

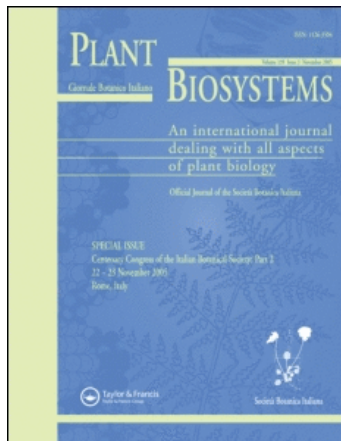
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ADVANCES FOR CELL AND TISSUE ANALYSES IN PLANTS. PART 1

Mass spectrometry in plant proteomic analysis

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Abstract

The current revolution in proteomics has been generated by the combination of very sensitive mass spectrometers coupled to microcapillary liquid chromatography, specific proteolysis of protein mixtures and software that is capable of searching vast numbers of mass measurements against predicted peptides from sequenced genomes. The challenges of post-genomic plant biology include characterization of protein function, post-translational modifications and composition of protein complexes as well as deciphering protein complements in intracellular compartments – proteomes of cell organelles. In this review we summarize the current mass spectrometry methods currently being used in plant proteomics and discuss the various tagging strategies that are being used for purification and proteomic analysis of plant protein complexes.

Abbreviations: BCCD, biotin carboxyl carrier protein domain; CBP, calmodulin-binding protein; CID, collision-induced dissociation; ESI, electrospray ionization; EST, expressed sequence tag; FT-ICR, Fourier transform ion cyclotron resonance; GFP, green fluorescent protein; GST, glutathione S-transferase; HA, haemagglutinin; HILEP, hydroponic isotope labelling of entire plants; His, histidine; HPB, HA-PreScission-Biotin; HPLC, high-performance liquid chromatography; ICAT, isotope-coded affinity tags; ICPL, isotope-coded protein label; iTRAQ, isobaric tag for relative and absolute quantification; LC, liquid chromatography; MALDI, matrix-assisted laser desorption ionization; MBP, maltose-binding protein; MS, mass spectrometry; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SILAC, stable isotope labelling with amino acids in cell culture; SILIP, stable isotope labelling *in planta*; Strep, streptavidin; TAP, tandem affinity purification; TBP, TATA-box-binding protein; TOF, time-of-flight; UPLC, ultraperformance liquid chromatography

Keywords: *Plant sample preparation, complex mixtures, protein tagging, TAP tag, LC-MS/MS*

Methods and instruments for MS-based proteomics

Introduction

The proteome is the complete set of proteins expressed by a genome, cell, tissue or whole organism at a given time under defined conditions and is the functional representation of the genome (Wilkins et al. 1997). The proteome size is much bigger than the genome size of an organism, as, for example, in human where the proteome is estimated at a million proteins whereas the number of genes is 20,000–40,000. This ratio differs from one organism to another essentially due to the prevalence of alternative or multiple splicing events (Harrison et al. 2002; Baerenfaller et al. 2008). The proteome is also highly

dynamic because of tissue-/developmental-specific mRNA expression and many post-translational modifications (Wilkins et al. 1997). A genome-scale proteome map for *Arabidopsis thaliana* has recently been constructed by Baerenfaller et al. (2008) who used different organs, development stages and undifferentiated culture cells to generate a complex dataset for evaluation of gene models and proteome dynamics. Therefore, one of the challenges in proteomics is to analyse thousands of proteins in a single sample, and mass spectrometry (MS) is currently the most powerful tool to achieve this (Pandey & Mann 2000). A major challenge for proteomic analysis is, however, the absence of methods for protein amplification in an equivalent way to polymerase chain reaction (PCR) for DNA amplification.

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Principles of mass spectrometry and current instruments

A mass spectrometer analyses the components of a sample by measuring the mass-to-charge ratio (m/z) of charged peptide ions and provides structural information by fragmentation analysis, leading to peptide sequence and protein identification. A typical mass spectrometer contains an ionization source, a mass analyser to measure the m/z of the ions and a detector to register the number of ions at each m/z value. Tandem mass spectrometers have two or more mass analysers and employ ion fragmentation (most commonly, collision-induced dissociation [CID]) to fragment a precursor ion after the first mass analyser. The first mass analyser detects the whole spectrum of peptide ions present in the sample (MS scan). The ions of interest can then be selectively allowed to progress into the collision cell, containing an inert gas that collides with the selected ion-inducing fragmentation. The resulting ions are then analysed as product ions (MS/MS scan; de Hoffmann & Stroobant 2007).

Matrix-assisted laser desorption ionization (MALDI; Tanaka et al. 1988; Karas & Kruger 2003) and electrospray ionization (ESI; Fenn 2002) are the two soft ionization techniques, most commonly used for proteomics. In typical MALDI sample preparation, the protein digest is mixed with a matrix solution and co-crystallized on a MALDI target plate. When the laser is directed onto the MALDI plate, the matrix absorbs the laser energy, becomes ionized and transfers the charge to the peptides while protecting them against the laser energy. The ions released are then analysed (Karas & Kruger 2003). MALDI-MS is easy to implement and is a reliable technology. It can be relatively inexpensive and extremely fast with regard to data acquisition which makes it ideal for large-scale high-throughput applications (Cramer et al. 2005).

In ESI, the analyte solution is sprayed through the electro spray needle towards the ion source that has a high potential difference with respect to the needle (Fenn et al. 1989). The solvent is evaporated from the droplets, and the remaining solutes are ionized under a strong electric field and temperature applied to the source (Mora et al. 2000; Fenn 2002). ESI is a very gentle ionization and can be easily coupled with liquid chromatography (LC) for fractionation of the sample. The advantage of electro spray spectra for large molecules is production of multiply charged component ions, which can be easily fragmented to produce ions for MS/MS analysis.

Several different types of mass analysers are routinely used in proteomic MS, such as linear quadrupole (or multipole) ion trap (Douglas et al. 2005; Payne & Glish 2005), time-of-flight (TOF) (Chernushevich et al. 2001; Cotter et al. 2007) and

Fourier transform ion cyclotron resonance (FT-ICR) analysers (Marshall et al. 1998). These differ in their sensitivity, resolution, mass accuracy and the resulting ability to generate clear ion mass spectra from peptide fragments (Domon & Aebersold 2006). For example, linear ion traps are robust and sensitive instruments, but have relatively low mass accuracy. Combination of two or more analysers is used in “hybrid” instruments that bring together the best of different analysers and allow tandem MS (MS/MS) analysis.

The Fourier transform-ion cyclotron resonance-mass spectrometer (FT-ICR-MS) is a highly sensitive instrument with an excellent mass accuracy and resolution compared with that of any other MS instrument. This is also a trapping instrument with an ultrahigh vacuum system that allows the ions to be trapped for long periods of time. The use of this machine includes intact protein analysis (top-down analysis), analysis of proteolytic digests (bottom-up analysis) as well as mapping 3D structure (conformational analysis; Heeren et al. 2004).

Recently, a new type of mass analyser called the Orbitrap has been developed (Hardman & Makarov 2003). The new LTQ–Orbitrap is a hybrid instrument composed of a fast linear ion trap and the new Orbitrap detector which combines high accuracy with very good sensitivity (Hu et al. 2005).

Sample preparation and fractionation

Sample fractionation is crucial for successful identification of low-abundance proteins by MS, since the most abundant peptides tend to dominate the mass spectrum and mask any minor peaks. There are several options for sample fractionation, including methods for organelle or subcellular fractionation (Dunkley et al. 2004; Pendle et al. 2005), 1D or 2D gel fractionation, non-gel-based fractionation and one- or two-dimensional LC, preferably HPLC (high-performance liquid chromatography) or nano-UPLC (ultraperformance liquid chromatography). Protein fractionation using either one- or two-dimensional gel electrophoresis (1DE and 2DE, respectively) is often used prior to LC-MS. 2DE is very commonly used before MALDI-TOF analysis to separate proteins. In this case, single spots from 2D gels are excised, digested and analysed by MS. This has been used to study the *Arabidopsis* nuclear proteome (Bae et al. 2003). This can be, however, a very time-consuming process that usually needs quite large amounts of starting material. Protein separation by 1D sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions is a simple and commonly used fractionation method. The protein bands are then excised and digested by trypsin, usually in the

gel pieces, to produce a peptide mixture for every gel band or section. This mixture is usually further fractionated on an LC chromatography column prior to MS (Rosenfeld et al. 1992; Wu et al. 2005). Trypsin is the most widely used proteolytic enzyme because it is very stable and specific for lysine and arginine residues, which aids the software in peptide and protein identification. Each peptide mixture sample can then be analysed by LC-ESI-MS/MS or MALDI-MS/MS.

Analysis of complex mixtures

A complex mixture may contain many thousands of peptides that must be fractionated before they can be passed to the mass spectrometer. The peptide mixtures are usually separated by gradient elution in an HPLC or UPLC system on the basis of their hydrophobicity on reverse phase columns. After elution from the column, the peptide fractions go through a fine capillary tip to be sprayed over the ESI of the mass spectrometer. LC-MS/MS generates large amounts of raw MS data. Algorithms have been created to analyse MS data, leading to development of search engines that use MS peptide data to identify proteins from primary sequence databases. A proteomic search engine generates hypothetical peptides and their theoretical m/z and tandem mass spectra from the protein sequence database. The program then compares each theoretical spectrum to the observed mass spectrum and determines the best match of the peptides which have the same mass as the precursor ion. SEQUEST (Eng et al. 1994; MacCoss et al. 2002) was the first such program algorithm developed. There are several other software packages to analyse MS/MS data such as X!Tandem (Craig et al. 2004), OMSSA (Geer et al. 2004) and PEAKS (Ma et al. 2003) for *de novo* sequencing, but Mascot (Perkins et al. 1999) is the most widely used search engine. The MS2 data are submitted to Mascot as a peak list, which contains, for each peptide, the mass of the precursor ion (MS1) and the masses of its fragment ions (MS2). Species with completely sequenced genome, such as *Arabidopsis* or rice, have comprehensive and well-annotated translated protein databases. Protein identification in species with unsequenced genomes is a more difficult, but not impossible, task, especially for cereal genomes such as wheat in which many proteins have considerable sequence identity with sequenced genomes such as rice and Brachypodium. Expressed sequence tag (EST) databases, such as the TIGR Plant Transcript Assemblies (TA) databases (Childs et al. 2007), can also be used when a complete genome sequence is not available. The TA databases contain the sequences of expressed transcripts collected from EST databases

and the NCBI GenBank nucleotide database (full length and partial cDNAs). However, virtually complete genome sequences will be available in the near future for many of the commonly studied plants.

Quantitative proteomics

Mass spectrometry allows identification of thousands of proteins and their modifications in the same mixture, but it also can be used for quantification and comparison of different samples (Mann 1999; Mann et al. 2001; Ong & Mann 2005). A powerful approach to performing this comparison is to label the protein samples differentially so that the source of each peptide in the whole complex mixture can be identified after MS of the two samples mixed together. For protein identification and quantification in complex mixtures, relative quantification based on the use of isotopes is the most accurate method (Steen & Mann 2004). A number of methods have been developed to label proteins/peptides prior to MS analysis. These methods can be divided into two categories: the post-extraction labelling category (isotope-coded affinity tags [ICAT], isotope-coded protein label [ICPL], isobaric tag for relative and absolute quantification [iTRAQ], ^{18}O) and *in vivo* labelling category (stable isotope labelling with amino acids in cell culture [SILAC], ^{15}N).

Post-extraction labelling. ICAT, developed in 1999, is a method based on labelling of cysteine residues in protein samples with a “light” or “heavy” reagent (Gygi et al. 1999). The labelled samples are then pooled, digested and compared by MS. ICAT can only distinguish between protein samples containing cysteine, which is a great limitation as cysteine is a rare amino acid and is only present in a fraction of proteins or peptides. A similar method called ICPL based on the labelling of the much more frequent free amino groups of intact proteins, which is applicable to any protein sample, has recently been developed (Kellermann 2008). iTRAQ is a method in which the peptides in a mixture after proteolysis are labelled at their N-termini. Sets of four or eight iTRAQ reagents have been developed, allowing the comparison of two to eight samples in the same run (Dunkley et al. 2006; Jones et al. 2006).

Oxygen isotope labelling is used in the “heavy water” labelling technique, which is based on incorporation of one or two ^{18}O atoms into the carboxyl termini of all tryptic peptides during proteolytic cleavage of proteins using ^{18}O rich water for digestion. The samples with ^{16}O and ^{18}O are then pooled and compared (Yao et al. 2001).

In vivo labelling techniques. SILAC is a method based on the incorporation of amino acids containing substituted stable isotopes (e.g. deuterium, ^{13}C , ^{15}N). The “heavy” or the “light” amino acid is incorporated *in vivo* via the normal metabolism of the cell and generates proteins of altered molecular mass, whose peptides can be easily differentiated in the MS (Ong et al. 2002). SILAC is a very straightforward method for analysing animal cell culture samples and has been widely used, but it is not always suitable for use on whole organisms and is less useful for plants. Plants are very versatile autotrophic metabolic specialists and are able to generate all 20 amino acids necessary for protein synthesis by themselves. Moreover, plants can interconvert different amino acids much more easily than animals, which causes difficulties for the use of SILAC in plants.

The ability of plants to synthesize amino acids from inorganic elements provides the opportunity for an easy and relatively inexpensive uniform labelling of their total amino acid and protein content by the stable isotope ^{15}N . A common approach is to grow the plants hydroponically and to label the proteins by incorporation of ^{15}N from supplied $^{15}\text{NO}_3^-$ and $^{15}\text{NH}_4^+$, which can then be compared with the normal ^{14}N proteins. Using Hoagland media, uniform labelling with ^{15}N isotope was first used successfully in potato plants (Ippel et al. 2004) where 98% of the total proteins were ^{15}N labelled. *Arabidopsis* plants can also be successfully labelled and labelling does not affect plant development (Hebeler et al. 2008). In another study, hydroponic isotope labelling of entire plants (HILEP), in combination with MS, was used for relative protein quantification of seven-week-old *Arabidopsis* plants submitted to oxidative stress (Bindschedler et al. 2008). An approach to the automated analysis of uniformly $^{14}\text{N}/^{15}\text{N}$ -labelled proteins was developed using MASCOT peptide identification in conjunction with the trans-proteomic pipeline (Palmlblad et al. 2007). In order to study the interaction between tomato plant-root-knot and nematode, Schaff et al. (2008) developed a method that they called stable isotope labelling *in planta* (SILIP). This method allows the plants to grow in a 99.3% ^{15}N -enriched nitrate soil-based medium. After two months' growth, the tomato tissues were extracted and analysed by LC/MS/MS. The results indicate that ^{15}N incorporation was almost 99% and was uniform throughout the plant (Schaff et al. 2008).

Label-free methods. Label-free methods are becoming increasingly popular to compare samples (Cunningham & Laing 2006; Ono et al. 2006; Wang et al. 2006; Bantscheff et al. 2007). There are currently two different label-free strategies. The first one is based on measuring and comparing the mass

spectrometric signal intensity of peptide precursor ions (MS1) belonging to a particular protein (Wang et al. 2003; Wiener et al. 2004). The second method is based on the counting and comparison of the number of fragment spectra identifying peptides of a given protein (Liu et al. 2004; Colinge et al. 2005; Old et al. 2005).

Affinity purification of plant protein complexes

There is a continuous quest for methods that allow rapid isolation of proteins and protein complexes from plant material with a high degree of purity. Affinity purification is a standard technique for isolation of protein complexes or protein purification for testing of protein–protein interactions. Originally developed for yeast and mammalian protein purification, the protein tagging strategies are becoming standard tools in plant molecular biology.

Single protein tags

A wide variety of different tags is currently available, which allows flexible choice of tagging and purification strategy for plant proteomics. Many tags have been successfully used in plants, including Protein A, green fluorescent protein (GFP), calmodulin-binding protein (CBP), glutathione S-transferase (GST), haemagglutinin (HA), His (histidines, 6 \times), maltose-binding protein (MBP), c-Myc, FLAG, TBP (TATA-box-binding protein) and Strep II (streptavidin; see Table I and references therein). In general, the efficiency of protein purification depends on the protein topology and location of the epitope tag in relation to the specific motifs and signal peptides. For the best results in affinity purification, the epitope tag has to be maximally exposed – on the surface of the protein complex.

A good example of a small and efficient tag is the Strep II tag, which is an eight amino acid peptide that is structurally similar to biotin, thus allowing purification using streptavidin and its derivatives (Witte et al. 2004). Another single tag, the FLAG tag, was sufficient for immunopurification of polyribosomal complexes of *Arabidopsis* for global analysis of gene expression (Zanetti et al. 2005). However, another study showed that FLAG tag on the C terminus can impair the function of the protein (Kato et al. 2003).

The biotin tag, which offers a simple and rapid method for protein complex purification *in planta*, was developed using a tomato biotin peptide and a tobacco etch virus (TEV) protease cleavage site as a single affinity tag to purify protein complexes from the whole protein extracts of rice suspension cell culture. The affinity purification of biotin-tagged proteins is performed on the basis of the biotin–avidin

Table I. Single epitope tags and TAP tags used for affinity purification of plant proteins.

Protein tag	Acronym	Binding domains	Size	Binding agent	Advantages	Disadvantages	References
<i>Single epitope tags</i>							
Protein A	Prot A	(IgG)-binding domains of protein A	14 kDa	IgG	Fast, efficient and inexpensive. Used for majority of TAP tags.	As a single tag, not suitable for MS proteomics because of abundant antibody fragments in eluate.	(Rohila et al. 2004, 2006; Rubio et al. 2005)
Green fluorescent protein	GFP	GFP	28 kDa	Anti-GFP monoclonal antibody	Can be used in parallel to protein localization studies.	As a single tag, not suitable for MS proteomics because of abundant antibody fragments in eluate. Antibodies are expensive.	(Peckham et al. 2006)
Calmodulin binding protein	CBP	CBP	26 aa, 4 kDa	Calmodulin (CAM)	Relatively small. Used for TAP tags.	Binds to any CAM-related protein in plants.	(Rohila et al. 2004, 2006)
Glutathione S-transferase	GST	GST	211 aa, 26 kDa	Glutathione-Sepharose 4B beads	Soluble protein with high affinity to substrate.	Large tag.	(Nakagami et al. 2002; Sridhar et al. 2006)
Hemagglutinin epitope	HA	HA, usually 4×	9 aa	Anti-HA monoclonal antibody	Small size.	As a single tag, not suitable for MS proteomics because of abundant antibody fragments in eluate. Host His-containing proteins are likely to co-purify.	(Koroleva et al. 2004)
Hexahistidine	His ₆	Six histidines	6 aa	Ni ²⁺ -NTA, Co ²⁺ -CMA (Talon)	Small size. Used for TAPa tags.	Strong positive charge; could result in oxidized protein.	(Nakagami et al. 2002; Sridhar et al. 2004; Witte et al. 2004; Rubio et al. 2005; Sridhar et al. 2006; Koroleva et al. 2009)
Maltose binding protein	MBP	MBP	396 aa, 40 kDa	Cross-linked amylose	Improves protein solubility; efficient and inexpensive.	Large tag.	(Sridhar et al. 2004; Sridhar et al. 2006; Koroleva et al. 2009)
c-Myc	c-Myc	Myc epitope, usually 9×	11 aa	Anti-Myc monoclonal antibody	Small size.	Used in TAPa tag. As a single tag, not suitable for MS proteomics because of abundant antibody fragments in eluate.	(Rubio et al. 2005)
FLAG	FLAG	FLAG	8 aa	Anti-FLAG monoclonal antibody	Small size.	As a single tag, not suitable for MS proteomics because of abundant antibody fragments in eluate.	(Nakagami et al. 2002; Zanetti et al. 2005)
Biotin	Biotin	Biotin from tomato	213 aa	Streptavidin, Strep-tactin	Biotin-streptavidin is a very strong interaction.	Need protease for elution.	(Zhong et al. 2003)
Biotin carboxyl carrier protein domain of 3-methylcrotonyl CoA carboxylase from <i>Arabidopsis</i>	BCCD	Biotin carboxyl carrier protein domain	80 aa	Streptavidin magnetic beads	Biotin-streptavidin is a very strong interaction.	Need protease for elution.	(Qi & Karagiri 2009)
Strep II	Strep II	Biotin-like epitope	8 aa	Streptavidin magnetic beads	Biotin-streptavidin is a very strong interaction.	Need protease for elution.	(Witte et al. 2004)

Table I. (Continued).

Protein tag	Acronym	Binding domains	Size	Binding agent	Advantages	Disadvantages	References
<i>Tandem affinity purification (TAP) tags</i>							
TAP (1)	NTAPi and CTAPi	Tandem Protein A domains; CBP (calmodulin-binding protein) with removed NLS domain	181 aa	IgG agarose - Calmodulin (CAM) agarose	Efficient; Gateway-compatible vector is available	Long procedure of purification, TEV proteolysis is slow at 4°C; non-specific binding to plant CAM-like proteins.	(Rohila et al. 2004, 2006; Witte et al. 2004; Koroleva et al. 2007; Van Aken et al. 2007; Van Leene et al. 2008; Takahashi et al. 2008; Zhao et al. 2008)
TAP (2)	NTAPa CTAPa	Protein A, His ₆ c-Myc ₉		IgG-agarose Ni ²⁺ -NTA, Co ²⁺ -CMA (Talon)	Efficient; Gateway-compatible vector is available	Long procedure of purification; NTAPa vector is not in a standard frame; HRV Precision protease is expensive.	(Rubio et al. 2005)
HA-PreScission-Biotin	HBP	HA epitope ; Biotin carboxyl carrier protein domain of 3-methylcrotonyl CoA carboxylase from <i>Arabidopsis</i>	106 aa	Streptavidin magnetic beads, anti-HA antibody	The first step of purification (biotin-based) is very efficient.	Proximal location of HA tag not suitable for MS proteomics because of abundant antibody fragments in eluate. One-step purification was preferred.	(Qi & Katagiri 2009)

or biotin–streptavidin interactions, which are the strongest non-covalent biological interactions known (Wilchek & Bayer 1990). The successful application of this system led to the isolation of TBP complexes in rice (Zhong et al. 2003). This method in combination with tandem MS resulted in successful identification of 86 unique proteins associated with the TBP protein. Besides the proteins known to be associated with TBP, many other proteins involved in pre-mRNA processing and chromatin remodelling were found in the purified biotin–TBP protein fraction (Zhong et al. 2003).

A popular reporter of protein localization, GFP, can also be used as an affinity purification tag. This purification scheme suggested by Peckham et al. (2006) exploits the hydrophobic nature of GFP while maintaining a gentle environment for integrity of labile complexes. The protocol entails a simple three-step purification procedure for GFP fusion proteins produced in tobacco suspension cells, with the intent of maximizing purity and yield under gentle conditions maintaining GFP solubility and increasing hydrophobicity (Peckham et al. 2006). However, this protocol cannot ensure that the GFP elution fraction will not include other hydrophobic proteins that are not genuine interacting partners of the tagged protein.

Sometimes more than one epitope tag is required to study protein interactions. For example, a combination of His-, GST- and MBP-tagged proteins was used to show that APETALA1 (AP1) and

SEPALLATA3 (SEP3), both MADS box DNA-binding proteins, interacted with SEU, which encodes a plant-specific regulatory protein (Sridhar et al. 2004, 2006).

Tandem affinity purification tags

Over the past few years, the tandem affinity purification (TAP) procedure with the use of complex protein tags (TAP tags) (Rigaut et al. 1999) has been used in many biological systems, including plants. The TAP strategy is usually based on two (or more) rounds of protein purification, in order to improve the purification of protein complexes for subsequent proteomic analysis. Incorporation of a specific protease site between two or more tags allows removal of the distal tag used in the first round of purification (usually Protein A), thus eliminating non-specific background. So far, three TAP tag applications (Figure 1) have been developed for plants (Rohila et al. 2004; Rubio et al. 2005; Rohila et al. 2006; Qi & Katagiri 2009). A successful example of TAP tag purification approach is isolation of the Cf-9 protein complex (420 kDa) in tobacco by Rivas et al. (2002). Essentially, any combination of epitope tags which includes a proteolytic site between the tags and a proximal tag not requiring antibody-based purification is suitable for proteomic analysis by MS.

The original TAP tag (Rigaut et al. 1999) has been modified by removing a cryptic splice site and

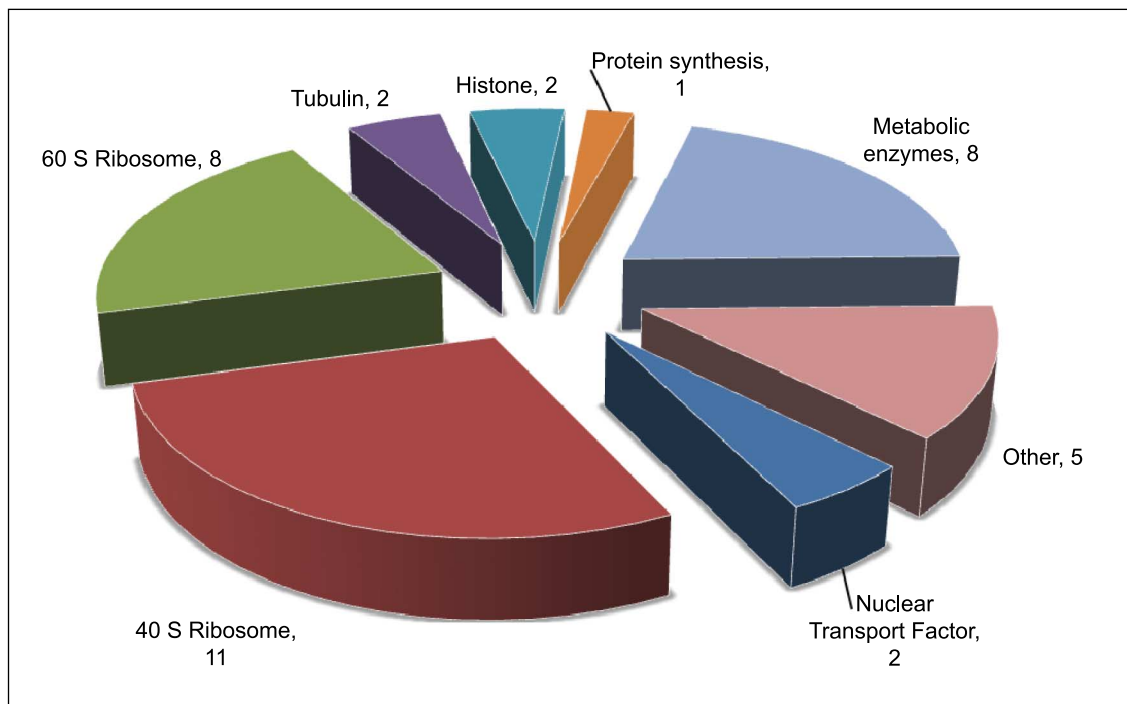


Figure 1. Proteins identified in TAP fraction of Nuclear Transport Factor p15h2, and their distribution between different functional groups.

a nuclear localization signal, polyadenylation sites and AT- or GC-rich regions, and by the inclusion of castor bean catalase intron 1 for improved expression in plants (Rohila et al. 2004, 2006). Creation of Gateway-compatible binary vectors, NTAPi and CTAPi, allow high-throughput cloning for expression of tagged proteins. This tagging strategy was applied to study interaction partners of 41 TAP-tagged rice protein kinases (Rohila et al. 2006). In total, all 41 rice kinases were purified and 23 of these were isolated as complexes with one or more interacting proteins (Rohila et al. 2006). In another study, a high throughput TAP/MS protocol was developed by Van Leene et al. (2007), using NTAPi tags to analyse cell cycle related protein complexes from *Arabidopsis* cell suspension cultures. TAP mapping of six known core cell cycle proteins followed by TAP and MALDI tandem MS led to identification of 28 new molecular associations and confirmation of 14 previously described interactions (Van Leene et al. 2007). The disadvantages of NTAPi and CTAPi tags include co-purification of native proteins with calmodulin-binding domains and a requirement for incubation at 16°C for proteolytic treatment by TEV protease (the reaction is very slow at 4°C), which may lead to proteolysis by endogenous proteases. An alternative TAP tag for the isolation of plant protein complexes, called pC-TAPa (Rubio et al. 2005), was developed to circumvent the problems with NTAPi and CTAPi tags. The pC-TAPa construct is also a Gateway-compatible vector, allowing convenient recombination of open reading frames from pre-existing Gateway entry clones. The TAPa tag includes three epitope tags (two Protein-A-binding domains, six His and nine Myc epitope repeats) and a 3C HRV protease site (Rubio et al. 2005).

Another TAP tag, originally developed to study mammalian protein, GS tag, which combines two IgG-binding domains of protein G with a streptavidin-binding peptide, separated by two TEV cleavage sites, was recently evaluated in comparison with NTAPi for its suitability for plants (Van Leene et al. 2008). The authors showed that the GS tag outperforms the traditional TAP tag in plant cells, both in specificity and yield. In addition, they replaced the TEV protease cleavage sites in the GS tag with the rhinovirus 3C cleavage site for improved protein complex stability during purification, thus combining the best features from several TAP tags.

A novel strategy of TAP tag protein purification was recently suggested, using the biotin carboxyl carrier protein domain (BCCD) of 3-methylcrotonyl CoA carboxylase (MCCA) from *Arabidopsis* (Qi & Katagiri 2009). This protein domain is readily biotinylated in plants and in *Escherichia coli* (Wang et al. 1994) and was used in development of the HA-

PreScission-Biotin (HPB) tag (Table I). The BCCD domain of the HPB tag is biotinylated *in vivo*, and the tagged protein can be captured by streptavidin beads on the basis of biotin-streptavidin interaction. The high affinity of this interaction allowed efficient recovery of tagged proteins and stringent purification to reduce non-specific binding of other proteins (Zhong et al. 2003; Qi & Katagiri 2009). The HPB tag was recently successfully applied to the challenging task of purification of low-abundance plasma-membrane protein complexes (Qi & Katagiri 2009). The purification method involved microsomal fractionation, chemical cross-linking, solubilization and TAP followed by protein identification using LC-MS/MS. However, one-step affinity purification based on the biotin-streptavidin interaction using magnetic streptavidin beads was preferred by the authors because it resulted in sufficient purity and allowed much higher yield of the tagged protein than a TAP procedure (Qi & Katagiri 2009). This choice was apparently made because of the configuration of this particular TAP tag, where the proximal location of HA does not allow protease cleavage for its removal. The presence of the HA tag will also leave associated antibody fragments at the second step of TAP, rendering the preparation unsuitable for LC-MS/MS due to high background level of peptides derived from the antibody.

Advantages and limitations of different protein tagging systems

Each tagging system has its advantages and disadvantages (Table I). The use of a particular tagging and purification strategy, whether employing a single tag or a TAP tag, has to be tailored to the needs of a particular experimental system. Apart from the cost and efficiency of protein purification, the most important considerations are that the tag should not interfere with the tagged protein function and/or biological system in question and should not be prone to non-specific interactions. For example, it has been found that the addition of a GFP or a FLAG tag to the C terminus of AtHKT1 impairs the function of this ion channel protein (Kato et al. 2003). The large protein tags are usually efficient for large-scale protein purification but have a greater chance of obstructing the binding sites or interfering with the protein function. The small peptide tags have a minimal impact on tertiary structure and biological activity of the fusion protein and are suitable for a higher degree of protein purification; however, the yield of purified protein is often compromised, thus resulting in low amounts of protein complex ligands, which are below the detection limit of MS. Moreover, each affinity tag has to be purified under specific buffer conditions, which

could affect the protein of interest. Often, TAP tags are preferred because higher degree of purity of protein complexes can be achieved through two steps of purification compared to the single-tag approaches. However, experimental data suggest that in some systems (especially when a fast protein purification step is required), a single tag may be as suitable and efficient as a TAP tag. In general, one-step affinity purification methods are easier and faster, and result in a higher yield of tagged proteins.

To compare protein purification efficiency of different tags, three purification strategies based on the Strep II, His(6) and TAP affinity tags were tested for purification of one membrane-anchored protein kinase and one soluble protein, from leaf extracts of *Nicotiana benthamiana* (Witte et al. 2004). Transiently expressed Strep II-tagged membrane protein was purified from it to almost complete homogeneity in less than 60 min and was directly suitable for enzymatic or MS analyses, allowing the identification of its phosphorylation sites. In contrast, purification of the same protein via His tag yielded partially oxidized protein of low purity. Soluble protein SGT1b was isolated with equal success as either TAP-tagged or Strep II-tagged protein with similar yield and high purity; however, purification of the Strep II-tagged protein was considerably easier and faster. Using either tagging strategy, the same co-purifying protein was identified, suggesting that both the Strep II and TAP tags are suitable for purification of protein complexes from plant material.

The common limitation of any affinity tag purification approach is that the total protein yield is dependent on the level of expression of the tagged protein. If the expression of the tagged protein is very low, neither protein purification protocol will provide enough proteins for MS analysis. A particular case is the potential competition between the tagged protein and the endogenous target protein for binding to the interacting partners. Therefore, 35S promoter rather than endogenous promoters may be a better choice to obtain enhanced gene expression. Another limitation is the potential effect of the epitope tag on conformation or the charge state of the tagged protein, which can make the binding of interacting protein less stable.

Methods for analysis of isolated protein complexes

The methods used for protein identification of affinity-purified protein complexes are mainly determined by the purpose of the experiment, the nature of the purification protocol and the complexity of the protein fraction. A gel image can provide semi-quantitative information of the composition of the affinity-purified fraction and a Western-blot-based

approach is good for confirmation of expected target proteins when a suitable antibody is available. Protein fractions isolated directly by immunoprecipitation are not usually suitable for straightforward LC-MS/MS or MALDI-MS, since the large amounts of immunoglobulins will mask other peptides present in the sample. However, a combination approach based on 2D gel separation with following in-gel digestion and MALDI fingerprinting or LC-MS/MS of digested fractions can be used (Van Leene et al. 2007, 2008).

In general, if the number of proteins is not expected to be high, then the mixture can be digested and analysed by MALDI fingerprinting or LC-MS/MS. If the interactors are more abundant (more than 20–100 proteins in a sample), a fractionation of proteins prior to MS analysis may be necessary. The fractionation can be done either on 1D or 2D gels (with following in-gel digestion of the cut-out gel bands or spots) or by non-gel separation techniques. The gel-loading capacity is usually limited and the procedure of cutting of gel spots in the absence of robotic spot-pickers can be very tedious. If numerous interactors (more than 100) including low abundance proteins are expected to be present in the purified fraction, a non-gel-based MS analysis is more suitable because it is more likely to give better coverage of the proteins in the sample. More complex analysis which includes in-solution digestion of the proteins followed by separation of peptides on two chromatography columns (e.g. MUDPIT), most commonly in a combination of cation exchange and reverse phase columns, has good potential for providing multiple protein identifications (more than 200) as has been demonstrated by analysis of the nucleolar proteome in plants (Pendle et al. 2005).

An example of TAP purification of proteins associated with nuclear transport factor p15h2

The TAP purification strategy was used for purification and proteomic analysis of proteins interacting with nuclear transport factor p15h2 (At1g27970). This small (14 kDa) protein can be expressed at a high level using transient expression in *Arabidopsis* cell culture. Expression of recombinant protein with attached NTAPi tag (Rohila et al. 2004) and purification following modified TAP protocol led to successful purification of the NTAPi-p15h2 bait protein altogether with isolation of associated proteins (Koroleva et al. 2007). Since another nuclear transport factor, p15h3 (At1g27310), was present among the co-purified proteins, it is likely that these proteins form a stable heterodimer or higher order complex associated with nuclear pores (Koroleva et al. 2007). The majority of the identified

proteins (Figure 1) were components of the large and small subunits of the ribosome, which confirms the function of p15h2 and p15h3 as nuclear transport factors. Similar numbers of 40S and 60S ribosomal proteins were present in the isolated fraction, indicating that the p15h2 and p15h3 are likely to transport both subunits.

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