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# The puzzle of protein location in plant proteomics

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**Abstract**

*Organelle proteomics allows the characterization of complex proteomes to understand the protein networks which regulate growth and development, as well as adaptation and evolution. Purification of organelles is of paramount importance and diverse protocols are published. Some organelles such as chloroplasts, mitochondria, and the nucleus are surrounded by membranes which facilitate their purification. Others have membranes easily disrupted (vacuoles and peroxisomes), or are complex systems for protein trafficking (endoplasmic reticulum, Golgi, and secretory vesicles). The cell walls present different difficulties since they have no physical limits allowing purification. The purity of the targeted cell compartment is usually evaluated by biochemical and/or immunological methods. Nevertheless, in any sub-cellular proteomic analysis, proteins from a different compartment can be detected and the difficulty is to decide whether it is a contamination, or the unexpected location is real and has a functional significance. Software to predict sub-cellular location of proteins is available. However, since not all the targeting signals are known at present, carefulness in the use of such tools is recommended. Different tactics to solve this puzzle are discussed in this commentary.*

Plants like other organisms depend on proteins to maintain their functions and to adjust to environmental changes. Proteins arrange themselves into metabolic and regulatory pathways and their accurate localization is essential for the organism. To understand the diverse protein functions, information on the identification of all the proteins, as well as the knowledge of their location, post-translational modifications, and quantitative changes in the cell are important. Proteomics can provide the basic information, but the main challenge is the biochemical complexity, and the dynamic range of proteins. Since the cell is structured in organelles with different but interconnected functions, proteomic analysis of purified cell organelles reduces sample complexity to a subset of functionally related proteins or pathway modules [51, 56]. Reliable protein localization by proteomics requires that either organelle preparations are free of contaminants or that techniques are used to discriminate between genuine organelle residents and contaminating proteins [23, 12, 34]. Nonetheless, plant organelle proteomics has been restricted by the difficulties in isolating pure sub-cellular compartments in sufficient amount.

**Organelle isolation**

Organelle isolation implies the choice of an appropriate method for the purification of the targeted organelle. Reasonably pure preparations of some organelles surrounded by a double membrane, such as chloroplasts and mitochondria, can be achieved [20, 27, 33]. Cells may be released from tissues by enzymatic treatment or mechanical disruption; the last one being the most frequently used in plant proteomics. The exposure time as well as the strength of the forces applied has to be optimized to avoid a progressive destruction of the biological supramolecular architecture; otherwise large-scale destruction of organelles will occur and organelle yield is compromised. Nevertheless, the purity of organelle preparations can be established using microscopic observations, marker enzymes or antibodies against known proteins. The difficulty is that the analysis tool (mass spectrometry) is 100 to 1000 times more sensitive than classical biochemical and immunological tests [25]. In certain cases, microscopic observations proved to be helpful to check the quality of a sample [5, 23, 6], but they can be subjective. It is then hazardous to use results of sub-cellular proteomics directly as a proof for the location of the identified proteins.

***Organelles surrounded by double membranes***

From the protein composition point of view, mitochondria and chloroplasts are quite complex and include both very soluble proteins (present in the matrix and the intermembrane space) and very hydrophobic membrane

proteins. In addition, there are membrane proteins of intermediate solubility, such as some subunits of the oxidative phosphorylation complexes or the outer membrane porins. This chemical heterogeneity is a real challenge for the proteomic analysis.

Most of the chloroplast proteomic studies started with the *Arabidopsis* genome sequencing. Predictions indicate that 2100 to 2700 proteins are located in the *Arabidopsis* chloroplast [57, 56]. However, only about half of these proteins have been experimentally identified [22]. The purification of intact chloroplasts by percoll gradient centrifugation produces reasonably pure preparations. However, chloroplasts are complex organelles composed of six distinct suborganellar compartments: three different membranes (the two envelope membranes and the internal thylakoid membrane) and three discrete aqueous compartments (the intermembrane space of the envelope, the stroma and the thylakoid lumen). As a result of this structural intricacy, the external and internal routing of chloroplast proteins is necessarily a complex process [27, 28]. Proteomics of membrane proteins is challenging since they are poorly resolved using classical 2D-electrophoresis techniques [53, 66]. Proteins predicted to be targeted to the endoplasmic reticulum (ER) and mitochondria have been identified in chloroplast proteomic studies [35, 62], pointing to the need for experimental determination of protein location and the relative value of proteomic analysis.

Mitochondria can be isolated from many tissues but *Arabidopsis* cell suspension cultures have been largely used. On average, samples of 90-95 % purity were obtained using a Percoll gradient method [61], and yeast mitochondrial preparations reached 98% purity using free-flow electrophoresis [65]. About one fourth of the expected 2000-2500 *Arabidopsis* mitochondrial proteins were identified by proteomics [39]. The sub fractionation of mitochondria into the four basic compartments (outer membrane, inter-membrane space, inner membrane, and matrix) involves a complex protocol. As for chloroplasts, the proteomic characterization of mitochondrial hydrophobic proteins is a difficult task because of their low abundance and insolubility [41, 8, 53]. Several pathways are presently explored to solve the problem of contamination by non-mitochondrial proteins [39, 6].

As other organelles delimited by two membranes, nuclei isolation and purification was performed on different plants by density gradient centrifugation [2, 9, 32]. Most of the proteins identified were verified nuclear proteins, but also non-classical nuclear proteins were identified in different plants. A comparative study of the nuclear proteomes of *Arabidopsis*, rice and chickpea was done; from the 382 proteins identified in the three proteomes, only a small proportion were orthologous proteins, the others being specific for each plant [9]. Most

of the nuclear proteins show a high level of divergence in the protein classes between the three plants, which is surprising for an organelle having similar functions in different organisms.

***Other organelles***

The isolation of other organelles such as peroxisomes [1], vacuoles [26, 48] and oil bodies [30] produced samples enriched in the selected organelle. The purity of the selected organelle can be evaluated by western blotting using antibodies specific against proteins from undesirable compartments [26]. In these preparations, the proportion of proteins known to be present in other compartments depends on the purification method. Some authors pretend that those are new proteins for the studied compartment, others just call those proteins contaminants. However it is clear that the location of such proteins should be verified by other methods than proteomics.

Although reasonably pure preparations of some organelles (chloroplast and mitochondria) can be achieved by centrifugation and density gradients, the isolation of other compartments can not be accomplished by centrifugation because they do not have specific buoyant densities. Such is the case of the endomembrane system which has proven recalcitrant to purification [19, 49]. An additional problem with the endomembrane system is that proteins move along the different organelles in a controlled way. Some are residents of a particular compartment such as the ER or the Golgi, and others are sent to specific compartments (plasma membrane, tonoplast, and vacuole) or are secreted outside the cell. A well-designed technique was developed for such organelles employing differential isotope labelling: LOPIT (Localization of Organelle Proteins by Isotope Tagging) [11, 13]. LOPIT is based on the partial separation of organelles by density gradient centrifugation followed by the analysis of protein distributions within the gradient by Isotope-Coded Affinity Tag (ICAT) labeling and mass spectrometry (MS). This technique does not depend on the production of pure organelles, and enables proteins from different sub-cellular compartments to be distinguished even if their distributions overlap.

***The case of the cell wall***

The cell wall cannot be defined as an organelle, but it is a very important cell compartment which requires designing specific strategies for its purification [24]. The lack of a delimiting membrane may result in the lost of proteins during its purification and in contamination by other cell compartments. The polysaccharide networks constitute potential traps for intracellular proteins. Two types of strategies were tried: non-destructive and destructive ones [24, 38]. In both cases, proteins not expected to be present in cell walls were found, leading to

the hypothesis of the existence of an alternative secretory pathway [60]. However, such a pathway, if it exists, probably targets only a few proteins to the cell wall. Indeed, intracellular proteins identified in cell wall proteomics vary from one experiment to another, and it is possible to reduce their number by maintaining the integrity of plasma membranes or improving the cell wall purification procedure [5, 6, 17].

Altogether, the results of sub-cellular proteomics contain a certain number of proteins for which a clear localization cannot be predicted. The relatively high number of proteins without a known function identified by proteomics also raises the question of their function and location. Proteomic results start to be incorporated into databases such as those listed in Table I. This will make easier comparisons between results obtained in different conditions, and both allow confirmation of sub-cellular localization and point at possible contaminants.

**Protein location**

Most proteins are synthesized on cytosolic ribosomes, except for chloroplast and mitochondria having their own synthesis capacities in addition to the nuclear-encoded proteins. After synthesis, proteins can be post-translationally modified, leading to differential location. The accurate protein location is essential for all living organisms and organelle proteomic analysis can generate very large candidate list of putative constituent proteins. Therefore independent approaches are required to verify if the candidate proteins can be included in the targeted organelle. To establish the location profiles of large set of proteins, new tools were developed that generated high-throughput localization data of a large number of proteins.

***Bioinformatic predictions***

Many proteins may contain within their amino acid sequence information that could be used for predicting their sub-cellular location, *e.g.* signal peptides, transit peptides, nuclear import and export signals [64]. Numerous computational programs have been developed with the aim of predicting the location of the proteins, most of which rely on the presence of these signals [15, 37, 45, 47]. It is important to understand the uses and limits of the bioinformatic methods developed lately, and recent reviews give a comprehensive approach on the available tools [14, 34]. Since computational methods are based on different algorithms, it is recommended to use several prediction programs to make a reasonable choice. Two tools available on line are listed in Table II. Both Aramemnon and *ProtAnnDB* use a series of available software predicting sub-cellular localization of proteins and compare their results [58, 59]. In addition, recent reports suggest the existence of alternative sorting

**Table I. Plant proteomic databases**

| Database Name | Cell compartment | Web site | Species | Reference |
|---|---|---|---|---|
| PPDB | any compartment | http://ppdb.tc.cornell.edu/ | *Arabidopsis thaliana*, *Zea mays* | [18] |
| SUBA | any compartment | http://www.plantenergy.uwa.edu.au/suba2/ | *Arabidopsis thaliana* | [22] |
| PRIDE | any compartment | http://www.ebi.ac.uk/pride/ | all organisms | [31] |
| NASC proteomics database | any compartment | http://proteomics.arabidopsis.info/ | *Arabidopsis thaliana* | |
| AtProteome | any compartment | http://fgcz-atproteome.unizh.ch/ | *Arabidopsis thaliana* | [3] |
| eSLDB | any compartment | http://gpcr2.biocomp.unibo.it/esldb/index.htm | all organisms | [50] |
| *WallProtDB* | cell wall | http://www.polebio.scsv.ups-tlse.fr/WallProtDB/ | *Arabidopsis thaliana*, *Oryza sativa* | [52] |
| PlantProteomics07 | plasma membrane | http://www.grenoble.prabi.fr/data/PlantProteomics07/ | *Arabidopsis thaliana* | [16] |
| plProt | plastid | http://www.plprot.ethz.ch/ | *Arabidopsis thaliana*, *Nicotiana tabacum* | [35] |
| AMPDB | mitochondrion | http://www.plantenergy.uwa.edu.au/applications/ampdb/ | *Arabidopsis thaliana* | [21] |
| AMPP | mitochondrion | http://www.gartenbau.uni-hannover.de/arabidopsis.html | *Arabidopsis thaliana* | [36] |
| AtNoPDB | nucleolus | http://bioinf.scri.sari.ac.uk/cgi-bin/atnopdb/home | *Arabidopsis thaliana* | [7] |

**Table II. Bioinformatic tools for plant sub-cellular proteomics**

| Database name | Cell compartment | Web site | Species | Reference |
|---|---|---|---|---|
| *ProtAnnDB* | any compartment | http://www.polebio.scsv.ups-tlse.fr/ProtAnnDB/ | *Arabidopsis thaliana*, *Oryza sativa* | [58] |
| Aramemnon | membranes | http://aramemnon.botanik.uni-koeln.de/ | *Arabidopsis thaliana*, *Oryza sativa, Vitis vinifera, Zea mays, Other seed plants* | [59] |

routes for proteins. For instance, the chloroplastic ceQORH protein was shown to be synthesized without a canonical cleavable chloroplastic transit sequence [42, 43]. Other proteins have been shown to be targeted to the chloroplast *via* the secretory pathway and undergo glycosylation [46, 54, 63]. Moonlighting proteins make the interpretation of proteomic results more difficult since those proteins are located in several cell compartments where they have different functions [29].

Proteins routed through the secretory pathway can be predicted using different tools [14]. No specific prediction program exists for vacuole, and therefore, localization can only be inferred *via* experimental data or homology searching referring to well-annotated proteins. Despite the knowledge of alternative secretion pathways, no current machine learning approach directly addresses the problem of predicting proteins entering the non-classical secretory pathway. However, prediction methods based on amino acid composition are in principle capable of foretelling proteins entering the non-classical secretory pathway [55]. A sequence-based method of prediction of non-classical-triggered secretion for mammalian and bacterial proteins has also been developed [4]. The assumption is that extracellular proteins share certain properties and features which can be related to protein function outside the cell, independently of the secretory process itself. Fifty proteins found in several proteomic studies of *Arabidopsis* cell walls and devoid of predicted signal peptide were analyzed. Only 14 were predicted as putative secreted proteins [24], suggesting that this alternative pathway is highly exceptional.

***Experimental location***

The experimental testing of protein targeting is the best way to verify the computational predictions. Several methods have been used and their accuracy is uneven. The first protein localizations were made by biochemical methods, and some abundant proteins were characterized and turned into marker enzymes for several cell compartments. The biochemical tools consist mainly in destructive approaches allowing cell fractionation. Fractionation has several restrictions since, as mentioned above, some compartments cannot be easily separated; still those organelles that can be fractionated are not free of contaminants [23]. Immunoprecipitation of tagged proteins may be useful to identify other proteins within a complex.

Cytological localization is a non-destructive technique allowing a good localization of proteins. Immunolabeling of cells supply a good resolution and high specificity, depending on the antibody. Proteins encoded by multigenic families may be difficult to localize precisely because highly specific antibodies are necessary to discriminate them. This is critical when different members of such protein families are targeted to

different compartments. The development of a small peptide tag for covalently label proteins such as *c*-myc or His is a good approach because a larger protein tags may affect the function of the protein of interest. This allows the use of commercial antibodies.

One of the most popular methods to localize proteins *in-vivo* is the Green Fluorescent Protein (GFP)-fusion technology. GFP is a small protein from jellyfish that can be visualized by fluorescence in a non-invasive way [10]. *In-vivo* uptake assays are widespread since they maintain the cellular environment, and targeting into all organelles can be tested at the same time. The principal drawback is that the tag may modify the location of the native protein and may even alter the condition of the wider system through dominant effects. It is important to test such constructs in a null mutant for the studied protein and with the native expression signals; if not there is inevitably a degree of overexpression. Another important point is to verify that the distribution of the GFP signal is consistent with gene expression visualized via *in situ* RNA hybridization. Localization of proteins in the endomembrane system is more complex than in chloroplasts or mitochondria since there is an exchange of membrane constituents. Furthermore the proteins of interest may be distributed between more than one compartment, and still cycle between them [40, 44]. The critical point is the subjective interpretation one puts on each observation in light of the experimental conditions and other pertinent data.

**Conclusion**

The results of sub-cellular proteomics cannot be considered sufficient to ensure the correct determination of protein location in cells. The use of several localization techniques is recommended, even if this seems to be redundant. Bioinformatic software based on experimental data provides valuable tools to predict sub-cellular localization of proteins. Even if a protein can accumulate at several places in the cell and be active at a place where it does not accumulate, combining approaches that take into account targeting and accumulation of proteins may give more confident localization [40]. Finally, the integration of localization data with expected biological function and within metabolic networks is important to confirm the location of a particular protein.

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