry and top-down analysis of PTMs using high-resolution mass spectrometry. This will define the true number of proteoforms (157) in plants and allow PTM interactions to be studied in detail. The most conservative estimate based on current knowledge of the five major classes of PTMs discussed (**Table 1**) is that more than 100,000 proteoforms exist with various combinations of modifications. However, on the basis of additional PTMs not reviewed here (such as methylation, acetylation, succinylation, and *S*-nitrosylation), the prospect of multiple modifications by the same PTM to a protein, and the timing of PTMs during the lifetime of proteins, we conservatively estimate more than 600,0000 proteoforms are likely to exist in *Arabidopsis*—20 times the number of protein coding genes.

### SUMMARY POINTS

1. Posttranslational modifications (PTMs) occur on amino acid side chains or at a protein's C or N termini and extend the chemical decoration and properties of the 20 standard amino acids by modifying existing functional groups, introducing new ones, or exposing new termini through polypeptide cleavage.
2. PTMs occur over the lifetime of most protein molecules, with various PTMs involved in their synthesis, assembly, localization, function, and degradation.
3. PTMs occur in most subcellular compartments through the targeting of isoforms of modifying enzymes and their targets throughout the cell.
4. PTMs can result in either activation or inactivation of functions and can also have localization effects, so the functional relevance of each event cannot be inferred directly from large-scale quantitative PTM data sets.
5. Disruption of PTMs has numerous consequences for many aspects of plant growth, development, and response to the environment, showing their importance for our understanding of the link between the proteome and the phenome in plants.

## DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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