Analytical technologies for identification and characterization of the plant N-glycoproteome

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INTRODUCTION

Characterization of the detailed structures of N-glycoproteins can provide valuable insights into basic aspects of cell and organismal biology and is also becoming increasingly important for pharmaceutical development and production (De Marchis et al., 2011; Xu et al., 2011; Yang et al., 2012). In plants, N-glycosylation is a common feature of secreted proteins and there is considerable interest in developing more advanced analytical platforms to characterize N-glycosylation in order to provide new insights in the plant cell wall proteome (Minic et al., 2007; Marino et al., 2010; Zhang et al., 2010; Catala et al., 2011; Ruiz-May et al., 2012). However, structural elucidation of glycans, glycopeptides, and glycoproteins is notoriously difficult as glycans are structurally complex, being branched, and containing a variety of types of intersaccharide linkages, and heterogeneous, with a wide range of abundance between glycoforms and occupancy of glycosylated sites. Ideally, a comprehensive analysis of a plant glycoprotein of abundance between glycoforms and occupancy of glycosylated intersaccharide linkages, and heterogeneous, with a wide range of complexity and variability in glycoprotein populations from any eukaryotic source still poses significant analytical obstacles. Sample preparation that allows enrichment of the targeted glycoproteins/glycopeptides is therefore an important step as it increases the concentration of the desired analyte while reducing sample complexity. A second technical hurdle is the hydrophilic nature of glycans and their relatively larger masses compared with the native tryptic peptides to which they are attached. This often results in poor retention on the reverse phase chromatographic materials that are typically used in peptide separations, reduced ionization efficiency and larger than optimal m/z detection range in MS analysis, all of which can contribute to poor sensitivity (An et al., 2009; Ytterberg and Jensen, 2010). Furthermore, the labile nature of the glycan–peptide bond often causes the neutral loss of individual carbohydrates and few fragmented ions from the peptide during the collision induced dissociation (CID), yielding little or no information for peptide identification, glycol-site determination, and full glycan sequence. As a result, deglycosylation is often required and the free peptide and glycan are subjected separately to tandem MS (MS/MS) analysis. Finally, due to the high glycan heterogeneity, determination of the glycan structure through bioinformatics tool-based database searching is often difficult and requires time-consuming manual interpretation.

To overcome these analytical challenges, many new strategies have been developed and implemented over the past decade involving the development of three major tools: (1) selective enrichment technologies for glycoproteins, glycopeptides, and oligosaccharides (e.g., sequence, linkages, and branching), the high degree of complexity and variability in glycoprotein populations from any eukaryotic source still poses significant analytical obstacles. Sample preparation that allows enrichment of the targeted glycoproteins/glycopeptides is therefore an important step as it increases the concentration of the desired analyte while reducing sample complexity. A second technical hurdle is the hydrophilic nature of glycans and their relatively larger masses compared with the native tryptic peptides to which they are attached. This often results in poor retention on the reverse phase chromatographic materials that are typically used in peptide separations, reduced ionization efficiency and larger than optimal m/z detection range in MS analysis, all of which can contribute to poor sensitivity (An et al., 2009; Ytterberg and Jensen, 2010). Furthermore, the labile nature of the glycan–peptide bond often causes the neutral loss of individual carbohydrates and few fragmented ions from the peptide during the collision induced dissociation (CID), yielding little or no information for peptide identification, glycol-site determination, and full glycan sequence. As a result, deglycosylation is often required and the free peptide and glycan are subjected separately to tandem MS (MS/MS) analysis. Finally, due to the high glycan heterogeneity, determination of the glycan structure through bioinformatics tool-based database searching is often difficult and requires time-consuming manual interpretation.

Keywords: glycoproteins, N-glycans, glycopeptides, lectins, mass spectrometry
Another practical, but sometimes less informative means to assess glycoprotein presence is the assessment of changes in the electrophoretic mobility of the constituent proteins. This is a relatively straightforward technique, but does not distinguish between different types of N-glycans, or between N- and O-linked glycans. The presence of glycophorins can also be suggested by the presence of sialic acid terminal non-reducing residues (Ruiz-May et al., 2012). Antibodies to horseradish peroxidase (HRP) can recognize glycoproteins with an epitope associated with β-1→3 fucose structures on complex-type N-glycans (Fitchette et al., 2007; Bardor et al., 2009; Figure 2).

Once an enriched glycoprotein sample has been prepared, a typical next step is to fragment the parent protein backbone and analyze it with MS. Depending on the protein complexity in the initial samples, the analysis of glycoproteins then often proceeds with the enrichment of glycopeptides following glycoprotein digestion. This can result in missed cleavages, yielding glycosylated polypeptides that are too large to be detected by some MS instruments. However, the use of less specific proteases, such as pepsin, thermolysin, Proteinase K, either alone or in combination, can reduce this problem (Chen et al., 2009). Nevertheless, no matter what the digestion procedure, the degree of heterogeneity of the resulting mixture of peptides and glycopeptides can complicate the subsequent analysis. Glycopeptides often ionize less efficiently than non-glycopeptides and may be less abundant than their unmodified counterparts. Therefore, in some cases, deglycosylation prior to or concurrent with enzymatic digestion is desirable (see below). If deglycosylation is not employed, an additional enrichment of glycopeptides following glycoprotein digestion is often implemented to improve specificity. An alternative chemical approach for glycoprotein/glycopeptide enrichment is the use of hydrazine released glycans, and improvements in separation science including the miniaturization of chromatographic formats, capillary electrophoresis (CE), and ion mobility technologies, (2) enhanced MS technology and development of more effective analysis workflows; and (3) newly developed software packages and algorithms for interpretation of MS fragmentation spectra. Most of these technologies have been initially developed to study mammalian glycoproteins and there are only a few reported examples of their successful application to characterize plant N-glycosylation (Fitchette et al., 2007; Bardor et al., 2009; Zhang et al., 2012), which is structurally distinct from that of mammalian or yeast proteins (Ruiz-May et al., 2012). Key structural differences, such as the absence of non-reducing terminal sialic acid in plant N-glycans and the presence of α-1,3 fucose (Fuc) in the innermost GlcNAc residue necessitate tailoring of existing analytical workflows.

In this review we present some of the analytical platforms or strategies, including new developments in MS instrumentation, which can be used for the systematic characterization of N-glycoproteins, with particular reference to the challenges of studying those from plants, as well as a perspective of future developments in the field. To our knowledge there are no published reports describing large- or even intermediate-scale analysis of plant protein glycosylation together with associated N-glycans analysis. However, given the new and emerging analytical pipelines we are soon likely to see a dramatic expansion in the identification and functional characterization of plant N-glycoproteomes, which in turn will be of great value for researchers studying the plant cell wall and associated secretory pathways.

**GLYCOPROTEIN DETECTION**

A summary of a typical workflow for characterizing N-glycoproteins/larger scale N-glycoproteomes is shown in Figure 1. A valuable first step is to confirm the presence of glycoproteins in a crude extract using 1D or 2D gel-based electrophoretic separations, followed by immunoblot analysis or direct visualization using a commercially available sugar detection stain (Fitchette et al., 2007; Bardor et al., 2009). Antibodies to horseradish peroxidase (HRP) can recognize glycoproteins with an epitope associated with β-1→2 xylose and/or α-1→3 fucose structures on complex-type N-glycans (Fitchette et al., 2007; Bardor et al., 2009; Figure 2).

GLYCOPROTEIN/GLYCOPEPTIDE ENRICHMENT AND PREPARATION

Depending on the protein complexity in the initial samples, the analysis of glycoproteins then often proceeds with the enrichment of glycoproteins or glycopeptides by specific lectins or immunosorbent chromatography (Budiger and Gabius, 2001) to remove non-glycosylated forms. Lectin chromatography using Concanavalin A (Con A) has been reported for the enrichment of N-glycoproteins from diverse sources including human serum, Caenorhabditis elegans plasma membrane glycoproteins (Bunkoeng et al., 2004; Fan et al., 2004), and plant tissues (Minic et al., 2007; Zhang et al., 2010; Catala et al., 2011). Various plant lectins are available with affinities for different sugars (Gabius et al., 2004) and such lectins have been used sequentially (Yamamoto et al., 1995, 1998), in parallel (Yang et al., 2006; Lee et al., 2009; Zelinsky et al., 2010), and as mixtures (Li et al., 2004) to increase the range of identified glycoproteins. When working with limited amounts of sample it is often necessary to miniaturize the chromatography. Both silica-based and monolithic materials incorporating a lectin-containing stationary phase are now commercially available that can be packed into micro or capillary columns, greatly increasing the number of glycoproteins that can be detected in low concentrations compared with standard column formats (Madiera et al., 2006; Feng et al., 2009).

Several such enrichment methods have been successfully applied in large-scale N-glycoproteome analyses, such as the combined use of lectin (Con A) affinity chromatography and enzymes such as PNGase-A can be used as an alternative (Ytterberg and Jensen, 2010).
FIGURE 1 | A schematic overview of experimental approaches for the systematic characterization of glycoproteins by mass spectrometry-based proteomic analysis. Regardless of the sample sources, the general workflow includes three major steps: enrichment of glycoproteins, glycopeptides, and released glycans; mass spectrometry analysis of glycopeptides, glycans, and peptides; and interpretation of MS fragmentation spectra, allowing identification of core amino acid sequences of the glycopeptides, glycosylation sites, and potential glycan structures.

FIGURE 2 | Organ-specific immunodetection of glycan epitopes. Protein extracts from different tomato organs were fractionated 12% polyacrylamide gels and visualized by SYPRO Ruby protein staining (A) or transferred onto nitrocellulose membrane for immunoblotting using antisera that recognize β-1,2-xylose (B) or α-1,3-fucose (C) residues.

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highly selective precursor ion scanning for sugar oxonium ions. The oxonium ions are often used as diagnostic marker ions, indicating the presence of specific sugar compositions. The oxonium ions are often used in mass spectrometry to identify and characterize peptides and glycopeptides. In this regard, two new dissociation techniques, electron capture dissociation (ECD; Syka et al., 2004) and electron transfer dissociation (ETD; Syka et al., 2004), provide valuable alternative dissociation techniques that induce backbone fragmentation at N-C bonds and create complementary c- and z-type ion series, yielding information that is complementary to conventional CID fragmentation (Zubarev et al., 1998; Syka et al., 2004; Coon et al., 2008). ETD is generally favored as chemical approaches often introduce unexpected modifications of peptide side chains (Fitchette et al., 2007; Bardor et al., 2009).

After deglycosylation, LC-MS/MS is used to identify the amino acid sequence of the deglycosylated peptide. Alternatively, the development of ion mobility spectrometry (IMS) has extended the ability to characterize glycoprotein/glycopeptides to the realm of isomeric and conformational structure (Plasencia et al., 2008). For example, Olivova et al. (2008) used IMS to resolve the light and heavy chains in the gas phase allowing the accurate mass measurement of each. Furthermore, the informative dual-collision cell design of this instrument was leveraged in a two-step fragmentation process to provide a detailed characterization of the glycan structures and the determination of the glycosylation site.

Other than direct MS analysis of enriched glycopeptides, deglycosylation steps can also be performed to gain additional information about peptide sequence and complete glycan structure. These enzymes are commonly used for enzymatic deglycosylation. PNgase F, an amidease that hydrolyzes the amide bond between the Asn residue on the peptide backbone and the innermost HexNAc unit, is active on high mannose and complex plant N-glycans, except those with an α-1,3-fucose residue linked to the innermost HexNAc (Fitchette et al., 2007; Bardor et al., 2009).

In this case, PNgase A can be used to release glycans from relatively short glycopeptides (Fitchette et al., 2007; Bardor et al., 2009). Alternatively, endoglycosidase H (Endo H) cleaves the glycosidic bond between the two GlcNAc residues on the high-mannose-type N-glycans of plant glycoproteins (Harvey, 2005). While these deglycosylation steps are time consuming it has been shown that pressure cycling and exposure to microwaves can greatly reduce deglycosylation times (Chen et al., 2009). Chemical deglycosylation can also be used, although enzymatic deglycosylation is generally favored as chemical approaches often introduce unexpected modifications of peptide side chains (Fitchette et al., 2007; Bardor et al., 2009).

Traditional, low-energy CID MS/MS has been the method of choice for peptide and glycopeptides sequencing operating in either automated data-dependent acquisition mode, or manual selection for MS/MS through infusion analysis. Another unique feature of CID MS/MS analysis of glycopeptides or glycans is the production of oxonium ions, such as m/z 163 (Hex + H)\(^+\), m/z 204 (HexHexNAc + H)\(^+\), and m/z 366 (Hex-HexHexNAc + H)\(^+\), which can be used as diagnostic marker ions, indicating the presence of specific sugar compositions. The oxonium ions are often monitored in precursor ion scanning mode for highly selective detection of glycopeptides in digestion mixture and subjected to MS/MS and MS/MS/MS of the selected glycopeptides ions, yielding complete peptide and glycan sequences (Sandia et al., 2004; Zhang and Williamson, 2005). It should be noted that these oxonium ions are produced by the incorporation of ECD-type fragmentation in more common LC enrichment coupled with nano-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) allows for low concentration of analytes, improves ionization efficiency and allows for low flow rates, which in turn makes it possible to use extended analysis times for MS/MS and multi-stage fragmentation (MS\(^n\)) oligosaccharide analyses (Bahr et al., 1997).

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released glycans can be further purified and analyzed by MALDI-TOF or LC-ESI-MS/MS analysis to identify the glycan structure (Au et al., 2009). In practice, MS/MS spectra derived from the glycosidic bond fragment ions, along with cross-ring fragmentation of glycans and treatments with different exo-hexosidases are required for complete structural elucidation (Zaia, 2004). In the past, per-derivation, such as permethylation, of glycans has often been used to improve sensitivity and has yielded more structurally relevant fragment ions during MS/MS. However, as new MS technologies are constantly being developed, the sensitivity improvements provided by derivatization has only been marginal and insufficient to compensate for the more complex sample preparation and the fact that derivatization makes it difficult to employ glycosidase treatment to determine linkages and identify saccharide units (Kang et al., 2007). Another technique, called isotope coded glycosylation site-specific tagging (IGOT), has been used for the large-scale identification of N-linked glycoproteins from complex biological samples of C. elegans (Kaji et al., 2003, 2007) and mouse (Zielinska et al., 2010). This approach involves protein tryptic digestion and enrichment of resulting glycopeptides followed by conversion of glycosylated Asn residues into 18O-labeled aspartic acids by PNGase F digestion in 18O water. The subsequent 3 Da mass difference created by this process is readily identified by LC-MS/MS analysis, indicating N-linked glycosylation sites. However, a potential pitfall of this approach is that a significantly high false positive rate (∼60%) has been reported (Angel et al., 2007), apparently due to the fact that the trypsin used for proteolysis was still active, leading to the incorporation of 18O into the C-termini of the peptides during the deglycosylation step.

Capillary electrophoresis has been used to analyze the heterogeneity of isolated glycoproteins with MS detection (Balaguer and Neusaus, 2006; Thakur et al., 2009; Szabo et al., 2010) and the incorporation of microfluidic devices can also greatly facilitate the analysis by increasing sensitivity and reducing analysis times (Zhuang et al., 2007, 2011). While these CE/MS methods have proven useful as a means to characterize glycoprotein heterogeneity and to investigate the structure of glycans at the N- and O-glycosylation sites of recombinant glycoproteins (Zaia, 2010), they have not yet proven sufficiently robust for the analysis of complex glycoprotein mixtures.

**QUANTITATIVE N-GLYCOPROTEIN ANALYSIS BY MS**

In principle quantitative glycoproteomics experiments may be carried out by the same assortment of techniques that have been developed and are currently in use for labeling and label-free approaches in other proteomics disciplines. There have been several excellent reviews (Bindschedler and Cramer, 2011; Matus et al., 2011; Pan et al., 2011) and the reader is referred to these for detailed descriptions of the various experimental strategies employed and methodologies in use.

Historically, the quantification of proteoglycans has been difficult as it involves both the structural elucidation of glycan and peptide moieties. While structural characterization of peptides is well developed and is facilitated by the existence of complex genomic databases for a growing number of species, the structural characterization of the glycan moiety remains a challenge as they are often exceedingly heterogeneous and chemically complex, with differing chemical configurations and abundant isoforms. Furthermore, glycans are frequently branched and contain an assortment of glycan linkages (Zaia, 2004). These complications combine in such a way that quantitative experiments must be carried out in different tiers with each providing a different level of quantitative information. The first tier involves determining glycosylation sites and their degree of occupancy. For N-linked glycans potential glycosylation sites can be recognized due to the presence of the consensus sequence Asn-X-Ser/Thr using any of several open access programs such as NetGlyc (http://www.cbs.dtu.dk/services/NetOGlyc/). However, not all potential sites are occupied and those that are occupied are often only partially so by a variety of differing glycans. Determining the number and types of glycoforms and their relative proportion present at each site represents the second tier of quantifications. The third tier of glycoprotein quantification involves comparing the state of glycosylation between different samples, i.e., control verses treated, control verses diseased, etc. Of the methods available, only MS allows for quantitative characterization at all three tiers.

Two-dimensional gel electrophoresis with glycan-specific staining has been the traditional method used to identify and quantify glycoproteins (Bardor et al., 2009). When coupled with MS of tryptic digests of excised spots it can provide quantitative characterization on all three levels. However, the heterogeneity and structural complexity of the glycans has important consequences on a protein’s mobility which complicate the interpretation of the electropherograms. Thus, LC-MS has emerged as the preeminent tool for the identification and quantitative characterization of glycoproteins and glycopeptides. The development of novel hybrid mass spectrometers incorporating ion mobility cells, high mass accuracy and resolution, improved sensitivity, and scanning capabilities has allowed detailed glycoprotein structural characterization, with accurate empirical information concerning the position and degree of occupancy of the glycosylation sites as well as the composition, structure, and distribution of glycoforms.

In the discovery phase of research, most quantitative MS strategies involve either a labeling approach (Gygi et al., 1999, Kaji et al., 2003; Zhang et al., 2003; Ross et al., 2004; Aggarwal et al., 2006; Atwood et al., 2008; Haqqani et al., 2008) or a label-free strategy (Hill et al., 2009; Rebecca et al., 2009; Zhang et al., 2012). These non-targeted approaches allow one to quickly survey complex proteomes to determine the features that vary between samples and more precise quantitative information can be obtained through a targeted approach involving specific reaction monitoring (SRM) as recently described by Zhang et al. (2012). New developments involving data independent acquisition (DIA) using approaches such as MSp (Levin et al., 2011; Gillet et al., 2012; Hopfgartner et al., 2012) could potentially simplify these procedures further by allowing the discovery and targeted data to be collected simultaneously greatly reducing the instrument time required.

**BIOINFORMATIC TOOLS**

One of the biggest challenges in the field of glycoproteomics is the development of bioinformatic tools for glycan analysis. As a consequence of the complex fragmentation behaviors of glycopeptides and glycans, which tend to vary under different CID
As described above, the characterization of the plant N-glycoproteome is methodologically challenging due to the extreme structural heterogeneity and the often low concentration of several glycoforms attached to each glycopeptide residing in the total glycoproteomic analysis: size exclusion chromatography facilitates identification of tryptic glycopeptides with N-linked glycosylation sites. J. Proteome Res. 7, 567–574.

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