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Review

Post-translation modification of proteins; methodologies and applications in plant sciences

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ABSTRACT

Proteins have the potential to undergo a variety of post-translational modifications and the different methods available to study these cellular processes has advanced rapidly with the continuing development of proteomic technologies. In this review we aim to detail five major post-translational modifications (phosphorylation, glycosylaion, lipid modification, ubiquitination and redox-related modifications), elaborate on the techniques that have been developed for their analysis and briefly discuss the study of these modifications in selected areas of plant science.

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1. Introduction

The range of protocols available to researchers studying the cellular proteome is wide and covers biochemical and cell biology analyses, the application of selective affinity materials and, increasingly, the utilisation of mass spectrometric analysis. The role of mass spectrometry (MS) in proteomic analysis has developed rapidly since early reports, expanding beyond mere protein identification via database MS/MS derived fragment ion matching to the study of variations in expression levels, the study of protein–protein interactions, the study of DNA–protein cross links and the selective study of proteins which have undergone specific post-translational modification. Whilst the study of differentially expressed proteins offers a significant insight into cellular responses to a variety of stimuli or changes in environmental conditions, it is increasingly recognised that many physiological responses, as represented in the proteome, occur via differential modification of the proteins rather than an alteration in their expression levels. Of the post-translational modifications, protein phosphorylation was one of the first to be studied as a major field within proteomic analysis as phosphorylation of proteins is a common mechanism for altering the cellular activity of the protein. More recently, further protocols have been developed in order to study other modification types which also have a significant impact on cellular activity. Whilst much of this research has developed primarily from mammalian cellular research laboratories, it is increasingly finding applications in plant proteomic studies. The review will not discuss in any detail the study of expression levels of modified proteins and the role of mass spectrometry in protein identification which have been discussed in previous review articles (Newton et al., 2004; Pan et al., 2009). Rather, the aim of this review is to describe protocols available for the study of specific post-translation modifications of proteins both as part of a global proteome analysis and as the selective study of the degree of modification of specific proteins or sub-proteomes. The protein modifications under review are phosphorylation, glycosylation, ubiquitination, prenylation/farnesylation and the specific study of the redox proteome (proteins altered by reactive oxygen species). As shown in Table 1 and Fig. 1a and b, more than 75% of the available literature is concerned with phosphorylation as a post-translational modification (the majority of which regarding mammalian proteomics). Fig. 1b indicates that within plant science phosphorylation is still the most prominently studied post-translational modification followed by glycosylation and ubiquitination, whilst Table 1 highlights the number of manuscripts in each area for different organisms (obtained via entering the organism name, modification type as topics covering the years 2008–2010 in web of science). For each of these modifications, the technologies available (encompassing two-dimensional electrophoresis, immunological protocols, chemical derivatisation methods and mass spectrometric analyses) will be described and selected examples where such analysis have been applied to plant proteomic investigations will be discussed.

Table 1
Bibliometric analysis of the scientific literature (2008–2010) based on topic references of "proteomics", the species/organisms under study and each post-translational modification (PTM).

PTM	Organism	No. papers
Phosphorylation	Mammalian	2277
	Yeast	1122
	Fungi	53
	Bacteria	302
	Plant	525
	Arabidopsis	460
	Tobacco	96
Glycosylation	Mammalian	285
	Yeast	231
	Fungi	36
	Bacteria	121
	Plant	129
	Arabidopsis	77
	Tobacco	42
Prenylation	Mammalian	22
	Yeast	9
	Fungi	3
	Bacteria	3
	Plant	7
	Arabidopsis	8
	Tobacco	0
Redox proteome	Mammalian	7
	Yeast	9
	Fungi	2
	Bacteria	5
	Plant	20
	Arabidopsis	22
	Tobacco	3
Ubiquitination	Mammalian	174
	Yeast	213
	Fungi	6
	Bacteria	16
	Plant	69
	Arabidopsis	75
	Tobacco	7

2. Phosphorylated proteins

2.1. The phosphoproteome

Phosphorylation of proteins is a key regulator of intra-cellular biological processes and is a reversible modification affecting both the folding (conformation) and function of proteins and regulating enzymic activities, substrate specificities and protein localisation, complex formation and degradation (Thingholm et al., 2009; Reinders and Sickmann, 2005). It has a major influence on various cell functions for example, signal transduction, homeostasis, proliferation, cell differentiation, metabolic maintenance and cell division. The relevance of phosphorylation is highlighted by the number of genes involved in phosphorylation processes. Regulation of this modification is tightly controlled by two enzymes, protein kinases (attaching of phosphate groups to amino acid residues) and phosphatases (removing of phosphate groups). The variety of functions phosphorylated proteins are involved in depends on the number (multiple residues within a protein can be phosphorylated by different kinases) and site of phosphorylation. Multiple sites of phosphorylation allow a protein to adapt several different functions, depending on which phosphorylation site is modified. This is highlighted by phosphorylation at one particular amino acid residue which may lead to conformational change that in turn allows for the phosphorylation of different amino acid residues within the same protein, or on the other hand, prevent the phosphorylation of nearby amino acids through sterical hinderance (Paradela and Albar, 2008). The four most common types of phosphorylation occurring on amino acids are: O-phosphates, N-phosphates, S-phosphates and acyl-phosphates. O-phosphates are the most

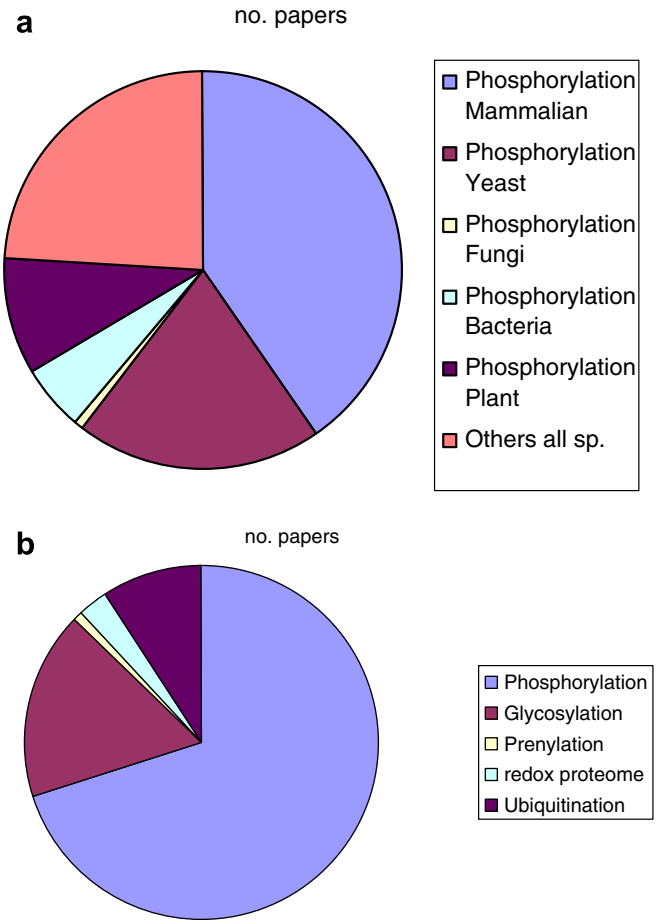

Fig. 1. (a) A summary of papers published (2008–2010) comparing phosphorylation in different species/organisms and all other modifications in all organisms. (b) A comparison of manuscripts published (2008–2010) for each modification in plants.

common class and they are mostly commonly attached to serine (Ser)-, threonine (Thr)- and tyrosine (Tyr)-residues. The occurrence of phosphorylation on Ser- and Thr-residues is more frequent than on Tyr-residues. N-, S- and acyl phosphorylations are far less common. N-phosphates are attached to hisitidine and lysine residues, S-phosphates are attached to cysteine (Cys) residues and acyl-phosphates are mostly on aspartic (asp) and glutamic (glu) residues (Sickmann and Meyer, 2001). However, phosphoproteins are generally present at relatively low abundance within the cell and most proteins are only transiently phosphorylated following their key cellular signals making detection and analysis a challenge.

2.2. Methods of phosphoprotein analysis

Several reviews have reported a variety of techniques and methodologies for phosphoprotein analysis (Reinders and Sickmann, 2005; Thingholm et al., 2009; Paradela and Albar, 2008; Parker et al., 2010). An overview of contemporary techniques is covered in this review. A comprehensive study of protein phosphorylation should include the identification of phosphoproteins and sites of phosphorylation, the identification of the proteins (kinases and phosphatases) involved in the phosphorylation process and a description of the biological events following on from the phosphorylation of the protein. The isolation of proteins from complex cell mixtures is a critical step. Upon lysis of a cell, proteases and phosphatases are released/activated, leading to the degradation of proteins and the phosphate groups on phosphoproteins being lost. Sample preparation should be conducted at 4 °C to minimise such enzymic activity, and protease and phosphatase inhibitor cocktails are required during sample solubilisation steps (Reinders and Sickmann, 2005). The choice of phosphatase inhibitor cocktail is important as each will have specific enzymic reactions; some cocktails may only inhibit the removal of phosphate groups from Ser/Thr-residues but allow the removal of phosphate groups from Tyr-residues. Protein kinases also need to be inhibited in order to identify phosphorylation sites of biological relevance. This is normally done by the addition of EDTA, EGTA or specific kinase inhibitors to the buffers. It is also important to consider how the buffers and reagents used during these initial sample preparation steps will affect subsequent analytical protocols (Hemmings, 1996). There are several methods for detecting phosphoproteins. Highly complex protein samples can be separated according to their p*I* and molecular weight using 2D gel electrophoresis (2D-PAGE). Phosphoproteins can be either directly visualised (Patton, 2002) on the gel using phosphospecific stains (Cutting and Roth, 1973) or by western blotting techniques (Kauffman et al., 2001). Commercially available phospho-stains are less sensitive but their use is more convenient when compared to techniques such as radiolabelling. The stains are mainly specific for certain types of phosphorylation such as O-phosphorylations. Other phosphospecific stains such as the fluorescent Pro-Q DPS or Pro-Q Diamond (Steinberg et al., 2003; Chitteti and Peng, 2007) bind directly to the phosphate moiety of phosphoproteins with high quantitative linearity and the stain is compatible with other staining methods and subsequent mass spectrometry analysis. Stasyk et al. (2005) used this method in the analysis of the regulation of endosome-specific phosphoproteins from EpH4 mammary epithelial cells following stimulation by epidermal growth factor. Western blotting following 2D-PAGE, using phospho-specific antibodies is widely used (Kauffman et al., 2001) and is able to detect femtomole levels of phosphoproteins, however, specificity and sensitivity is dependent on the respective antibodies (Berwick and Tavare, 2004), and detection with such antibodies provides no information of specific phosphorylation sites. One of the most sensitive methods is to radiolabel the phosphate groups using ^{32}P or ^{33}P (either *in vivo* or *in vitro*) followed by radioimmunoblotting (Bendt et al., 2003). The major advantage to this radiolabelling technique is the ability to detect all the different types of phosphorylations and the signals can be quantitated, but ^{32}P is toxic for many cells and will, over time, cause damage to the cell.

One of the major problems with using 2D-gel electrophoresis quantitative phosphoproteomics is that only a relatively limited number of proteins are accessible for detection and identification. Large-scale profiling of phosphoproteins requires a considerable number of high resolution 2D-PAGE maps to achieve significant data which can be difficult to achieve, owing to the lack of reproducibility between gels and considerable amount of time each gel takes to run and process. Further analysis requires the protein spots of interest to be excised from the gels, destained, digested an desalted, prior to peptide analysis by MS for further validation. This is also problematic due to the presence of co-migrating proteins that can occur and give false-positive results. Given that 2D-gel electrophoresis can not provide evidence for the phosphorylation of distinct protein spot, specific enrichment of phosphorylated proteins and/or peptides was developed and is now the most widely used technique in phosphoproteomics (Reinders and Sickmann, 2005). Site-specific analysis of phospho-sites is usually performed by MS, requiring the phosphoprotein first to be cleaved enzymatically or chemically into peptides of a size suitable for sequence analysis. The detection of phosphopeptides by MS is usually hindered by suppression effects, so many different enrichment methods for separating phosphorylated from non-phosphorylated

proteins/peptides have been established depending on the sample type and study targets (Fig. 2). Commericial kits have made the enrichment process easy, fast and reproducible (Paradela and Albar, 2008), but it has been demonstrated that the different methods differ in specificity towards phosphoproteins and phosphopeptides isolated (Bodenmiller et al., 2007).

Immunoprecipitation can be used, binding of phospho-specific antibodies, such as anti-phosphotyrosine antibodies, to specific phosphorylated peptide sequences. Following immunoprecipitation the sample can be separated by SDS–PAGE and the protein bands of interest can be excised for MS analysis. Antibodies against phosphorylated Ser-, Thr- and Tyr-residues can be used for overall enrichment of phosphoproteins from a complex mixture, although this method is limited by the specificity of the antibodies (Gronborg et al., 2002). The enrichment of phosphopeptides utilising phospho-specific antibodies has also been reported by Rush et al. (2005), where they applied anti-phosphotyrosine antibodies for the enrichment of peptides containing phosphotyrosine residues. Immunoprecipitation of phosphotyrosine containing proteins is more widespread than that of phosphoserine or threonine (Gronborg et al., 2002), owing to the expensive mixtures of Ser- and Thr-antibodies and the better quality of Tyr-antibodies. Immobilised Metal-Ion Affinity Chromatography (IMAC) allows for the purification of phosphoproteins and peptides from complex mixtures by their affinity towards positively charged metal ions (Fe^{3+}, Al^{3+}, Ga^{3+} or Co^{2+}) chelated to a solid support. It (IMAC) is a widely used technique for the enrichment of phosphopeptides prior to MS analysis and its enrichment effect is demonstrated in Fig. 3 (Nuhse et al., 2003, 2007; Bond et al., 2007). The phosphorylated peptides and proteins are bound to the stationary phase by electrostatic interactions from their negatively charged phosphate group with the positively charged metal-ions that are bound to the column material via iminodiacetic acid (IDA), nitriloacetic acid (NTA) or Tris-(carboxymethyl)-ethylendiamine (TED) linkers. Non-phosphorylated species can be washed away and the phosphopeptides or proteins can be eluted by a salt- and/or pH gradients. A major problem of IMAC-based separations is the non-specific binding of peptides containing acidic amino acids such as Glu and Asp, and the strong binding of multiply phosphorylated peptides. IMAC experimental conditions are very variable, requiring great care as small variations in pH and/or ionic strength can affect the selectivity of the IMAC stationary phase. Development of this technique has lead to the sequential elution from IMAC or SIMAC protocol. This method allows the mono- and multi-phosphorylated peptides to be efficiently enriched from highly complex samples using both IMAC and other similar techniques such as titanium dioxide chromatography (TiO_2), thus allowing further separation than just IMAC alone (Thingholm et al., 2009). The widespread use of IMAC procedures is partially explained due to its compatibility with further separation and detection techniques – to high performance liquid chromatography (HPLC) – electrospray (ESI) MS or matrix assisted laser desorption ionisation (MALDI) time-of-flight (ToF) MS (Bond et al., 2007; Parker et al., 2010).

Titanium dioxide chromatography allows for the highly selective enrichment of phosphopeptides from complex samples by their affinity toward TiO_2-coated beads packed into a micro-column (Thingholm et al., 2008). TiO_2 has an affinity for phosphate ions in aqueous solutions based on the selective interaction of water-soluble phosphates with porous TiO_2 microspheres.

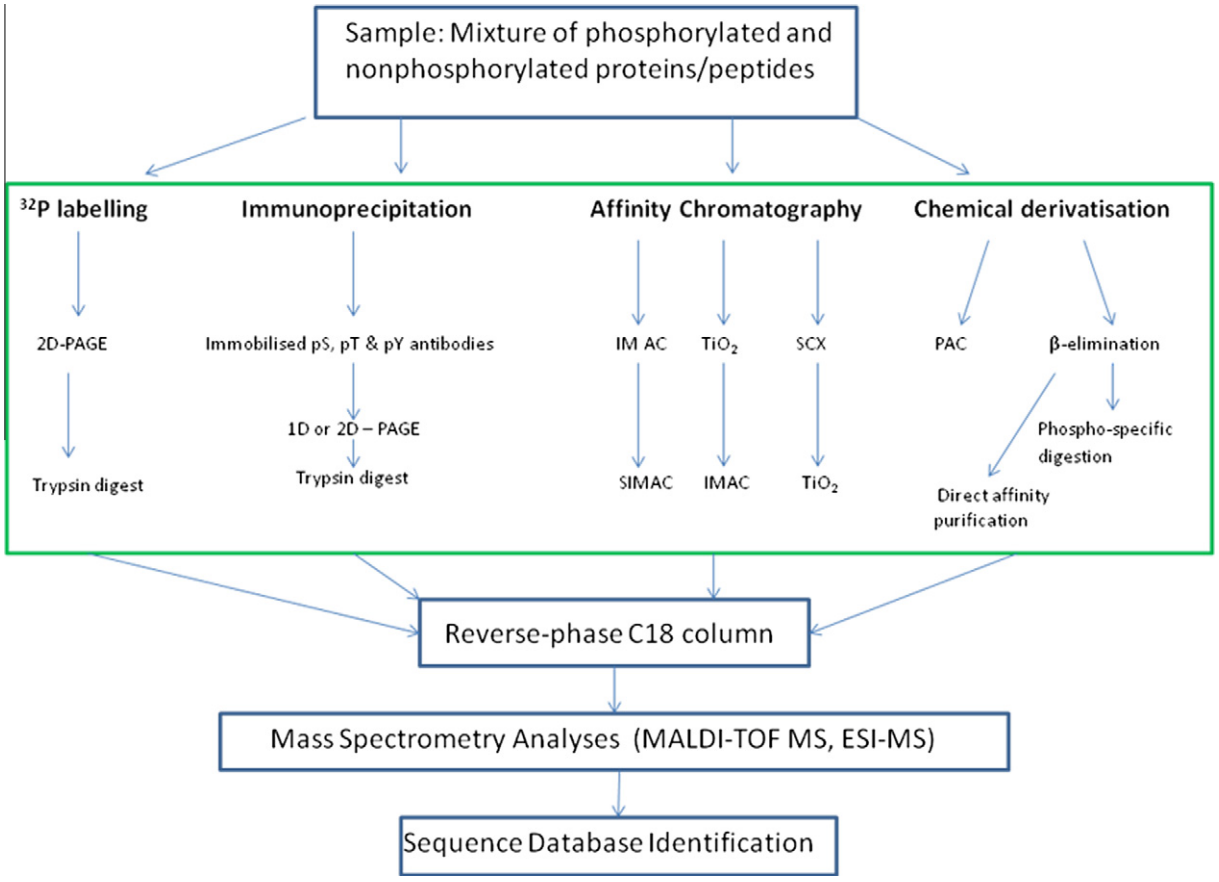

Fig. 2. Flowchart summarising various methods for phosphoprotein separation, detection and analysis.

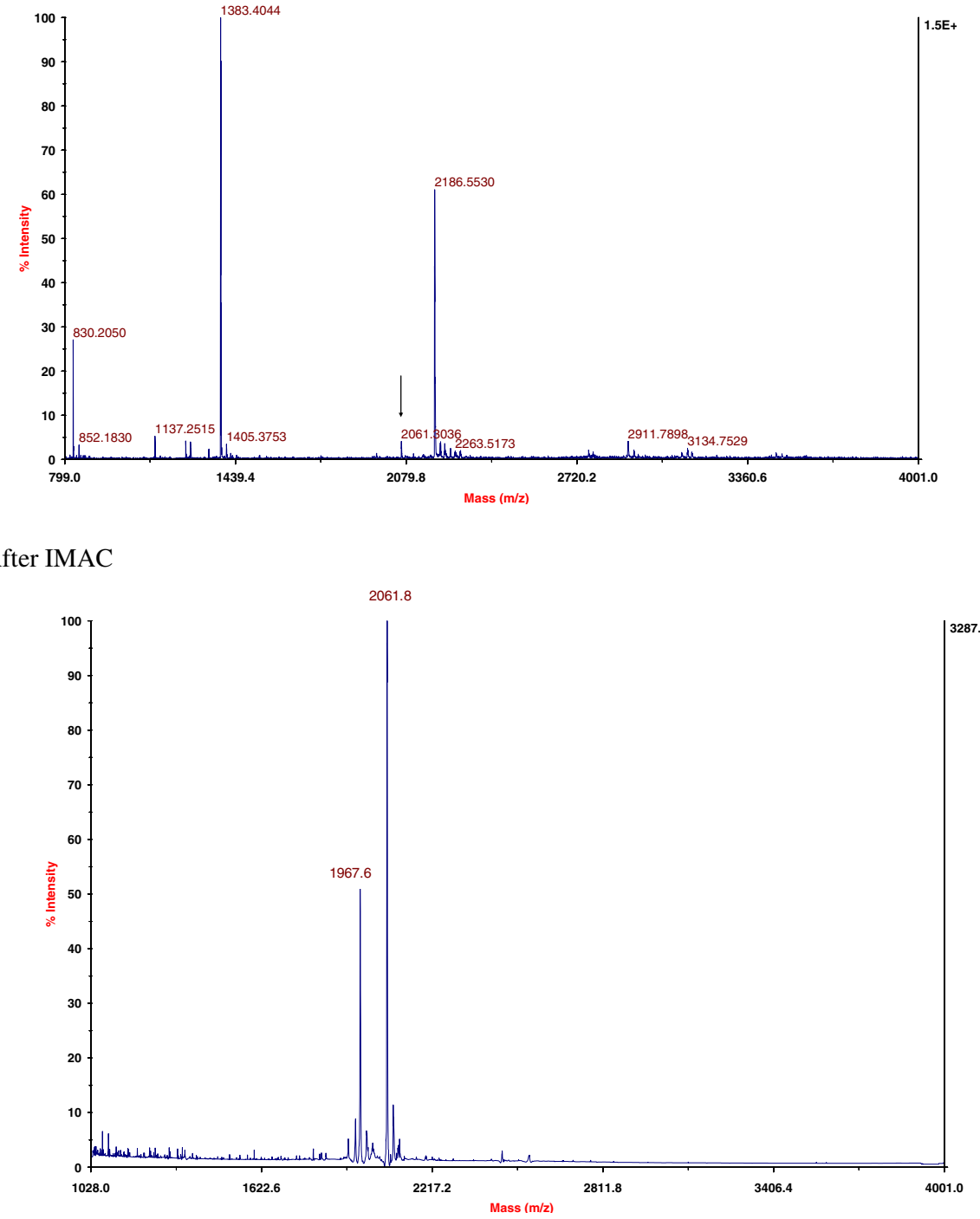

Fig. 3. MALDI spectrum of β-casein trypsin digested peptides mixture before and after IMAC Ziptip. With the amino acid sequence FQ*p*SEEQQQTEDELQDK. Arrow is phosphopeptide.

Phosphopeptides are trapped in a TiO_2 precolumn under acidic conditions and desorbed under alkaline conditions. Peptide loading in 2,5-dihydroxybenzoic acid (DHB) reduces the amount of non-phosphorylated peptides binding whilst retaining the high affinity for phosphorylated peptides (Larsen et al., 2005). This method has been found to be more selective than IMAC, and can be easily coupled with a LC–ESI-MS or an offline LC system followed by MALDI-ToF MS. Strong cation exchange (SCX) chromatography can also be used in the enrichment of phosphorylated peptides. The procedure utilises the fact that under acidic conditions (pH 2.7) tryptic phosphorylated peptides are singly positively charged (+1) and bind weakly to the cations in the SCX particles, they can be further separated from non-phosphorylated tryptic peptides that usually have a charge of +2 at a low pH. The main advantage to this technique is that complex tryptic mixtures can be analysed directly since the development of multidimensional protein identification technology (MuDPIT) LC protocols (Olsen et al., 2006 and Wolters et al., 2001). Hydrophilic Interaction Chromatography (HILIC) has been utilised as a prefractional stage of peptides prior to phosphopeptide enrichment such as IMAC. HILIC separates polar biomolecules (in an organic mobile phase) by their ability to hydrogen bond to a neutral, hydrophilic stationary phase. The

bonds can then be disrupted by decreasing the organic environment in the mobile phase and the peptides will subsequentially elute according to their polarities. In 2008, McNulty and Annan, showed that prefractionation using HILIC improved phosphopeptide selectivity of IMAC to more than 99%. HILIC was compared to SCX and IMAC as well as combined with IMAC for a more efficient method of phosphopeptide recovery McNulty and Annan (2008).

Chemical derivatisation can also be applied to phosphopeptide enrichment. The β-elimination with Michael addition reaction involves phosphoserine and phosphothreonine being converted into dehydroalanine and β-methylhydroalanine respectively by β-elimination. Subsequently this generates aminoethylcystiene from dehydroalanine by Michael addition (and β-methylaminoethylcystiene from β-methylethylalanine), which are isosteric with lysine and are used as targets for lysine specific digestion to aid the assignment of phosphorylation sites. This protocol allows phosphopeptide mapping by making phosphorylated threonine and serine residues recognisable by a protease (Oda et al., 2001). The disadvantages of β-elimination include the potential contamination from peptides with other modifications that can also undergo β-elimination (such as *O*-glycosylation) and the fact that derivatisation can not be used to investigate tyrosine phosphorylated peptides or the tagging of non-phosphorylated serine residues. Alternatively, Phosphoramidate Chemistry (PAC) is used to link phosphate groups to immobilised iodoacetyl groups for purification. Zhou and co-workers (2005) demonstrated that PAC is efficient for the isolation and identification of Ser-, Thr- and Tyr-phosphorylated proteins from highly complex samples by using a multi-step chemical modification method. The first step is to O-methyl-esterify the carboxyl groups on the peptides (to prevent their interaction in subsequent steps) and then to derivatise the phosphopeptides by a sulphydryl group which binds to and iodoacetyl group immobilised on a synthetic polymer (through phosphoamidate chemistry). The phosphate groups are reconstituted by acid hydrolysis, therefore allowing the identification of the phosphorylation sites by MS. Another method for phosphorylated protein analysis is COmbined FRActional DIagonal Chromatography or COFRADIC. This technique consists of two consecutive identical separations, with a modification step targeted to a subset of peptides between the two separations (Gevaert et al., 2005). In a complex mixture, the modified peptides obtain different chromatographic properties and separate from the bulk of un-modified peptides in the second run. Not only can COFRADIC be used for the global analysis of phosphopeptides, it can also be used to separate other types of peptides such as methionyl, cysteinyl and amino (N) terminal peptides. In the first or primary run, a peptide mixture is fractionated on a reverse-phase HPLC column. The fractions are then subjected to chemical or enzymatic modification to alter the column retention properties of a class of peptide present in each primary fraction. In the second run, these modified primary fractions are reloaded on the same column and the peptides shift out of the original collection interval and can be specifically isolated for further MS analysis (Gevaert and Vandekerckhove, 2004). The advantages of COFRADIC are its flexibility in selecting specific chemistries or enzymatic modifications, reproducibility and sensitivity. However, like other techniques investigating phosphopeptides by MS, the identification of a phosphoprotein from a complex mixture and from a single peptide sequence is still reliant on the peptide identification software as well as a high resolution separation of the peptide mixture prior to MS analysis (Gevaert et al., 2006, 2007). Most phosphorylation sites are identified by tandem MS and sequencing of the phosphopeptides by MS/MS following any of the separation techniques mentioned previously. Both MALDI- and ESI-sources can be used to perform the analysis

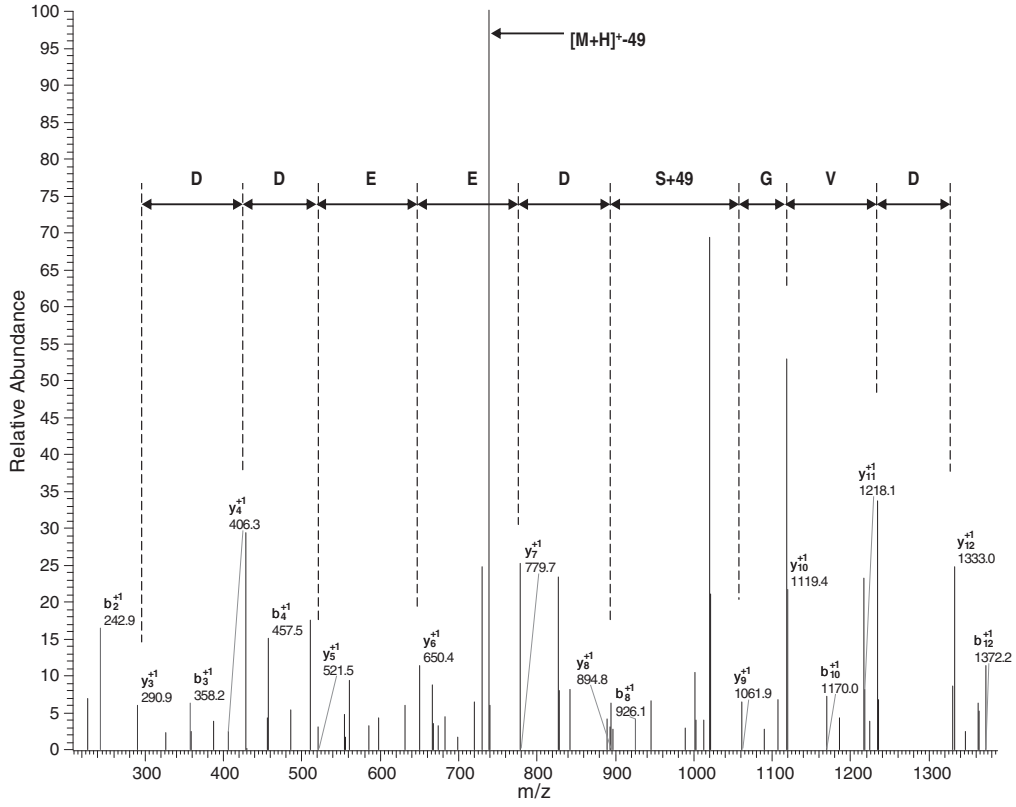

Fig. 4. MS/MS spectrum of doubly charged phosphopeptide with sequence K.IEDVGS*DEEDDSGK.D precursor ion 787.85 indicating the normally detected *neutral loss of 98 Da from phosphopeptide precursor ions (MH_2^{2+} −49) representing the loss of the phosphate group as a neutral moiety.

but usually localisation of the phosphorylation sites is conducted using double or triple charged ions in MS/MS mode produced by ES-ionisation. Using MALDI-TOF MS, serine- and threonine-phosphorylated peptides can be identified, as they tend to lose phosphoric acid ($-H_3PO_4$) under mass spectrometric conditions. Peptide ions fragmented by collision-induced dissociation (CID) ESI-MS produces a series of b and y ions, allowing the peptide sequence to be interpreted. Phosphorylation on Ser- and Thr-residues is often susceptible and conventional CID will typically result in the partial loss of phosphoric acid due to the gas-phase β-elimination of the phosphoester bond. Partial neutral losses are also observed for phosphotyrosine residues (−80 Da, HPO_3), however, the phosphate groups on tyrosine residues are much more stable than on the other two phosphoamino acids. Phosphotyrosine residues do not undergo β-elimination but do produce a characteristic immonium ion at *m/z* 216.

Fig. 4 shows the loss of phosphoric acid (−98Th) from a doubly charged peptide with a sequence K.IEDVGS*DEEDDSGK.D, in this spectrum, the most abundant peak is the *m/z* of precursor ion (787.88) minus 49Th at *m/z* 739.88 which is of high abundance as shown in the spectrum. In this spectrum, all the y ions of this peptide can be observed so the sequence can also be determined. The phosphorylation site can be assigned to serine as the mass difference between y_9 and y_8 ions are 87 + 49. A disadvantage of this analysis is the decreased ionisation rates of phosphopeptides in complex mixtures due to suppression effects. This can be compensated by measuring the phosphopeptides in negative ion mode as their ionisation is better in this mode, however changing to negative ion mode has its own problem, as negative-polarity MS/MS spectra are mostly of poor quality when compared to positive ion mode (Amoresano et al., 2006). With the phosphate groups on Ser- and Thr-residues being labile to CID, the ion originating from the loss of phosphoric acid can be selected for further fragmentation by MS^3 (Alcolea et al., 2009). This method provides more phosphopeptide sequence information and helps the specific assignment of the phosphorylation sites on Ser- or Thr-residues. Two recent fragmentation techniques have been developed to avoid the prodominant loss of the phosphate group seen during CID. Both techniques, electron capture dissociation (ECD) and electron transfer dissociation (ETD) have been implemented on FT-ICR or LTQ-Orbitrap MS. ECD or ETD fragmentation can be used for large peptides and in the analysis of multiple charged peptides and the assignment of precise phosphorylation sites (Mikesh et al., 2006) as there is no loss of the modification as seen with CID analysis. Molina et al. (2007) evaluated the use of ETD for the global phosphoproteome analysis. When compared to CID in this paper, it was shown that CID yielded 60% less phosphorylation site identifications with an average of 40% more fragment ions per fragmentation spectrum. However, even though the number of phosphorylation sites was lower, a significant set of phosphopeptides were identified with CID, indicating that both CID and ETD should be combined for more comprehensive analysis. As mentioned earlier, protein identification is dependent on the peptide sequence software. With recent developments in database algorithms and mass spectrometric sensitivity (Alcolea et al., 2009), a recent publication by Wang et al. (2010) investigated the software databases used for phosphoprotein/peptide identification and developed a program that permits the databases to run in parallel, which allows efficient identification of phosphorylated peptides and proteins.

2.3. Studies of phosphorylated proteins in plants

Extensive research has been carried out by the Kersten et al. research group investigating plant phosphoproteomics, with two major reviews being produced by the group in 2004 and 2009. In 2004, they reported on papers investigating *in vivo* and *in vitro* plant phosphoproteomic studies Kersten et al. (2004). The identification of AtMAPK3 and AtMAPK6 substrates using protein microarray system, mapping over 300 *in vivo* phosphorylation sites on 200 putative *Arabidopsis* plasma membrane proteins, and identification of more than 130 phophopeptides belonging to 151 putative intra-cellular Arabidopsis proteins were discussed. Another paper reviewing available techniques and their uses in plant phosphoproteomics was published by Laugesen et al. (2004). The 2009 paper (Kersten et al., 2009) reports on the developments made in plant phosphoproteomics including the development of depositories of experimentally determined phosphorylation sites, quantitative profiling of phosphorylation sites and phosphoproteins, plant peptide microarray and peptide chips platforms to link substrates to specific kinases or for comparative kinome profiling, and development of software databases for predicting phosphorylation sites in plants. The paper reviews several systematic phosphoproteomic analyses which had recently been performed to optimise *in vitro* and *in vivo* technologies which identify components of the plant phosphoproteome. The development of this new tool has enabled rapid identification of potential kinase substrates such as kinase assays using plant protein microarrays. Progress has also been made in quantitative and dynamic analysis of mapped phosphorylation sites. Increased quantity of experimentally verified phosphorylation sites in plants has prompted the creation of dedicated web-resources for plant-specific phosphoproteomics data. This resulted in development of computational prediction methods yielding significantly improved sensitivity and specificity for the detection of phosphorylation sites in plants when compared to methods trained on less plant-specific data. Nakagami et al. (2010) reported a large-scale phosphoproteome analysis in the model monocot, rice (*Oryza sativa* L. cv. *japonica* cultivar Nipponbare), as an economically important crop. Using unfractionated whole-cell lysates of rice cells, they identified 6919 phosphopeptides from 3393 proteins. The investigation of the conservation of phosphoproteomes between plant species was also investigated and a novel phosphorylation-site evaluation method was developed. It was found that the ratio of tyrosine phosphorylation in the phosphoresidues of rice was equivalent to those in *Arabidopsis* and human. The data suggests that plants have conserved regulatory mechanisms based on phosphorylation. Nuhse et al. (2007) demonstrated the use of a stable isotope-based quantitative approach for pathway discovery and structure function studies in *Arabidopsis* cells treated with the bacterial elicitor flagellin. The quantitative comparison identified individual sites on plasma membrane proteins that undergo rapid phosphorylation or dephosphorylation. The data revealed both divergent dynamics of different sites within one protein and coordinated regulation of homologous sites in related proteins. On RbohD, it was found that both unchanging and strongly induced phosphorylation sites existed and by complementing an RbohD mutant plant with non-phosphorylatable forms of RbohD, the sites that undergo differential regulation are required for activation of the protein.

The role of protein kinases and phosphatases and their limitations were discussed by Fuente van Bentem and Hirt (2007). Novel mass spectrometry-based techniques have enabled the large-scale mapping of *in vivo* phosphorylation sites in plants and alternative methods based on peptide and protein microarrays have revealed protein kinase activities in cell extracts, in addition to kinase substrates. The paper reported a combined phosphoproteomic approach of mass spectrometry and microarray technology that enhanced the construction of dynamic plant signalling networks that determines plant biology. Protein phosphorylation and degradation are also known to occur during plant defence signalling cascades. Although the number of completely sequenced genomes is constantly growing, many plants have not had their genomes completely sequenced, impacting upon the number of proteins that can

be identified (Quirino et al., 2010). This paper also investigated the relationship between known pathogen genomes and potential protein identifications to determine protein function in plant defence signalling. Laugesen et al. (2006) proposed an integrated procedure taking the crude protein extract that consists of sequential purification steps and finishing with the identification of phosphorylation sites. This involved the enrichment of phosphoproteins with a commercially available chromatography matrix, 2D PAGE analysis of the enriched fraction followed by the selective staining with the phosphospecific fluorescent dye Pro-Q Diamond, phosphopeptide capture, from the tryptic lysate of 2D spots, using IMAC microcolumns, and finally, the identification of the phosphoproteins and their corresponding phosphorylation sites by MALDI-TOF-TOF spectrometry. The method was applied to contrasting samples prepared from cell suspension cultures of *Arabidopsis thaliana* and roots of *Medicago truncatula*. The results obtained, demonstrated the robustness of the combination of two enrichment stages, sequentially at the protein and at the peptide levels, to analyse phosphoproteins in plants. Bacterial LPS have the ability to act as modulators of the innate immune response in plants. Complex and largely unresolved perception systems exist for LPS on the plant cell surfaces that lead to the activation of multiple intracellular defence signalling pathways. Gerber and Dubery in 2004, investigated the perception mechanism of cultured *Nicotiana tabacum* cells towards LPS from *Burkholderia cepacia* (LPS (B.cep.)), and during signal transduction the rapid phosphorylation of several proteins with the hyperphosphorylation of two proteins of 28 and 2 kDa was determined. Using 2D analysis, significant differences and de novo LPS-induced phosphorylation were also observed, and staurosporine (a protein kinase inhibitor) inhibited the extracellular alkalinisation response induced by LPS Inhibition of protein phosphatase activity by calyculin A intensified the LPS responses. The results indicated that perception and signal transduction responses during LPS elicitation of tobacco cells require a balance between the actions of certain protein kinases and protein phosphatases (Gerber and Dubery, 2004). To overcome the low abundance and high abundance interference, a method was developed in 2009 that is capable of removing 85% of the extremely abundant Rubisco enzymes from soybean leaf soluble protein extract. This method allowed for roughly 230 previously inconspicuous protein spots in soybean leaf to be more easily detectable (3-fold increase in vol.%) using fluorescent detection and allowed 28 phosphorylated proteins previously undetected, to be isolated and identified by MALDI-TOF-MS (Krishnan and Natarajan, 2009).

3. Glycosylated proteins

3.1. Glycoproteins

Glycoproteins are proteins with covalently bound sugars and represent the most complex form of protein post-translational modification. Over 50% of all proteins are substituted with glycans attached to one or several sites (Apweiler et al., 1999). Some examples of proteins that are both biologically active and glycosylated are hormones, enzymes and toxins and glycoproteins can occur inside the cell, both in the cytoplasm and subcellular organelles within cell membranes and in extracellular fluids and matrices. The carbohydrates attached to proteins are involved in molecular recognition, inter- and intra-cellular signalling, embryonic development, fertilisation, immune defence, inflammation, cell adhesion and division processes, viral replication and parasitic infections (Geyer and Geyer, 2006; Collins and Paulson, 2004). The glycan chains can significantly alter protein conformation and consequently modulate the functional activity of a protein and protein/protein interactions (Dwek, 1996; Slawson et al., 2006). Structural diversity of oligosaccharides in glycoproteins is vast due to their biosynthesis being a non-template driven process coordinated by many enzymes. The structure of oligosaccharides is dependent on the polypeptide backbone and the substrate levels and enzyme availability, which may change during cell growth, differentiation and development. There are two main types of glycosylation: *N*-glycosylation and *O*-glycosylation. In *N*-glycosylation the glycan is β-glycosidically attached via N-acetylglucosamine (GlcNAc) to the amide group of asparagines (Asn) within an Asn-X-Ser/Thr motif, where X is any amino acid apart from proline. All N-glycans have a $Man_3GlcNAc_2$ pentasaccharide core and can be divided into three classes by the number of monosaccharides attached: oligomannosidic glycans; complex-type glycans and hybrid-type glycans. *O*-glycosylation is commonly known as mucin-type glycosylation, in which the carbohydrate unit is α-glycosidically linked via *N*-acetylgalactosamine (GalNac) to serine (ser) or threonine (thr) residues. O-linked glycans do not share a distinct core like N-glycans but have a number of different core regions with some common motifs. Mucin-type O-glycans vary in size ranging

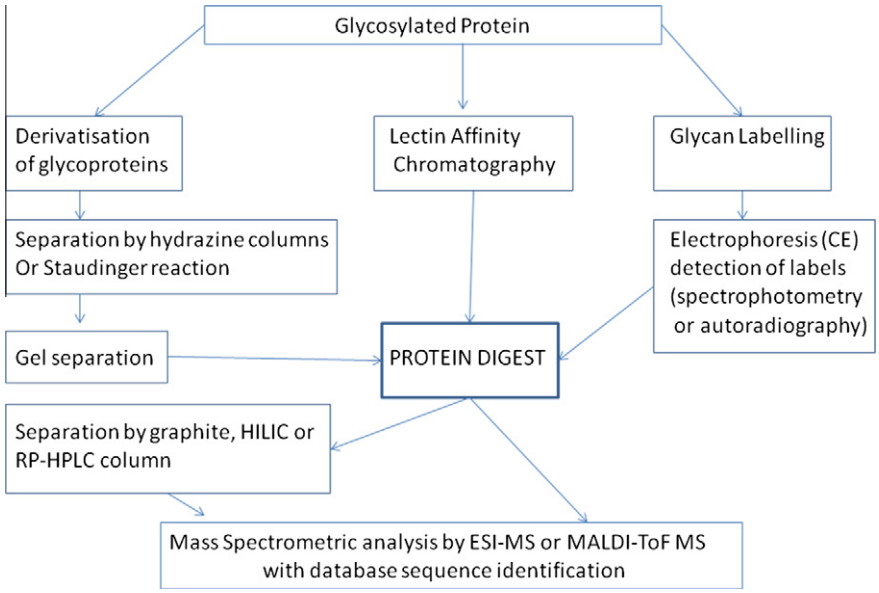

Fig. 5. Flowchart summarising various methods for glycoprotein separation, detection and analysis.

from a single residue to extended oligosaccharide chains similar to complex-type N-glycans (Geyer and Geyer, 2006). In each glycoprotein, potential *N*- or *O*-glycosylation sites may be occupied in varying proportions. A wide range of structurally different glycans may occur at a particular glycosylation site. Glycoproteins can consist of many glycoforms (consisting of a number of different glycans attached to an individual glycosylation site with variable degrees of site occupancy). This heterogeneous glycosylation pattern causes many problems when trying to isolate and identify glycosylated proteins and glycosylation sites.

3.2. Methods of glycoprotein analysis

Many different methods have been documented with the majority being detailed in Post-translational Modification of Proteins: Tools for Functional Proteomics (Kannicht, 2002). Geyer and Geyer (2006) discussed the methods for investigating intact glycoproteins. An overview of these techniques is given in Fig. 5. The initial steps include separation utilising SDS–PAGE or 2D-electrophoresis, however these techniques pose resolution problems due to the heterogeneous glycosylation pattern and many membrane proteins can be glycosylated and these are in low representation on an electrophoresis gel. Enrichment methods can also be utilised for the separation of glycosylated proteins and peptides, with the main technique being lectin affinity chromatography. Lectins are proteins that specifically interact with carbohydrates without modifying them. They interact with specific glycan motifs with the structural domain which interacts with the glycan varying between lectins allowing selective enrichment for particular subsets of glycoproteins from a complex protein mix (Nawarak et al., 2004). A series of lectin columns with different specificities allows the fractionation of oligosaccharides into structural subsets prior to enzymic digestion (Cummings, 1994). Lectin affinity chromatography can aid separation of structural isomers and provides substantial information on their structural features when in combination with other separation techniques such as RP-HPLC (Geyer et al., 2005; Budnik et al., 2006). Chemical derivatisation of the glycan has also been used to separate the glycoproteins. There are two reactions that are generally used: Schiff-base reaction of aldehydes with a hydrazine (Khidekel et al., 2004) and Staudinger ligation between a phosphine and an azide (Saxon and Bertozzi, 2000). This type of enrichment is specific to the glycan modification and can provide information about the peptide/protein identity that is modified, but it does not provide information on the site or structure of glycosylation. A further chemical derivatisation technique developed from the Schiff-base reaction is Periodate-acid-Schiff coupled affinity (PAS) chromatography (Budnik et al., 2006). The periodate oxidation reaction utilizes the vicinal diol functionality, which is unique to glycans. The periodate oxidation with the coupling to a hydrazine via the Schiff-base reaction can be employed in a variety of ways using different coupling agents such as biotin hydrazides, digoxigenin hydrazides or hydrazide column, this reaction can also be performed on proteins or on peptides (Jebanathirajah et al., 2002). Labelling of glycans at the reducing end-aldehyde group by means of chemical derivatization has also been developed. Using reductive amination, chromogenic or fluorescent groups are introduced which allows sensitive detection during chromatographic separation. The two most common labelling agents used for chromatographic profiling of glycans are 2-aminopyridine (PA) and 2-aminobenzamide (2-AB) (Geyer and Geyer, 2006; Wohlgemuth et al., 2009). Capillary electrophoresis (CE) has also been documented for glycoform analysis (Pacakova et al., 2001; Kakehi et al., 2001) and can obtain complete resolution of the separated glycans unlike SDS–PAGE and 2D electrophoresis. The other benefit of CE is the high separation efficiency and the speed of analysis. Charged saccharides, can be separated directly based on their electric charge/molecular size ratio (Hermenti et al., 1994), however capillary electrophoresis does not show the nature of the glycan attached, so it has been concluded that a multi-step approach has to be conducted to obtain a complete mapping and characterising of the oligosaccharides and determination of carbohydrate composition. An example of this multi-step approach can be seen in Hagglund et al. (2004) where using human plasma, lectin purification was followed by hydrophobic interaction chromatography (HILIC) and partial deglycosylation to determine the sites of *N*-glycosylation (Wohlgemuth et al., 2009). HPLC has proved to be of great value in profiling and separation of glycans because of the range of adsorbents available and the speed, reproducibility of separation allowing quantification of relative proportions of glycans. Anion exchange chromatography separates glycans on the basis of the number of charged groups present, and depending on the conditions used the size of the glycans allowing within one class of similarly charged glycans larger structures to be eluted prior to smaller ones (Guile et al., 1994). An extension to anion chromatography for the separation of glycoprotein-glycan is high-performance/high-pH anion exchange chromatography (HPAEC), coupled to a pulsed amperometric detector (PAD). Advantages of this technique include: the fact that labelling of the glycans is not required, it is a comparatively high throughput technique and efficiently separates glycans according to size, structure, linkage and branching. Disadvantages of HPAEC-PAD are the increased rate of epimerization of reducing GlcNAc to ManNAc at pH 12, which requires reduction of the oligosaccharides prior to analysis and the high-salt content makes a direct coupling to mass spectrometry difficult (Lee, 1996). Reverse Phase (RP)-HPLC separates sugars on the basis of their hydrophobicity, however derivatization with a hydrophobic label (such as 2-AB, PA or others) is required (Rudd et al., 2001). Graphite or HILIC columns can also be used to fractionate oligosaccharides due to hydrophobic interactions and can be used for efficient separation of glycan isomers and closely related compounds (Novotny and Mechref, 2005). Karnoup et al. (2007) investigated a novel HPLC-UV-MS method investigating glycosylation of plant-derived glycoproteins using antibody-derived glycopeptides and obtained quantitation of non-glycosylated proteins, de-glycosylated proteins and total glycosylated proteins. There are two main ionisation procedures used for the characterisation of carbohydrates by mass spectrometry: ESI and MALDI (Fenn et al., 1989; Karas and Hillenkamp, 1988). MALDI mass spectrometry has been commonly used to determine structures of glycoproteins and carbohydrates cleaved from glycoforms of small glycoproteins with single glycosylation sites. Limited numbers of glycosylation sites can often be resolved by MALDI-ToF mass spectrometers (Yang and Orlando, 1996). To analyze larger proteins that have complex carbohydrate modifications by MALDI, the proteins must be enzymically cleaved into smaller fragments and the glycans have to be treated with glycosidases to remove the carbohydrate modifications from the protein. The fragmentation patterns of both N-linked and O-linked glycopeptides are mainly derived from the glycan fragmentation and peptide backbone fragmentation (Budnik et al., 2006). MS instruments capable of performing MS^n experiments could be used in MS^3 mode in order to determine specific glycan losses during fragmentation of both the cleavage of the glycosidic bonds between the sugar rings and the cleavage within the ring, called cross-ring cleavages (Domon and Costello, 1988). Glycosidic cleavage ions provide information on carbohydrate sequence and branching as well as non-carbohydrate constituents, whereas the cross-ring cleavage ions can provide extended information on branching and monosaccharide linkage positions (Sheeley and Reinhold, 1998). An accompanying technique to MS is Nuclear Magnetic Resonance (NMR) spectroscopy. NMR methods enables a complete identification of all structural features of glycans including

monosaccharide constituents, ring size and anomeric configurations, the types of linkage between monosaccharides, their conformational preferences and the type and position of possible non-carbohydrate substituents (Vliegenthart et al., 1983). The main disadvantage of this technique, is the relatively large amount of material which is required to obtain high-resolution spectra (Kalidhar, 1998).

3.3. Protein glycosylation in plants

N-glycosylation is a major modification of proteins in plant cells. In the biotechnology industry, transgenic plants are emerging as a key process for the production of recombinant glycoproteins to be used for therapeutic purposes and an improved understanding of protein glycosylation in plants has become vital to the quality control of these emerging drugs. The number of therapeutic glycosylated proteins that are produced in plants is steadily increasing with *N*-glycosylation being essential for their stability, folding and biological activity. The *in vivo* applications of pharmaceutical recombinant glycoproteins produced in transgenic plants are strongly dependant on the *N*-glycosylation patterns of a recombinant therapeutic glycoprotein (Bardor et al., 1999a, 1999b). Shimoda et al. (2007) investigated the glycosylation of capsaicin and 8-nordihydrocapsaicin by cultured cells of *Catharanthus roseus* in an attempt to reduce their pungency and enhance their solubility in water. The sugar conjugation of drugs reduces toxicity and enhances oral absorption, it was reported that water-soluble capsaicinoid glycosides are useful as potential prodrugs of capsaicinoids, and the high capacity of *C. roseus* cells for the glycosylation of capsaicinoids would also be beneficial for the production of highly water-soluble capsaicinoid glycosides (Shimoda et al., 2007). Investigations into the *N*-glycosylation of the isolectin L of bean phytohemagglutinin (PHA-L) was performed in 1999, and was carried out on the native PHA-L as well as on the N-glycans released from this lectin. Two other glycopeptides containing the potential *N*-glycosylation sites were also prepared, by means of proteolytic cleavage of PHA-L and purified by HPLC, analysed by MALDI-ToF MS. This study confirmed that PHA-L is *N*-glycosylated by two populations of oligosaccharides, high-mannose-type N-glycans and paucimannosidic-type N-glycans, located on Asn-12 and Asn-60, respectively, and has pointed out the microheterogeneity of the glycans N-linked on both Asn residues (Bardor et al., 1999a,b). The analysis of new glycosylation mutants and the diasbling of glycosyltransferases aided the identification of the biological functions of N-glycans in plants, allowing the potential to engineer plant cells by knocking out part of their glycan processing machinery or by complementing this machinery with heterologous glycosyltransferases to produce recombinant glycoproteins with mammalian-like glycans at a lower cost than their mammalian counterparts (Lerouge et al., 1998). The activities of two de-*N*-glycosylation enzymes, PNGase (pep tide *N*4(*N*-acetyl-glucosaminyl) asparagine amidase) and ENGase (endo *N*-acetyl-b-D-glucosaminidase), involved in the release of N-glycans from *N*-glycoproteins, has been observed in several organs of tomato plants (*Lycopersicon esculentum*, Mill., cv. Dombito) using fluorescence-HPLC and a resofurin-labelled N-glycopeptide substrate. PNGase and ENGase activities were detected in every organ assayed but with quantitative differences. It was shown that both de-*N*-glycosylation activities were associated with high levels of proteins and protease activities, and during fruit growth and ripening, these activities decreased notably. The detection of these enzyme activities in the different organs is most likely associated with the previously characterised unconjugated N-glycans in tomato plants and that the mechanism of de-*N*-glycosylation are important in differentiating and growing tissues and organs (api-cal buds, leaves and flowers). It was also shown that the protein degradation occurs when cells (Lhernould et al., 1994) or excised root tips (James et al., 1993) are deprived of sugar, and under these conditions, an increase in de-*N*-glycosylation activity was shown (Lhernould et al., 1994) correlating with high protease activity. The actively growing and dividing tissues may also be subject to an insufficient sugar source if photosynthetic products are not abundant, allowing glycoproteins recycling such as de-*N*-glycosylation to counteract this lack of sugars (Faugeron et al., 2006). The global analysis of glycoproteins in plants has also been undertaken, for example Minic et al. (2007) used lectin affinity purification followed by 2D PAGE and mass spectrometric protein identification to study the glycosylation of proteins within mature stems of *Arabidopsis*.

4. Lipid-modified proteins

4.1. The lipid-modified proteome

Protein modification via the addition of a lipid moiety to the protein has been reported in a wide range of organisms and can be sub-divided into distinct modification types based on the nature of the lipid attached, with each type having its own specific biosynthesis enzymes for the formation of the post-translationally modified protein. Myristoylation involves the addition of a 14 carbon acyl chain ($C_{14}H_{26}O$), primarily to glycine residues within the protein structure. However, a comparatively less abundant location for the myristoyl group is on lysine and arginine amino acids. A second type of lipid modification is the addition of a palmitoyl group ($C_{16}H_{30}O$) to the protein at cysteine amino acid residues, generally via the sulphur atom of the cysteine side chain. This modification of proteins is also referred to as palmitoylation or S-acylation. The final type of lipid modification of proteins is termed prenylation and results in the addition of either a farnesyl ($C_{15}H_{25}$) or a geranylgeranyl isoprenoid group ($C_{20}H_{33}$) to the protein. Prenylation occurs at a conserved amino acid motif at the C terminus of the proteins modified, CAAX, where C is a cysteine amino acid (and the C terminus), A is an aliphatic amino acid and X can be any amino acid. Modification by lipids has been implicated in a number of diverse biological roles, mainly having a role in membrane binding and membrane targeting of proteins. Prenylation of proteins has been extensively studied, primarily due to the fact that small GTPases involved in the development of cancer require farnesylation for their localisation at the membrane, which has been shown to be a pre-requisite for the diseases progression (Wang and Sebti, 2005). Therefore, a number of pharmaceutical agents that can interfere with the prenylation of these proteins have been developed as oncology treatments.

4.2. Methods of protein-lipid modification analysis

The standard methodology for the study of protein-lipid modification is confined to targeting both the protein of interest (which is suspected as being potentially modified) and also the type of lipid modification itself. The protocol involves the incorporation of the gene for the protein into a gene construct and the expression of this, usually via the addition of a cellular lysate, typically rabbit-reticulocyte lysate, providing the required cellular mechanisms to produce the protein from the construct, is carried out (Hancock, 1995). Added to this is a labelled lipid precursor (such as H^3-labelled farnesyl diphosphate) which acts as the modification group for any reaction. The reaction mixture can then be separated on a 1D-SDS–PAGE gel and the protein of interest identified via western blotting and the presence of the modification detected by autoradiography. Usually such experiments are also combined with site-directed mutagenesis experiments to show that mutagenesis of

the C terminus (in the case of prenylation) causes a loss of the incorporation of the radiolabelled lipid and also cellular localisation experiments to demonstrate that lipid incorporation results in the proteins translocating to the membrane. Whilst these types of experiments have been successful in the past, they have been noted to require a significant amount of experimental work (taking weeks or months to complete). A recent study sought to reduce the experimental time required for such analyses utilising a Thin Layer Chromatography (TLC) linear analyser (Benetka et al., 2006). In this refined protocol, GST-fusion protein cDNA was used rather than the protein alone and after incubation with the appropriate radiolabelled lipid precursor, the protein was extracted using glutathione Sepharose beads before application to the TLC analyser for separation. Radiolabel lipid content was quantified by radiography as before whilst a GST antibody was used to visualise the protein in question. Another recently developed method for the study of the S-acylation of specific proteins has been reported that requires the protein of interest to be purified in its native (potentially modified) state. Following purification, the lipid modification (if present) is cleaved from the protein using platinum (IV) oxide, thereby releasing the lipid with an ethyl group added to the carboxylic acid. This ethyl addition allows for the efficient analysis and quantitation of the lipid modification of the protein using gas chromatography linked to mass spectrometric detection (Sorek and Yalovsky, 2010). The analysis of a known lipid-modified proteins modification status is possible using SDS–PAGE gel-shift analysis. This approach makes use of the mass shift before and after lipid-modification and is commonly applied to prenylated protein analysis. The prenylated protein has a lower mass and therefore migrates further through the 1D-SDS–PAGE gel, whilst the unprenylated protein has a slightly higher mass (due to a series of changes in the protein during the prenylation process). This mass shift is significant enough to monitor selectively by western blot visualisation of the protein band and comparison to the standard protein molecular weight ladder on the gel. A further technique to identify whether a selected protein is modified, requires a pre-requisite knowledge regarding the effect on cellular localisation of the protein before and after modification. For example, many farnesylated proteins, after modification, are found solely in the membrane, whilst prior to modification they exist only in the cytosol. The approach therefore monitors the location of the protein by creating a GFP-tagged protein whose cellular location can be easily visualised, with the tag being located at the N terminus and so not interfering with the addition of the lipid at the C terminus (Keller et al., 2005). Alternatively, the proteome can be fractionated into different cellular fractions (cytosol and membrane) and the protein of interests location detected via western blotting.

With respect to targeting lipid-modified proteins in a global sense (without choosing a selected protein of interest in advance), one approach is to apply bioinformatics analysis to the increasingly available protein sequence data available. Given that some lipid modifications occur at conserved sequence motifs, this approach would appear to be feasible (for example, within the human genome, approximately 100 genes have the required CAAX sequence for prenylation). A prenylation prediction suite of bioinformatic software has been developed (PrePS) and applied to plant and mammalian protein information databases (Maurer-Stroh et al., 2007). Furthermore, a study developed this approach and suggested that, in order to avoid a large number of false positive identifications, sequence recognition of the conserved motifs be combined with surrounding sequence data (Eisenhaber et al., 2004). The report rationalised that, as well as the expected motif sequence, this specific region of the protein to be modified must have the required flexibility (via what the researchers term a linker region) to enter the active site of the enzyme responsible for the formation of the modified protein. Hence a probability score that excluded conserved motifs that lacked such flexibility improved the confidence that proteins identified were lipid-modified. Whilst it is noted that such an approach only serves to identify potentially lipid-modified proteins, it does allow for the more directed further experimental research (via those protocols already discussed) as to these modification types. A recent approach to study lipid-modified glutamate residues via a protocol that involves selective cleavage at modified sites followed by trans-esterification (via sodium methoxide addition) has been reported. The further reaction of the peptide modification site to produce acylpyrrolidinone moiety can then be assayed for (Sawatzki et al., 2005). Whilst interesting, such modifications are known to occur only in mammals.

Approaches have also been developed that rely upon the selective extraction from total proteome samples of only those proteins with the defined lipid modification with a number of studies having utilised an ‘‘azide labelling approach’’. This has also been termed a ‘‘tagging-via-substrate’’ (TAS) approach and one such example is a method utilised to selectively purify farnesylated proteins (Kho et al., 2004). In the experiment, a synthetic farnesyl substrate which contains an azide moiety within its structure is added to a cell culture (in excess amounts compared to the normal farnesyl substrate) and is incorporated into the cells required lipid-modified proteins during the normal farnesylation process. After the proteins are extracted from the sample, the azide group of the newly farnesylated proteins is chemically reacted in order to add a further chemical moiety onto the farnesyl group which contains a biotin group as part of its structure. Once reacted in this way the lipid-modified proteins can either be selectively detected (using a streptovidin linked reporter – usually peroxidise) or selectively purified (using agarose affinity beads). A similar approach was later developed for geranylgeranylated proteins, again using an azide-modified lipid substrate (Chan et al., 2009). In this study, the azide incorporated into the modified protein was then reacted with a tetramethylrhodamine (TAMARA) dye for the selective visualisation of the lipid-modified proteins after electrophoretic separation. A separate study used a biotin switch method to study plant protein palmitoylation from cell lysates (Hemsley et al., 2008). The method makes use of the fact that palmitoylation occurs on the sulphur atom of the cysteine amino acid of the protein modified. The proteins of the cell lysate are treated with a sulphydryl reagent (*N*-ethylmaleimide – NEM) which blocks all free cysteines (i.e. those not acylated). Each sample is then divided into two and a S-acyl specific cleavage is undertaken by the addition of hydroxylamine before ahydryl reactive biotin agent is added which attaches biotin to any free sulphur residues (i.e. only those that were acylated prior to acyl cleavage). Again biotin can then be used to purify the palmitoylated proteins and alongside the appropriate control samples. These selective purification methods generally use 1D- or 2D-SDS–PAGE for further separation of the lipid-modified proteins. However, a number of recent reports have highlighted issues regarding the application of PAGE separation techniques to the lipid-modified proteins and investigated how these may be overcome. One report studied the non-specific lipid transfer protein 1 (LTP1) from barley which is know to be lipid-modified at a specific peptide sequence and noted that the modified peptide could be identified after using size exclusion chromatography for protein separation (followed by digestion and mass spectrometric analysis) but not by 1D-PAGE and similar analysis (Zídková et al., 2007). It was concluded that the alkaline pH used during PAGE and the SDS itself (via detergent action) destabilised the lipid modification and these should be avoided as much as is possible during any such study. A separate study noted that lipid-modified peptides after in-gel digestion exhibit low extraction recovery and the researchers therefore developed a separate extraction protocol (using *n*-dodecyl-β-D-maltoside,

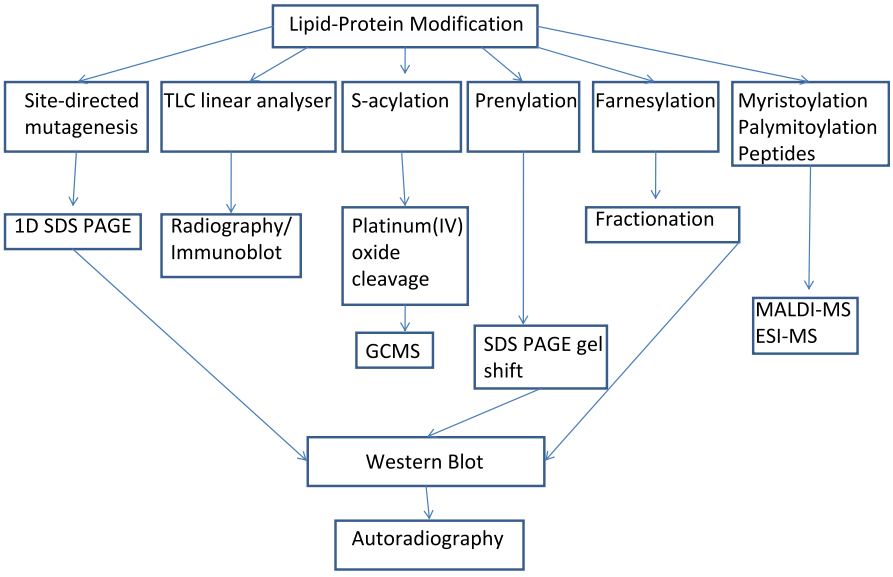

Fig. 6. Flowchart summarising various methods for lipid-modification of proteins via selective separation, detection and analysis.

followed by a chloroform/methanol extraction) which greatly increased the extraction of the test lipid-modified peptide chosen to assess the effectiveness of the technique (Ujihara et al., 2008). The application of a global mass spectrometry driven analysis technique for the study of lipid-modified proteins has also been reported (Hoffman and Kast, 2006). For the study a number of example peptides were synthesised and lipid-modified representing myristoylation, farnesylation and palmitoylation. The study analysed the behaviour of the peptides during MS/MS fragmentation in both an electrospray and MALDI mass spectrometer and also studied the effect on the tandem mass spectra produced of the various possible charge state of the chosen precursor ion. The researchers were looking to find either diagnostic product ions in the MS/MS spectra produced or diagnostic mass losses from the precursor ion. For each of the lipid-modifications, both a diagnostic marker ion and loss were detected but some were only shown in either electrospray or MALDI. The techniques available for lipid-modification analysis are outlined in Fig. 6.

4.3. Studies of protein-lipid modification in plants

As discussed previously, a major focus of the analysis of lipid-modified proteins has been on the modification of small GTPases involved in the development of cancer. There have however also been some cases of such protein modification and its role in plants. A prenylation prediction suite has been applied to genomic databases available and highlighted a large group of plant proteins which exhibited a high degree of homology (Maurer-Stroh et al., 2007). The group of proteins has been suggested, by further annotation work, to be a group of chaperones involved in metal chelation and a further protein identified by the same study was linked to protein ubiquitination and subsequent degradation. Palmitoylation has been both predicted and experimentally verified in a number of plant proteins. As might be expected the lipid-modification of GTPases in plants has been identified and linked to cellular development and signalling via their effect on stomatal closure induced by abscisic acid (Sorek et al., 2007). In this case, the protein Rho of plants 1 (ROP 1) was shown to be acylated, with GCMS being used to identify the nature of the lipid modifications. Palmitoylation has also been implicated in regulating calcium signalling in plants via the lipid-modification of calcinurin B-like proteins (CBLs) which bind both calcium and specific kinases which they regulate dependent on calcium levels (Batistic et al., 2008). Furthermore, the calcium dependent protein kinases themselves have also been shown to be N-myristotylated. Other plant proteins known to be palmitoylated include proteins involved in plant defence systems (Kim et al., 2005) and tubulin (Caron et al., 2001) suggesting a wide range of roles for the modification. In plants, farnesylation has also been determined in a number of proteins. Nucleosome assembly protein 1 (NAP 1) can be farnesylated and the modification causes the localisation of the protein in the nucleus and correlates with cell proliferation (Galichet and Gruissem, 2006). Membrane anchored ubiquitin fold (MUB) protein, whose specific role is still unclear, has been shown to be both farnesylated and gernaylgernaylated (Downes et al., 2006), whilst an enzyme involved in cytokine biosynthesis (IPT3) is farnesylated, with lipid-modification being linked to differential cellular localisation of the enzyme (Galichet et al., 2008). Gernaylgeranylation has also been identified in plant proteins (as mentioned above alongside farnesylation), however the sole gernaylgeranyl-modification of a protein has been noted. Plant calmodulin (CaM53) is gernaylgernaylated and the modification allows for the localisation of the protein in the plasma membrane, further suggesting a role for the modification in cellular signalling (Rodríguez-Concepción et al., 1999). Prenylation has also been shown to occur on a protein (APETALA 1) involved in controlling flower development in which the protein modified acts as a selective transcription factor (Yalovsky et al., 2000) and also a protein again involved in metal binding, as previously stated for palmitoylation (ATFP3). Finally, lipid-modification has also been associated with the plants innate immune response and defence system via farnesylation (Goritschnig et al., 2008). These few studies alone (although not all inclusive) demonstrate the wide range of roles that the modification has within plants.

5. Redox proteomics

5.1. The redox proteome

Redox proteomics describes the post-translational modification of proteins by numerous reactive oxygen species (ROS) and is a more recent addition to the catalogue of modifications to consider when undertaking proteomic analysis. The area of redox proteomics is a complex area for wide scale analysis due both to the diverse number of ROS that form the modifications and also the range of individual amino acids within the protein sequence that

can undergo such modification. The modification causing agents can loosely be grouped into the ROS such as hydroxyl, peroxide and superoxide radical species and mixed nitrogen–oxygen species (RNS) which include nitric oxide and peroxynitrite. Oxidative damage caused by these species can alter the structure of many cellular moieties, however it is thought that proteins absorb up to 70% of the ROS produced (Davies, 2005). In relation to plants, nitric oxide is especially important as it is itself produced by plants themselves and its production is thought to relate to challenge to the plant by pathogenic organisms. The oxidative damage to proteins is dealt with by the cell by the ubiquitin proteasome pathway (Davies, 2001), involving the further modification of oxidised proteins by ubiquitin, a further post-translation modification discussed in this review. Whilst the production of ROS and RNS is usually thought to be related to oxidative stress with organisms, it is also becoming recognised that the active production of such species in plants has a role in cellular signalling mechanisms and may be beneficial to the organism when controlled as part of the usual cell cycle and development. This has led to the development of the oxidative signalling hypothesis (Foyer and Noctor, 2005). As mentioned, a variety of amino acids can be modified by ROS and RNS and the most commonly modified are amino acids whose R sidechain contain sulphur atoms – cysteine and methionine. In the case of cysteine, the thiol element of the amino acid undergoes modification and can be oxidised to one of four modification products: a disulphide, sulphenic acid, sulphinic acid or a sulphonic acid. Furthermore, nitric oxide can also cause the formation of a S-nitrosothiol group and nitric oxide intermediates can form S-glutathioylation products in addition to those listed above. Methionine, also a sulphur containing amino acid, can also undergo modification, however it forms fewer and less common modifications, namely methionine sulphoxide and, less commonly, a sulphone derivative. The amino acid tyrosine can also be oxidised to either crosslinked dimers (bi-tyrosine) or 3,4-dihydroxytyrosine by ROS and RNS can also lead to the formation of nitrotyrosine, whilst tryptophan is oxidised to kynurenine and its precursor, N-formylkynuenine. Finally, lysine, arginine, proline and threonine side chains can be oxidised via carbonylation, during which aldehyde or ketone groups are formed dependent on the original sidechain group. This wide range of amino acid substrates for oxidation and diversity of endproduct modification via the oxidative process makes the analysis of the redox proteome a complex endeavour. The analysis is complicated further due to the labile nature of some of the oxidative modifications, for example considering the oxidation of cysteines by ROS alone, the formation of disulphides and sulphenic acids is an easily reversible process whilst the formation of sulphinic and sulphonic acids from the same amino acid residue are markedly less easy to remove. For this reason, a major undertaking when studying the redox proteome is to ensure that the modification process is effectively prevented from altering further the status of proteins after the defined experimental time point (Hansen and Winther, 2009). Therefore, quenching of such pathways during protein extraction is essential and a number of mechanisms to undertake this have been recently reviewed for studying the oxidative nature of thiol containing proteins and reagent groups allowing the retention of the proteomes oxidative status include *N*-ethylmaleimide (NEM), iodoacetamide (IAM) and methylmethanethiosulfonate (MMTS).

5.2. Methods of redox proteome analysis

The techniques available for the analysis of the redox proteome are highlighted in Fig. 7, as might be expected given its comparatively new status in the grouping of protein post-translational modifications, the methods for the analysis protein oxidisation-derived modification is less diverse when considered in light of the methods available for phosphoprotein analysis for example. The study of the oxidative modification of a selected protein can be studied by mass spectrometry, with digestion and mass spectrometric analysis providing peptide sequence and molecular weight data. The shift in mass of the peptide from that expected of the unmodified amino acid sequence can be used to identify the presence and identity of the modification, for examples conversion to SO_3H adds 32 Da to the peptide mass (Sheehan et al., 2010). In order to improve this identification, it is also possible to improve the clarity with which the mass shift resulting from oxidative modification is observed by chemically reacting such groups. Maeda et al. (2005) showed that reaction with iodoacetamide of all free thiols followed by reduction of disulphides and then reaction with 4-vinylpyridine of the now-reduced (previously disulphide) thiol groups produced an increased mass shift of 57 Da for free thiols and 105 Da thiols which were modified in the native protein. Furthermore, the MS/MS data obtained can identify the amino acid within the sequence which is modified by virtue of the addition mass loss between the MS/MS derived b and y fragment ions formed during MS/MS of peptides. A universal redox proteome analysis protocol allowing high throughput analysis of the modification status of cellular proteomes for all types of oxidative modification has yet to be

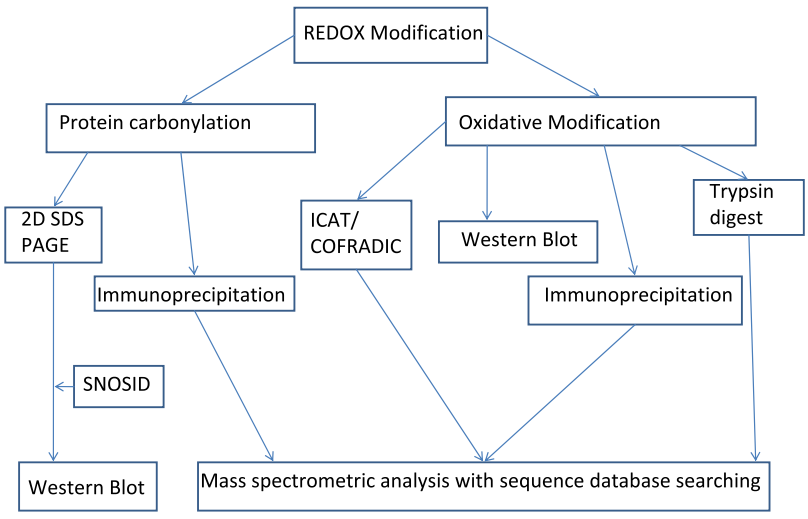

Fig. 7. Flowchart summarising various methods for the analysis of the redox proteome.

established. A common methodology for redox proteome analysis utilises 2D SDS–PAGE separation analysis of proteins followed by selective detection of a specific oxidised amino acid sidechain type (Sheehan, 2006). One example of this methodology is the study of protein carbonylation via 2D-SDS–PAGE separation, followed by a western blotting of the proteins via transfer of the proteome to nitrocellulose membranes, reaction of protein carbonyl groups with hydrazine-dinitrophenol (DNP) and detection of the oxidatively-modified proteins using an antibody against the DNP selectively added to the carbonylated proteins (Nakamura and Goto, 1996). The labelling of carbonylated proteins using the same DNP labelling process has also utilised immunoprecipitation of the oxidised proteins, followed by nano-LC-MS/MS analysis (with and without 2D SDS–PAGE prefractionation) in order to identify the modified proteome (Kristensen et al., 2004). Antibodies for other oxidative modification groups such as nitrotyrosine are also available and can be used in a similar fashion (Kanski et al., 2005). An alternative methodology for the study of proteins with oxidised thiol groups, relies upon the reaction of those thiol groups which are not oxidised with activated thiol Sepharose. Different experimental groups proteomes are treated in this manner and functionalised beads used to extract the un-modified thiol containing proteins. By comparison of experimental groups with the relevant control samples (using 2D-SDS–PAGE), those proteins missing from the experimental group, but present in the control, can be observed to be modified via an oxidative process (Hu et al., 2009, 2010). The same principle undertaking analysis in a "gel-free" manner uses isotope-coded affinity tag techniques, which are more often associated with quantitative proteomics rather than the application of mass spectrometric analysis to protein modification (Gygi et al., 1999). The basis of the application to oxidative modification analysis (specifically thiol modification) relies upon the fact that ICAT reagents selectively "mass label" and then extract cysteine amino acid containing peptides. Hence, any modified cysteines are not modified and represent differentially intense signals in the mass spectrometric data produced (however, the experiment assumes that there is no quantitative difference in the proteins expression as the ICAT response will also be affected by this factor). The exact nature of the modification (which of the four types of available modification of cysteines for example) however is not elaborated by this type of analysis. So-called "diagonal" analysis, where an experiment is repeated twice with a selective chemical reaction modifying specific modification groups between the two reaction, has also been applied to cysteine oxidative modifications. These fall into two categories – diagonal gel analysis (Samelson, 2001) and diagonal chromatography (see Gevaert et al., 2006, review) (COFRADIC – see also the phosphoproteome analysis section of this review). In diagonal gel analysis1D gel separation is undertaken in non-reducing conditions, followed by reduction of any disulphide modification groups. The experiment is then repeated after applying the gel lane at a 90° angle to the first gel. Any non-cysteine modified proteins will migrate identically in both gel experiments and occur on the "diagonal" of the gel, whilst newly reduced disulphides migrate further in the second experiment and occur below the diagonal. Similarly, COFRADIC – mainly applied to peptides – relies on different reverse phase chromatography retention after chemical reaction of the thiol containing peptides. Another technique applied to redox proteome analysis (and other post-translational modifications as described earlier in this review), is the application of biotin labelling of the modification in question or alternatively, biotin switch analysis. Biotinylated, oxidised glutathione has been applied to the analysis of S-glutathionylation of proteins whereby an ester of biotin and glutathione was used and biotin used for the determination of the modified proteins due to its unique recognition by streptovidin as discussed previously (Ito et al., 2003). A "biotin switch" methodology has also been applied (for example for the analysis of S-nitrosylation), in which free thiols are chemically blocked by MMTS followed by the reaction of any remaining nitrothiol groups to free thiol groups using ascorbate. These "new" free thiol groups are then reacted with a sulphydryl-specific biotin-containing agent and either visualised on a 2D-SDS–PAGE using streptovidin linked antibodies or purified using a streptovidin affinity column (Jaffrey and Snyder, 2001). When using 2D-SDS–PAGE in such analysis, it has been demonstrated that using urea (rather than SDS) during thiol reactions improves the signal intensity of the ions produced. A further development of this methodology was the SNOSID (SNO Site Identification) method (Hao et al., 2006), which utilises a trypsin digestion step after the second chemical modification and before affinity purification, hence purifying single – previously modified – peptides rather than full proteins, however this produces only a single peptide per protein. Alternatively, the biotin reaction has also been replaced with the intact protein (after blockage of free thiols) having "chemically un-modified" thiols selectively reacted with a His-tag protein allowing easy purification. The His-tag linker is designed such that when the purified protein extract is digested with trypsin, S-nitrosylated amino acids exhibit a 217.12 Da mass shift making their recognition in peptide sequence MS/MS data easier to detect (Camerini et al., 2007). A further development in nitrosylation analysis is the replacement of the biotinylation reagent with a selective fluorescence dye (as used in DIGE analysis), where different dyes can be added to different samples (binding solely to modified proteins), the samples mixed and separated on 2D-SDS–PAGE and then each sample visualised individually via its dyes specific excitation wavelength (Sun et al., 2007).

5.3. Studies of oxidative protein modifications in plants

The selective analysis of the effect of oxidation of mitochondrial proteome of rice leaf has been undertaken, studying the carbonylation protein composition of proteins of the organelle following *in vitro* oxidation. DNP was used to selectively tag the oxidised proteins and subsequent mass spectrometric and 2D-SDS–PAGE separation followed by mass spectrometric analysis was applied to study the oxidised proteins formed. Differentially oxidised proteins were found in both the treated sample and control sample suggesting that there is an innate oxidation process as well as oxidation via elevated levels of ROS and RNS. A further study of protein carbonylation of mitochondrial proteins demonstrated that the oxidation status of the proteome increased during the senescence of peach fruit (Qin et al., 2009), suggesting that the specific damage of some mitochondrial proteins was the cause of the observed effects. Proteins oxidised in relation to the treatment included proteins of the TCA cycle, respiratory complex proteins, chaperones and redox proteins (Kristensen et al., 2004). The effect of methyljasmonate on the redox proteome of *Arabidopsis* leaves and roots has been investigated via the mechanism of chemically labelling oxidised cysteines and indicated that stress and defence related proteins exhibited the most predominant response (Alvarez et al., 2009). The study also used MS/MS data to map the oxidised amino acid residues within the protein sequence. The study of the specific oxidation substrates of the enzyme thioredoxin in Barley seeds was investigated using ICAT reagents and indicated a number of target proteins which had already been characterised as well as new proteins, such as ribosomal proteins, and indicated that the most commonly oxidised protein was dehydroascorbate reductase, therefore linking thioredoxin to the ascorbate–glutathione cycle (Hägglund et al., 2008). The role of thioredoxin in the chloroplast lumen has also been studied and indicated that the redox proteomic status of the proteins identified were important in the degradation of photosystem subunits of PSII and adaption to differing light intensities (Hall et al., 2010).

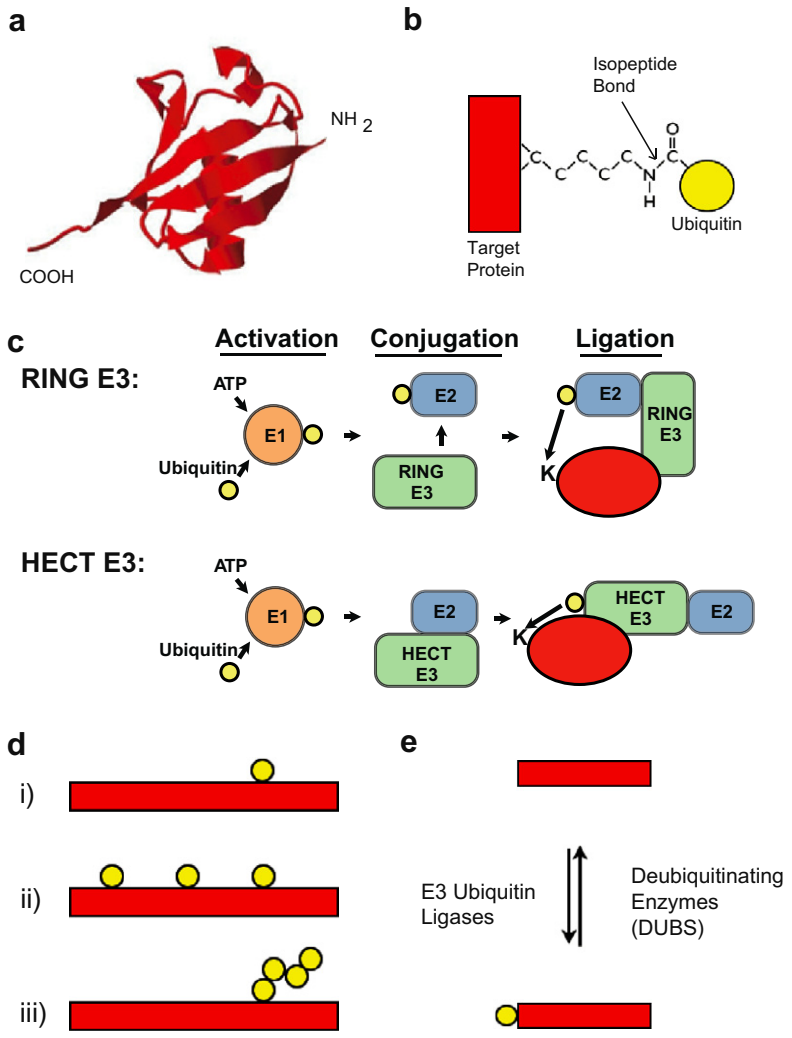

Fig. 8. (a) The structure of ubiquitin (PDB code 1UBI; Ramage et al., 1994), showing the C-terminal tail. (b) Ubiquitin is attached to target proteins via an isopeptide bond. (c) A cascade of enzymes attaches ubiquitin in either of two ways. E1 enzymes activate ubiquitin, using ATP, and transfer it to an active site cysteine residue on an E2 conjugating enzyme. An E3 ubiquitin ligase enzyme recognises the target protein and binds to the E2 enzyme. In the case of RING E3 ligases, the E2 enzyme transfers the ubiquitin onto the target protein whereas HECT E3 ubiquitin ligases ubiquitinate the target proteins themselves. (d) Proteins can be (i) monoubiquitinated, (ii) multimonoubiquitinated, or (iii) polyubiquitinated. (e) Ubiquitination is reversible; ubiquitin is attached by E3 ubiquitin ligase enzymes and removed by deubiquitinating enzymes.

6. Protein ubiquitination

6.1. Ubiquitination of proteins

Ubiquitin is a highly conserved 76 amino acid protein (see Fig. 8) that becomes attached covalently to target proteins by an isopeptide bond between the C-terminal carboxyl group of ubiquitin on glycine 76, and an epsilon amino group of a lysine in the target protein (Ciechanover, 2005); this is called ubiquitination, or ubiquitylation. Ubiquitin moieties are attached by a cascade of E1, E2 and E3 enzymes (Pickart, 2001; Staub and Rotin, 2006). The E1 activating enzyme forms a thioester bond between its active site and the C-terminal carboxyl group of ubiquitin and passes the ubiquitin onto an E2 conjugating enzyme, which also forms a thioester bond with the ubiquitin. An E3 ubiquitin ligase enzyme binds to the E2 and recognises the substrate protein; together the E2 and the E3 attach ubiquitin to the target protein. The human genome contains two E1 enzymes, 37 E2 enzymes and >600 E3 enzymes (Deshaies and Joazeiro, 2009; Markson et al., 2009; Michelle et al., 2009; Groettrup et al., 2008), whereas *Arabidopsis* contains two E1 ubiquitin activating enzymes, 34–37 E2 conjugating enzymes and hundreds of E3 ubiquitin ligases (Kraft et al., 2005). Ubiquitination is a reversible modification, with ubiquitin being attached by E3 ubiquitin ligases and removed by deubiquitinating enzymes (DUBs) (Komander et al., 2009). Proteins can be monoubiquitinated, multimonubiquitinated (in which case several lysine residues have a ubiquitin moiety attached to them) or polyubiquitinated (a second ubiquitin moiety is attached to a lysine residue within the first ubiquitin moiety on the target protein, to form a polyubiquitin chain). There are seven different lysine residues within ubiquitin: lysines 6, 11, 27, 29, 33, 48 and 63, thus there are seven different types of polyubiquitin chain, of which all are found in cells (Xu et al., 2009). Forked ubiquitin chains are possible, containing a mixture of ubiquitin chain linkages (Kim et al., 2007).

The most widely studied type of ubiquitin modification is the K^{48}-linked polyubiquitin chain in which the second ubiquitin moiety is attached to lysine 48 of the first ubiquitin moiety. A chain of four or more K^{48}-linked ubiquitin moieties targets proteins for destruction by the proteasome (Ciechanover, 2005). Ubiquitin can also act as a signalling modification, more akin to phosphorylation, and is recognised by at least 20 ubiquitin binding domains (Hurley et al., 2006). Ubiquitin has many diverse roles in the cell. Monoubiquitination plays a role in transcriptional control via monoubiquitination of histones (Vissers et al., 2008; Weake and Workman, 2008; Laribee et al., 2007)

and sorting of cell surface proteins for degradation by the mammalian lysosome, since several members of the mammalian endocytic machinery are monoubiquitinated (Polo et al., 2002; Urbé et al., 2003; McCullough et al., 2004. Cell surface receptors that are destined for degradation by the lysosome have been shown to be multimonoubiquitinated (Mosesson et al., 2003; Haglund et al., 2003; Carter et al., 2004) The ubiquitinated receptors are degraded in the lysosome in a process called downregulation (reviewed by Clague and Urbé, 2006; Williams and Urbé, 2007; Luzio et al., 2009; Pryor and Luzio, 2009; Woodman, 2009). K^{63}-linked polyubiquitin chains were also found to be present on epidermal growth factor (EGF) receptors that were destined for lysosomal degradation (Huang et al., 2006). Plant cells also have a highly dynamic endocytic pathway (reviewed by Otegui and Spitzer, 2008; Irani and Russinova, 2009; Richter et al., 2009) and they too internalise cell surface receptors for degradation in the vacuole, although the situation is complicated by the fact that at least some plant cells contain two vacuoles, a degradative one, and a storage organelle (Paris et al., 1996; Swanson et al., 1998; Frigerio et al., 2008). Much of the endocytic machinery is conserved between plants and animals, for instance in *Arabidopsis*, two homologues of Rab5 have been found that act in delivery of proteins to the vacuole (Kotzer et al., 2004; Haas et al., 2007). Many plant homologues are known of the yeast ubiquitination machinery (Bachmair et al., 2001) and ATP dependent ubiquitin conjugation has been demonstrated in extracts from a large number of plants (Vierstra, 1987). Evidence for a role of ubiquitin in endocytosis and degradation of cell surface receptors in plants is, as yet, sparse; however, plant homologues have been found of the endosomal sorting complex required for transport (ESCRT) machinery, which directs ubiquitinated proteins to the lysosome in mammalian cells (Winter and Hauser, 2006; Spitzer et al., 2006) and *Arabidopsis* mutants lacking the deubiquitinating enzyme AMSH3 were shown to lack a lytic vacuole, and to secrete vacuolar proteases (Isono et al., 2010). Polyubiquitination plays a role in degradation of proteins with K^{48}-linked polyubiquitin chain formation causing degradation of the target protein by the proteasome (Ciechanover, 2005). Recognition of K^{48}-linked polyubiquitin chains by the proteasome has also been demonstrated in *Arabidopsis* (Fatimababy et al., 2010). K^{63} linked polyubiquitin chains have been shown to be involved in DNA repair (Spence et al., 1995; Hofmann and Pickart, 1999), cell signalling, for instance of NFKB (Sun and Chen, 2004) and membrane trafficking (Hicke, 1999; Huang et al., 2006). K^{11}-linked chains are involved in specifying proteins for degradation by ER associated lysosomal degradation (Xu et al., 2009).

6.2. Methods of analysis for ubiquitinated proteins

Fig. 9 overviews the different protocols for the study of protein ubiquitination. Firstly, Inhibitors can be used since protein degradation by the proteasome can be inhibited with lactacystin and MG132 (Maki et al., 1996) or ubiquitination itself can be inhibited by using a temperature sensitive E1 activating enzyme. Since there are two E1 enzymes but many E3 ubiquitin ligases, inhibiting an E1 enzyme will inhibit ubiquitination by many different E3 ubiquitin ligases. A mutant Chinese hamster ovary cell line called ts20 has been shown to be incapable of modifying proteins with ubiquitin at the non-permissive temperature (Kulka et al., 1988). Whilst there are currently no reports of a temperature sensitive plant E1, a similar effect could theoretically be achieved by using the recently developed chemical inhibitor of E1 activating enzymes (Yang et al., 2007). Small molecule inhibitors are currently being developed against members of each class of enzymes in the ubiquitin proteasome system, namely the E1s, the E2s, some E3 ubiquitin ligases, the proteasome itself and deubiquitinating enzymes, reviewed by Guedat and Colland, 2007. Ubiquitination is an extremely labile modification (even in lysis buffer at 4 °C) and thus it is important to be able to inhibit deubiquitinating enzymes. Most are cysteine proteases (Komander et al., 2009), and can be inhibited with iodoacetamide (Maor et al., 2007), *N*-ethylmaleimide or ubiquitin aldehyde (Hershko and Rose, 1987) although these are known not to work for at least one JAMM domain deubiquitinating enzyme (Cooper et al., 2009). Alternatively, cells can be lysed with boiling SDS (Urbé et al., 2003). Information about whether endocytosed proteins have reached a lysosome or a plant vacuole can be gathered by treating the cells with protease inhibitors, and determining whether the protein accumulates. Leupeptin and E64 have been used to inhibit both lysosomal proteases (Davidson and Watts, 1989), and vacuolar proteases in plant cells (Moriyasu and Inoue, 2008).

Ubiquitination is commonly studied by immunoprecipitating the protein of interest and immunoblotting for ubiquitin, taking care to inhibit deubiquitinating enzymes. Addition of one ubiquitin moiety (monoubiquitination) results in a discrete band on the blot 8.5 kDa above the protein of interest. Multimonoubiquitination or polyubiquitination results in a smear of ubiquitin above the band corresponding to the protein of interest, or possibly a ladder of bands. DNA encoding tagged versions of ubiquitin can be

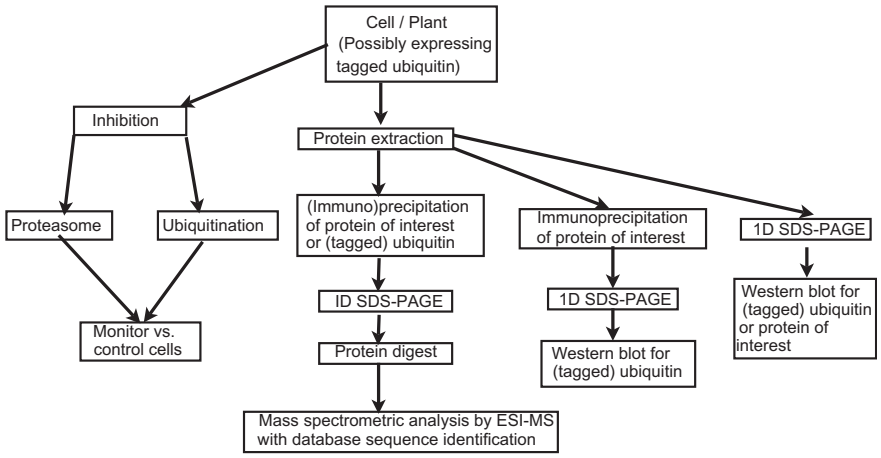

Fig. 9. Flowchart summarising various methods for ubiquitinated protein separation, detection and analysis.

introduced into cells, to lead to expression of tagged ubiquitin, and samples can be blotted with an antibody against the tag, for instance HA-tagged ubiquitin (Mosesson et al., 2003) or Flag-tagged ubiquitin (Row et al., 2006). Polyubiquitination and multimonoubiquitination of a given protein are indistinguishable by western blotting with most ubiquitin antibodies, however the issue can be addressed with the use of the antibodies FK1 and FK2, FK1 only recognises polyubiquitinated proteins whereas FK2 recognises mono and polyubiquitinated ubiquitin conjugates (with K^{29}, K^{48} and K^{63} chain linkages). This approach was used to show that the EGF receptor and Met were multimonoubiquitinated (Haglund et al., 2003; Carter et al., 2004), although it did not reveal polyubiquitination of the EGF receptor. Alternatively, ubiquitin mutants can be expressed that lack some or all of the seven lysine residues and therefore cannot form polyubiquitin because they lack the acceptor sites for the next ubiquitin moiety (Haglund et al., 2003; Mosesson et al., 2003). Recently, reagents have been developed that can discriminate between different types of polyubiquitin chain by western blotting. HWA4C4 is specific for K^{63}-linked polyubiquitin chains (Wang et al., 2008). Further linkage specific antibodies have been developed, which specifically recognise K^{48} or K^{63} linked chains (Newton et al., 2008) and K^{11}-linked polyubiquitin chains (Matsumoto et al., 2010). These reagents will facilitate analysis of ubiquitin editing.

MS has also been applied to the study of protein ubiquitination (reviewed by Kirkpatrick et al., 2005; Xu and Peng, 2008; Peng, 2008; Jeram et al., 2009). Two approaches are possible: either a given protein can be purified and its ubiquitin modifications examined, as has been carried out in the study that showed that phosphoenolpyruvate carboxylase becomes monoubiquitinated in castor oil seeds (Uhrig et al., 2008) or all of the ubiquitinated proteins in the sample can be purified and the ubiquitinated proteins identified, as has been carried out for *Arabidopsis* (Maor et al., 2007). Proteomic analysis by MS requires tryptic peptides (cleaved at arginine and lysine residues) to be produced. Since ubiquitin is attached to lysine residues, trypsin cannot cleave after a lysine residue that has been ubiquitinated. Thus two peptides would be effectively joined together to form a longer peptide and the peptide would have a higher mass because of extra mass from the ubiquitin. The ubiquitin, being itself a protein, is cleaved too and the C-terminal sequence of ubiquitin KESTLHLVLRLRGG gives rise to fragments of: -K, ESTLHLVLR, LR and GG after trypsin digestion. Of these only the C-terminal diglycine is still attached to the peptide fragment of the ubiquitinated protein adding 114.043 Da. During tandem MS fragmentation of the peptide, the b and y ions generated can be used to determine both the peptide sequence and (using the 114 Da shift) the location of the ubiquitination. Since, in a polyubiquitin chain, one ubiquitin moiety is a substrate for another, the ubiquitination sites on the ubiquitin moieties can be determined, whether it is lysine 48 or lysine 63, for instance, and so the type of polyubiquitin chain can be identified. Thus Huang et al. (2006) were able to identify the type of polyubiquitin chain present on the epidermal growth factor receptor as K^{11}, K^{29}, K^{48} and K^{63} linked chains.

It is also possible to use MS to identify all of the proteins that are ubiquitinated after a given stimulus – the "ubiquitinome", "ubiquitome" or ubiquitylome. In one study, suspension cultures of *Arabidopsis* were lysed in the presence of iodoacetamide and 8 M urea to inhibit deubiquitinating enzymes, and ubiquitinated proteins were precipitated using GST-tagged recombinant ubiquitin binding domains, namely a tandem UBA domain from *Arabidopsis* isopeptidase, or a triple UIM domain of the S5 subunit of the *Arabidopsis* 26S proteasome (Maor et al., 2007). Multidimensional protein identification technology (MUDPIT) MS analysis identified 294 ubiquitinated proteins, some of which were polyubiquitinated with K^{48}, K^{63}, K^{11}, K^{33} or K^{29} polyubiquitin chains (in decreasing order of abundance). The proteins included some that were involved in metabolism, transcription, RNA processing, translation and chromatin structure. More recently, *Arabidopsis* seedlings were lysed in the presence of ubiquitin aldehyde and MG132 and the ubiquitinated proteins were purified by precipitation with a UBA domain and analysed by MS (Manzano et al., 2008). In this case, 200 proteins were identified as potential ubiquitinated proteins, with functions similar to those identified by Maor et al. (2007), although there were only seven proteins that were identified by both studies. Often, studies to identify the ubiquitome involve expressing ubiquitin that is tagged in some way and then precipitating the tagged ubiquitin. Overexpression of His-tagged ubiquitin has been particularly successful (Xu et al., 2009) since it circumvents the problem that ubiquitin antibodies do not, in general, work well for immunoprecipitation, and also reduces the amount of irrelevant peptides in the reaction, because His-tagged proteins can be purified on nickel affinity columns. A tandem affinity method has been used to purify ubiquitinated proteins from *Arabidopsis* (Saracco et al., 2009). *Arabidopsis* lines were created that overexpressed His-tagged ubiquitin. Seedlings were lysed in the presence of iodoacetamide, and the ubiquitinated proteins were purified first by precipitation with a tandem UBA domain, followed by nickel beads. MS MUDPIT analysis identified 54 candidate ubiquitinated proteins including some that were polyubiquitinated with every type of polyubiquitin chain apart from K^{27} (Saracco et al., 2009). Another recent technological advance has been to use limiting concentrations of trypsin to digest the ubiquitinated proteins under native conditions (Xu and Peng, 2008). The bulk of the tightly folded ubiquitin molecules does not get cleaved – only the C-terminal trypsin cleavage site is sensitive to cleavage under these conditions because of its extended conformation. This partial digest approach has been termed "Middle down mass spectrometry". Use of the signature mass shift of 114 Da is not without its problems because trypsin digestion of proteins that are modified with ubiquitin and two ubiquitin-like proteins, namely ISG15 and Nedd8 (reviewed by Welchman et al., 2005) all produce an identical signature fragment, because their C-terminal peptide sequences are the same. Thus some proteins may have been identified erroneously as being ubiquitinated, but may in fact be isgylated or neddylated. Another cause for concern is the finding that iodoacetamide can derivatize peptides to produce a mass shift that mimics the 114 Da signature peptide from trypsin digestion of ubiquitinated proteins (Nielsen et al., 2008; Lapko et al., 2000). The use of alternative proteases, such as Lys C, or combinations on replicate samples (reviewed by Jeram et al., 2009) shows promise.

6.3. Protein ubiquitination in plants

An interesting aspect of ubiquitination in plants is the direct binding of a hormone (Auxin) to an E3 ubiquitin ligase. Auxin has many actions, including elongation of oat coleoptiles (the protective sheath around the new leaf), the bending of stems towards light, and the formation of adventitious roots (Woodward and Bartel, 2005). Recently, insights into auxin's mode of action have revealed a surprising turn of events (reviewed by Santner and Estelle, 2009, 2010; Tan and Zheng, 2009). Auxin exerts its effects through transcriptional control. In the presence of auxin, hundreds of genes are transcribed by transcription factors called ARFs (Auxin Response Factors; Hagen and Guilfoyle, 2002). In the absence of auxin, transcription is inhibited because proteins called the Aux/IAA proteins bind to the ARF transcription factors and block transcription (IAA stands for Indole-3-acetic acid, one of the auxins). If auxin is present, the Aux/IAA protein binds to TIR1 (Dharmasiri et al., 2005; Kepinski and Leyser, 2005), the "F-box" component of an E3 ubiquitin ligase complex called the SCF (Skp1-Cullin-F-box) complex, reviewed by Yu et al. (2007). The Aux/IAA protein

becomes polyubiquitinated and is then degraded by the proteasome (Gray et al., 2001). This loss of the Aux/IAA protein alleviates the transcriptional repression, so that ARF can transcribe the gene in question. The transcriptional control described above is not unusual. The striking feature of the process is the way that the SCF ligase (and TIR1 in particular) is controlled, so that the Aux/IAA protein is ubiquitinated and degraded only when auxin is present. It was expected that there would be an auxin receptor, which would set up a signalling cascade, which would lead to the activation of the TIR1 ligase. This was not the case; the auxin receptor is TIR1, part of the E3 ubiquitin ligase itself. This is highly unusual – as yet there is no example of this from the mammalian literature. Another unusual feature is that the complex binds to a lipid that was unknown before the elucidation of this pathway, namely inositol hexakisphosphate (IP6).

Other studies of ubiquitination in plants have shown that in *Arabidopsis*, the E3 ubiquitin ligases UBC1 and UBC2 mediate ubiquitination of histone H2B, upregulating the expression of *FLOWERING LOCUS C* (*FLC*), which in turn represses the flowering process (Gu et al., 2009). However, somewhat confusingly, an *Arabidopsis* mutant lacking the DUB *UBP26*, that cannot deubiquitinate H2B and so accumulates ubiquitinated H2B (and would thus be expected to express *FLC*), instead produces low levels of mRNA for *FLC* and has an early flowering phenotype (Schmitz et al., 2008). Other research has indicated a role for ubiquitination in receptor degradation akin to the degradation of EGFR in mammalian models. *Arabidopsis* was transformed with a GFP fusion protein of the plant pattern receptor FLS2 (Robatzek et al., 2010). Incubation of the leaves with the ligand for FLS2, a fragment of bacterial flagellin called flg22 and analysis of the leaves by immunofluorescence showed that the FLS2 was taken up into intra-cellular vesicles within 10 min and degraded after longer incubation times (Robatzek et al., 2010). FLS2 becomes ubiquitinated by an E3 ubiquitin ligase called AvrPtoB (Göhre et al., 2008).

7. Concluding remarks

It seems clear that the major focus of proteomic analysis has been towards mammalian systems, however the technologies utilised in these analyses have also been applied to the area of plant science. Protein modification, as described in this review, provides the cell with the ability to modulate protein activity, localise proteins and control their rate of degradation and therefore it can be argued that protein modification has as great an impact on cellular as the level of protein expression (quantitative proteomics). The ability to study the intentional modification of proteins and the occasionally unintentional (redox based) modification has led to a greater understanding in a number of areas regarding plant growth, defence, signalling and control of events such as flowering. The low stoichiometry and lability of some modifications adds to the problems in obtaining an accurate dataset regarding which proteins are modified. A further hurdle in proteomic analysis as a whole in plants (impacting also on protein modification analysis) is the lack of a fully sequenced and annotated genome sequence for some species of plants. Without such information proteomic analysis requires complete homology between any plant under investigation and a plant species whose genome has been sequenced in order to identify proteins. As the number of groups actively working in studying plant proteomics and protein modification increases, it will be possible for large datasets from the same species of plant to be compared in order to determine intra-laboratory differences, overlaps and the reproducibility of proteomic data as has been achieved for human protein analysis via the Human Proteome Organisation (HUPO: http://www.hupo.org/).

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