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The mitochondrial proteome of the moss *Physcomitrella patens*

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ABSTRACT

Extant basal land plants are routinely used to trace plant evolution and to track strategies for high abiotic stress resistance. Whereas the structure of mitochondrial genomes and RNA editing are already well studied, mitochondrial proteome research is restricted to a few data sets. While the mitochondrial proteome of the model moss *Physcomitrella patens* is covered to an estimated 15–25% by proteomic evidence to date, the available data have already provided insights into the evolution of metabolic compartmentation, dual targeting and mitochondrial heterogeneity. This review summarizes the current knowledge about the mitochondrial proteome of *P. patens*, and gives a perspective on its use as a mitochondrial model system. Its amenability to gene editing, metabolic labelling as well as fluorescence microscopy provides a unique platform to study open questions in mitochondrial biology, such as regulation of protein stability, responses to stress and connectivity to other organelles. Future challenges will include improving the proteomic resources for *P. patens*, and to link protein inventories and modifications as well as evolutionary differences to the functional level.

1. Introduction

1.1. Similar, yet different

About 500–450 million years ago plants began to colonize the land masses of Earth (Kenrick and Crane, 1997; Lang et al., 2010) and evolved to organisms so diverse as to include large towering trees and small ground-covering mosses, shaping our current ecosystems. Common for all land plants are selective pressures such as temperature fluctuations, the availability of water and nutrients, the levels of oxygen and CO_2, as well as sunlight. In addition, plant survival strategies include the need to balance emerging stresses such as e.g. drought, flooding or high UV irradiation.

Many open questions remain, both fundamental and applied, regarding the dynamic organization of plant metabolism. Main interests are the organelles governing plant energy metabolism and many biosynthetic pathways, namely chloroplasts and mitochondria. During normal conditions as well as abiotic stresses, these electron transport chain-containing compartments are crucial partners in maintaining plant ROS balance, vitality and growth (Mittler, 2002; Møller, 2001; Schwarzländer and Finkemeier, 2013). To date, plant mitochondria are less well investigated than their chloroplast partners and merit attention to unravel their exact roles and organization in plants.

As representative species from several early-diverging land plant lineages are available as model organisms, they represent a valuable resource to study mitochondria for several reasons. First, they provide a necessary reference point to reconstruct land plant evolution. Second, their study can unravel the mechanisms of alternative metabolic organization and plant survival strategies facing stresses. Third, they can offer technical advantages, such as amenability to microscopy techniques (Furt et al., 2012; Mueller et al., 2014; Wu and Bezanilla, 2014) and the ease of precise genome engineering via a high rate of homologous recombination, such as in the model moss *Physcomitrella patens* (Reski, 1998).

Interestingly, comparative studies regarding mitochondrial genomes have already revealed that they have undergone extreme changes during land plant evolution. There is a trend for increasing size and complexity paired with the gain of RNA-editing sites and introns (Li et al., 2009; O'Toole et al., 2008; Rüdinger et al., 2012). DNA transmission, maintenance and transcription as well as RNA processing have been modulated considerably.

On the other hand, mitochondrial proteomic data regarding model organisms outside angiosperms such as the green alga *Chlamydomonas*

Abbreviations: ABA, abscisic acid; ATE, arginyl-tRNA protein transferase; COX, cytochrome *c* oxidase; DIGE, differential in gel electrophoresis; MS/MS, tandem mass spectrometry; OAS-TL, *O*-acetylserine (thiol) lyase; PCA, principal component analysis; PINK, PTEN-induced putative kinase 1; ROS, reactive oxygen species; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; SDS PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; TEM, transmission electron microscopy; TOM, translocase of the outer mitochondrial membrane.

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reinhardtii (Atteia et al., 2009) and non-vascular land plants are still scarce. This review summarizes the available proteome information for the model moss *Physcomitrella patens* and gives a perspective on future interests in moss mitochondrial biology.

1.2. A moss view on mitochondria

Mosses are basal land plants with a wide distribution in different habitats. Their different survival mechanisms are partly still enigmatic compared to vascular plants. Most tissues of *Physcomitrella patens* are single cell layers or filaments, which are poikilohydric (i.e. equilibrate with the humidity of the environment), yet this moss is more resistant to several abiotic stresses such as salt stress and dehydration than most vascular plants (Mishler and Oliver, 2009; Oliver et al., 2005). Thus, *P. patens* can recover from a water loss of up to 75–92% of its fresh weight (Cui et al., 2012; Frank et al., 2005), a dehydration tolerance that can be further increased by treatment with the plant stress hormone ABA (Khandelwal et al., 2010; Koster et al., 2010). Under these adverse conditions, the protection of membrane integrity and the limitation and repair of damage caused by reactive oxygen species (ROS) are likely among the main issues in mitochondria (Koster et al., 2010; Møller, 2001; Schwarzländer and Finkemeier, 2013), similar to the situation in mature seeds (Wang et al., 2015).

Notably, it is already known that some aspects of moss mitochondrial biology, such as morphology and movement are differing from known patterns: moss mitochondrial form is very variable and ranges from elongated network-like morphology to small round structures (Fig. 1A, B), whereas movement is an order of magnitude slower than in the model flowering plant *A. thaliana* (Furt et al., 2012; Mueller and Reski, 2015).

2. Land plant organelles in the evolutionary spotlight

2.1. Resources: prediction and comparative genomics platforms

The *Physcomitrella patens* genome has been available since 2008 (Rensing et al., 2008) with the genome annotation V1.6 (Zimmer et al., 2013) encompassing 32,275 protein-coding genomic loci while annotation is continuously being improved (V3.3, www.cosmoss.org, Lang et al., 2016). The *P. patens* gene models have been readily integrated into comparative genomics platforms such as PLAZA, Phytozome, KEGG and OrthoMCL (Chen et al., 2006; Goodstein et al., 2012; Kanehisa et al., 2014; Van Bel et al., 2012) which allow for the convenient search of homologous genes between many plant species with high-throughput genomics data.

In order to automatically allocate predicted moss proteins and thereby metabolic functions to subcellular compartments (Zimmer et al., 2013), annotation can be either transferred from protein models of other previously annotated organisms, and/or subcellular localization assigned by prediction algorithms (Emanuelsson et al., 2007; Mitschke et al., 2009; Hooper et al., 2014). Both approaches suffer from some pitfalls, as sequence similarity is no guarantee for similar function or localization, and thus transfer of e.g. GO terms between species may even mask evolutionary changes in eukaryotic metabolic compartmentation. Moreover, prediction algorithms can only be as good as their training sets and often do not provide congruent results (Heazlewood et al., 2004; Hooper et al., 2014). Thus, the increasing amount of annotations inferred by electronic annotation (labelled *IEA*) needs to be balanced by experimental datasets in order to improve gene annotation and conduct sensible cross-species comparative analyses.

Currently, the predicted mitochondrial proteome of *P. patens* consists of the 42 proteins encoded by the mitochondrial genome (Terasawa et al., 2006), and proteins encoded by 1947 (6%) of the 32,275 protein-coding genomic loci either predicted by *pred2goa* (Zimmer et al., 2013) or based on manual annotation (cosmoss.org, Nightly-build downloaded 20160303). *Pred2goa* combines the predictions of several computational localization prediction algorithms to decide on a consensus for the predictions.

By comparison, protein isoforms encoded by 2549 of 27,206 protein-coding genomic loci (TAIR 10) are annotated to localize to mitochondria in *Arabidopsis* (see also Rao et al. in this issue) according to SUBAcon (SUBAIII 20160307, Tanz et al., 2013) representing approximately 9.4% of all nuclear-encoded protein models. The SUBAcon algorithm (Hooper et al., 2014) integrates both experimental data (GFP localizations and MS/MS identifications) and localization predictions to derive subcellular localization of proteins, which are still mostly lacking for *P. patens*.

However, initial MS/MS datasets are available for *P. patens* including analyses integrating different environmental conditions and

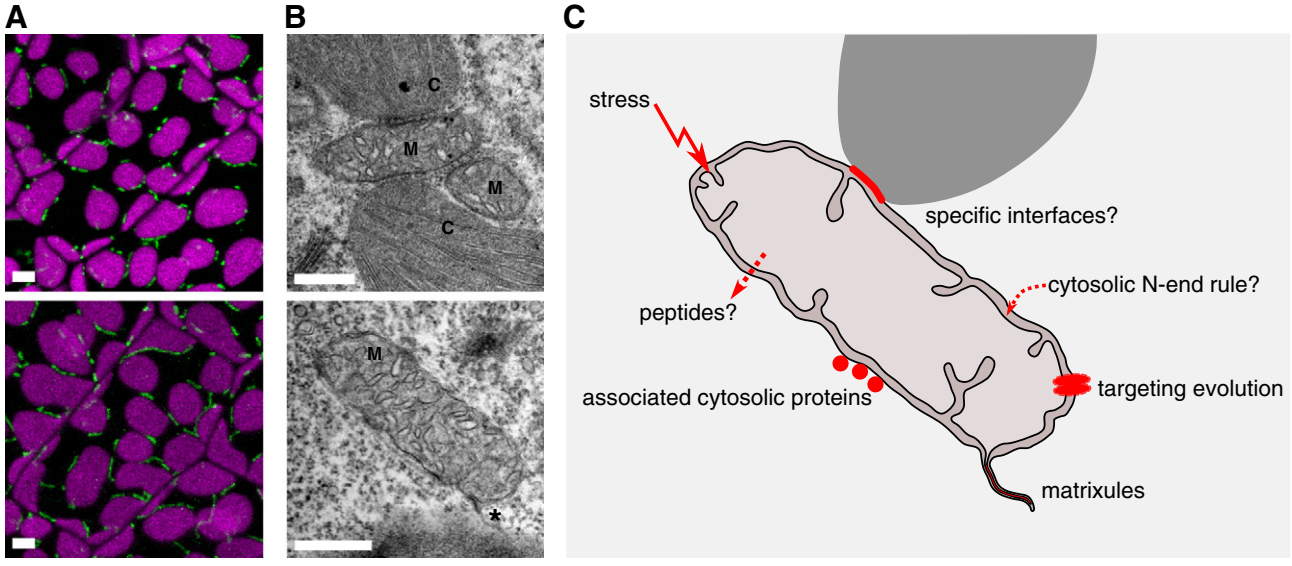

Fig. 1. Moss mitochondria: Morphology and current research topics. (A) Maximum intensity projections of *P. patens* mitochondria labelled with mtmEOS (green) (Mueller and Reski, 2015) and chlorophyll autofluorescence (magenta): moss mitochondria can exhibit small and round morphologies (top) or long elongated structures (bottom). Scale bars 5 μm. (B) TEM images of *P. patens* mitochondria (prepared as described in Schuessele et al. (2016)). Proximity between different subcellular compartments (top, C chloroplast, M mitochondrion) and tubular mitochondrial extensions (matrixules, *bottom) can be observed, but the functional significance is unclear. Scale bar 0.5 μm. (C) Schematic representation of current research topics in mitochondrial biology with an emphasis on moss as model system.

developmental stages (Cui et al., 2012; Skripnikov et al., 2009; Wang et al., 2014; 2010, 2009, 2008), as well as one peptidomics study discussing the export of peptides from mitochondria (Fesenko et al., 2015). Regarding purified organelles, the proteomes of isolated chloroplasts were addressed by two studies (Mueller et al., 2014; Polyakov et al., 2010), and purified mitochondria by one study (Mueller et al., 2014).

To date, the largest study identified proteins encoded by 1352 genomic loci in isolated (Lang et al., 2011) mitochondria and plastids and used a quantitative MS strategy employing ^{15}N metabolic labelling to distil the 284 genomic loci most confidently encoding mitochondrially localized proteins in *P. patens* (PCA cluster I and II, Mueller et al., 2014). This study combined MS identification and quantitation data as well as predictions, to assign proteins to mitochondrial and plastid protein clusters by attributing reliability values (fit uncertainty) based on multivariate statistical analysis (Mueller and Reski, 2014).

2.2. Comparative mitochondrial proteomics

2.2.1. Coverage and redundancy

The number of different proteins present in mitochondria of multicellular organisms is estimated to be higher than 1000, ranging from 1100 to 2000 (Meisinger et al., 2008; Millar et al., 2006; Møller, 2016), including probable spatio-temporal variations of the mitochondrial proteome (see also Palmfeldt and Bross, as well as Rao et al., both in this issue). To date, well characterized mitochondrial proteomes such as in baker's yeast (Meisinger et al., 2008; Reinders et al., 2006; Vogtle et al., 2012) are approaching 100% coverage by experimental data. The number of confirmed plant mitochondrial proteins is still comparably low, though rapidly increasing (Lee et al., 2013; Salvato et al., 2014). To date, in *Arabidopsis*, proteins encoded by 598 out of 2549 genomic loci with the assigned GO-term mitochondrion were identified experimentally either by MS and/or GFP localization (SubaIII (Tanz et al., 2013)), corresponding to 23.5% (Fig. 2A). In comparison, 277 (14%) of 1947 predicted mitochondrial proteins were identified in a study of isolated moss mitochondria (Mueller et al., 2014), of which 142 where quantified and assigned to mitochondria based on multivariate statistics and a clustering algorithm (based on PCA Fig. 2B).

However, for both *A. thaliana* and *P. patens* a large fraction of 44% (471) and 67% (515) of experimentally observed mitochondrial proteins did not have a mitochondrial prediction (Fig. 2), underlining the need for continuous improvement of prediction algorithms, but also for a careful evaluation of experimental observations (see Section 3.). In total, the coverage of the *P. patens* mitochondrial proteome can be estimated to be about 15–25% of the putative moss mitochondrial proteome assuming a size of 1500–2000 proteins.

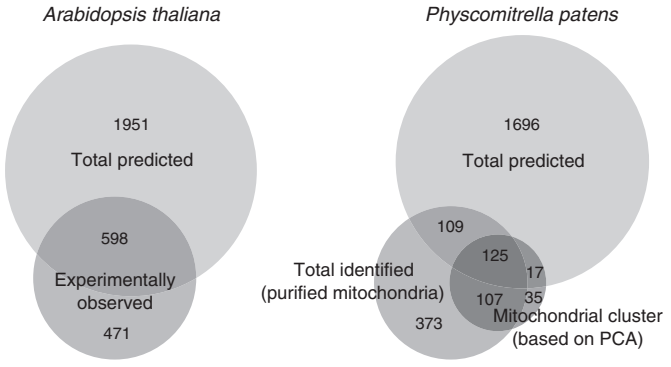

Fig. 2. Comparison of prediction and experimental evidence for nuclear-encoded mitochondrial proteins. (A) *A. thaliana* predicted localization (by SUBAcon) or experimentally observed via GFP or MS/MS. (B) Overlap of *P. patens* proteins (total predicted) with annotated localization via pred2goa and/or based on manual annotation (www.cosmoss.org nightly-build downloaded 20,160,303) and proteins identified via MS/MS in Mueller et al. (2014) in samples containing purified mitochondria (total identified, without further discrimination between true mitochondrial localized and contaminating proteins). Mitochondrial cluster: most confident mitochondrial proteins based on multivariate statistics and cluster analysis using MS/MS identifications and metabolic labelling data (Mueller et al., 2014). Diagram created using BioVenn (Hulsen et al., 2008).

Notably, in moss proteomics, special attention should be given to the fact that many similar genomic loci exist due to a high metabolic redundancy (Lang et al., 2005; Rensing et al., 2008) and retained paralogs from a whole genome duplication, which likely occurred 30 to 60 million years ago (Rensing et al., 2007). This results in a high percentage of peptides that cannot be assigned to a single locus on the *P. patens* genome, but to a group of loci summarized in "protein sets". For *P. patens*, it is thus expected that 30–50% (Mueller et al., 2014) of identified protein isoforms are members of protein sets. Approximately 36% of these protein sets (Mueller et al., 2014) contain gene models which were only differing in UTR splicing, and not in coding sequences. It is thus important to pay particular attention to unique peptides of protein models in order to avoid biased results, especially using quantitative proteomics.

2.2.2. Variability and evolution

Based on phylogenomics, the presence of several plant-specific, nuclear-encoded mitochondrial proteins (Klodmann et al., 2010; Millar et al., 2004) in *P. patens* was already expected. To date, proteomic evidence (Mueller et al., 2014) is available for plant-type (Perry et al., 2006) TOM20 homologs (Pp1s276_23V6.2, Pp1s15_226V6.1), respiratory chain complex subunits (COX X2 Pp1s392_46V6.1/Pp1s392_49V6.1 (Millar et al., 2004); carbonic anhydrases Pp1s207_12V6.1, Pp1s31_190V6.1, Pp1s38_289V6.1 (Sunderhaus et al., 2006); complex 1 putative membrane arm subunits Pp1s117_102V6.1, Pp1s59_321V6.1, Pp1s338_47V6.1 (Klodmann et al., 2010); a putative homolog to human C20orf7 complex I assembly Pp1s198_55V6.1 (Sugiana et al., 2008)), and alternative respiration components (alternative oxidase Pp1s183_11V6.2/V6.3); uncoupling protein (Pp1s54_186V6.2)); putative internal (Pp1s120_70V6.1) and putative external (Pp1s65_201V6.1) NAD(P)H dehydrogenases (Clifton et al., 2006; Elhafez et al., 2006; Michalecka et al., 2003; Sweetlove et al., 2006).

However, the more challenging question is to trace the evolution of protein targeting to different organelles as well as the partitioning of metabolic pathways (Martin, 2010). Mass-spectrometry identifications can provide a general overview, but need further validation using either quantitative proteomics or single-protein studies using e.g. fluorescent reporters (Carrie and Small, 2013; Mueller et al., 2014; Salvato et al., 2014). One example for modified compartmentation in land plants is the cysteine biosynthesis, which has evolved a complex regulatory mechanism involving OAS-TL localization to mitochondria in *A. thaliana*, whereas it is restricted to cytosol and plastids in *P. patens* (Birke et al., 2012). Further differences in amino acid and redox metabolism were suggested by MS/MS identifications (Mueller et al., 2014) but still await additional experimental confirmation.

Using the high-throughput moss dataset and phylogenetic analyses, an evolutionary variability in subcellular localization can be traced for around 4% of the investigated gene families (Mueller et al., 2014). This includes proteins such as beta-carbonic anhydrases, prolyl-cis trans isomerases, heat shock proteins and enzymes of the ascorbate-glutathione cycle. During evolution, proteins may undergo loss or acquisition of targeting sequences and acquire tissue- or development-specific targeting (Carrie and Small, 2013; Mueller et al., 2014). The relevance of these mechanisms to organelle biology is still largely unknown and may be studied using comparative analyses in several model organisms (Xu et al., 2012). Proteins exhibiting a high evolutionary variability in localization could be particularly interesting, as their retargeting may have affected the evolution of organellar function.

Moreover, as the mitochondrial proteome dataset of *P. patens* (Mueller et al., 2014) was further validated using quantification of proteins via labelling with ^{15}N, it provides information about the relative abundance of protein isoforms. As gene duplication can lead to sub- or

neofunctionalization of paralogs (Duarte, 2005), protein abundance data can hint at the functional importance and redundancy of the encoded protein isoforms. In moss mitochondria, protein isoforms with distinct abundance levels were found for heat shock proteins, ADP/ATP carriers, and several subunits of respiratory chain complexes.

In addition, several proteins of unknown function were experimentally confirmed to exhibit a conserved mitochondrial localization between moss and flowering plants, suggesting yet unknown important roles in mitochondrial biology. The domains present in these proteins suggest putative functions in diverse processes such as stress and autophagy (HVA-like proteins), RNA editing (MAM33), protein-protein interactions (MA-3, TPR), regulation and signalling (CBS, GRAM) and morphology (mitofilin) (Mueller et al., 2014).

2.3. Moss mitochondria under stress

The high resilience of many mosses to abiotic stresses has elicited interest into the molecular mechanisms of this stress resistance. To date, several moss-specific stress responses are known, including the presence of two NPQ (non-photochemical quenching) systems, many LEA-domain (late embryogenesis abundant) containing proteins, a "bryoporin", a Na^+-ATPase and several moss-specific proteins with unknown functions (for review, see Müller et al. (2016)). In addition, a series of publications addressed moss stress tolerance using 2D-SDS-PAGE and subsequent identification of proteins from excised protein spots in *P. patens* (Cui et al., 2012; Wang et al., 2010; 2008, 2009). In these datasets several proteins identified in the mitochondrial MS dataset (Mueller et al., 2014) are present, which might give hints at the moss mitochondrial response to abiotic stresses. Notably, protein abundances of the ROS scavenging enzyme ascorbate peroxidase (Pp1s152_82V6.1, CAD38154.1) and of an ATP synthase beta subunit (Pp1s24_254V6.1, gi|168008180, BAC85045.1) were repeatedly found to be altered significantly. Wang et al. (2008) found the abundance of the ATP synthase beta subunit isoform decreased (Pp1s24_254V6.1) under ABA treatment of moss gametophores (72 h 50 μM, Wang et al., 2010), as well as under salinity stress (72 h 250–350 mM NaCl), and proposed a downregulation of cell growth and respiration under salinity stress. Interestingly, the same protein isoform was upregulated in a DIGE (differential in gel electrophoresis) study (Cui et al., 2012) of dehydrated gametophores (72 h, up to 25% of their fresh weight). The putative mitochondrial ascorbate peroxidase abundance was upregulated during a long-term dehydration experiment (Wang et al., 2009) as well as during ABA treatment (72 h 50 μM, Wang et al., 2010). In summary, there are hints in literature regarding the importance of regulation of respiration and the mitochondrial ascorbate-glutathione cycle during abiotic stresses, but no study has yet provided functional data.

Future challenges will also include linking transcriptome data (Cuming et al., 2007; Frank et al., 2007) observing fast changes in the amount of nuclear transcripts of mitochondrial proteins, such as TSPO1 (tryptophan-rich sensory protein involved in the transport of tetrapyrrole precursors), to proteomics data. For instance, *P. patens* TSPO1 (Frank et al., 2007) was linked to the oxidative burst after treatment with a fungal elicitor (Lehtonen et al., 2012).

2.4. Posttranslational protein modifications in mitochondria

Protein modifications can change protein properties, protein activity, as well as protein stability, but may only be present on a small fraction of protein isoforms, multiplying isoform diversity (Olsen and Mann, 2013). To date, many posttranslational modifications such as lysine sidechain acetylation (König et al., 2014), arginylation (Wong et al., 2007) and phosphorylation (Leitner, 2016) have become accessible via targeted enrichment strategies prior to MS/MS analysis (see Hosp et al., as well as Kruse and Højlund, both in this issue). Indeed, a first phosphoproteomics study had been conducted in *P. patens* before the full genomic sequence was available (Heintz et al., 2006), whereas a more extensive screen on filamentous protonema and regenerating protoplasts was realized in 2014 (Wang et al., 2014). Analysis of this dataset led to 49% of the about 2000 identified phosphopeptides matching *P. patens* V1.6 protein models with a low fraction annotated with the GO-term mitochondrion. Candidate mitochondrial phosphoproteins include proteins related to quality control and iron metabolism (Pp1s319_35V6.1 prohibitin, Pp1s333_11V6.1 NifU-like protein), as well as two respiratory chain subunits (Pp1s53_211V6.1 succinate dehydrogenase flavoprotein subunit SDH1, Pp1s104_73V6.1 NADH-ubiquinone oxidoreductase 20 kDa subunit/Fe-S protein 7).

Regarding lysine acetylation in moss mitochondria, none has been reliably identified so far, indicating the need for enrichment strategies (König et al., 2014).

Recently, we identified the mitochondrial protein PpATAD3.1 (Pp1s106_174V6.1), a putative AAA-type ATPase, to be arginylated (Hoernstein et al., 2016). This N-terminal posttranslational modification constitutes a destabilizing residue in the context of the cytosolic N-end rule pathway of protein degradation. Here, proteins bearing destabilizing residues such as N-terminal arginine can become recognized by a certain class of ubiquitin ligases triggering polyubiquitination and subsequent rapid degradation via the 26S proteasome (Gibbs et al., 2014). Accessibility of organelle proteins to the cytosolic N-end rule pathway could be based on a topology with the N-terminus facing the cytosol or protein re-translocation from the organelle to the cytosol. Similar scenarios are known for several important proteins in animal systems such as the mitochondrially-localized PINK (PTEN-induced putative kinase 1, Yamano and Youle, 2013) and the ER chaperone BiP (GRP78, Cha-Molstad et al., 2015). Notably, the apparent N-terminus of PpATAD3.1 identified by MS/MS was proteolytically processed exposing Q30 as N-terminal amino acid which was in turn converted to E30 via deamidation followed by subsequent N-terminal arginylation. The enzyme responsible for arginylation (ATE) localizes exclusively to the nucleus and the cytoplasm in *P. patens* (Schuessele et al., 2016). Further, this sequential modification series is only known from the N-end rule pathway localized in the nucleus and the cytosol. Thus, the arginylation of PpATAD3.1 suggests a novel link of the cytosolic N-end rule pathway to the regulation of mitochondrial protein stability in plants (Hoernstein et al., 2016).

3. Quantitative proteomics, contaminants and organellar contact sites

Isolating organelles for MS/MS studies poses the challenge to reduce the number of contaminants from other organelles to a minimum in order to avoid false positive identifications. Arguably, the aim of purifying an organelle to 100% can never be fully met, especially as several proteins may specifically co-purify for several reasons. This poses the question of the specificity and exact origins of contaminations in organelle proteomes. Quantitative mass spectrometry enables the assignment of abundance levels in addition to simple identifications and thus can give the amount of co-purification when comparing protein extracts from distinct cellular fractions to each other (Huang et al., 2014; Lilley and Dunkley, 2008). As no non-photosynthetic material is available in moss, metabolic labelling of whole moss cultures with ^{15}N was employed to characterize the mitochondrial and chloroplast proteomes and to remove reciprocal contaminants from the datasets (Mueller et al., 2014). Protein extracts from isolated "light" mitochondria were mixed with protein extracts from isolated "heavy" chloroplasts in a 1:1 ratio. This approach enabled 86% of the overlap of protein identifications from both organellar extracts to be quantified.

In addition to aiding the assessment of contaminations by highly abundant proteins such as RuBisCO, protein abundance ratios can hint at other reasons for a co-purification with mitochondria. First, true dual or multiple targeting can lead to similar relative abundances in several organellar extracts, as e.g. for presequence peptidase PreP (Mueller et al., 2014; Xu et al., 2012). Dual targeting can be biased towards one

organelle or change during development, resulting in distinct relative abundances, requiring targeting validation via e.g. fluorescent reporter fusions (Mueller et al., 2014). Second, co-purifying proteins can originate from a third organelle (such as ER) contaminating both organellar fractions. Third, co-purification can mean association to the organelle of interest, even if protein targeting clearly addresses another organelle or the cytosol, such as for glycolytic proteins (Giegé et al., 2003) or mitochondrially associated membranes (MAMs) in yeast and animals (Wieckowski et al., 2009).

Thus several experimental strategies should be carefully combined in order to utilize and screen quantitative proteomics data to unravel a yet neglected feature of eukaryotic cells: organelle heterogeneity and connectivity at contact sites. In plants the identity of most interorganellar contact sites remains unknown, whereas biochemical as well as microscopic evidence exists for e.g. ER/chloroplast and ER/mitochondria interfaces (Andersson et al., 2007; Jaipargas et al., 2015; Mehrshahi et al., 2013; Mueller and Reski, 2015).

4. Conclusion and future perspectives using the *Physcomitrella* model

In conclusion, mitochondrial proteomics in *Physcomitrella patens* is still in its infancy but has shown promising developments. The existing mitochondrial datasets should be seen as a foundation to build on, using more sensitive mass spectrometers, expanding labelling strategies such as metabolic labelling, and targeted enrichment strategies regarding posttranslational modifications. Starting from proteomic datasets, *P. patens* offers an amenable platform for orthogonal approaches using biochemistry, in vivo labelling with isotopes, targeted reporter tagging or mutagenesis of endogenous genomic loci, as well as advanced fluorescent microscopy techniques in uniform and single-layered moss tissues. Studying moss mitochondria on a functional level will also include the challenge to link special features of moss mitochondria to (moss-)specific proteins.

Mitochondrial functionality is organized on many levels from gene expression over protein targeting to the morphology, context and connectivity of a single organelle at a certain time point. The emerging picture shows mitochondria as linked and dynamic structures with proteomic techniques as promising tools to unravel yet unknown diversity between species, as well as the interplay between their organelles, especially under stress conditions.

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