Towards the analysis of mitochondrial physiology in nitrogen fixing root nodules

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Abstract

Mitochondria contribute essentially to cellular metabolism by covering the bulk of its ATP requirements. However, mitochondria also engage in many catabolic as well as some anabolic pathways, most of which are conserved among eukaryotes. Additional mitochondrial functions of plants take place in the backdrop of photosynthesis. As such, plant mitochondria have extra functions when compared to their animal counterparts, for example in supporting chloroplast nitrogen assimilation by providing 2-oxoglutarat for the production of glutamate. A possible nitrogen source, apart from the uptake of soil derived nitrate and ammonium, is the fixation of molecular nitrogen by symbiotic nitrogen fixation. Symbiotic nitrogen fixation takes place between several plant species of the legume family and rhizobial bacteria and is based on an exchange of metabolites between the two symbiotic partners. The bacteria provide ammonia to the plant in exchange for reduced carbon compounds. This process takes place in specialized plant organs, the root nodules. Mitochondria have some particular functions in the N_2-fixing cell. However, it is currently unclear if the interactions of mitochondria and bacteroids are of a supportive or an antagonistic nature. In this thesis, special emphasis is placed on the physiology of mitochondria in the context of symbiotic nitrogen fixation and hinges on the proteomic investigation of root nodules. Our results support the view that mitochondria are of great importance for the fixation of atmospheric nitrogen (manuscript 1). In the course of this study a mitochondrial alanine aminotransferase has been identified, which may catalyze the transfer of the amino group from bacteroid derived alanine to 2-oxoglutrarate, thus supplying pyruvate for ATP production and glutamate for ammonium assimilation. To investigate the role of plant mitochondria in SNF in more detail, it is necessary to conduct further studies on isolated organelles of high purity. The steps required for such a procedure, including further sub-fractionation to improve protein coverage in proteomic studies, are documented in manuscript 2. The workflow described in this manuscript will also allow extending the definition of plant mitochondria aside from SNF by in-depth mass spectrometry. The current knowledge on the protein content of higher plant mitochondria is summarized by highlighting two model species, potato and Arabidopsis (publication 1).

Keywords: mitochondrial physiology, proteomics, symbiotic nitrogen fixation

Zusammenfassung

Mitochondrien tragen maßgeblich zum Zellstoffwechsel bei, indem sie den Großteil des dafür benötigten ATPs bereitstellen. Darüber hinaus sind sie an vielen katabolischen und anabolischen Stoffwechselwegen beteiligt, von denen die meisten gleichermaßen in allen Eukaryoten vorkommen. In Pflanzenzellen sind die Mitochondrien zusätzlich in den Photosynthesestoffwechsel eingebunden. Verglichen mit Mitochondrien aus Tieren, haben Pflanzenmitochondrien zusätzliche Funktionen. So unterstützen sie zum Beispiel die Stickstoff-Assimilierung, indem sie 2-Oxoglutarat zur Herstellung von Glutamat beisteuern. Eine mögliche Stickstoffquelle, neben der Aufnahme von Nitrat und Ammoniak aus dem Erdreich, ist die Fixierung von molekularem Stickstoff (N_2) mittels symbiotischer Stickstofffixierung. Symbiotische Stickstofffixierung findet zwischen Pflanzenarten aus der Familie der Leguminosen und den Rhizobien-Bakterien statt und basiert auf einem Metabolitaustausch zwischen den Symbiosepartnern. Die Bakterien versorgen die Pflanze mit Ammonium, während die Pflanze den Bakterien reduzierte Kohlenstoffverbindungen zur Verfügung stellt. Dieser Prozess findet in spezialisierten Pflanzenorganen, den Wurzelknöllchen, statt. Die Mitochondrien übernehmen auch im Kontext der N_2-fixierenden Zelle Spezialaufgaben. Bisher ist allerdings unklar, ob diese Interaktionen unterstützend oder antagonistisch sind. Der Fokus dieser Arbeit liegt auf der physiologischen Charakterisierung der Mitochondrien, insbesondere im Kontext der symbiotischen Stickstoffixierung. Ein zentrales Element ist dabei die proteomische Untersuchung von Wurzelknöllchen. Die gewonnenen Ergebnisse unterstützen die Auffassung, dass Mitochondrien auch für die stickstofffixierende Zelle von herausragender Bedeutung sind (Manuskript 1). Es konnte eine mitochondriale Alanin-Aminotransferase identifiziert werden, die den Transfer einer Aminogruppe von bakteriell produziertem Alanin auf 2-Oxoglutarat katalysiert. In den Mitochondrien wird dadurch Pyruvat für die ATP-Synthese und Glutamat für die Assimilation von Ammonium bereitgestellt. Um die Rolle der Pflanzenmitochondrien in der symbiotischen Stickstofffixierung noch genauer erforschen zu können, ist es notwendig, weitere Untersuchungen an isolierten, reinen Organellen durchzuführen. Die dafür erforderlichen Schritte, einschließlich einer anschließenden Subfraktionierung zur Erhöhung der Proteinabdeckung in proteomischen Studien, beschreibt Manuskript 2. Das dargestellte Protokoll ermöglicht außerdem eine erweiterte Charakterisierung von Pflanzenmitochondrien mittels Massenspektrometrie über SNF hinaus. Der aktuelle Kenntnisstand über die Proteine in Mitochondrien höherer Pflanzen wird in Publikation 1 exemplarisch anhand der Modellorganismen Arabidopsis und Kartoffel zusammengefasst.

Schlüsselbegriffe: Mitochondriale Physiologie, Proteomik, Symbiotische Stickstofffixierung

Contents

Abbreviations

2

2OG · *2-Oxoglutarate*

2PG · *2-Phosphoglycerat*

3

3PGA · *3-Phosphoglyceric acid*

A

Acetyl-CoA · *Acetyl-coenzyme A*

AD · *Alanine dehydrogenase*

ADP · *Adenosine diphosphate*

AlaAT · *Alanine aminotransferase*

AOX · *Alternative oxidase*

AS · *Asparagine synthetase*

AspAT · *Aspartate aminotransferase*

ATP · *Adenosine triphosphate*

B

BPGA · *Bisphosphoglycerat*

C

CIP · *Carrier import pathway*

CS · *Citrate synthase*

F

Fd · *Ferredoxin*

Fe–S · *Iron- sulfur*

G

GDC · *Glycine decarboxylase complex*

GIP · *General protein import pathway*

GOGAT · *Glutamine oxoglutarate aminotransferase*

GS · *Glutamine synthase*

H

HSP70 · *70 kilodalton heat shock protein*

I

IBM · *Inner boundary membrane*

ICDH · *NADP-dependent isocitrate dehydrogenase*

IDH · *Isocitrate dehydrogenase*

M

MCF · *Mitochondrial carrier family*

MDH · *Malate dehydrogenase*

MS · *Mass spectrometry*

N

N_2 · *Molecular nitrogen*

NADH · *Nicotinamide adenine dinucleotide*

NH_3 · *Ammonia*

NH_4^+ · *Ammonium, ammonium cation*

NiR · *Nitrite reductase*

NO · *Nitric monoxide*

NR · *Nitrate reductase*

O

OAA · *Oxaloacetic acid*

OXPHOS · *Oxidative phosphorylation*

P

PCD · *Programmed cell death*

PDC · *Pyruvate decarboxylase complex*

PEP · *Phosphoenolpyruvate*

PEPC · *Phosphoenolpyruvate-carboxylase*

P_i · *Inorganic phosphate*

PR · *Photorespiration*

Q

Q · *Ubiquinone*

QH_2 · *Ubiquinol*

R

ROS · *Reactive oxygen species*

RuBisCO · *Ribulose-1,5-bisphosphate carboxylase/oxygenase*

S

SAM · *Sorting and assembly machinery*

SNF · *Symbiotic nitrogen fixation*

T

TCA · *Tricarboxylic acid*

TIM · *Translocase of the inner membrane*

TOM · *Translocase of the outer membrane*

V

VDAC · *Voltage dependent anion channel*

1. The mitochondrion – a special organelle

1.1 General properties of mitochondria

Nearly all eukaryotes contain mitochondria, which originated from endosymbiotic bacteria in early ancestor cells (Gray 1999). This explains their common structure and function in eukaryotes. Mitochondria have a pivotal position in the cell. Equipped with their own genome and protein synthesis apparatus, they are considered as semi-autonomous organelles (Westermann 2010). Initially, mitochondria were solely regarded as ATP (adenosine triphosphate) producing organelles. Since ATP is the most important energy transmitter for energy consuming processes in the cell, this alone exemplifies the significance of mitochondria. ATP generated by mitochondria drives a large number of physiological processes such as synthesis of biomolecules, active transport through membranes, movement, development, and reproduction. Besides ATP-production, mitochondria are also important for the biosynthesis and catabolism of several amino acids, ubiquinone, Fe-S centers and heme, as well as for fatty acid metabolism. More recently the functions of mitochondria derived molecules in signaling, for example reactive oxygen species (ROS) in programmed cell death, are discussed (Wang 2001). Future research is expected to add further entries to the already impressive list of mitochondrial functions.

1.2 Mitochondrial structure

Mitochondrial functions are based on the structural properties of this organelle. Electron microscopy allows the detailed investigation of the dynamic and variable morphology of mitochondria. Mitochondria are spherical to ellipsoid organelles of 1-2 µm length and are encompassed by two membranes. The outer membrane separates mitochondria from the cytosol and is characterized by integral membrane proteins, the porins (which are also known as voltage-dependent anion channels, VDACs, (Young *et al.* 2007). The inner membrane is impermeable to most metabolites and forms the cristae protruding into the mitochondrial matrix. Refined mitochondrial structures show an additional subdivision of the inner membrane into the inner boundary membrane (IBM) and the cristae membrane (Vogel *et al.* 2006). The inner cristae membrane contains the protein complexes of the so-called oxidative phosphorylation (OXPHOS) system, which couples the reduction of O_2 into water to the phosphorylation of ADP (adenosine diphosphate) into ATP. The space between inner and outer mitochondrial membrane is the intermembrane space, containing only few proteins. It is connected to the cristae lumina by constrictions at the cristae bases, the cristae junctions. The space enwrapped by the inner membrane is referred to as the matrix. The matrix contains diverse soluble proteins and is the site of the tricarboxylic acid (TCA) cycle in addition to other metabolic pathways. It also contains the mitochondrial genetic apparatus.

Mitochondrial morphology, as well as the mitochondrial number per cell and mitochondrial distribution, is dynamic and variable. Metabolically active liver cells contain 1000-2000 mitochondria (Lemieux and Hoppel 2009). Plant cells generally have less mitochondria. For example, root cap cells of maize seedlings contain about 200 mitochondria (Juniper and Clowes 1965). Fusion and fission events control mitochondria numbers. The organelles can fuse to 10 µm long filamentous structures. Mitochondrial filaments forming a mitochondrial reticulum were observed in animals and plants (Westermann 2010, Logan 2006). It has been suggested that the enzymatic organization of the mitochondria influences their ultrastructure and function (Hackenbrock 1966). For instance, the mitochondria are able to change their internal structure by modifying the shape of the cristae (Cogliati *et al.* 2016).

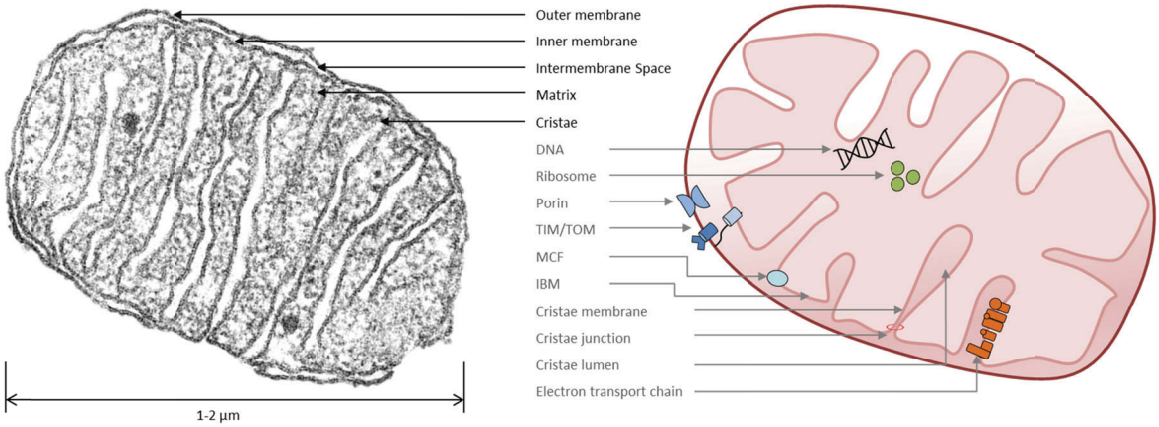

Figure 1:

Mitochondrial structure. Left panel: electron microscope picture of a mitochondrion from a pancreas cell (× 190.000, Scheffler 2008). The organelle consists of the outer- and inner membrane and the intermembrane space. The inner membrane is folded and forms cristae, which protrude into the matrix. Right panel: simplified drawing of a mitochondrion including some functional components like the matrix-located ribosomes and mitochondrial DNA. The outer membrane consist of porins and pre-protein translocases of the outer membrane (TOM), which are functionally connected to pre-protein translocases of the inner membrane (TIM). The inner membrane possesses carriers of the mitochondrial carrier family (MCF), the protein complexes of the electron transport chain and the ATP synthase complex. It can be subdivided into the inner boundary membrane (IBM) and the cristae membrane. Cristae junctions (highlighted by a red circle) separate the cristae lumen from the mitochondrial intermembrane space.

1.3 Mitochondrial transport processes

The outer mitochondrial membrane is not much of a barrier for metabolites. It includes voltage dependent anion channels (VDAC) which have an inner diameter of about 3 nm and are permeable for molecules up to 5 kDa in mass (Vander Heiden *et al.* 2000). In contrast, the inner mitochondrial membrane is widely impermeable for metabolites thus retaining the proton gradient generated by the electron transfer chain. Transporters within the inner membrane are therefore of prime importance for mitochondrial physiology since they mediate the exchange of metabolites between the mitochondrial matrix and the cytosol and enable metabolic interactions between mitochondria and the rest of the cell. The mitochondrial carrier family (MCF) members are substrate specific and transport numerous metabolites like ADP, ATP, inorganic phosphate (P_i), pyruvate, malate, 2-oxoglutarate (2OG), citrate, glutamate or oxaloacetate (Kuan and Saier 1993). Moreover, the inner mitochondrial membrane also contains non-MCF transporters, for example for the passage of Ca^{2+} ions (Carraretto *et al.* 2016).

Since the mitochondrial genome only encodes for a very small percentage of the total mitochondrial proteins (many genes encoding mitochondrial proteins are localized in the nucleus), mitochondria have to import the bulk of their proteins. The general protein import pathway (GIP) is responsible for the import of most proteins synthesized in the cytoplasm (Murcha *et al.* 2014). The majority of nuclear-encoded mitochondrial proteins possess an N-terminal targeting sequence which is recognized by receptors of the Translocase of the Outer Membrane (TOM). The TOM complex is associated with its counterpart in the inner mitochondrial membrane (the Translocase of the Inner Membrane, TIM). Before import, the proteins destined for import are kept unfolded by chaperons such as the cytosolic 70 kDa heat shock protein (HSP70). Initially, the targeting sequence binds to the TOM receptor complex and is subsequently gated through the outer and then the inner mitochondrial membrane (Neupert and Herrmann 2007). In the matrix, the targeting sequence is cleaved off for protein maturation. Specific pathways insert proteins into the outer and inner membrane. Metabolite carriers are integrated into the inner mitochondrial membrane using a specialized TIM complex of the carrier import pathway (CIP). The import of ß-barrel proteins into the outer mitochondrial membrane is mediated by the TOM complex and the Sorting and Assembly Machinery (SAM, Chacinska *et al.* 2009).

1.4 Central mitochondrial metabolism

The main function of mitochondria is the production of ATP. However, they are also engaged in many catabolic as well as some anabolic pathways conserved among eukaryotes to be discussed in the following paragraphs.

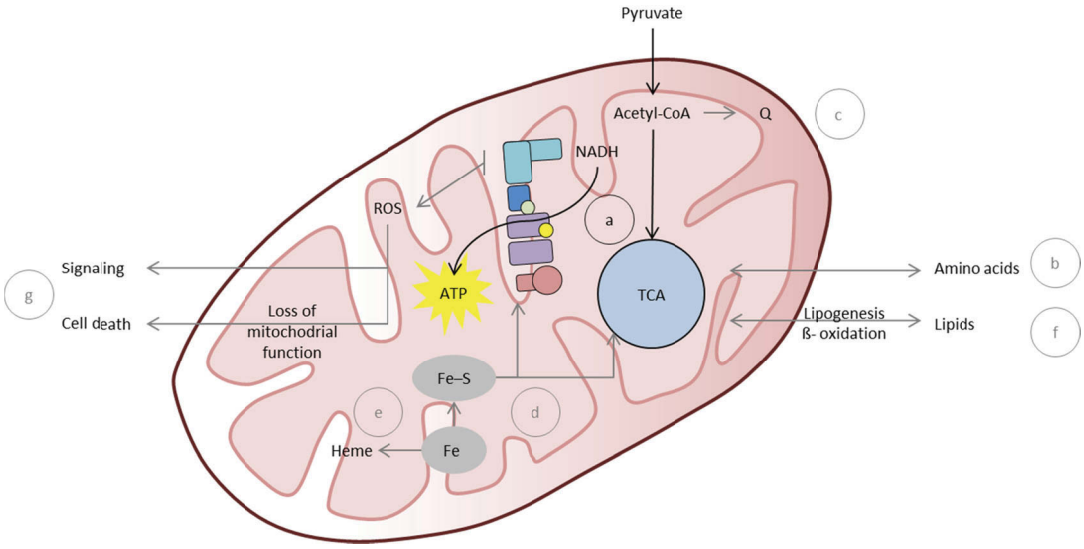

Figure 2:

Central metabolic pathways of mitochondria. Pyruvate is imported into mitochondria, oxidized and decarboxylated in the TCA cycle to generate NADH. Electrons from NADH are subsequently transported along the electron transport chain to molecular oxygen (O_2) which indirectly drives ATP production via the generation of a proton gradient across the cristae membrane to be utilized by the ATP synthase complex for the phosphorylation of ADP (a). Mitochondria are also a major site of amino acid metabolism, particularly of their catabolism (b). The acetyl-CoA based synthesis of ubiquinone (Q, c), as well as the formation and incorporation of Fe-S clusters into mitochondrial proteins (d) are additional mitochondrial functions. Iron is the central cofactor of heme and at least the final steps of heme biosynthesis take place in the mitochondria (e). As a by-product mitochondria produce reactive oxygen species (ROS) which are involved in signaling pathways (f). ROS also play a major role in programmed cell death (PCD). ATP, adenosine triphosphate; Q, ubiquinone; CytC, cytochrome c; Fe-S, iron-sulfur cluster; NADH, nicotinamide adenine dinucleotide; ROS, reactive oxygen species; TCA, tricarboxylic acid

1.4.1 Tricarboxylic acid cycle

Cellular respiration generates energy in the form of ATP and is based on three major metabolic processes: 1) glycolysis 2) the tricarboxylic acid (TCA) cycle, and 3) oxidative phosphorylation (OXPHOS). Glycolysis takes place in the cytosol, whereas the TCA cycle and OXPHOS are located in mitochondria (Figure 2a). Pyruvate, derived from cytoplasmic glycolysis, is imported into the mitochondrial matrix where it is decarboxylated by the pyruvate decarboxylase complex (PDC) to form acetyl-coenzyme A (acetyl-CoA). This is fed into the TCA cycle where it initially reacts with oxaloacetate, catalyzed by the citrate synthase (CS). In a series of downstream steps, citrate is then decarboxylated and electrons are transferred to NAD^+ and FAD to produce NADH and $FADH_2$. Finally oxaloacetate is regenerated to start a new round of acetyl-CoA oxidation (Burton and Krebs 1953).

1.4.2 Oxidative phosphorylation

Among almost all eukaryotes the electron transport chain of the inner mitochondrial membrane is conserved and consists of four membrane bound protein complexes. The process of election transport across the respiratory chain follows a uniform scheme. However, the plant electron transport chain features some special properties described in chapter 2. At the entry sites of the respiratory chain, $NADH+H^+$ and $FADH_2$ are oxidized. Complex I (NADH:ubiquinone oxidoreductase) transfers two electrons from NADH to ubiquinone (Q). During this process, Q is reduced to ubiquinol (QH_2) and protons (H^+) are transported into the cristae lumen. Complex II (succinate:ubiquinone reductase) oxidizes succinate to fumarate in the course of the TCA cycle. Analogous to complex I, Q is reduced into QH_2 by this complex, albeit without transfer of protons across the cristae membrane. Electrons from QH_2 are used by complex III (ubiquinone: cytochrome c-oxidoreductase) to reduce cytochrome-c, which is localized at the outer surface of the inner mitochondrial membrane. Complex III also transfers H^+ across the membrane. Complex IV (cytochrome c oxidase) is the terminal oxidase of the respiratory chain. Cytochrome c is oxidized and the electrons are used for the reduction of O_2, producing water. Overall, the activities of three of the four protein complexes of the electron transfer chain contribute to the proton gradient across the inner mitochondrial membrane. Reflux of the protons back into the matrix drives the phosphorylation of ADP by the ATP synthase (complex V, Chance and Williams 1956).

Most ATP generated by this process is exported from the mitochondria and used to drive energy-demanding cellular processes (Saraste 1999).

1.4.3 Amino acid metabolism

Protein synthesis and several other metabolic processes require ongoing amino acid synthesis and degradation (Figure 2b). Plants can synthesize all of the 20 proteinogenic amino acids themselves, whereas most animals are unable to produce the complete set of amino acids. For example, humans have to take up phenylalanine, valine, threonine, tryptophan, methionine, leucine, isoleucine, lysine, and histidine with their diet (Massey *et al.* 1998).

The carbon skeletons for the biosynthesis of amino acids are derived from Calvin cycle-, glycolysis-, and TCA cycle-intermediates (Schatz and Mason 1974). Amino acids are not only important for protein biosynthesis, but also have additional metabolic roles. Arginine, for instance, is used in the mitochondria for nitric monoxide (NO) formation, which is an important signaling compound (Kozlov *et al.* 1999). Glutamate, instead, is a central component of ammonia assimilation, in plants as well as in animals.

The catabolism of amino acids is common to all species but especially important in animals. All degradation processes share the formation of α-keto acids, NADH, and ammonium cations (NH_4^+). Amino acid degradation results in the release of NH_4^+, which is cytotoxic in high concentrations. The urea cycle is specific for animals and takes part in eliminating ammonium from the cell. Ammonium is mainly metabolized in liver mitochondria and afterwards excreted in the form of urea via the kidneys (Krebs and Henseleit 1932, Morris, JR 2002). The ammonia metabolism of plants is described in chapter 3. Degraded amino acids can be categorized according to the metabolic fate of the resulting α-keto acids. Glucogenic amino acids are precursors for TCA cycle intermediates or pyruvate. Ketogenic amino acids are converted into acetyl-CoA and acetoacetyl-CoA and contribute to the formation of fatty acids or ketone bodies. It follows that mitochondria are essential for amino acid synthesis, as well as for their degradation (Jungas *et al.* 1992).

1.4.4 Biosynthesis of organic cofactors- Ubiquinone

Mitochondria are involved in the biosynthesis of coenzymes such as ubiquinone (Q). Q is a lipid and ubiquitous in cell membranes. The enzyme is an electron carrier in the respiratory chain, but other possible functions were also shown. For example, Q is involved in antioxidative processes and is also a cofactor for uncoupling proteins (Turunen *et al.* 2004). Starting from acetyl-CoA, Q is presumably synthesized at the inner mitochondrial membrane (Bentinger *et al.* 2010, Figure 2c).

1.4.5 Biosynthesis of inorganic cofactors- Iron sulfur clusters

The assembly of iron-sulfur (Fe–S) clusters starts in mitochondria (Lill and Kispal 2000). Iron is transported into the cytosol by transferrin proteins before its import into mitochondria. The mitochondrial matrix is the cell compartment with the lowest oxygen concentration and is therefore suitable for the oxygen sensitive process of Fe-S cluster assembly by the iron–sulphate cluster assembly machinery (Rouault and Tong 2005). Fe-S clusters are important cofactors for Fe-S metalloproteins such as aconitase, subunits of the respiratory complexes I, II, and III as well as for the ferredoxin (Fd)/Fd reductase system (Abdel-Ghany *et al.* 2005). Accordingly, they are among the most important electron carriers in nature and contribute to processes such as respiration, photosynthesis, and nitrogen fixation (Figure 2d).

1.4.6 Heme biosynthesis

The mitochondrial Fe–S enzyme ferrochelatase, which contains a Fe-S cluster (Crooks *et al.* 2010), incorporates iron into protoporphyrin to form heme. This is the final step of heme biosynthesis in mammals. Heme is an iron chelator and used in respiration, oxygen transport, detoxification, and signaling (Ajioka *et al.* 2006). Probably the best known example of a heme function is its role in hemoglobin, where it binds and transports oxygen (Gibson 1973). Furthermore, all cytochromes include heme groups (Guo *et al.* 2016, Figure 2e). Plants also contain heme, for example in the form of leghemoglobin.

1.4.7 Fatty acid metabolism

Fatty acids are main components of lipids which serve as structural elements of cell membranes and in energy storage. Both, fatty acid biosynthesis and degradation rely on acetyl-CoA. Fatty acid biosynthesis in mitochondria is of minor importance (it mainly is required for biosynthesis of lipoic acid biosynthesis, a co-factor of some mitochondrial enzymes), but mitochondria contribute indirectly to fatty acid biosynthesis by exporting the TCA intermediate citrate. Citrate can be converted into acetyl-CoA which is the starting-point for fatty acid biosynthesis (Hellerstein *et al.* 1996). Also, cardiolipin, a lipid present in mitochondrial membranes, is synthesized in mitochondria (Schlame and Hostetler 1997).

The degradation of fatty acids takes place at different locations in animal and plant cells. In animals, mitochondrial fatty acid degradation is of fundamental importance for energy production. Catabolism of fatty acids is mediated by the ß- oxidation pathway and generates acetyl-CoA which can be used for the TCA cycle (Gerhart-Hines *et al.* 2007). By contrast, in plants ß- oxidation of fatty acids is restricted to peroxisomes and primarily takes place in germinating oilseeds or in senescent tissue (Graham and Eastmond 2002, Figure 2f).

1.5 Other mitochondrial functions

Mitochondria are involved in retrograde signaling. Several cellular communication pathways transmit mitochondrial signals to regulate the nuclear gene expression, which alters cellular metabolism (Liu and Butow 2006, Rhoads and Subbaiah 2007). Another important function of mitochondria is their role in programmed cell death (Figure 2g). The demise of cells is, amongst other processes, caused by the permeabilization of mitochondria and the generation of reactive oxygen species (Green and Kroemer 2004).

2. Special functions of mitochondria in plants in the context of photosynthesis

Mitochondrial functions in plants take place in the background of photosynthesis, the most important biochemical process on earth. The light reaction of photosynthesis contributes to ATP production. However, in contrast to mitochondria, this ATP is not provided to other cellular compartments but rather used for processes taking place in the chloroplasts themselves, most notably the Calvin cycle. Furthermore, chloroplast ATP formation only takes place in the light. This has far-ranging implications for the mitochondria, which have to provide ATP for the entire plant cell at night, but to the cell excluding the chloroplast compartment at daytime. Mitochondria are important for balancing the cellular redox state and for photorespiration, as outlined below.

2.1 Photosynthesis and the enzyme RuBisCo

Photosynthesis is of fundamental importance for life. Its products, organic compounds and O_2, are the prerequisite of most living beings. Plant photosynthesis requires specialized organelles: the chloroplasts. Similar to mitochondria they are surrounded by two membranes and possess a highly structured interior. Chloroplasts contain a folded thylakoid membrane system which harbors the protein complexes of the electron transfer system (the two photosystems, the cytochrome b_6f complex and some additional components) as well as the chloroplast ATP synthase complex. The 'stroma' is the hydrophilic subcompartment of chloroplasts (Lichtenthaler *et al.* 1981). Metabolically, photosynthesis is divided into two processes, which occur at the same time but in different locations. The first process is referred to as 'light reaction' and is located in the thylakoid membranes of the chloroplast. Light energy is used for the photolysis of water. The electrons liberated by this reaction are transported through the chloroplast electron transport chain to their final electron acceptor $NADP^+$, which is reduced to NADPH+ H^+. Other products of the light reaction are O_2 and ATP. Both, NADPH and ATP are needed for the second process of photosynthesis, the Calvin cycle. This process depends considerably on its key enzyme, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). Besides ribulose-1,5-bisphosphate RuBisCO has two other substrates, CO_2 and O_2. In the Calvin cycle, RuBisCO mediates the binding of CO_2 to ribulose-1,5-bisphosphate to yield two molecules of 3-phosphoglyceric acid (3PGA). By consuming ATP, 3PGA is reduced into triosephosphates. These triosephosphates can either be used to regenerate ribulose-1,5-bisphosphate, or for the formation of building blocks for starch biosynthesis (within chloroplasts) or sucrose biosynthesis (in the cytoplasm).

2.2 Interactions of mitochondria and chloroplasts

Mitochondria and chloroplasts strongly interact metabolically as well as physically (Raghavendra *et al.* 2003). Chloroplasts use H_20, CO_2, and light energy to produce carbohydrates and O_2. In contrast, mitochondrial ATP production depends on carbohydrates and O_2 and releases H_2O. Furthermore, both organelles exchange energy (ATP), reduction equivalents (e.g. via the malate-oxaloacetate shuttle) and carbon compounds.

2.2.1 Photorespiration

The second most prominent metabolic pathway on earth is photorespiration (PR, also termed glycolat cycle, C_2 cycle). It is initiated by the oxygenase side reaction of RuBisCO. Three different subcellular compartments are involved in the photorespiratory pathway: chloroplasts, peroxisomes, and mitochondria (Dellero *et al.* 2016, Bauwe *et al.* 2010). The oxidation of ribulose-1,5-bisphosphate by RuBisCO in the chloroplasts results in 3-phosphoglyceric acid (3PGA) as well as 2-phosphoglycolate (2PG) formation. 2PG is dephosphorylated to glycolate and imported into peroxisomes. Here, glycolate is converted to glyoxylate and subsequently to glycine. Glycine is transported into the mitochondria. Two molecules of glycine are converted into serine, with a concomitant loss of CO_2 and NH_3. This reaction is catalyzed by the concerted action of glycine decarboxylase complex (GDC) and serine hydroxymethyltransferase, both of which accumulate in high rates in leaf cells at daytime (Douce *et al.* 2001). Serine is subsequently transferred back in the peroxiosomes. There it is used for the production of glyceric acid, which is phosphorylated into 3PGA in the next step, taking place in the chloroplasts again (Maurino and Peterhansel 2010). Since PR also indirectly exports reduction equivalents from chloroplasts it may also be important for the prevention of photoinhibition under stress conditions (Wingler *et al.* 2000). Chapter 3 describes the connection of PR and nitrogen assimilation.

2.2.2 The cellular redox state is kept in a balance by the mitochondria in leaf cells

Respiratory electron transport is based on the complexes I to IV in animals and plants. However, in plants, several additional enzymes participate in respiratory electron transport. Alternative NADH/NADPH dehydrogenases are located at the outer and inner surface of the inner mitochondrial membrane. Compared to complex I, they have a lower affinity with respect to NADH/NADPH and do not translocate protons across the inner mitochondrial membrane. Another non-proton pumping respiratory oxidoreductase in the mitochondria of plants is the alternative oxidase (AOX, Moore *et al.* 2002). As such, electron transport in plants can be completely uncoupled from ATP formation (Wagner and Krab 1995). A tight regulation of the activities of these enzymes on the gene and the biochemical level is required.

Both AOX and the alternative NADH/NADPH dehydrogenases are important to keep the redox balance in the cell, especially during stress conditions. ROS induces the expression of the AOX genes (Vanlerberghe and McLntosh 1996). AOX, in turn, participates in controlling the level of mitochondrial signaling molecules such as superoxide and peroxide by the mitochondrial ETC. The formation of these signal molecules is modulated by AOX, because AOX activity shortens the electron transfer chain (Vanlerberghe 2013).

2.2.3 Mitochondrial activity supports photosynthesis

Photosynthesis depends on mitochondrial support. Mitochondrial oxidative metabolism has been suggested as a prerequisite for maintaining high rates of photosynthesis (Padmasree *et al.* 2002). Mitochondria are sink organelles for excess reduction equivalents from photosynthesis and can provide ATP to the chloroplasts, which is important in the absence of the photosynthetic light reaction, e.g. at night. Reduction equivalents cannot directly be transferred from one organelle to another since the membranes lack suitable transporters for NADH and NADPH. Instead, they are indirectly transported by exchanging reduced organic compounds for oxidized ones. For instance, NADPH can be used to reduce oxaloacetic acid (OAA), oxoglutarate and bisphosphoglycerate (BPGA) to malate, glutamate and triose phosphates, respectively. All these compounds can be exported from the chloroplast and are used to regenerate NADH from NAD^+ in the cytoplasm or in mitochondria by complementary biochemical reactions.

Another aspect of the metabolic interaction between mitochondria and chloroplasts is the recycling of respiratory CO_2 for carbon fixation by the Calvin cycle. CO_2 is released in mitochondria by the decarboxylation of photorespiratory glycine and by the TCA cycle. An intra-cellular CO_2 recycling pathway has been suggested to prevent diffusion of respiratory/photorespiratory CO_2 into the atmosphere. It is based on the conversion of CO_2 into bicarbonate by the action of mitochondrial carbonic anhydrases, which is supported by the alkaline pH of the mitochondrial matrix (Llopis *et al.* 1998, Zabaleta *et al.* 2012). Gene expression studies showed the upregulation of carbonic anhydrases in the presence of elevated CO_2 in Chlamydomonas (Spalding *et al.* 2002). Furthermore, characterization of an Arabidopsis mutant lacking the gene encoding a mitochondrial-localized carbonic anhydrase supports the suggested CO_2 recycling pathway (Soto *et al.* 2015).

2.3 Metabolic flexibility of the TCA cycle

Plants are exceptionally flexible in terms of their metabolism. In animals, pyruvate is the main end product of glycolysis and imported into the mitochondria, but in plant cells malate is an alternative end product of glycolysis (Day and Hanson 1977, Raghavendra and Padmasree 2003). It is formed from phosphoenolpyruvate (PEP) by the action of the enzymes PEP-carboxylase (PEPC) and a cytoplasmic malate dehydrogenase. Activity of the latter enzyme requires NADH. Malate can also originate from photosynthesis and its transport into mitochondria may be used to export excess reduction equivalents as outlined above. After import of malate into the mitochondria, malate can either be re-oxidized to OAA by the mitochondrial malate dehydrogenase or converted to pyruvate by malic enzyme. Both reactions produce reduction equivalents that can be used for ATP-production and provide entry points into the TCA cycle.

Starting with OAA and acetyl-CoA, the TCA cycle produces reduction equivalents for oxidative phosphorylation. However, during the day the mitochondrial TCA cycle seems to be largely inhibited since mitochondrial CO_2 efflux is decreased by up to 80% (Atkin *et al.* 2000). At the same time, the TCA cycle reactions were shown to be relevant for photosynthesis (Nunes-Nesi *et al.* 2008). Current findings suggest a non-cyclic mode of the TCA cycle to provide carbon skeletons for other cellular processes. Depending on the cell's needs, the TCA cycle switches from the cyclic to non-cyclic modes and uses the intermediates for amino acid, secondary metabolite, and purine biosynthesis, ammonia assimilation, and the glyoxylate cycle (Sweetlove *et al.* 2010).

3. Special functions of mitochondria in plants in the context of N_2 fixation

As outlined in the previous chapters, mitochondria are involved in a wide range of cellular processes common to most eukaryotes, but have special functions in plants. Additional features of plant mitochondria are related to the assimilation of molecular nitrogen (N_2), which is based on the symbiosis with rhizobial bacteria. The role of mitochondria in the context of N_2 fixation has received little attention, despite its enormous economic importance.

3.1 Nitrate uptake and assimilation

Air is composed of approximately 78% molecular nitrogen (N_2), 20% oxygen (O_2), and a small quantity of other gases (Frey and Mannella 2000). Nitrogen is a prerequisite for the synthesis of essential plant components like nucleic acids, amino acids and proteins. Although air consists mainly of nitrogen, its molecular form cannot be utilized by plants. Instead, plants have to take up inorganic nitrogen from the soil as ammonium (NH_4^+) and nitrate (NO_3^-). Natural sources of nitrate and ammonium, such as lightning or decomposition of biomass, are not sufficient to support intensive agriculture on a global scale. Therefore, modern agriculture relies on nitrogen fertilizer mainly originating from the Haber-Bosch process[1]. However, the disadvantages of extensive use of fertilizer are the high costs and it is negative environmental impact.

[1] $N_2 + 3H_2 \rightarrow 2NH_3$

Nitrogen fertilizer generally contain nitrate, which is taken up by the plant. Afterwards it is either stored in vacuoles or transported to the leaves. The enzyme nitrate reductase (NR) reduces nitrate to nitrite (NO_2^-, Scheible *et al.* 1997). Nitrite is then further reduced to ammonium by the nitrite reductase (NiR) in the chloroplast (Vincentz *et al.* 1993). Additionally, there are internal sources of ammonium, such as photorespiration, the degradation of storage compounds, and protein turnover (Stumpf *et al.* 1989). The glutamine synthase/ glutamine oxoglutarate aminotransferase (GS/GOGAT) pathway is the main ammonium assimilation pathway in plants. GS incorporates the ammonium into glutamine. Subsequently, GOGAT transfers the amino group to 2OG (2-oxoglutarate), which results in the production of two glutamate molecules (Figure 3). GOGAT is exclusively located in plastids. Interestingly, 2OG is provided by mitochondria either directly or indirectly. The mitochondria localized isocitrate dehydrogenase (IDH) converts isocitrate to 2OG in the course of the TCA cycle (Martínez-Rivas and Vega 1998, Gálvez *et al.* 1999). Alternatively, a cytosolic NADP-dependent isocitrate dehydrogenase (ICDH) produces 2OG from isocitrate exported from mitochondria (Galvez *et al.* 1996).

Glutamate and glutamine are the primary assimilation products of ammonium. Both are the starting point of several metabolic reactions leading to the formation of other amino acids or nucleotides. Glutamate is the precursor of glutamine, proline, and arginine (Delauney and Verma 1993, Wu and Morris 1998). Using glutamate as a substrate, an aspartate aminotransferase (AspAT) transfers the amino group to OAA, which produces aspartate. Aspartate is very reactive and can transfer its α-amino group to other compounds, mainly 2-oxoacids, by several aminotransferases (Liepman and Olsen 2004). Aspartate is also the precursor for amino acids of the aspartate family, including methionine, lysine, and threonine (Azevedo *et al.* 1997, Wadsworth 1997). Finally, aspartate can be aminated by an asparagine synthetase (AS) to yield asparagine, a transport and storage form of nitrogen. In legumes, transported nitrogen is up to 86% asparagine (Lea *et al.* 2007).

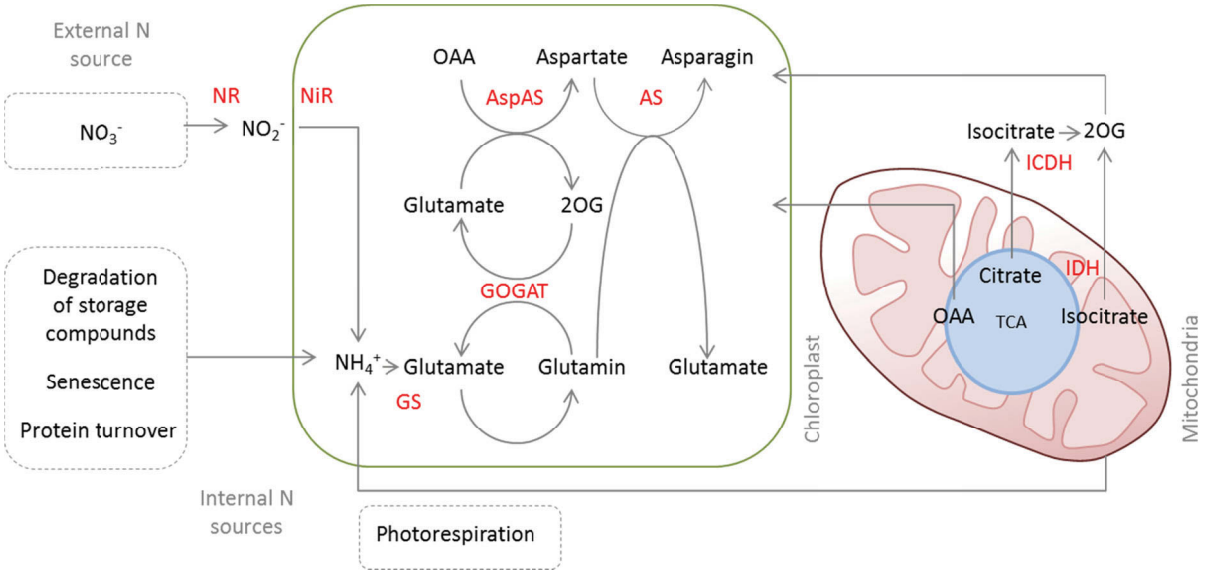

Figure 3:

Ammonium assimilation by the GS/GOGAT pathway. Plants can take up nitrate (NO_3^-) from external sources, such as fertilizers. Nitrate is reduced by a nitrate reductase to nitrite, which is imported into the chloroplast. There, another reduction step is carried out by nitrite reductase (NiR) resulting in the production of ammonium. Other sources for ammonium are photorespiration, degradation processes from storage compounds, senescence or protein turnover. In each case, the ammonium is incorporated into glutamine using the glutamine synthetase (GS). Glutamine 2-oxoglutarate aminotransferase (GOGAT) transfers the amino group from glutamine to 2OG, thereby completing the GS/GOGAT cycle. The enzyme aspartate aminotransferase produces aspartate. Aspartate is aminated to yield asparagine. 2OG and OAA can be provided by the mitochondrion. 2OG is produced from isocitrate either by a cytosolic ICDH or mitochondrial IDH. 2OG, α-Ketoglutaric acid; GOGAT, Glutamine oxoglutarate aminotransferase; GS, Glutamine synthase; ICDH, NADP-dependent isocitrate dehydrogenase; IDH, isocitrate dehydrogenase; NH_4^+, ammonium ion; NiR, nitrite reductase, NO_2^-, nitrite; NO_3^-, nitrate; NR, nitrate reductase; OAA, oxaloacetic acid; (modified from: Gálvez *et al.* 1999)

3.2 Symbiotic nitrogen fixation

In addition to the previously described internal and external nitrogen sources, ammonium can also originate from symbiotic nitrogen fixation (SNF). SNF refers to the conversion of N_2 into NH_3 (ammonia) by the nitrogenase enzyme of Rhizobial bacteria (Willems 2006). Several plants of the legume family are able to engage in symbioses with Rhizobia. During the establishment of this symbiosis, the bacteria penetrate the plant root and the plant initiates the growth of new root organs, the nodules. Once inside the plant cell the bacteria change into nitrogen fixing bacteroides. In return of being provided with carbon compounds from the plant the bacteroides reduce molecular nitrogen from the surrounding air to ammonia (Eq. 1). The symbiosis thus facilitates plant growth and the production of protein rich seeds without the need for artificial nitrogen fertilizer, although at the price of losing reduced carbon compounds to the bacteroides. Besides the economically important crop production, legumes are also a valuable green fertilizer and are often used in crop rotation practices in which they also have a beneficial impact on soil quality (Biederbeck *et al.* 1998).

Equation 1: $N_2 + 8e^- + 16\ ATP + 8H^+ \rightarrow 2NH_3 + 16\ ADP + 16\ Pi + H_2$ (Igarashi 2003)

The energy (ATP, electrons) necessary for the nitrogenase reaction most likely comes from malate oxidation by the bacteroid TCA cycle and OXPHOS. The NADH from the TCA cycle is furthermore used to produce reduced ferredoxin, which deliver electrons to the nitrogenase complex. However, alternatively, energy for the nitrogenase reaction also could be provided by the mitochondria.

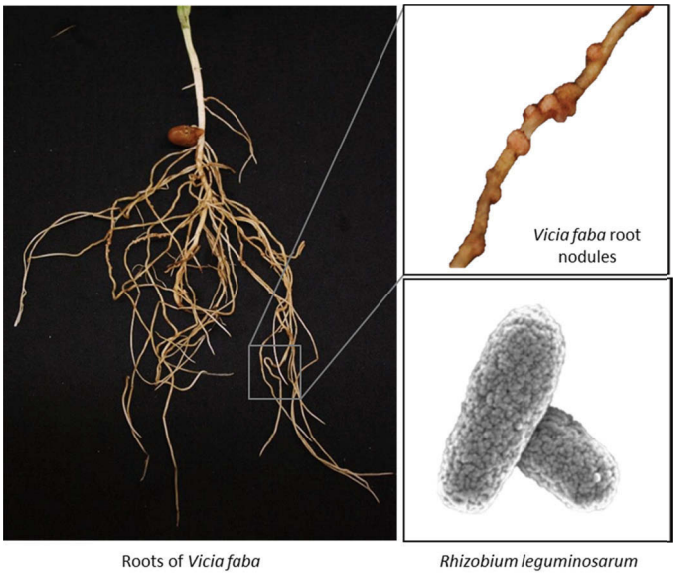

Figure 4:

Vicia faba roots with nodules containing its symbiotic bacterium *Rhizobium leguminosarum*. Left panel, roots of a four week old *Vicia faba* plant (Thal *et al.*, unpublished); upper right panel, roots with nodules (Thal *et al.*, unpublished); lower right panel, *Rhizobium leguminosarum*; (Huang *et al.* 2014)

Clearly, the symbiotic relationship requires significant adaptations from both partners. For instance, a multi-step signal exchange between plant and bacteria initiates the formation of the symbiosis (Garg and Geetanjali 2007). Once established, SNF is based on a transfer of metabolites between the plant and the bacteroides (Udvardi and Poole 2013). Despite of the immense importance of SNF, it is surprising to find that its molecular basis is not understood in great detail. For example, it is unclear in which form the fixed nitrogen is exported from the bacteroides (White *et al.* 2007).To gain a broader understanding of SNF it is necessary to investigate the biochemical processes taking place within root nodules. This may also foster efforts to transfer the benefits of SNF to non-legume crop plants.

3.3 Interactions of mitochondrial metabolism with nitrogen fixing bacteroides in the legume nodule

The mitochondria may play an important part in the symbiosis. It has been shown that mitochondria are located at the periphery of infected cells, thereby forming a mitochondrial layer between gas-filled intercellular spaces and the bacteroides (Bergersen 1982). Additionally, the structure of mitochondria is changed in root nodules since 30-40% of them are bigger and contain more cristae (Summerfield *et al.* 1995). However, it is currently unclear if the interactions of mitochondria and bacteroides are supportive or antagonistic. Mitochondria might provide ATP and/or precursors for ammonium assimilation in SNF. In contrast, bacteroides as well as mitochondria use malate for energy production. Thus, both compete for organic acids. Furthermore, the mitochondrial respiration requires O_2, which is of limited availability in the hypoxic nodule tissue.

3.3.1 Mitochondrial energy supply in the nodule

The number of mitochondria is increased in infected cell in comparison to root cells, indicative of higher energy requirements (Fred *et al.* 2002). SNF needs large quantities of ATP for ammonia assimilation and to maintain the proton motive force across the peribacteroid membrane which could be covered by mitochondria (Millar *et al*. 1995). Besides ATP, mitochondria also produce reduction equivalents. These are transported indirectly, for example using the malate-OAA shuttle. This involves malate dehydrogenase (MDH) to convert malate to OAA. The identification of an induced nodule-specific plastid NAD-MDH points to the significance of the malate-OAA shuttle to fulfil the changed requirements during N_2 fixation (Scheibe 2004).

3.3.2 Mitochondrial metabolism provides support for ammonium assimilation in nodules

Root nodules can be categorized according to their morphology. Plants from the tropics, like Glycine, develop determinate nodules with a spherical form (Parniske 1994). These plants incorporate the ammonium from SNF into ureides prior to storage or transportation (Tajima *et al.* 2004). Almost all other legume plants grow nodules of cylindrical shape containing a persistent apical meristem, referred to as indeterminate nodules. The xylem sap of these plants, such as Pisum, mostly contains asparagine (Peiter *et al.* 2004). Bacteroides of determinate and indeterminate nodules also export diverging nitrogen compounds. E*x planta R. leguminosarum* bacteroides of soybean mainly export NH_3/NH_4^+, whereas pea bacteroides exclusively released alanine (Waters *et al.* 1998, Li *et al.* 2002). It is likely that both alanine and ammonium are exported from the bacteroides *in vivo* but that the ratio varies between legume species (Allaway *et al.* 2000).

Bacteroid derived ammonium may be incorporated into glutamine, analogous to the GS/GOGAT pathway in leaves (Groat and Vance 1981). Glutamate synthase (GOGAT) was identified to be a key enzyme for the assimilation of symbiotically fixed N_2 into amino acids in Alfalfa (Anderson *et al.* 1989). The 2OG required for the GS/GOGAT pathway, as well as OAA for asparagine synthesis is suggested to be provided by mitochondria (Nunes-Nesi *et al.* 2011). Alternatively, a bacterial alanine dehydrogenase (AD) may produce alanine from pyruvate and ammonium. Alanine might then be transported into the cytosol of the plant cell (Poole and Allaway 2000). The production of glutamate from alanine is then performed by an alanine aminotransferase (AlaAT), which catalyzes the reversible transfer of an amino group from alanine onto 2OG to form glutamate and pyruvate. A mitochondrial form of AlaAT has been identified (Liepman and Olsen 2003), suggesting that this step may take place in mitochondria. The AlaAT derived pyruvate could be receycled in the TCA cycle and glutamate might be exported from the mitochondria as reaction partner of AspAT or GS.

3.3.3 Oxygen supply to mitochondria in root nodules

Low cellular oxygen concentrations are a prerequisite for nitrogen fixation since the nitrogenase is extremely sensitive to this gas. However, oxygen is the final acceptor for the electrons derived from reduction equivalents being oxidized by the mitochondrial and bacteroid electron transfer chains. Therefore, sufficient concentrations of oxygen are a prerequisite for ATP production and SNF. Aeration of the root cell leads to the simultaneous uptake of oxygen and nitrogen. To protect the nitrogenase from oxygen the plant produces the oxygen binding protein leghemoglobin, which is also responsible for the pink color of the nodule (Figure 4). Leghemoglobin is located in the plant cytoplasm in large quantities (e.g. soybean $3 \cdot 10^{-3}$ mol/l, Heldt and Piechulla 2015). Bacteroid ATP production is less affected by reduced O_2 concentration since its cytochrome c oxidase has a higher affinity to oxygen compared to its mitochondrial counterpart (Lodwig and Poole 2003). Interestingly, in addition to its oxygen buffering capacity, leghemoglobin maintains a high oxygen flux for respiration of mitochondria (Ott *et al.* 2005). Supported by leghemoglobin the respiration of mitochondria in infected cells is enhanced compared to uninfected cells (Polacco and Todd 2011). The glycolysis intermediate 1,3-bisphosphoglyceric acid mediates the oxygen release from the heme group of hemoglobin in blood (Stefansson *et al.* 2016), a similar function for leghemoglobin in nodules can be hypothesized. As mitochondria are involved in key regulatory steps (Fernie *et al.* 2004), they may regulate the oxygen concentration in the cell according to demand.

3.3.4 Mitochondria and bacteroides compete for malate

The bacteroides are supplied by the plant with organic acids, mainly malate. Photosynthesis derived sucrose enters the glycolytic pathway and is degraded to phosphoenolpyruvate (PEP) or pyruvate. PEP may be carboxylated to oxaloacetate and finally reduced to malate. Malate is transported into the bacteroides and oxidized in the citric acid cycle to produce NADH. In this trade-off between plant and bacteria the malate/fumarate metabolism of the plant is affected and the metabolic pathways and key enzymes of malate and fumarate synthesis are altered after the formation of nodules (White *et al.* 2007). At the same time malate and other organic acids are also used for ATP production in the host cell mitochondria. Malate partitioning between mitochondria and bacteroides thus needs to be regulated carefully.

4. Objectives

This thesis focuses on expanding our understanding of mitochondrial functions in the cell, especially with respect to symbiotic nitrogen fixation. A closer investigation of root nodule mitochondria will potentially unravel additional special features of these organelles and broaden the understanding of the biochemical processes taking place within root nodules. This, in turn, may form the basis for future research. The new insights are relevant for the long-term goal to improve crop yield by enhancing SNF efficiency and may ultimately help in integrating nitrogenase into the mitochondrial metabolism to produce self-fertilizing non-legume crops (Oldroyd and Dixon 2014).

Proteomic tools are highly suited to gain insight into the metabolism of SNF. The analysis of protein content and protein abundances allows conclusions to be drawn on their functions in SNF. This may be even further improved by the analysis of posttranslational modifications. Moreover, the study of the proteomes from both symbiotic partners nicely complements existing transcriptome studies because protein and transcript abundances are often not correlated (Sousa Abreu *et al.* 2009).

In the following sections, three research projects are briefly introduced, which complement each other to address the above-mentioned research objectives. They also form the basis of three manuscripts comprising this PhD thesis.

4.1 Characterization of mitochondrial functions in root nodules during SNF (Manuscript 1)

Total cell extracts from *Vicia faba* nodule and root extracts were analyzed by shotgun mass spectrometry (MS) to investigate the symbiosis and nitrogen fixation specific alterations in the root nodule physiology. For this, the proteome of nodules capable and incapable of SNF were compared to fertilized and nitrogen depleted roots. The data obtained in these comparisons support the view that mitochondria are of central importance for the symbiosis. Nodules contained numerous mitochondrial proteins with increased abundances. One of them, a coproporphyrinogen III oxidase, is involved in the biosynthesis of heme (Madsen *et al.* 1993), which is required for leghemoglobin production and function. Furthermore, evidence is presented that suggests a participation of the mitochondria in the catabolism of alanine, which is potentially exported from the bacteroides. A mitochondrial alanine aminotransferase identified in the course of this project is a candidate enzyme catalyzing the transfer of the amino group from alanine to 2OG, thus supplying pyruvate for ATP production and glutamate for ammonium assimilation at the same time. Similarly, mitochondrial 2OG may also be used for glutamate production (Lancien *et al.* 2000). Our study thus supports the notion that mitochondria are of great importance for the biochemistry of the N_2-fixing cell.

4.2 Improved isolation and sub-fractionation of mitochondria (Manuscript 2)

To gain new insights into mitochondrial physiology it is desirable to identify all members of plant mitochondrial proteome since this would allow to comprehensively understand mitochondrial functions in plants and their potential to support SNF. A prerequisite for characterizing the plant mitochondrial proteome is the preparation of pure organelles since organellar contaminations inevitably lead to the false positive assignment of non-mitochondrial proteins to the mitochondrial compartment. The manuscript carefully compiles and documents all steps necessary for the generation of pure mitochondrial fractions. Furthermore, protocols to subdivide isolated mitochondria into subfractions for subsequent proteomic analysis are presented. The methods described in this publication thus allow the characterization of plant mitochondria in so far unprecedented depth.

Extended definition of the plant mitochondrial proteome (Publication 1)

Mitochondrial presumably contain 2000-3000 proteins to maintain its diverse functions (Millar *et al.* 2005). Some of these proteins are tissue specific and related to specialized functions only required in a particular cell type or under specific conditions. Modern proteomic methods to analyze the mitochondrial proteome are based on mass spectrometry (MS) and allow the identification and quantification of thousands of proteins from complex samples (Aebersold and Mann 2003). To better define the proteome of the mitochondria from plants, mitochondrial fractions of two different species – potato and Arabidopsis - were characterized in parallel by in-depth mass spectrometry (Salvato *et al.* 2014, Lee *et al.* 2013). Our study profoundly reviewed the datasets and led to the discovery of several new proteins important for plant mitochondrial functions. For instance, the comparison of the comprehensive Arabidopsis and potato mitochondrial proteomes enabled us to identify two novel carriers of the MCF family.

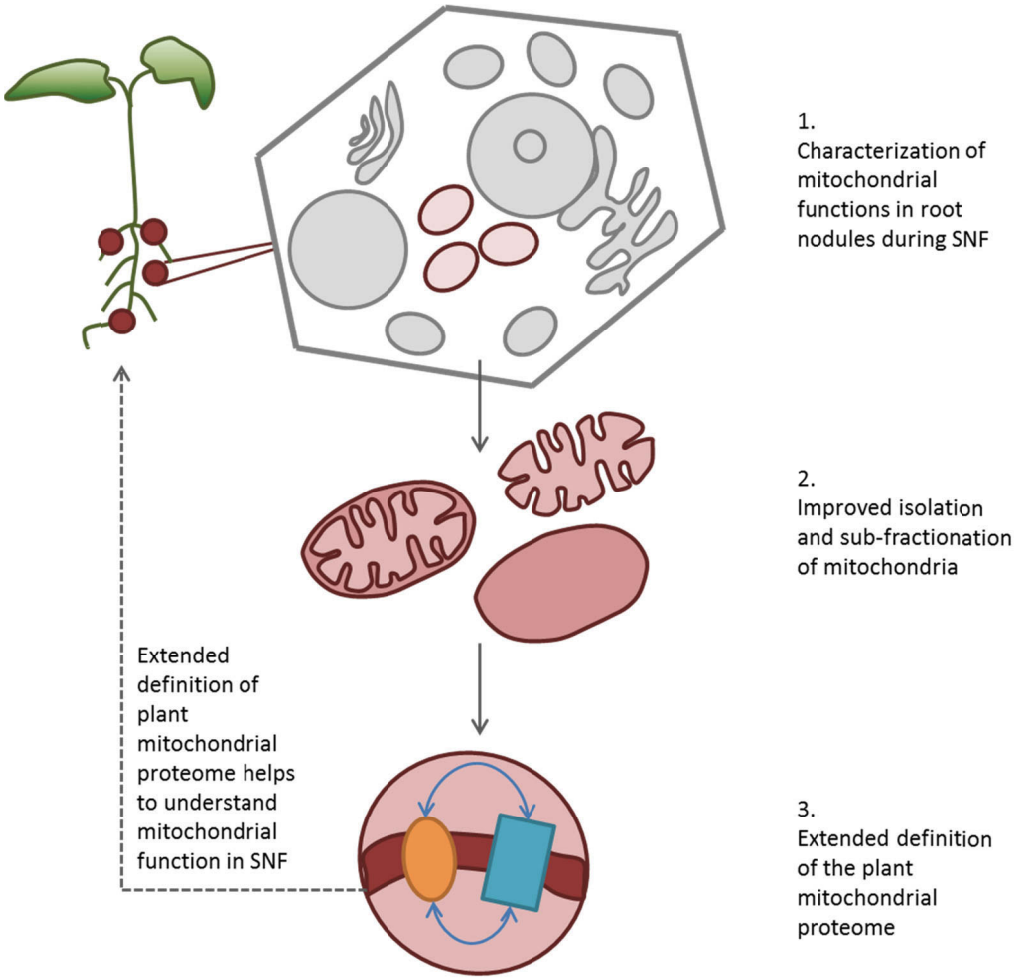

Figure 5:

Graphical summary of the three objectives. 1) The identification of possible interactions between mitochondrial functions in root nodules and SNF, 2) The improved isolation and sub-fractionation of mitochondria, and 3) The extended definition of the plant mitochondrial proteome.

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6. List of figures

7. Publications and Manuscripts

7.1 Manuscript 1

Proteomic analysis shows the specific impact of nodulation and biological nitrogen fixation on *Vicia faba* root nodule physiology, to be submitted

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Proteomic analysis shows the specific impact of nodulation and biological nitrogen fixation on *Vicia faba* root nodule physiology

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Running title

Physiology of symbiotic nitrogen fixation in plants

Keywords

Symbiotic nitrogen fixation, metabolism, *Vicia faba, Rhizobium leguminosarum*, root nodule, shotgun mass spectrometry

1. Abstract

Root nodules are specialized plant organs housing and regulating the mutual symbiosis of legumes with nitrogen fixing Rhizobia. As such, these organs fulfill unique functions in plant metabolism. However, the differentiation between the enzymes executing the nitrogen fixation step itself and those merely involved in sustaining the Rhizobia:plant symbiosis is a challenging task and requires a suitable experimental setup to be resolved. Shotgun mass spectrometry is a convenient tool to monitor proteomes and comparative analyses thus provide information on the physiological differences between samples.

Using *Vicia faba* and *Rhizobium leguminosarum* we performed quantitative proteome analyses of nitrogen fixing and non-nitrogen fixing nodules as well as fertilized and non-fertilized roots. Pairwise comparisons between these samples revealed common altered enzymes in active nodules. Similarly, general differences between nodules and roots were observed. Together, this allows differentiating between nitrogen fixation and symbiosis related proteins. The data additionally suggest that enzymes in sulfur metabolism are upregulated in nodules, providing further evidence for previously reported correlation of nitrogen and sulfur fixation in these plant organs. Further observations relate to the, mainly hormonal, control of nodulation. Most importantly, a new model of symbiotic nitrogen fixation in indeterminate nodules is suggested which covers previous, contradicting reports on nitrogen export from bacteroids and combines them into a coherent pathway in which both, ammonia and alanine are supplied to the plant. A mitochondria localized alanine aminotransferase may have a crucial role in symbiotic nitrogen fixation and thus classifies mitochondria as organelles with important functions in SNF.

2. Supporting Information

Supp. Table 1:

Top 5 proteins derived from pairwise comparisons of Fix+/Fix-, Fix+/N, Fix-/0, and N/0

Supp. Table 2:

Protein IDs and Fasta headers of proteins unique to the groups Fix+ and Fix-

3. Introduction

Biological nitrogen fixation of several plants from the *Fabaceae* family is of considerable significance for both natural ecosystems and agriculture. Especially important are legume plants as peas, lentils, soybean, peanuts and other legume crops providing 27% of the world's food and forage production (Graham 2003). The ability of legumes for fixing molecular nitrogen is based on a symbiosis with bacteria of the genus Rhizobium. This symbiosis enables the plants to grow and to produce protein rich seeds without the need for artificial nitrogen fertilizer. Besides crop production, legumes are also a valuable green fertilizer and are often used in crop rotation practices in which they also have a beneficial impact on soil quality (Biederbeck *et al.* 1998).

The establishment of the legume:Rhizobia symbiosis is a complex process which is initiated by signaling events between the host plant and its bacterial partner and ends with the formation of new root organs, the nodules. Within the nodules the symbiotic bacteria (now termed bacteroids) reduce the atmospheric dinitrogen (N_2) with the enzyme nitrogenase to produce ammonia or amino acids to be provided to the plant in exchange for organic nutrients (Hoffmann *et al*. 2014). The plant invests carbohydrates from photosynthesis to support the energy intensive processes required for the initiation and maintenance of symbiosis, such as nodule growth and senescence, assimilation of the fixed nitrogen in the host cell into nitrogen-containing organic compounds for the export from the nodule, production of leghemoglobin (a protein regulating the oxygen concentration of the N_2-fixing cell), and methionine/cysteine biosynthesis (Schubert 1986). As yet, many details of these processes are unknown, even the primary export compound of the of the bacteroide N_2 fixation. Considering the profound impact SNF has on the plant it is not surprising that the accompanying alterations in root tissue morphology and metabolism are driven by significant changes in the abundances of several enzymes (Marx *et al.* 2016, Rolfe *et al.* 2003). However, given that root nodules constitute a plant organ on their own, which is different to the root tissue it originated from, it is often difficult to tell if altered protein abundances are tissue specific or the consequence of nitrogen fixation.

Here, we aim at elucidating tissue-specific and SNF-dependent alterations in *Vicia faba* root/nodule physiology by analyzing the proteomes of nodules capable and incapable of SNF and compare them to fertilized and nitrogen depleted roots. The selection of the sample groups and the pairwise comparisons conducted in this study thus enable differentiation between symbiosis-specific and SNF-specific processes.

4. Material and Methods

4.1 Plant material and plant cultivation conditions

Vicia faba (var. 'Fuego') seeds were sterilized (70% EtOH 2 min, H_20 10 min, NaClO 10 min) and soaked in ddH_20 for 24 h in the dark. Plants were then transferred to pots (12 cm ID) filled with broken foamed clay (Lamstedt Lecaton, 2 – 4 mm, Lamstedt, Germany). The substrate Lecaton was chosen, because the material is free of nutrients and can be removed from root material at harvest leaving only minor residues. Plants were grown for two weeks under 8 h light/ 16 h dark regime at 20 °C. Plant were fertilized with 0.5x Hoagland nutrient solution without nitrogen (1 mM $MgSO_4*7H_2O$, 40 µM KH_2PO_4, 50 mM NaFeEDTA, 0.1 mM $CaCl_2$, 0.2 µM $Na_2MoO_4*2H_2O$, 10 µM H_2BO_3, 0.2 µM $NiSO_4*6H_2O$, 1 µM $ZnSO_4*7H_2O$, 2 µM $MnCl_2*4\ H_2O$, 0.5 µM $CuSO_4*5\ H_2O$, 0.2 µM$CoCl_2*H_2O$, Hoagland D. R. and Arnon D. I. 1950) twice a week and watered with demineralized water according to demand. After the first growing period bacteria were added to the groups Fix+ (*Rhizobium leguminosarum* biovar. Viciae) and Fix- (*Rhizobium leguminosarum* mutant deficient in nitrogen fixation, NifH78::Tn5, Ma *et al.* 1982). No bacteria were added to groups N and 0. Only N plants were fertilized after 2 weeks with 0.5x Hoagland (without 0.1 mM $CaCl_2$ but including 2.5 mM $Ca(NO_3)_2*4H_2O$, and 2.5 mM KNO_3). Group 0 plants were neither inoculated with bacteria nor fertilized with NO_3^-. Harvest took place four weeks after planting. Fix+ and Fix- nodules were harvested after four weeks whereas roots were used for N and 0.

4.2 Element analysis

Leaf material from three plants of each group was dried and ground with a ball mill at RT. The carbon and nitrogen content of the powder was determined in an element analysator by dry oxidation at 1500 °C and excess O_2 (Vario EL, Elementar, Hanau, Germany).

4.3 Protein extraction and MS sample preparation

5 g of roots of N and 0 plants and nodules from Fix+ and Fix- plants were shock frozen in liquid nitrogen and ground in a ball mill to fine powder before proteins were extracted with SDS buffer [4% (w/v) SDS, 125mM Tris-HCl (pH 6.8), 20% (v/v) glycerol] for 5 min at 60 °C. After centrifugation (10 min at 18.000 g) the protein concentration of the supernatant was determined using the Pierce BCA-200 Protein Assay Kit (Thermo Fisher Scientific, Dreieich, Germany). For purification and concentration of the proteins a volume corresponding to 50 µg of protein was mixed with β-mercaptoethanol and bromophenol blue to yield final concentrations of 5% [v/v] and 0,05% [w/v], respectively) and were loaded on a glycine/ sodium dodecyl sulfate (SDS) polyacrylamide gel according to to Leammli (Laemmli 1970). When the proteins reached the border between the 4% acrylamide stacking and the 14% acrylamide separating gel, the run was stopped and the gels were incubated in fixation solution (15% [v/v] ethanol, 10% [v/v] acetic acid) for 30 min and stained for 1 h with Coomassie Brilliant Blue G250 (Neuhoff *et al.* 1985). Then the gel bands were cut into cubes with edge lengths of approximately 1 mm, transferred into low-binding Eppendorf caps (Eppendorf, Wesseling-Berzdorf, Germany) and dried in a vacuum centrifuge. Proteins were in-gel Trypsin digested and resulting peptides were extracted according to Fromm *et al.* (2016).

4.4 Label-free quantitative shotgun mass spectrometry and protein identification

Tandem mass spectrometry (MS/MS) analysis was performed using a Q-Exactive (Thermo Fisher Scientific, Dreieich, Germany) mass spectrometer coupled to an Ultimate 3000 (Thermo Fisher Scientific, Dreieich, Germany) UPLC as described by Fromm *et al.* (2016). In brief, 2 to 5 µl of sample solution were injected onto a 2 cm, C18, 5 µm, 100 Å reverse phase trapping column (Acclaim PepMap100, Thermo Fisher Scientific, Dreieich, Germany) at a rate of 4 µl min^{-1}. Peptide separation was achieved on a 50 cm, C18, 3 µm, 100 Å reverse phase analytical column (Acclaim PepMap100, Thermo Fisher Scientific, Dreieich, Germany). Peptides were eluted by a non-linear 5% to 30% [v/v] acetonitrile gradient in 0.1% [v/v] formic acid at a flow rate of 300 nl min^{-1} and over a period of 60 mins and at 35 °C. Transfer of eluted peptides into the mass spectrometer was performed in a NSI source (Thermo Fisher Scientific, Dreieich, Germany)

equipped with stainless steel nano-bore emitters (Thermo Fisher Scientific, Dreieich, Germany). The spray voltage was set to 2.2 kV, capillary temperature to 275 °C, and S-lens RF level to 50%. The MS was run in positive ion mode, MS/MS spectra (top 10) were recorded from 20 mins to 100 min. Full MS scans were performed at a resolution of 70,000 whereas 17,500 was used for MS/MS scans. Automatic gain control (AGC) targets for MS and MS/MS were set to 1E6 and 1E5, respectively. Only peptides with 2, 3, or 4 positive charges were considered.

4.5 MaxQuant Evaluation

MS data were evaluated using the MaxQuant software package (version 1.5.5.1) including the Andromeda search engine (Cox and Mann 2008) against an in-house database of combined *Medicago truncatula* and *Rhizobium leguminosarum* protein sequences as well as common contaminants. For the Andromeda search, allowed variable modifications were oxidation of methionine residues and N-terminal acetylations. Carbamidomethylation of cysteine residues was selected as fixed modification. The false discovery rate at the peptide spectrum match level, determined by target decoy approach, was set to 0.01. For identification, information on precursor mass and retention time of other runs were superimposed on ions for which no MS/MS information was possible in individual runs. Label free quantification (LFQ) values were applied for relative quantitation of proteins. In detail, Max Quant and Andromeda settings were chosen as follows:

A) Group specific parameters: A1) General: Standard LC-MS run, Multiplicity 1, variable modifications, acetyl (N-term), oxidation (M); A2) Instrument: Instrument type: Orbitrap; first search peptide tolerance, 20; main search tolerance, 4.5; peptide tolerance unit, ppm; individual peptide mass tolerance, chosen; isotope match tolerance, 2 (ppm); centroid match tolerance, 8 (ppm); centroid half width, 35 (ppm); time valley factor, 1.4; isotope time correlation, 0.6; theoretical isotope correlation, 0.6; recalibration unit, ppm; use MS1 centroids, not chosen; use MS2 centroids, not chosen; intensity dependent calibration, not chosen; min. peak length, 2; max. charge, 7; min. score for recalibration, 70, digestion mode, specific; enzyme, Trypsin/P; max. number of missed cleavages, 2; match type, match from and to; number of threads, 3; max. instrument type, cut peaks, chosen; gap scans, 1; advanced peak splitting, not chosen; intensity threshold 0, intensity determination, value at maximum A3) Label free quantification

(LFQ): LFQ min. ratio count, 2; Fast LFQ, chosen; LFQ min. number of neighbors, 3; LFQ average number of neighbors, 6 A4) Advanced: Max. number of modifications per peptide, 5; min. time, NaN; max. time NaN; additional var. mods for special proteins, not chosen; separate variable modifications for first search, not chosen; separate enzyme for first search, not chosen.

B) Global parameters were chosen as follows: B1) General: a fasta in-house file of combined *Medicago truncatula* and *Rhizobium leguminosarum* protein sequences including common contaminants; fixed modifications, carbamidomethyl (C); re-quantify, not chosen; match between runs, chosen; B2) Sequences: decoy mode, revert; special Aas, KR; include contaminants, chosen; I=L, not chosen; max peptide mass, 4600Da; min. peptide length for unspecific search, 8; max. peptide length for unspecific search, 25; B3) Identification: PSM FDR, 0.01; protein FDR, 0.01; Site decoy fraction, 0.01; min. peptide length, 7; min. peptides, 1; min. razor + unique peptides, 1; min. unique peptides, 0; min. score for unmodified peptides, 0; min. score for modified peptides, 40; min. delta score for unmodified peptides, 0; min. delta score for modified peptides, 6; base FDR calculation of delta score, not chosen; razor protein FDR, chosen; split protein groups by taxonomy ID, not chosen; filter labelled amino acids, not chosen; second peptides, chosen; dependent peptides, not chosen; B4) Protein quantification: min ratio count, 2; peptides for quantification, unique + razor; use only unmodified peptides and selected modifications, chosen; modifications used in protein quantification, acetyl (N-term), oxidation (M); discard unmodified counterpart peptide, chosen; B5) LFQ: separate LFQ in parameter groups, not chosen; stabilize large LFQ ratios, chosen; require MS/MS for LFQ comparisons, chosen; iBAQ, not chosen, advanced site intensities, chosen; B6) MS/MS- Fourier transformation mass spectrometry (FTMS): match tolerance 20 ppm, de novo tolerance 10 ppm, de-isotoping tolerance 7 ppm, top peaks per 100 Da 12, FTMS de-isotoping, higher charges, water loss, ammonia loss, dependent losses all chosen, FTMS recalibration not chosen B7) Advanced: Use normalized ratios for occupancies, advanced ratio estimation chosen, top x mass window (Da) 100.

4.6 Data processing with Perseus

From the MaxQuant output file, LFQ values were used for statistical analysis with the software Perseus (version 1.5.5.3, Tyanova *et al.* 2016). The imported data were reduced by removal of proteins which were only identified based on a peptide including a modification, if the protein group was found to be a protein derived from the reversed part of the decoy database or if the protein was a common contamination. In a next step the dataset was log transformed. The twelve samples groups include three biological repetitions respectively. Next, the data were classified into 'valid' or 'invalid', depending on at least three positive identifications in one group. The resulting dataset was utilized to perform statistical tests: analysis of variation (ANOVA) and two- sample t-test. For both tests the truncation was based on p- values with a threshold of <0.05. The valid values were employed to perform a blast search to link the identified *Medicago truncatula* proteins to *Arabidopsis thaliana*, enabling transfer of Arabidopsis protein localization data on the proteins found in the MS runs.

5. Results

5.1 Plant cultivation and treatment evaluation

Vicia faba plants were grown in foamed clay in short day conditions and harvested after 4 weeks. Plants were either supplied with mineral fertilizer (group N) or infected with Rhizobia capable or incapable of SNF (Fix+ and Fix-, respectively). Control plants (0) were neither fertilized nor infected with Rhizobia (**Figure 1**).

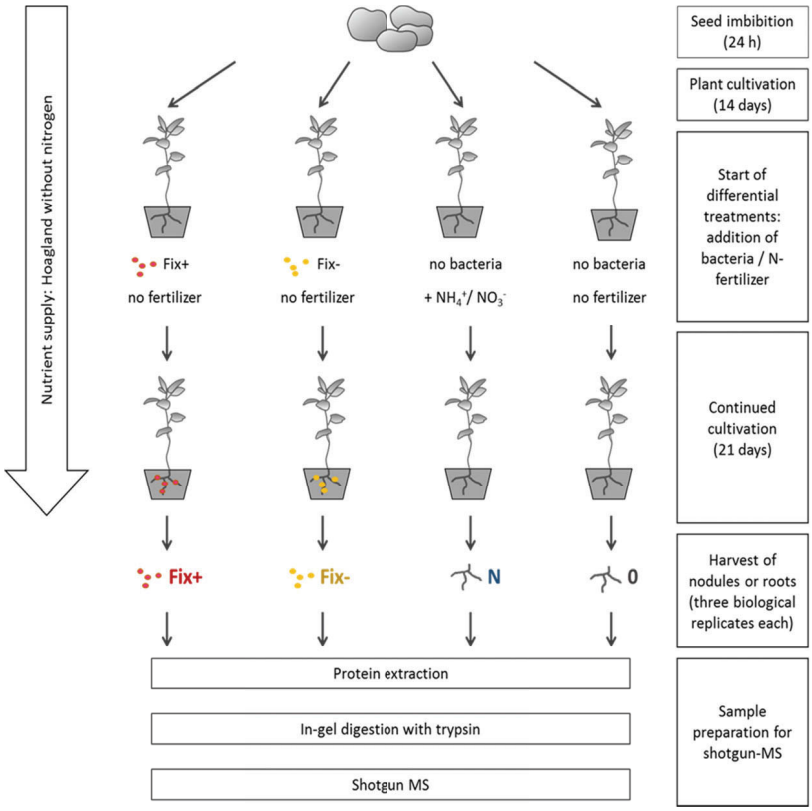

Figure 1:

Experimental setup. *Vicia faba* seeds (genotype Fuego) were divided into four treatment groups: cultivation in the absence of N-fertilizer but in the presence of nitrogen fixing bacteria (Fix+); cultivation in the absence of N-fertilizer but in the presence of bacteria deficient in nitrogen fixation (Fix-); cultivation in the presence of N-fertilizer and absence of bacteria; cultivation in the absence of N-fertilizer and bacteria. All four groups were cultivated for 14 days under standard conditions (see material and methods). Infection with Fix+ or Fix- bacteria (*Rhizobium leguminosarum biovar. Viciae* and *Rhizobium leguminosarum* IMA78, Ma *et al.* 1982*)* or fertilization with nitrogen took place at day 15 and plant cultivation continued for another 13 days during which all four groups were fertilized with 0,5x Hoagland nutrient solution (Hoagland D. R. and Arnon D. I. 1950) without nitrate twice a week and watered with demineralized water according to demand. Subsequently, nodules and roots were harvested, followed by protein extraction and sample preparation for LC-MS/MS analyses.

At harvest time (two weeks of treatment) the nodules were fully developed and plants in all four groups displayed the same growth rate and no phenotypical signs of nitrogen starvation. Fix+ nodules showed a reddish color whereas Fix- nodules as well as N and 0 roots where white and light brown, respectively (**Figure 2A**). If the duration of the four treatments was extended beyond two weeks, phenotypic differences became evident (not shown). We conclude that molecular analysis after two weeks is optimal because secondary effects of the four treatments still are limited.

To evaluate the nitrogen supply to the plant, shoots were harvested, dried and their carbon-to-nitrogen ration (C/N) was determined by dry oxidation at 1500 °C in the presence of excess O_2 employing an element analyzer. The calculated C/N ratio provides information on nitrogen supply to the plants by SNF or mineral fertilization. As expected, plants of group 0 possess the highest C/N ratio, followed by Fix-, Fix+, and N (**Figure 2B**).

These results indicate that plants across the four groups are similar in growth and development at harvest while treatments induced differing C/N ratios. We conclude that the selected experimental set-up is suited for the envisioned proteomic analyses.

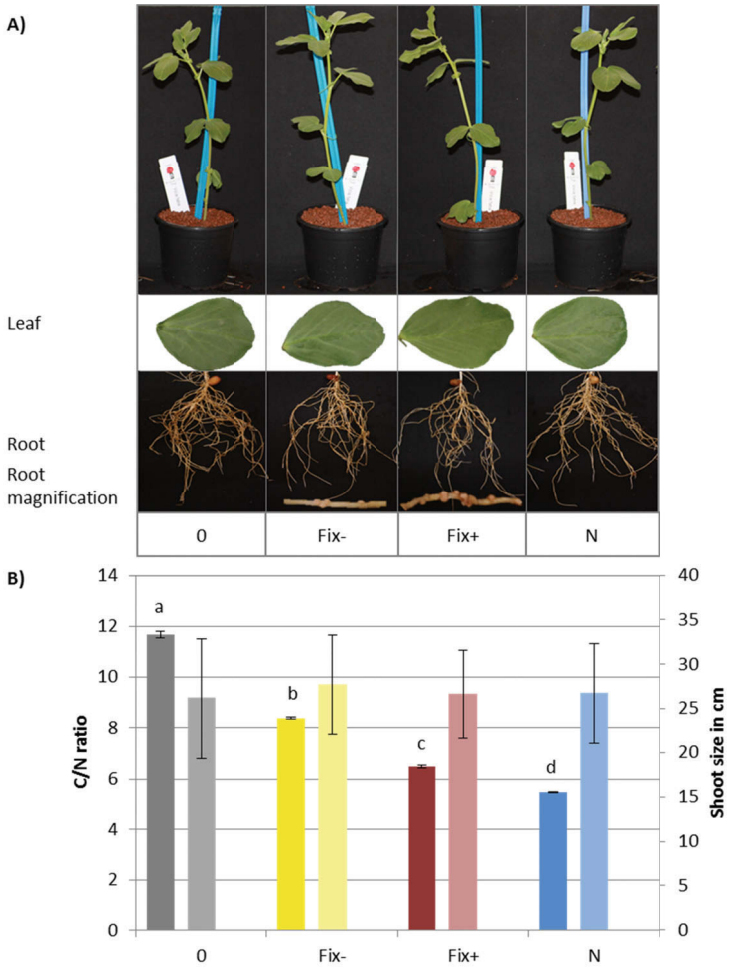

Figure 2:

Phenotypic comparison and nitrogen content of Fix+, Fix-, N, and 0 *Vicia faba* plants. A, shoots (upper panel), individual leaves (third leaf from the bottom, middle panel) and roots (lower panel) with nodules (inserts lower panel) of four weeks old *Vicia faba* plants. Fix+ plants develop red colored nodules, whereas Fix- grows more but smaller nodules of white color. N and 0 are non-nodulating. B, C/N ratio (left bars, axis) and shoot length (right bars, axis) of plants. Error bars of shoot lengths indicate standard deviation of >100 individual plants, error bars of C/N ratios indicate standard deviation of 3 biological replicates (letters indicate $p<0.00001$ in ANOVA).

5.2 Proteomic strategy and evaluation of raw data

Nodules from Fix+ and Fix- were picked by hand and root material from N and 0 plants was cut into pieces approximately 3 cm length before being stored in liquid nitrogen. Frozen samples were homogenized and proteins were subsequently extracted for proteomic analyses. Three biological replicates were prepared for each group. Using the Andromeda search engine implemented in the MaxQuant software (Cox and Mann 2008) and a database of *Medicago truncatula* and *Rhizobium leguminosarum* protein sequences, 2695 protein groups were identified in total and are referred to as 'raw dataset'. The genome of the model plant *Medicago truncatula* is sequenced and well annotated. Due to the close phylogenetic relationship to *Vicia faba* it represents a valuable data background for protein indentifications of *Vicia faba* proteins (Young and Udvardi 2009, Wojciechowski *et al.* 2004).

5.2.1 Principal component analysis (PCA)

To check for intra group and inter group variance a three-dimensional Principal Component Analysis (PCA) was performed on the raw dataset obtained by MaxQuant using the Perseus software (Tyanova *et al.* 2016, **Figure 3**). The analysis revealed tight clustering of the biological replicates within each group but, with the exception of N and 0, a clear separation between the individual groups. The distance of Fix+ and Fix- from N and 0 is expected, given that nodules and roots are different tissues, which also explains the relative close proximity of N and 0. However, the pronounced difference between Fix+ and Fix- is surprising.

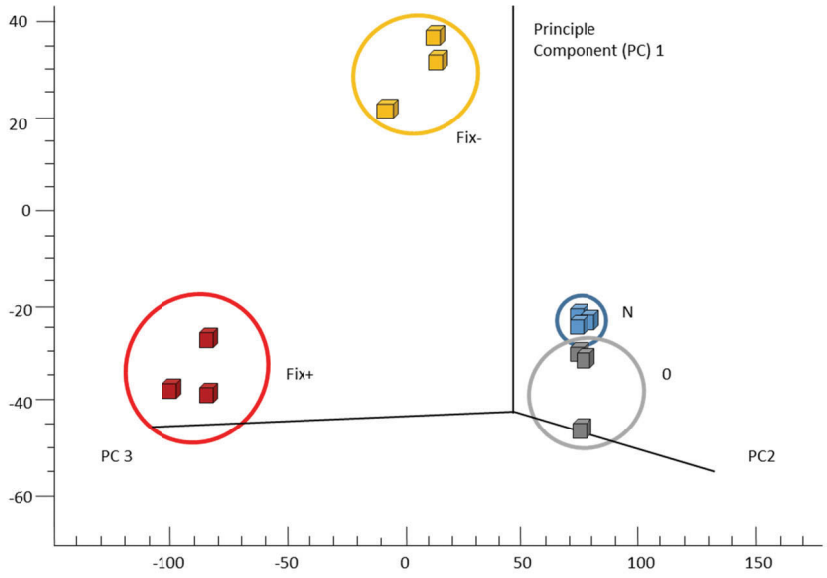

Figure 3:

Three-dimensional Principal Component Analysis (PCA) of shotgun MS datasets of three biological datasets in each of the four sample groups (Fix+, Fix-, N, 0). Abundance profiles (LFQ-values) for each identified protein in the unprocessed data obtained from MaxQuant were analyzed by the Perseus software (Tyanova *et al.* 2016). Cubes are color coded to identify of sample groups: Fix- (yellow), Fix+ (red), N (blue) and 0 (grey), the axes represent the three principal components.

5.2.2 ANOVA

The bulk of the 2695 identified proteins (1982 proteins, 74%) were assigned to *Medicago truncatula* and only a minor part (621 proteins, 23 %) are proteins of rhizobial origin. Ninety-two proteins (3%) are most likely contaminations or were excluded for other reasons (proteins only identified by one modified peptide, are decoy proteins, or common contaminations**, Figure 4**). Next, a filter requiring three positive protein IDs within at least one of the four sample groups (Fix+, Fix-, N, or 0) was applied, reducing the number of valid proteins to 658 and 159 for the plant and bacterial datasets, respectively. Valid plant proteins were further characterized by sample group appearances (Fix+, Fix-, N and 0): 434 proteins (65 %) appear in all four groups, 554 proteins (17 %) are found in min. three and 95% (781) appear in min. to two of the four groups.

Table 1: Valid Proteins broken down by appearance in sample groups

Amount of valid proteins per sample groups	
Min. in all four Groups (Fix+, Fix-, N and 0)	434
Min. in three Groups	554
Min. in two Groups	781
Min. in one Group	817

Proteins differing significantly between the groups, either in abundance or by occurrence, are of interest for further analyses. ANOVA analyses resulted in the identification of 301 and 55 proteins differing significantly in the plant and the *Rhizobium* fractions, respectively. As such, approximately 45% of the total valid plant proteins are altered in response to the cultivation conditions. Their subcellular localization was evaluated by querying their nearest *Arabidopsis* homologs against the SUBA-con database (Hooper *et al.* 2014, **Figure 4**).

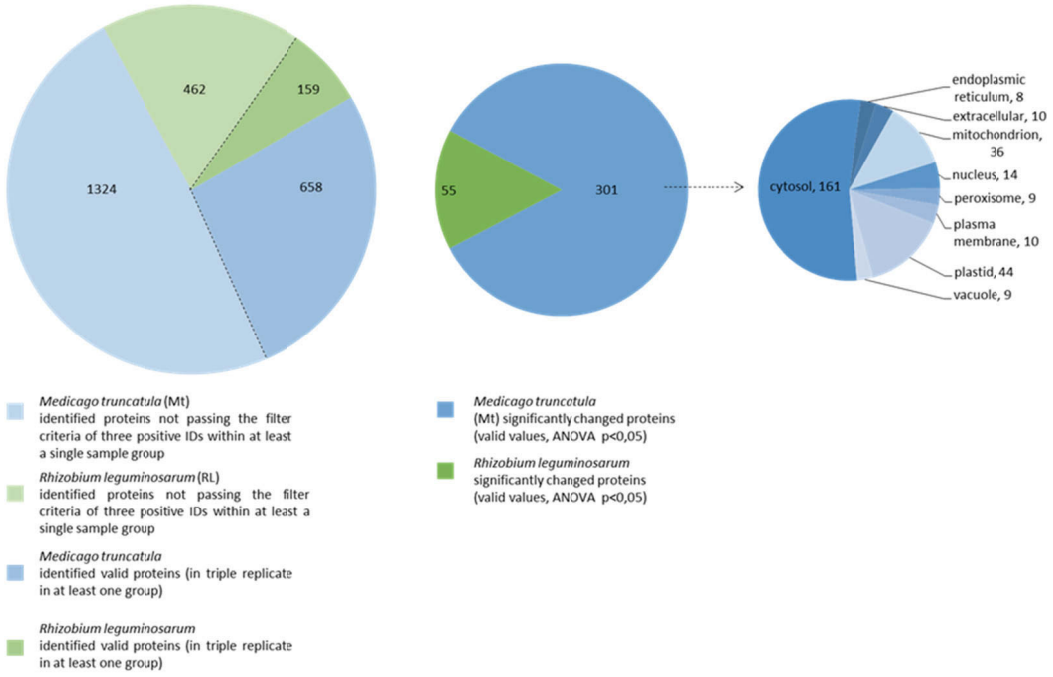

Figure 4:

Total number of identified plant and bacterial proteins within the four sample groups and the number of plant and bacterial proteins with significantly altered abundances and their expected subcellular localizations. A total of 2695 plant and bacterial proteins are identified across the biological replicates of the four sample groups (left diagram). Among them, 92 proteins are only identified by one modified peptide, derived from the reversed part of the decoy database or are common contaminations and are therefore excluded from the analysis. Of the remaining 2603 proteins, 1982 proteins are of plant origin and are highlighted in dark and light blue, whereas the 621 rhizobial proteins are shown in dark and light green. Proteins are considered as valid when they were identified three times in at least one of the sample groups and are shown in dark blue and green. They consist of 658 plant proteins (dark blue) and 159 bacterial proteins (dark green) and amount to 31.4% of all proteins. Among the validated proteins 301 plant and 55 bacterial proteins are identified by an ANOVA analysis to be significantly increased or decreased in abundance in at least one of the four sample groups (middle diagram). The 301 plant proteins were analyzed using the SUBA-con algorithm (Hooper *et al.*, 2014) to reveal their putative subcellular localizations (right diagram).

More than half of the proteins (161) are of cytosolic origin and are mainly involved in protein synthesis and degradation, RNA metabolism and basic sugar metabolism, including glycolysis and UDP-glucose biosynthesis. Furthermore, parts of the pentose phosphate pathway (Ito *et al.* 2011) and several enzymes of amino acid metabolism are also associated with the cytosol (Oehrle *et al.* 2008). Plastid proteins constitute the second largest dataset with 44 proteins involved in N-assimilation, starch biosynthesis and lipid metabolism (Daher *et al.* 2010, Rawsthorne 2002). The third biggest group consists of 36 mitochondrial proteins. The mitochondria produce and export ATP to drive energy dependent biochemical reactions in other cellular compartments, are an important site of amino acid metabolism and are involved in balancing the redox state of the plant cell. They also take part in leghemoglobin synthesis (Dubinin *et al.* 2011) and provide 2-oxoglutarate (2OG), a precursor for ammonia assimilation, to the cell. To a lesser extent, proteins of other subcellular compartments were found, accounting for 2-4% each of the analyzed proteins: endoplasmic reticulum, peroxisome, vacuole, extracellular compartment, plasma membrane, and nucleus. On a quantitative scale, these compartments seem to play a less pronounced role in nitrogen fixation.

5.3 Comparative analyses

Pairwise comparisons between the protein sets of the four sample groups were carried out to distinguish between core SNF enzymes and those involved in establishing and sustaining the Rhizobia:plant symbiosis (**Figure 5**-**Figure 7**; Table 2- Table 4). Meta-comparisons (identification of commonly regulated proteins in two or more pairwise comparisons) complement the pairwise comparisons as these allow identification of nitrogen fixation-specific and symbiosis-specific proteins.

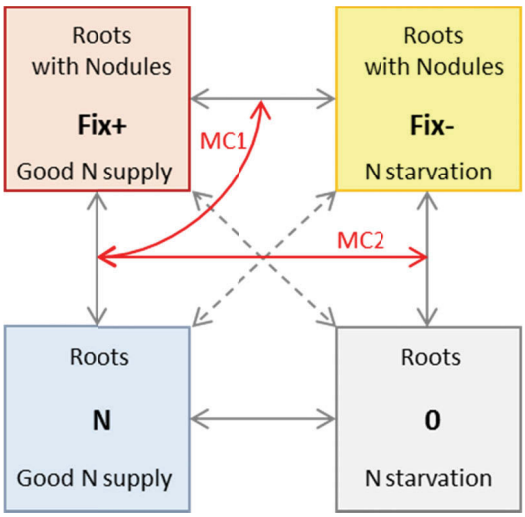

Figure 5:

Pairwise comparisons between the four sample groups and meta-comparisons. The four sample groups differ in two aspects i) nodule formation (true for Fix+ and Fix-, not the case for N and 0) and ii) nitrogen status (high for Fix+ and N, low for Fix- and 0). Six comparisons are conceivable between the four groups (grey full and dashed arrows) but only the full arrows allow comparison of groups differing only in a single aspect (nodulation or nitrogen status). Pairwise comparisons were thus limited to these combinations. Most interesting are the comparisons of Fix+ with Fix- and of Fix+ with N as these allow the identification of proteins specific for nitrogen fixation via a meta-comparison (MC) approach (M1). In a second meta-comparison (M2) the nodule forming groups are compared with N and 0 to further ascertain symbiosis related proteins.

In a first step, proteins with significantly changed abundances in four of the six conceivable comparisons are identified using a t-test (**Figure 6**). Only those proteins with p-values ≤ 0.05 were considered.

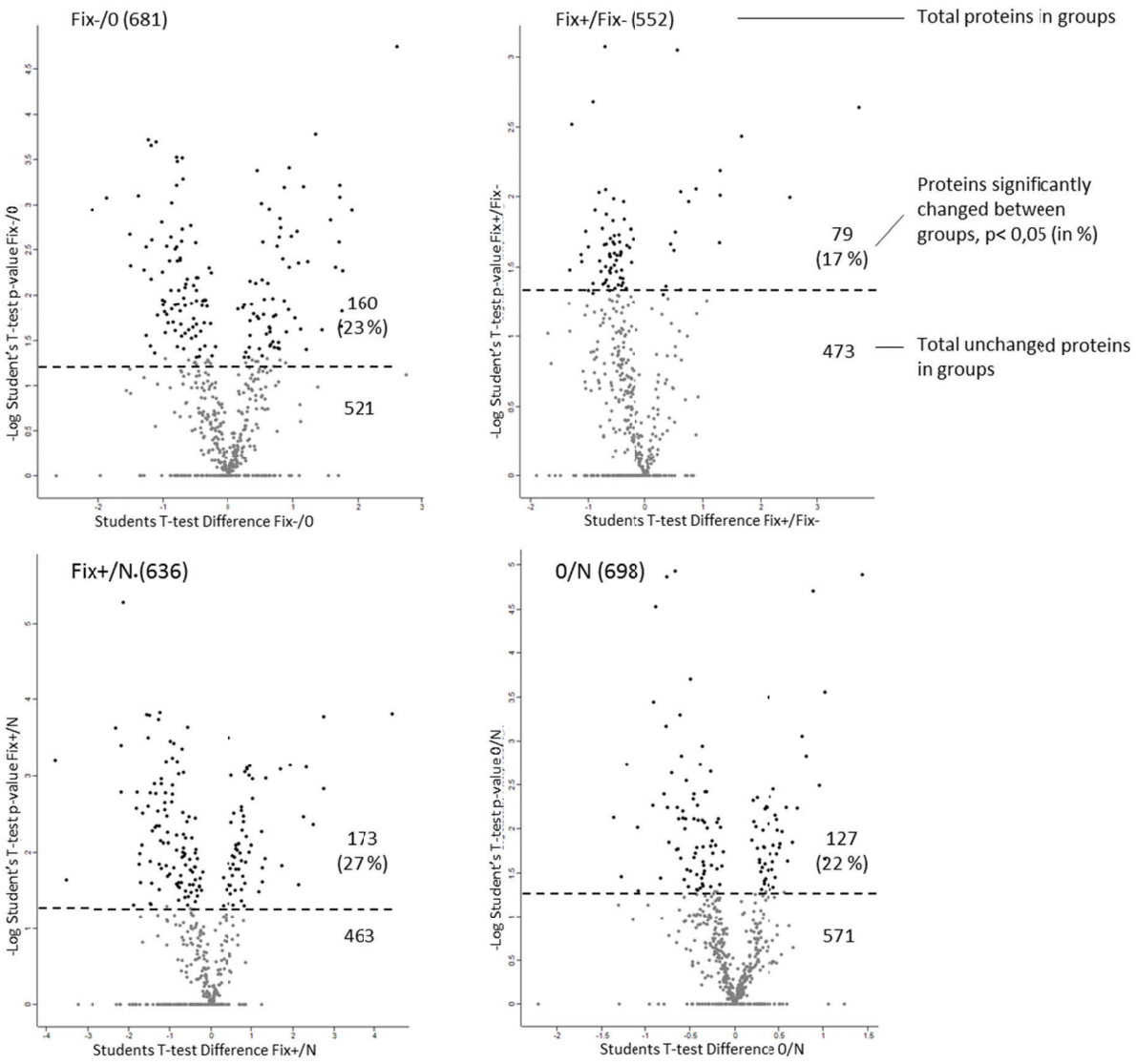

Figure 6:

Volcano plots of pairwise Student's t-test results. Four different combinations were used for pairwise t-tests (Fix-/0; Fix+/Fix-; N/0; N/Fix+). Each diagram contains all valid proteins occurring across the two groups as dots. The y-axis shows the negative decimal logarithm of Students p- value, the x-axis the Student's t-test difference results while the dashed line marks the significance p-value cutoff ($p \leq 0.05$). All proteins above this line are considered as differently abundant proteins. Numbers under the dashed line indicate the total sample size, those above the dashed line tell the number of proteins matching the significance threshold limit. Numbers in brackets indicate the percentage of changed proteins on the sample size. The larger the distance of the dots from the horizontal center of the graph, the bigger is its difference in abundance.

Within each comparison the bulk of proteins (73%-83%) did not differ significantly, suggesting a broadly similar metabolism between nodules and roots, as well as between nitrogen availability and nitrogen deficiency within each tissue type. The most striking changes can be observed between Fix+ and N (173 proteins, 27%) and Fix- and 0 (160 proteins, 23%). Both comparisons document the differences between root and nodule tissue, albeit at different nitrogen availabilities (high in N and Fix+, low in 0 and Fix- , **Figure 2**). When comparing within the same tissue type (Fix+/Fix- and N/0), the numbers of altered proteins were less uniform. Only 79 proteins (17%) changed between Fix+ and Fix- while 127 proteins (22%) were of altered abundance between N and 0 and both of these values are lower than was observed for the cross-tissue comparisons. The top five highest regulated proteins were evaluated separately (**Figure 7**).

5.4 Top-five upregulated and downregulated proteins in pairwise comparisons

To assess the most significant changes in the root and nodule proteomes, the five proteins with the highest (upregulated) and lowest (downregulated) abundance ratios in each of the four pairwise comparisons were summarized in **Figure** 7 (Table 3). Within this set of 40 proteins, 26 accessions are unique to one comparison while seven appear in more than one comparison, thus reducing the set to 33 non-redundant accessions. As expected, enzymes of aspartate and asparagine metabolism are upregulated in Fix+, regardless if compared to Fix- or N, while the regulation of other enzymes is remarkable because not expected beforehand.

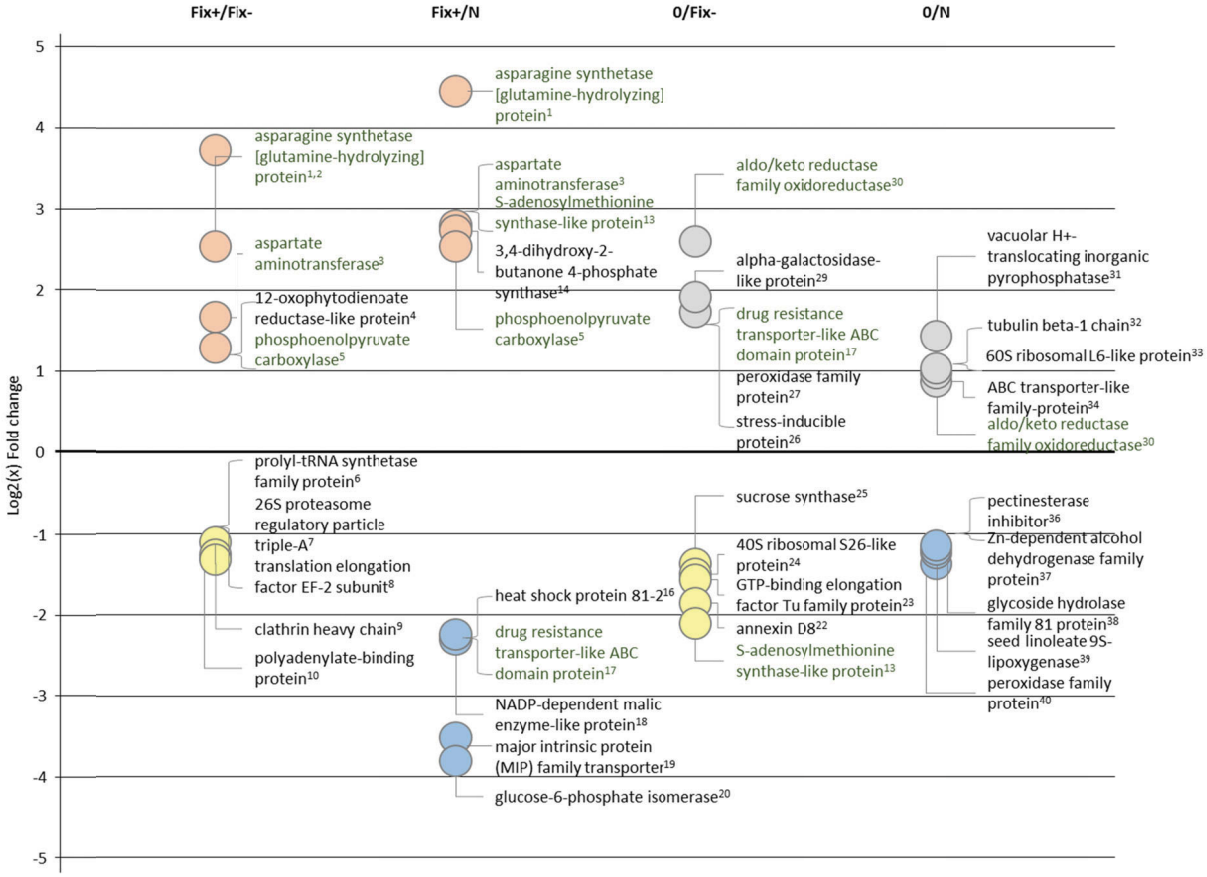

Figure 7:

Top-five upregulated and downregulated proteins. Proteins are presented as circles. The y- axis shows the log2(x) fold change of the proteins from the pairwise t-tests, which are shown on the x- axis. Proteins are labeled and numbered (see supplemental material Table 3). Protein names, which appear in more than one comparison, are highlighted in dark green.

Each of the four comparisons allows following different aspects of SNF. Although it is insufficient to analyze these aspects individually, they enable first insights and provide a valuable starting point for further analyses. Comparing Fix+ and Fix- allows identification of plant proteins involved in fixation of atmospheric nitrogen such as asparagine synthetase [glutamine-hydrolyzing] protein (1, 2) and aspartate aminotransferase (3), both of which are upregulated in Fix+. Phosphoenolpyruvate carboxylase (PEPC, 5) supports nitrogen fixation by converting the glycolytic intermediate PEP into oxaloacetate, a precursor for malate, to feed the bacteroid energy metabolism (among other processes). The enzyme thus links nitrogen fixation to carbon metabolism and respiration (Nomura 2006). The finding that four of the top five proteins upregulated in Fix+ are directly connected to SNF strongly indicates that the 12-oxophytodienoate reductase-like protein (OPR2, 4) is involved in SNF as well.

Proteins upregulated in Fix- may be linked to nodule formation and senescence, since four proteins are involved in protein biosynthesis and protein fate: prolyl-tRNA synthetase family protein (6), 26S proteasome regulatory particle triple-A ATPase protein (7), translation elongation factor EF-2 subunit (8), and polyadenylate-binding protein (10), while the fifth protein (Clathrin heavy chain, CHC, 9) is a central component of clathrin-mediated endocytosis in eukaryotic cells and therefore possibly involved in the uptake of bacteria into the host cells. A CHC1 gene has been found to be highly expressed in Rhizobium-infected root hairs (Wang *et al.* 2015).

The comparison of Fix+ and N reveals differences between nodule and root tissue, both of which being supplied sufficiently with nitrogen. Proteins more abundant in Fix+ contain typical enzymes of SNF (1, 3, and 5). In addition, S-adenosylmethionine synthase-like protein (13), and 3,4-dihydroxy-2-butanone 4-phosphate synthase (DHBP, 14) are upregulated. The S-adenosylmethionine synthase contributes to the biosynthesis of S-adenosylmethionine (AdoMet), while DHBP could be related to the formation of flavines and coenzymes derived from intermediates of the pentose phosphate pathway (Herz *et al.* 2000). Among the higher abundant proteins in N, no typical enzymes of $N0_3^-$ assimilation are found, most likely because the bulk of $N0_3^-$ assimilation is located in leafs (Canvin and Atkins 1974). Interestingly, two proteins related to energy metabolism are found: NADP-dependent malic enzyme-like protein

(18), and glucose-6-phosphate isomerase (20), indicative of an altered energy physiology in Fix+ nodules when compared to N.

The comparison between 0 and Fix- also showcases tissue specific differences, albeit this time under nitrogen deficiency (**Figure 2B**), which could explain the upregulation of stress related proteins in 0, for instance the stress-inducible protein (26) and the peroxidase family protein (27). The other proteins upregulated in 0 (17, 29 & 30) are involved in several unspecific metabolic and transport processes. Fix- contains upregulated proteins involved in protein biosynthesis (GTP-binding elongation factor Tu family protein, 23 and 40S ribosomal S26-like protein, 24). The sucrose synthase (SuSy, 25) hydrolyzes sucrose to provide energy and carbon skeletons for SNF. As observed in Fix+/N, the S-adenosylmethionine synthase-like protein (SAM, 13) is upregulated in the nodule sample. Also upregulated in Fix-is annexin D8 (22).

The last comparison of 0 and N can be used to evaluate the differences induced to root tissue by nitrogen status. In general, alterations in protein abundances are rather low and no coherent picture of the physiological impact of nitrogen deficiency emerges.

A particularly conspicuous aspect is clustering of proteins to assigned cellular functions: sucrose catabolism, starch synthesis/ degradation, sulfur metabolism, ammonium assimilation, cell growth and control mechanisms of nodulation as well as signaling.

5.5 Proteins with altered abundances participate in diverse cellular functions

The reference group of the comparative analyses is Fix+. A broader overview of the physiological differences between nodules capable and incapable of SNF as well as between nodules and roots is achieved by narrowing the study down to two comparisons Fix+/Fix- and Fix+/N. Proteins with significantly altered abundances are grouped according to cellular processes (**Figure 8**).

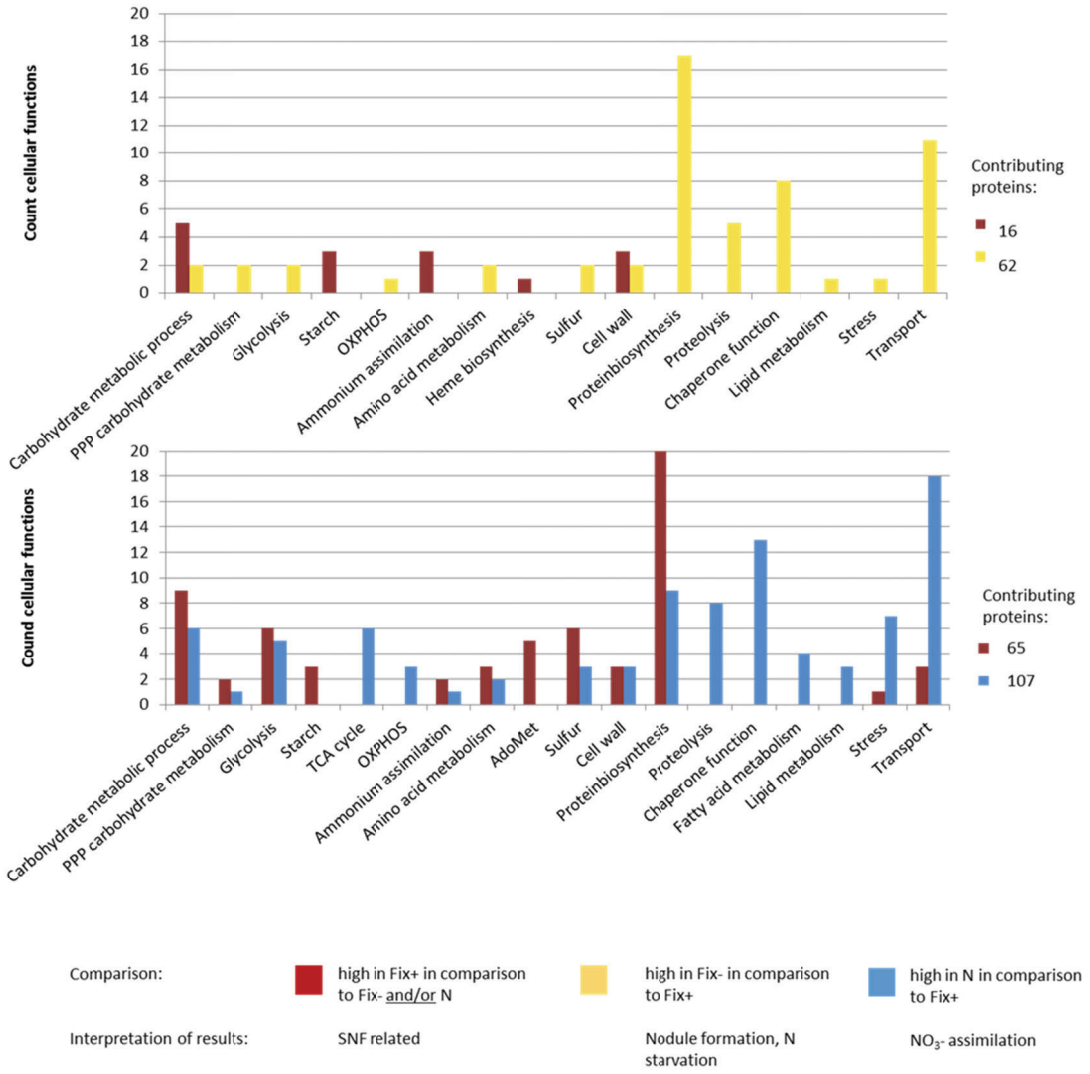

Figure 8:

Altered cellular functions in Fix+ compared to Fix- and N. Bars indicate the number of proteins with changed abundance associated with a cellular function. Upper panel: Fix+ (red bars) opposed to Fix- (yellow bars). Lower panel compares Fix+ with N (blue bars). AdoMet, S-Adenosyl methionine; OXPHOS, oxidative phosphorylation system; TCA, tricarboxylic acid cycle; PPP, pentose phosphate pathway

Proteins involved in metabolic processes involving carbohydrates, starch, ammonium, amino acids, S-adenosyl methionine (AdoMet), and sulfur are often more abundant in Fix+ when compared to Fix- and N. Carbohydrate metabolic process, ammonium assimilation and amino acid metabolism are directly involved in the assimilation of the fixed nitrogen and the provision of energy to the bacteroides. Interestingly, starch and AdoMet related biological processes are exclusively found in Fix+.

The term 'protein biosynthesis' is noticeable more often associated to nodules (Fix+, Fix-) indicating a possible role of this process in the formation of these organs. In Fix- and, even more so in N, proteins participating in proteolysis and chaperone function are more abundant than in Fix+, suggesting a higher protein turnover rate in nodules incapable of SNF and in roots sufficiently supplied with nitrogen fertilizer. In addition, N shows a higher abundance of TCA cycle members and components of the OXPHOS pointing towards an altered mode of mitochondrial energy metabolism in Fix+ plants. Similarly, fatty acid and lipid metabolism seems to be enhanced in N.

5.5.1 Meta comparisons

Based on the pairwise comparisons, meta-comparisons (MC) are helpful in identifying proteins essential to the fixation of atmospheric nitrogen or the maintenance of the symbiosis, respectively, by omitting those members of the proteomes specific for a single comparison. Proteins upregulated in Fix+ compared to Fix- and N (MC1, **Figure 5**) are very likely involved in biological nitrogen fixation. Similarly, proteins upregulated in nodule forming sample groups, when compared to the non-nodule forming groups, are considered necessary for the maintenance of the symbiosis (MC2, **Figure** 5).

In total, eight proteins are always found upregulated in Fix+ in the first meta-comparison (MC1, **Figure 5**, Tab. 2), while 42 proteins were identified in MC2. Interestingly, seven of the eight MC1 derived proteins were also identified in MC2, explained by the similarity between Fix+ and Fix-. Both groups share central processes such as bacteroid insertion and nodule formation. Fix- nitrogenase may be partially defective with residual nitrogen fixing capacity. Five of these proteins (asparagine synthetase [glutamine-hydrolyzing] protein, aspartate aminotransferase,

phosphoenolpyruvate carboxylase, neutral/alkaline invertase, and sucrose synthase) have been related to SNF by previous studies (Küster *et al.* 1997, Farnham *et al.* 1990, Nomura 2006, Gordon *et al.* 1999, Welham *et al.* 2009). The two remaining proteins (UDP-glucuronic acid decarboxylase and glycogen/starch/alpha-glucan phosphorylase family protein) can therefore be expected to be of great importance in SNF as well.

Table 2: Proteins involved in fixation of atmospheric nitrogen and maintenance of symbiosis as identified by meta-comparisons one and two (MC1/MC2). The 43 proteins are listed in alphabetical order with their corresponding majority protein ID and description. The protein abbreviation is given in column 4. The presence of the protein ID in MC1 and/or MC2 is indicated by a '+' in the columns MC1 and MC2. Grey, protein biosynthesis-related; pink, SNF related; yellow, sulfur fixation related; blue, amino acid metabolism; bold, found in MC1 and MC2.

	Majority protein IDs	**Description**	**Name**	**MC1**	**MC2**
1	Medtr7g074570.2;Medtr7g074570.1	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	PGM		+
2	Medtr1g066860.2;Medtr7g100720.1; Medtr1g066860.1;Medtr7g061155.1	40S ribosomal protein S23-1			+
3	Medtr7g107380.1;Medtr1g054310.1	40S ribosomal protein S5-2			+
4	Medtr7g053160.2;Medtr7g053160.1	40S ribosomal protein S6-2			+
5	Medtr5g010025.1;Medtr8g098850.1; Medtr8g098850.2	40S ribosomal S10-like protein			+
6	Medtr5g097200.1;Medtr3g094220.1; Medtr3g094220.2;Medtr5g097200.2	40S ribosomal S26-like protein			+
7	Medtr8g052030.1;Medtr5g018940.2; Medtr5g018940.1	40S ribosomal S4-like protein			+
8	Medtr8g046140.1;Medtr4g080740.1; Medtr3g007700.1	50S ribosomal protein L5P			+
9	Medtr4g059400.1;Medtr1g064500.1	60S ribosomal L12-like protein			+
10	Medtr1g069905.1;Medtr0067s0060.; Medtr7g107420.1	60S ribosomal L21-like protein			+
11	Medtr8g028125.1	60S ribosomal protein L5-2			+
12	Medtr3g093110.1	60S ribosomal protein L6			+
13	Medtr8g023140.1	alanine aminotransferase	ALAT, ALT		+
14	Medtr8g038210.2;Medtr8g038210.1	annexin D8	ANN		+
15	Medtr3g464580.1;Medtr3g464580.2; Medtr3g464580.3	asparagine synthetase [glutamine-hydrolyzing] protein	AS	+	
16	**Medtr1g013050.1**	**aspartate aminotransferase (HC)**	**AST**	**+**	**+**

17	Medtr5g011910.1;Medtr4g102170.1; Medtr4g024550.1;Medtr4g024265.1	cytoplasmic ribosomal protein S13			+
18	Medtr1g043040.1	cytoplasmic-like malate dehydrogenase	MDH		+
19	Medtr4g005880.1	D-3-phosphoglycerate dehydrogenase family protein	PHGDH		+
20	Medtr3g105430.1	eukaryotic translation initiation factor 3 subunit B (HC)			+
21	Medtr7g021870.1	eukaryotic translation initiation factor 3h			+
22	Medtr2g065470.3;Medtr2g065470.1; Medtr2g065470.2	glyceraldehyde-3-phosphate dehydrogenase	GAPDH		+
23	**Medtr0288s0040.1**	**glycogen/starch/alpha-glucan phosphorylase family protein**	**GPase**	**+**	**+**
24	Medtr4g092620.1	LL-diaminopimelate aminotransferase	DAP		+
25	Medtr1g096140.1;Medtr1g095970.1	neutral/alkaline invertase	INV		+
26	Medtr5g006340.4;Medtr5g006340.3; Medtr5g006340.2;Medtr5g006340.1; Medtr1g103690.1	O-acetylserine (thiol) lyase	OAS-TL		+
27	Medtr4g087520.1	O-acetylserine (thiol) lyase	OAS-TL		+
28	Medtr2g098950.1	pfkB family carbohydrate kinase			+
29	**Medtr2g076670.3;Medtr2g076670.2; Medtr2g076670.1**	**phosphoenolpyruvate carboxylase**	**PEPC**	**+**	**+**
30	**Medtr4g079860.1**	**phosphoenolpyruvate carboxylase**	**PEPC**	**+**	**+**
31	Medtr7g103620.1;Medtr7g103620.2	phosphopyruvate hydratase	Eno		+
32	Medtr3g088220.1	protein disulfide isomerase			+
33	Medtr2g073240.1	rhamnose biosynthetic-like enzyme	RH		+
34	Medtr2g086500.2;Medtr2g086500.1; Medtr3g070930.1	ribosomal protein L1p/L10e family protein			+

35	Medtr4g117470.1	ribosomal protein S8			+
36	Medtr3g084340.1	S-adenosyl-L-homocysteine hydrolase	AdoHcyH		+
37	Medtr2g046710.1	S-adenosylmethionine synthase-like protein	SAM		+
38	Medtr7g110310.1	S-adenosylmethionine synthase-like protein	SAM		+
39	**Medtr2g044070.2;Medtr2g044070.1**	**sucrose synthase**	**SuSy**	**+**	**+**
40	Medtr4g124660.4;Medtr4g124660.2; Medtr4g124660.1;Medtr4g124660.3	sucrose synthase	**SuSy**		+
41	Medtr4g077190.1	sulfite reductase [ferredoxin] protein			+
42	Medtr3g110720.1;Medtr1g106005.1; Medtr4g097830.1	tubulin beta-1 chain			+
43	**Medtr4g063600.2;Medtr4g063600.4; Medtr4g063600.3;Medtr4g063600.1**	**UDP-glucuronic acid decarboxylase**		**+**	**+**

In contrast to the other seven MC1 proteins, asparagine synthetase is not found in MC2 pointing towards an upregulation of this protein in response to SNF activity. Among the residual 35 proteins in MC2 17 (40%) are involved in protein biosynthesis (grey background in Table 2). As inderterminate nodules are growing tissue, the presence of protein biosynthesis related proteins is well explainable. Fourteen proteins (32%) participate in carbohydrate metabolism and are most likely involved in redirecting carbon flow within infected cells. However, six proteins (14%) are related to sulfur metabolism (OAS-TL, 26, 27; SAM, 36, 37, 38; sulfite reductase, 42), therefore suggesting a correlation between nodule metabolism and sulfur fixation. Indeed, such a link was reported recently (Kalloniati *et al.* 2015). Four proteins are engaged in amino acid metabolism, two of which have been reported to be involved in SNF. Aspartate aminotransferase was found in both, MC1 and MC2, while asparagine synthase was only present in MC1. Alanine aminotransferase was found in MC2 exclusively (just above the threshold in MC1, p=0.065), indicative of a function in symbiosis and nitrogen fixation.

6. Discussion

6.1 Experimental setup

The complexity of SNF is derived from its presence within specialized plant organs, the nodules, and the cooperation with symbiotic bacteria. Profound changes in cellular metabolism accompany the formation of the nodules as well as their maintenance, redirecting cellular metabolism to support the bacteria and nitrogen assimilation. A vast number of proteins have already been associated with SNF. However, it is often not clear, if proteins are directly involved in fixation of atmospheric nitrogen or if their altered abundancies are related to nodulation. Research has mainly focused on the comparison of either two different tissues (roots and nodules) or nitrogen states (sufficient/insufficient) and is thus limited in discriminating between SNF and nodulation related proteins. The pairwise comparisons and the derived meta analyses within the set of four sample groups, as presented in this study, provides the means to assign altered protein abundances to either process more precisely.

Next to amino acid metabolism also sucrose catabolism, starch synthesis and degradations as well as sulfur metabolism were discovered to represent the cornerstones of nodule metabolism and will be discussed in more detail in the following.

6.1.1 Ammonium assimilation in *Vicia faba: rhizobium leguminosarum* symbioses

Assimilation of ammonium derived from nitrate is performed by the plant enzymes glutamine synthetase (GS), glutamate synthase (GOGAT), and aspartate amino transferase. While this pathway is mostly performed in leaf chloroplasts, a minor pathway is also located in root plastids (Lancien *et al.* 2000).

Despite of evidence supporting the presence of this pathway also in root nodules, many issues are still unresolved. It is currently not clear in which form (NH_3, NH_4^+ or amino acids) the fixed nitrogen is exported from the bacteroides (Patriarca *et al.* 2002). *R. leguminosarum* excretes both alanine and NH_4^+ in differing ratios (Poole and Allaway 2000). Similarly, alanine was reported to be the main exported product of the bacteroides from pea (Waters *et al.* 1998), but this result was directly challenged by the finding that NH_3/NH_4^+ are the major products of determinated soybean bacteroid SNF (Li *et al.* 2002). Previous models were mainly based on studies performed on *ex planta* bacteroides which bear the risk of not properly representing the

in vivo situation. We here present an extended model on ammonium assimilation in *Vicia faba*. According to our proteome data, both alanine and ammonium might be exported from the bacteroides. Aspartate aminotransferase (AspAT) as well as asparagine synthase (AS) are upregulated in Fix+, which is in concordance with the finding that the indeterminate nodules mostly export asparagine into the xylem sap (Peiter *et al.* 2004). GOGAT was not found in the meta-comparisons thus indicating that symbiotic nitrogen fixation follows a different pathway to that of nitrate-derived nitrogen fixation. Aspartate synthesis from oxaloacetate requires glutamate. Given the absence of GOGAT from the meta-comparisons, glutamate must be produced from a different source. Asparagine synthase transaminates aspartate by consuming glutamine, thus producing glutamate. This glutamate can then be used for aspartate production. However, glutamine production depends on glutamate. In the absence of increased abundances of glutamate dehydrogenase in the meta-comparisons (or in Fix+ in general), this raises the question how the large amounts of glutamate necessary to drive asparagine production are produced in nodules. Evaluation of the dataset suggests alanine as an alternative source of glutamate. A bacterial alanine dehydrogenase (AD) is exclusively present in the Fix+ bacteria dataset producing alanine from pyruvate and ammonia. The important role of bacterial alanine production has been shown previously, but could not be sufficiently explained. Allaway *et al.* (2000) utilized a mutant strain defective in alanine catabolism of *Rhizobium leguminosarum* for the infection of pea. Infected plants grew more slowly than plants nodulated by the wild-type. Alanine is expected to be transported from the bacteroids into the cytosol of the plant cell (Day *et al.* 2001). The production of glutamate from alanine is established by an alanine aminotransferase (AlaAT) identified in the meta comparison analysis. AlaAT catalyzes the reversible transfer of an amino group from alanine onto 2-oxoglutarate to form glutamate and pyruvate. The identified AlaAT shows strong homology to ALAAT2 from *Arabidopsis thaliana*, which is predicted to be localized in the mitochondria (Liepman and Olsen 2003). Pyruvate produced by AlaAT could be used for the TCA cycle, while glutamate could then be exported from the mitochondria as reaction partner of AspAT or GS (**Figure 9**). Taken together we propose a pathway of nitrogen fixation in *Vicia faba* which requires ammonia and alanine to be exported from bacteroids in equal amounts, thus unifying previously conflicting results in a single model. However, the question remains why AlaAT, a central enzyme within this model

has not been detected in MC1 but only in MC2. LL-diaminopimelat aminotransferase, an enzyme involved in lysine production from aspartate, shares this feature with AlaAT and has unknown functions in nodule metabolism.

6.1.2 Allocation of sucrose into starch synthesis and glycolysis in nodules

Roots and nodules are heterotrophic tissues and rely on the provision of carbohydrates from photosynthesis transported by the phloem, mostly in the form of sucrose. In the sink cells sucrose is either used to fuel glycolysis or is turned into starch. Starch accumulation in nodules was observed in *Vicia faba* (Fred *et al.* 2002). In *Phaseolus vulgaris* starch is mainly present in uninfected cells of the nodule (Tate *et al.* 1994) and is remobilized when the shoot does not deliver sucrose to the root (Vance and Heichel 1991). The activity of starch-bound starch synthase in 'fix+' nodules declines over time in soybean nodules and a positive correlation between N_2 fixation and starch degradation in nodules was reported. Concordantly, starch accumulates in young soybean nodules and is gradually metabolized during the progression of symbiosis, maybe to fulfil the energy demand of the bacteroides (Forrest *et al.* 1991). In contrast, it was also shown in soybean that plastids are emptied of starch granules not until nodule senescence (Puppo *et al.* 2005).

Our results provide additional information on the concept of starch utilization in nodules, which is yet not exhaustively answered. Starch related processes appear to be especially important in Fix+. This is indicated by the finding of starch related enzymes upregulated in Fix+ (**Figure** 8). However, starch metabolism is also altered in Fix- as it can be seen in the example of the granule bound starch synthase (Medtr8g024340.2) which was identified in Fix+ and Fix- (but not in N and 0). Likewise, the meta-comparisons contain proteins involved in starch synthesis and degradation, comprising a neutral/alkaline invertase (INV) as well as a glycogen/starch/alpha-glucan phosphorylase family protein (GPase). In summary, the findings imply an important role for starch in SNF, one which is yet to be specified in more detail. As nitrogen fixation follows a 24-h rhythm it is possible that stored starch in nodules is degraded to support SNF and bacteroid metabolism in the dark in the same fashion it is used to drive respiration during the night (Wheeler 1969, Weise *et al.* 2011).

Besides starch synthesis, sucrose is fed into glycolysis and other pathways branching off at this substrate. Sucrose synthase (SuSy) is highly expressed in nodules. Due to low fructose concentrations it is expected to operate predominantly in the cleaving direction (Morell and Copeland 1985). SuSy is rated among the essential enzymes for SNF (Gordon *et al.* 1999). Our results are therefore in concordance with previous results, hence SuSy is of increased abundance in Fix+ and Fix- in the dataset. Furthermore, in many plant species SuSy is present in several isoforms which is expected to channel sucrose into different sink pathways such as starch synthesis or cell wall formation (Barratt *et al.* 2001).

Another protein presumably involved both in nodule growth as well as carbon partitioning is UDP glucoronic acid decarboxylase (UXS) since it is among the common seven proteins of the two meta-analyses. The enzyme catalyzes the conversion of UDP-glucoronic acid to UDP-xylose. The latter is required for the synthesis of plant cell wall polysaccharides such as xyloglucans and plant glycoproteins. Another upregulated protein in Fix+ and Fix- is the rhamnose biosynthetic-like enzyme (Medtr2g073240.1, RH). It is as well involved in cell wall biosynthesis. The actively growing indeterminate nodules of Vicia have an increased need for newly synthesized cell walls. Lignin, a cell wall component, is a metabolic sink for S-adenosylmethionine (Shen *et al.* 2002). Correspondingly, other enzymes related to lignin biosynthesis were found. One of them, a caffeic acid O-methyltransferase (Medtr4g038440.2), was also nodule specific (Fix+ and Fix-).

The glycolytic pathway is the primary route leading from sucrose to pyruvate but several other pathways are branching off. The meta-analysis detected several enzymes of glycolysis (GAPDH, Medtr2g065470.3;Medtr2g065470.1, glyceraldehyde-3-phosphate dehydrogenase; PGM, Medtr7g074570.2;Medtr7g074570.1, 2,3-bisphosphoglycerate-independent phosphoglycerate mutase and ENO, Medtr7g103620.1;Medtr7g103620.2, phosphopyruvate hydratase. All of them are high abundant in Fix+ and Fix-. The GAPDH product 1,3-bisphosphoglyceric acid mediates the oxygen release from the heme group of hemoglobin in blood (Stefansson *et al.* 2016), a similar function in relation to leghemoglobin in nodules can be hypothesized. 3-phosphoglycerateserves as a precursor for serine production, which seems connected to SNF. Another glycolysis intermediate is 2-phosphoglycerate, which is converted by phosphopyruvate hydratase to phosphoenolpyruvate. Phosphoenolpyruvate (PEP) may then be metabolized to oxaloacetate by phosphoenolpyruvate carboxylase (PEPC) or to pyruvate by pyruvate kinase.

PEPC was found to be positively correlated with nitrogenase activity (Nomura 2006) and is featured in both meta-analyses. It is therefore conceivable that the glycolytic pathway in root nodules is drained to a large extend before completion with potentially negative effects for downstream metabolic pathways, i.e. the TCA cycle and respiration. Concordantly, plants in the sample group N possess higher amounts of TCA cycle enzymes and OXPHOS subunits than Fix+.

6.1.3 Sulfur and sulfur-related metabolism

As reported by Kalloniati *et al.* (2015) nitrogen fixing nodules are an important source of reduced sulfur. This correlates with the frequent occurrence of enzymes involved in sulfur metabolism in the meta-analyses. Among these proteins the increased amount of D-3-phosphoglycerate dehydrogenase (PHGDH, Medtr4g005880.1) is striking since it is controlling the biosynthesis of serine, which in turn, is a precursor for glycine, tryptophan and cysteine, the first organic form of reduced sulfur. Cysteine is formed from O-acetyl-L-serine and hydrogen sulfide (provided by sulfite dehydrogenase) by the O-acetylserine (thiol) lyase (OAS-TL) of which three isoforms are more abundant in Fix+ and also identified in the meta comparisons. Kalloniati *et al.* (2015) measured gene expression and metabolites and proposed the incorporation of sulfate into glutathione. Our study provides no evidence for elevated levels of enzymes for glutathione synthesis. The enzymes of glutathione biosynthesis were either found equally expressed in all four plant groups (glutamate-cysteine ligase B, Medtr8g098350.1) or did not occur in the dataset (glutathione synthetase). We therefore suggest the conversion of serine, with cysteine and methionine as intermediates, into S-adenosylmethionine (AdoMet) which is used as a general donor for methyl groups. AdoMet is synthesized by S-adenosylmethionine synthase (SAM), which was found twice in the meta-comparisons. AdoMet related proteins are general feature of the proteome of Fix+ plants (**Figure 6**, **Figure 8**). SAM is a precursor for a diverse group of metabolites including cyclopropyl fatty acids, biotin, tRNA, lignin, ethylene, and polyamines (PA) (Fontecave *et al.* 2004, Ravanel *et al.* 1998). The methyl transferases involved in these processes also produce S-Adenosyl-L-homocysteine (AdoHcy) which can be regenerated to AdoMet by the action AdoHcy-Hydrolase (AdoHcyH), found in MC2 (Tab. 2). Together, this suggests an important role of sulfur metabolism in root nodules aimed at the production of AdoMet, but the downstream utilization of this compound is unclear.

6.1.4 Control mechanisms of nodulation, signaling

To avoid unnecessary energy losses nodule growth (and activity) is tightly controlled. Instead of maturing, the development of nascent Fix- nodules is stopped and the formation of new nodules is initiated, resulting in numerous, small and white nodules (**Figure 2A**). The various processes of nodulation control are complex and partly unknown.

Two proteins potentially involved in this process were identified in the meta comparisons: Annexin D8 (MtAnn8) and 12-oxophytodienoate reductase-like protein (OPR). Transcripts of an annexin (Mtann1) are upregulated in root tissues in response to rhizobial Nod factors (Carvalho-Niebel *et al.* 2002). An analogous protein from Medicago, MtAnn8, was upregulated in nodules in comparison to roots and is hypothesized to play a role in nodule growth and development (Marx *et al.* 2016). The appearance of annexin D8 in the present data set supports this suggestion.

Another regulatory function is linked to phytohormones. OPR appears in the Top five comparison Fix+/Fix- and in MC1 (**Figure 7**, Tab. 2). The protein is most likely involved in the conversion of linolenic acid to jasmonic acid (Li *et al.* 2009), as a response to pathogen attack or infection by rhizobial bacteria (van Wees *et al.* 2008). Jasmonic acid negatively affects nodulation and transcriptome studies showed a high induction of jasmonate biosynthesis related genes, such as allene oxide cyclase and 12-oxophytodienoate reductase in early infection stages (Sun *et al.* 2006, Kouchi 2004).

As described in the section concerning sulfur and sulfur-related metabolism (6.1.3) the metabolic fate of AdoMet is unclear. However, polyamine (spermidine, spermin) synthesis requires AdoMet and polyamines accumulate in *Lotus japanicus* nodules, providing a possible explanation for the increased AdoMet production in Fix+ and Fix- (Flemetakis *et al.* 2004). Additionally, spermidine and spermin have also been associated with autoregulation, a mechanism controlling nodule number in legumes (Terakado *et al.* 2006).

6.1.5 Summary: Metabolic pathways new scheme

In summary, the proteomic comparisons performed during the course of study largely confirm results from previous studies but also extends them and provides new angles for further targeted analyses of nodule physiology. Furthermore, it allows to differentiate between effects imposed by the fixation of atmospheric nitrogen fixation and those which are a consequence of the bacteria:plant symbiosis (**Figure 9**).

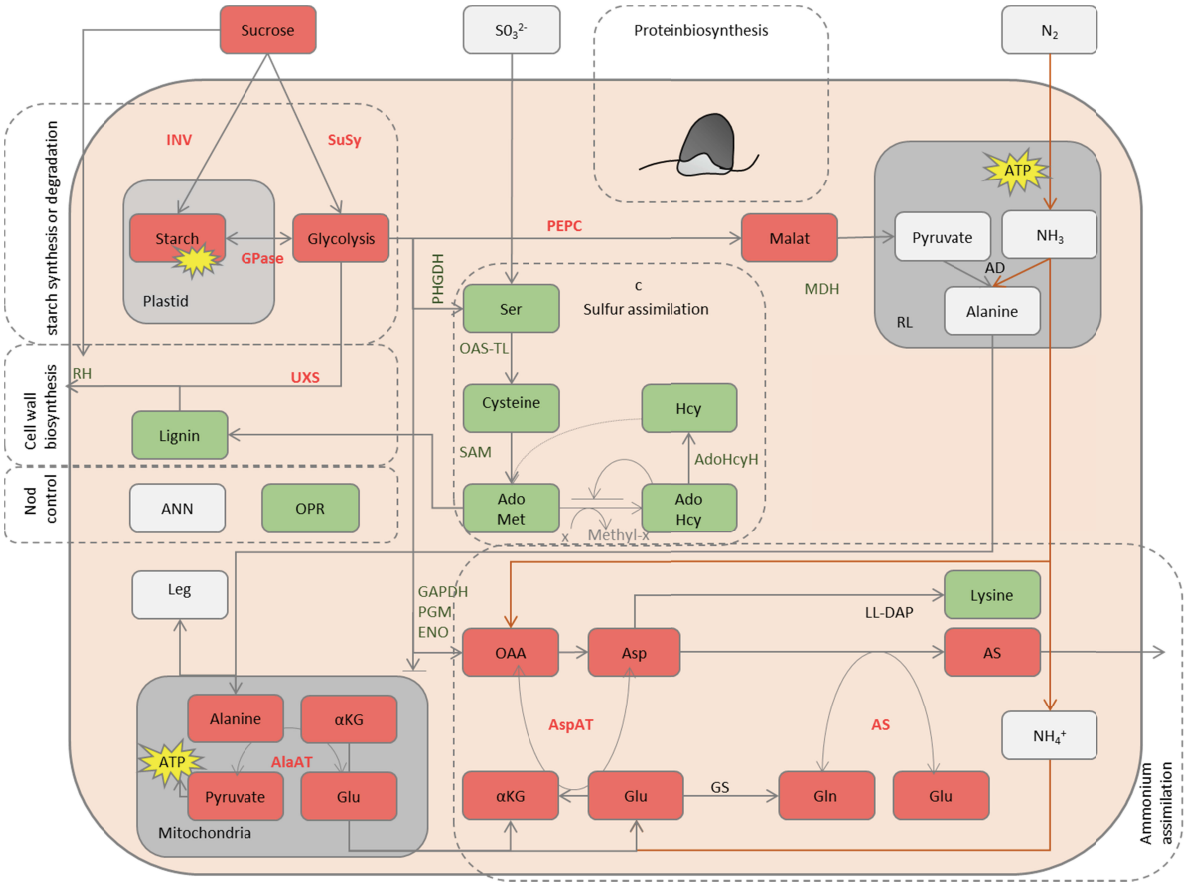

Figure 9:

Key metabolic processes in a Fix+ root nodule cell. For the sake of simplicity and a lack of spatial resolution, all physiological changes are depicted in an infected cell which not necessarily reflects the *in vivo* situation where alterations may also/exclusively affect non-infected, neighboring cells. Metabolites and enzymes induced by the fixation of atmospheric nitrogen fixation are colored in red and those which are a consequence of the bacteria:plant symbiosis are highlighted in green. Sucrose is imported and cleaved by INV or SuSy. INV is the starting point for the ATP (yellow star) dependent starch formation. Starch can be stored in the plastid and may be used at a later time point for glycolysis. Originating from SuSy, glucose forms cell wall components via UXS or precursors for malate production (via oxaloacetate, OAA) by glycolysis. OAA is also a precursor for aspartate. The bacteroides fix the atmospheric nitrogen (N_2) and release, in turn for malate, NH_4^+ (ammonium cation) and alanine into the cell. Ammonium (NH_4^+) is then incorporated into aspartate to form asparagine by asparagine synthase (AS), the transport form of reduced nitrogen. Alanine may be converted to pyruvate and glutamate by alanine aminotransferase (AlaAT) in the mitochondria. Serine metabolism also seems important in nodules. Reduced sulfur is assimilated by serine to form methionine and, subsequently, AdoMet. AdoMet is a precursor for several components such as lignin and polyamines. OPR, as well as ANN might be related to nodulation control mechanisms. INV, neutral/alkaline invertase; SuSy, sucrose synthase; UXS, UDP-glucuronic acid decarboxylase; PEPC, phosphoenolpyruvate carboxylase; DHBP, 3,4-dihydroxy-2-butanone 4-phosphate synthase; RL, *Rhizobium leguminosarum*; AdoMet, S-adenosylmethionine; SAM, S-adenosylmethionine synthase; ANN, annexin D8; OPR, 12-oxophytodienoate reductase-like protein; Leg, leghemoglobin; OAA, oxalacetate; Glu, glutamat; Asp, asparagin; AS, aspartat; AST, aspartate aminotransferase, AlaAT, alanine aminotransferase

6.1.6 Nodule cell organelles

Cell organelles have specific functions in the cell. The reduction of the sample complexity by sub fractionation, for example, on organelle level could increase the amount of identified proteins and it might be possible to get advanced insight into the nodule metabolism. Captivatingly, also bacteroides can be considered as organelle- like structures, albeit not as permanent ones. The analysis of the subcellular localization of proteins with altered abundances showed an over-representation of the cytosol, as well as plastids and mitochondria. The role of the plastids is hypothesized to be related to starch synthesis, whereas the function of mitochondria remains cloudy in regard to SNF and should be further studied. Mitochondria in infected cells are located in the periphery of the cell and are rich in cristae. Furthermore, four proteins are restricted to nodule mitochondria, which further highlights their impact on SNF (Šamaj and Thelen 2007). Two of these emerged in the present dataset (phosphoserine aminotransferase and coproporphyrinogen III oxidase). Phosphoserine aminotransferase is related to serine metabolism (Reynolds *et al.* 1988) whereas coproporphyrinogen III oxidase is part of the heme biosynthetic pathway (Madsen *et al.* 1993). The hypothesized function of the alanine aminotransferase is taking place in the mitochondria, thus supplying TCA cycle intermediates for ATP production. Furthermore, α-ketoglutarate is needed for ammonium assimilation, which might also be provided by mitochondria (Lancien *et al.* 2000). The key role of mitochondria in plants is hence extended to SNF hosting cells and the organelles are a vital issue for future research.

6.2 Outlook

Understanding SNF in detail is of high value for agricultural applications since it promotes improvements in legume crops directly. Future perspectives to harness the benefits of SNF in non-legume crop plants is directly linked to the identification of the enzymatic infrastructure supporting the nitrogenase function and the factors enabling and regulating the Rhizobia symbiosis, which may foster the establishment of legume SNF to non-legume plants. Several important aspects of nodule metabolism besides amino acid metabolism have been highlighted in this study: allocation of sucrose into starch synthesis and glycolysis, sulfur metabolism and related pathways, and the control of nodulation. Proteomic studies struggle with delivering hard evidence for the occurrence or absence of metabolic pathways. Instead, they excel in generating interesting candidate proteins for further, more targeted research. The strong participation of plastids in SNF turned out to be not related to classic nitrogen fixation via GOGAT and must therefore be based on other processes. Likewise, the role of mitochondria was also found to be unexpected. Focusing on the topics and the cellular compartments outlined above, the results produced in this manuscript now need to be validated by further studies employing the full suite of biochemical and genetic tools available today.

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9. Supplemental Tables

9.1 Numbers from top 5 Protein list

Table 3: Top 5 proteins derived from pairwise comparisons of Fix+/Fix-, Fix+/N, Fix-/0, and N/0. The proteins are listed in correspondence to **Figure 7** with their corresponding majority protein ID and description. The matching Arabidopsis genes were determined using the blast algorithm (column 4).

Nr. in Graph	Majority protein IDs	Description	Arabidopsis Gene
1	Medtr3g464580.1;Medtr3g464580.2;Medtr3g464580.3	asparagine synthetase [glutamine-hydrolyzing] protein	AT3G47340.1
2	Medtr5g071360.1;Medtr5g071360.2	asparagine synthetase [glutamine-hydrolyzing] protein	AT3G47340.1
3	Medtr1g013050.1	aspartate aminotransferase	AT4G31990.3
4	Medtr5g006780.1;Medtr5g006800.1	12-oxophytodienoate reductase-like protein	AT1G76680.2
5	Medtr4g079860.1	phosphoenolpyruvate carboxylase	AT2G42600.1
6	Medtr7g110660.2;Medtr7g110660.1	prolyl-tRNA synthetase family protein	AT3G62120.1
7	Medtr3g112260.1;Medtr3g117230.1	26S proteasome regulatory particle triple-A ATPase protein	AT1G45000.2
8	Medtr1g048000.1	translation elongation factor EF-2 subunit	AT1G56070.1
9	Medtr5g082900.1;Medtr5g082900.2	clathrin heavy chain	AT3G11130.1
10	Medtr1g019150.1	polyadenylate-binding protein	AT2G23350.1
13	Medtr7g110310.1	S-adenosylmethionine synthase-like protein	AT4G01850.2
14	Medtr2g009270.1	3,4-dihydroxy-2-butanone 4-phosphate synthase	AT5G64300.1
16	Medtr6g452990.1	heat shock protein 81-2	AT5G56010.1
17	Medtr8g014360.2;Medtr8g014360.1	drug resistance transporter-like ABC domain protein	AT1G59870.1
18	Medtr8g102620.1	NADP-dependent malic enzyme-like protein	AT1G79750.1
19	Medtr4g059390.2;Medtr4g059390.1	major intrinsic protein (MIP) family transporter	AT3G54820.1
20	Medtr5g065880.1	glucose-6-phosphate isomerase	AT4G24620.1
22	Medtr8g038210.2;Medtr8g038210.1	annexin D8	AT1G35720.1
23	Medtr1g024175.1;Medtr8g008740.1	GTP-binding elongation factor Tu family protein	AT1G07920.1
24	Medtr5g097200.1;Medtr3g094220.1;Medtr3g094220.2;Medtr5g097200.2	40S ribosomal S26-like protein	AT3G56340.1
25	Medtr2g044070.2;Medtr2g044070.1	sucrose synthase	AT1G73370.2
26	Medtr5g012030.1	stress-inducible protein, putative	AT1G62740.1

27	Medtr4g046713.1	peroxidase family protein	AT3G01190.1
29	Medtr3g094160.1	alpha-galactosidase-like protein	AT5G08370.2
30	Medtr7g021820.1;Medtr7g021680.1	aldo/keto reductase family oxidoreductase	AT1G60750.1
31	Medtr4g115970.1;Medtr4g115970.2	vacuolar H+-translocating inorganic pyrophosphatase	AT1G15690.1
32	Medtr3g110720.1;Medtr1g106005.1;Medtr 4g097830.1	tubulin beta-1 chain	AT4G14960.1
33	Medtr8g091910.1	60S ribosomal L6-like protein	AT1G18540.1
34	Medtr8g040620.1;Medtr8g016000.1	ABC transporter-like family-protein	AT3G21250.2
36	Medtr7g050870.1	pectinesterase/pectinesterase inhibitor	AT5G53370.1
37	Medtr8g095220.1	Zn-dependent alcohol dehydrogenase family protein	AT5G63620.1
38	Medtr6g016820.1	glycoside hydrolase family 81 protein	AT5G15870.1
39	Medtr8g018510.1	seed linoleate 9S-lipoxygenase	AT3G22400.1
40	Medtr2g029800.1	peroxidase family protein	AT5G06720.1

9.2 Unique proteins

Table 4: Protein IDs and Fasta headers of proteins unique to the groups Fix+ and Fix-. The table lists all proteins, which are exclusively found in the groups Fix+ and/ or Fix -. The first column contains the protein IDs and the second column the associated Fasta header. The columns Fix+ and Fix- include information on the occurrence of the protein. 'X' means that the protein was found, whereas a '-' indicates absence of the protein in the group. The next column shows functions of the protein.

Nr	Protein ID	Keyword function Fasta header	Fix +	Fix -	1) Function 2) Relation to SNF	Citation
1	Medtr4g038440.2	Cell wall caffeic acid O-methyltransferase	x	x	1) biosynthesis of monolignols, (building blocks of plant lignin→ cell wall) lignin, lignin-like compounds 2) isolated and purified from root nodules of alfalfa; hydrophobic barriers in cell walls, may provide a possible diffusionbarrierfor O2	(SELLSTEDT *et al.* 1991), (Vance and Bryan 1981)
2	Medtr0045s0110	Cell wall DUF642 family protein	x	x	1) DUF642 protein, associated with cell wall 2) Yet unknown function	(Vázquez-Lobo *et al.* 2012)
3	Medtr1g088480.1	Cell wall UDP-glucuronic acid decarboxylase-like protein	x	x	1) Catalyzes the NAD-dependent decarboxylation of UDP-glucuronic acid to UDP-xylose, important in the processes of growth and development in plant tissues 2) Substrate found in soybean nodules	(Stewart and Copeland 1999)
4	Medtr3g077940.5	Oxygen Carbonic anhydrase family protein	x	x	1) Catalyzes reaction: carbon dioxide and water to bicarbonate and protons (or vice versa) 2) Nodules contain CA protein in the IC increasing malate production and, concomitantly, their water uptake and cell volume, resulting increase of the number and volume of intercellular spaces facilitates oxygen diffusion inside the nodule	(Gálvez *et al.* 2000)
5	Mdtr3g087590.1	Diverse Myo-inositol 1-phosphate synthase	x	x	1) Catalyzes conversion of D-glucose 6-phosphate to 1L-myo-inositol-1-phosphate, first step in the production of all inositol-containing compounds: including phospholipids (membrane trafficking, signalling pathways, auxin storage, transport, cell wall biosynthesis, production of stress-related molecules) 2) no known connection, not shown in nodules	(Abreu and Aragao 2006)
6	Medtr5g071360.1	Energy metabolism Asparagine synthetase [glutamine-hydrolyzing] protein	x	x	1) Synthesis of aspartate and asparagine 2) Nitrogen assimilation	(Temple *et al.* 1998)

7	Medtr8g006450.1	Membrane structure and function Obtusifoliol-14-demethylase	x	x	1) Plant sterol biosynthesis 2) Nodule-induced metabolic gene (Lotus japonicas)	(Colebatch *et al.* 2004), (Knights *et al.* 1977)
8	Medtr8g024340.	Energy metabolism Granule bound starch synthase	x	x	1) Synthesizing crystalline structures within starch 2) Starch might play an important role source of energy for N fixation	(Forrest *et al.* 1991)
9	Medtr8g072010.2	Cell wall Pectinacetylesterase (PAE) family protein	x	x	1) PAEs catalyzes deacetylation of pectin, a major compound of primary cell walls 2) Nodule-enhanced expression, deacetylationized pectin more accessible to pectin-degrading enzymes, modulation of cell growth by loosening the primary cell wall and middle lamella in plant roots	(Györgyey *et al.* 2000), (Vercauteren *et al.* 2002)
10	Medtr4g047610.	Nodule senescence Papain family cysteine protease	-	x	1) Degradation of proteins 2) Cysteine proteases play a role in nodule senescence	(van Wyk *et al.* 2014)
11	Medtr8g090000.1	DNA replication Minichromosome maintenance	-	x	1) Process of DNA replication, specifically the formation and elongation of the replication fork 2) No known connection	(Lee and Hurwitz 2001)
12	Medtr4g011340.1	Energy metabolism Cytochrome C oxidase subunit 5b	x	-	1) Enzyme in the respiratory electron transport chain of mitochondria 2) NO is inhibitor of respiratory cytochrome c, NO can be generated as a byproduct of nitrogen fixation and/or respiration, regulatory function SNF?	(Durner and Klessig 1999)
13	Medtr7g090510.2	UDP-sulfoquinovose synthase	x	-	1) Sulfolipid biosynthesis (adding sulfite to UDP-glucose) 2) Proposed interaction: FMN domain of Fd-glutamate synthase is also involved in the delivery of sulphite to the reaction centre of uridine diphosphate (UDP) sulphoquinovose synthase (SQD1), thus linking nitrate and sulphate assimilation	(Shimojima *et al.* 2005)

7.2 Manuscript 2

Sample preparation for analysis of the plant mitochondrial membrane proteome

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Sample preparation for analysis of the plant mitochondrial membrane proteome

Running head: Mitochondrial membrane proteome

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Summary

Containing plastids and vacuoles in addition to those organelles also found in other (heterotrophic) cells, the plant cell displays an extraordinary level of compartmentalization, largely obtained by the utilization of membranes. These membranes not only confine reaction spaces but must also facilitate cross-talk between organelles and other cell compartments. They also host important components of the plant energy metabolism, i.e. the electron transport chains of mitochondria and chloroplasts. Characterization of the proteomes of these membranes requires isolation of pure and intact organelles from plant tissues followed by subsequent purification of their respective membranes. Membrane fractions are then amenable for further analyses using gel electrophoresis procedures or gel-free proteomic approaches. Here, we describe the preparation of intact mitochondria from *Arabidopsis thaliana* cell-culture, the isolation of outer and inner mitochondrial membranes and downstream proteomic applications for analyzing their membrane protein content.

Keywords: mitochondria, isolation, subfractionation, BN-PAGE, trypsin digestion, mass spectrometry

1. Introduction

Mitochondria are coated by two distinct membranes, the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM). The OMM surrounds the organelle and mainly functions in controlling the transport of proteins into the organelle. The folding of the IMM into cristae leads to an increase of membrane area and facilitates accommodation of higher amounts of the protein complexes involved in oxidative phosphorylation (OXPHOS). A substantial proportion of the adenosine triphosphate (ATP) consumed by the plant cell is produced by the action of these protein complexes. Recently, new results on the protein-protein interactions of OXPHOS subunits provided interesting insights into the functioning of the branched electron transport chain of plants (*1,2,3*). Analyzing the protein content especially of the inner mitochondrial membrane and the association patterns therefore is a promising approach to investigate processes related to key mitochondrial functions.

The first crucial step is the isolation of pure mitochondrial fractions, a task which is particularly challenging from leaf material due to the strong presence of chloroplasts in these organs. For the

analysis of leaf mitochondria, it is advisable to use young rosette leaves which are easier to disrupt and allow a better extraction of intact mitochondria. Arabidopsis plants grown hydroponically on wire mesh (*4*) may also be used for this. These systems offer the potential to harvest green shoots and non-green roots at the same time and allow a direct comparison of their mitochondria. Non-green organs, such as roots, usually yield isolates of higher purity. However, due to the absence of photosynthesis and photorespiration in these organs, their mitochondria have different functions which are expressed in their protein content (*5*). As an alternative to roots, cell or callus cultures grown in darkness can be used. An advantage of the cell culture is the ease with which cells can be manipulated by effector molecules or subjected to other treatments. In addition, these cells also possess softer cell walls making them less resistant to mechanical disruption. The selection of plant material therefore has a profound impact on mitochondrial yield and purity and is an important factor to be considered for the experimental setup. In terms of organelle isolation we here focus on the procedure used in our laboratory for the preparation of mitochondria from cell suspension cultures. While this method may require modification for other plant material, the downstream procedures described in this chapter (subfractionation, BN/SDS-PAGE, MS sample preparation) are transferable.

The procedure of isolating mitochondria can be broken up into three parts: cell disruption, differential centrifugation (to gain crude mitochondria) and density gradient centrifugation (to further eliminate organellar contaminants) (*6*,*7*). For analyzing the total mitochondrial membrane proteome (IMM and OMM) subpartitioning into membrane and soluble fraction can be performed. In-depth analyses of OMM and IMM proteomes require removal of the OMM from intact mitochondria by hypo-osmotic conditions causing swelling and OMM rupture (7). At the same time, the cristae membranes connected to the inner boundary membrane (collectively denominated as IMM in the following) grant a higher degree of elasticity to this membrane system, thereby retaining its integrity under the conditions applied. OMM fragments and the remaining mitoplasts (IMM and matrix) can now be separated by centrifugation (7). To disrupt the mitoplasts and isolate the IMM, a combination of swelling and sonication is applied, followed by centrifugation to pelletize the IMM. It should be noted that contact sites between OMM and IMM (8) prevent quantitative removal of the outer membrane from the

mitoplasts. Hence, while it is possible to obtain OMM fractions largely devoid of IMM, this does not apply *vice versa*.

Proteomic analyses can therefore be performed either focusing on a total mitochondrial membrane fraction, the OMM or the (OMM-contaminated) IMM (**Figure 1**). Two approaches aiming at either the general identification of mitochondrial membrane proteomes or the analysis of protein-protein interactions with IMM and OMM will be presented here. Shot-gun mass spectrometry enables the identification of thousands of proteins in a given sample and is therefore an appropriate tool for investigating entire proteomes, including those of the mitochondrial membranes ***(9,10)***. Key for successful identification of membrane proteins is the sample preparation. Sodium dodecyl sulfate (SDS) is widely used for solubilization of membrane proteins prior to MS analysis. However, SDS is not compatible with enzymatic digestion of proteins due to its highly denaturing properties and must be depleted prior to this step. Filter aided sample preparation ***(11)*** has been successfully applied for this. Alternatively, free SDS can be depleted from samples by SDS-PAGE which may additionally be used for pre-fractionation of the sample prior to MS. If no fractionation is required, the gel run is aborted as soon as the running front reaches the separation gel (**Figure 2A**). Proteins concentrate at this position and, after Coomassie staining, can be cut *en bloc* from the gel to be subjected to standard in-gel digestion procedures ***(12)***. For prefractionation of the sample the gel is run until the desired degree of separation is achieved. The gel lane will then be cut into fine slices, each of these to be treated separately in respect to digestion and peptide extraction **(Figure 2B, *12*)**.

Since the proteomes of OMM and IMM are organized in multi-protein complexes to a high degree, it may be desirable to analyze the subunit compositions of these complexes. For this, a gel-based approach combining a Blue Native polyacrylamide gelelectrophoresis (BN-PAGE) with a denaturing SDS–PAGE is a highly valuable tool **(13,*14*)**. BN/SDS-PAGE allows the separation of native protein complexes in the first dimension ***(15,16)*** followed by separation of their subunits in the second dimension. It thus enables the assignment of proteins to multiprotein complexes.

2. Materials

All Buffers are prepared with analytical grade chemicals and with ultra-pure de-ionized water (0.055 µS/cm). Stock solutions can be stored at 4°C for several weeks. All other solutions are prepared freshly on the day of use or the day before use.

2.1. Mitochondria Isolation from Arabidopsis cell cultures

1. 1 M 3-(N-morpholino)propanesulfonic acid (MOPS, stock)
2. 100 mM ethylene glycol tetra-acetic acid (EGTA, pH 7,2, stock)
3. 200 mM phenylmethanesulfonylfluoride (PMSF, in EtOH, stock)
4. 10 % (w/v) bovine serum albumin (BSA, stock)
5. Disruption buffer: 450 mM sucrose, 15 mM MOPS, 1.5 mM EGTA, 6 g/l PVP40, 0.2 % (w/v) BSA, 10 mM sodium ascorbate, 10 mM cysteine, 0.2 mM PMSF, pH 7.4 (adjusted at 4 °C with KOH)
6. 5x Gradient buffer: 1.5 M sucrose, 50 mM MOPS, pH 7.2 (adjusted at 4 °Cwith KOH)
7. Percoll (GE Healthcare Life Sciences, Solingen, Germany)
8. Percoll solutions (for 3/4/6 gradients see Table 1)
9. Washing buffer: 300 mM sucrose, 10 mM MOPS, 1 mM EGTA, 0.2 mM PMSF, pH 7.2 (adjusted at 4 °C with KOH)
10. Resuspension buffer: 400 mM mannitol, 1 mM EGTA, 10 mM tricine, 0.2 mM PMSF, pH 7.2 (adjusted at 4 °C with KOH)
11. Muslin cotton gauze, filter (~ 26 x 26 cm, mesh size: 180 µm), Miracloth (Merck Millipore, Darmstadt, Germany)
12. Waring Laboratory Blender
13. Fine paintbrush
14. Dounce homogenizer (15 ml)
15. Glass pipettes (1 – 5 ml), Pasteur pipettes with long neck (230 mm; Brand, Wertheim, Germany)

16. Ultracentrifuge tubes: clear, 25 x 89 mm (e.g. Beckman 1 x 3 ½ UC tubes; Beckman Coulter, Krefeld, Germany)
17. Swing-out rotor (e.g. Surespin 630; Thermo Fisher Scientific, Waltham, MA, US)
18. Ultracentrifuge (e.g. Thermo Sorvall WX Ultra 80; Thermo Fisher Scientific, Waltham, MA, US)
19. Large (approximately 500 ml) and small (approximately 50 ml) volume centrifuge tubes
20. Fixed-angle rotors (e.g. F12 – 6 x 500 LEX, A27 – 8 x 50; Thermo Fisher Scientific, Waltham, MA, US)
21. Refrigerated centrifuge: (e.g. Thermo Sorvall LYNX 6000; Thermo Fisher Scientific, Waltham, MA, US)

2.2. Subfractionation into mitochondrial membranes and matrix

1. 100 mM ethylene glycol tetra-acetic acid (EGTA, pH 7,2, stock)
2. 200 mM phenylmethanesulfonylfluoride (PMSF, in EtOH, stock)
3. Resuspension buffer without mannitol: 1 mM EGTA, 10 mM tricine, 0.2 mM PMSF, pH 7.2 (adjusted at 4 °C with KOH)
4. Sonicator (probe with 3 - 5 mm diameter)
5. Ultracentrifuge

2.3 Subfractionation into outer mitochondrial membrane and mitoplasts

1. 100 mM ethylene glycol tetra-acetic acid (EGTA, pH 7,2, stock)
2. 200 mM phenylmethanesulfonylfluoride (PMSF, in EtOH, stock)
3. 100 mM potassium dihydrogen phosphate (KH_2PO_4, pH 7.2 by titration with 100 mM potassium hydrogen phosphate, K2HPO4, stock)
4. Swelling buffer: 5 mM KH_2PO_4 (pH-adjusted stock), 0.2 mM PMSF.
5. 10 x Gradient buffer: 10 mM EDTA, 100 mM MOPS, 1 mM PMSF, pH 7.2 (adjusted at 4 °C with KOH).
6. Solutions for sucrose gradients (see Table 2).

7. Ultracentrifuge: Thermo Sorvall WX Ultra 80 (Thermo Fisher Scientific, Waltham, MA, US)
8. Swinging bucket rotor: Surespin 630 (Thermo Fisher Scientific, Waltham, MA, US)
9. Ultracentifuge tubes: Ultra-Clear Centrifuge Tubes, 14 x 95 mm (Beckman Coulter, Krefeld, Germany)

2.4 Subfractionation into inner mitochondrial membrane and matrix

1. 100 mM ethylene glycol tetra-acetic acid (EGTA, pH 7.2, stock)
2. 200 mM phenylmethanesulfonylfluoride (PMSF, in EtOH, stock)
3. Swelling buffer: 10 mM tricine, 1 mM EGTA, 1 mM PMSF, pH 7.3 (adjusted at 4 °C with NaOH)
4. 10 x Gradient buffer: 10 mM EDTA, 100 mM MOPS, 1 mM PMSF, pH 7.2 (adjusted at 4 °C with ?).
5. Storage buffer: 10 % (v/v) 10 x gradient buffer, 10 % (v/v) glycerol, 0,4 mM PMSF

2.5 Gel-based shotgun-MS

1. 0.5 M Tris-HCl, pH 6.8 (stock)
2. 1.5 M Tris-HCl, pH 8.8 (stock)
3. 10 % (w/v) sodium dodecyl sulfate (SDS, stock)
4. 10 % (w/v) ammonium persulfate (APS, stock)
5. Tetramethylethylenediamine (TEMED)
6. 2 x sample buffer: 4 % (w/v) SDS, 125 mM Tris-HCl pH 6.8, 20 % (v/v) glycerol
7. 10 x Tris-glycine SDS buffer: 248 mM Tris, 1.92 M glycine, 1 % (w/v) SDS
8. 1 mg/ml bromophenol blue (in 2-mercaptoethanol)
9. BioRad Protean II gel unit (BioRad, Richmond, CA, US)

2.6 2D BN/SDS-PAGE

1. 5 x BN - cathode buffer: 250 mM tricine, 75 mM Bis-Tris, 0,1 % (w/v) Coomassie blue 250 G, pH 7.0 (adjusted at 4 °C with HCl)

2. 6 x BN - anode buffer: 300 mM Bis-Tris, pH 7.0 (adjusted at 4 °C with HCl)
3. 6 x BN – gel buffer: 1.5 M aminocaproic acid (ACA), 150 mM Bis-Tris, pH 7.0 (adjusted at 4 °C with HCl)
4. 5 % Serva blue G: 750 mM ACA, 5 % (w/v) Coomassie blue 250 G
5. Solubilization buffer without digitonin: 30 mM HEPES, 150 mM potassium acetate, 10 % (v/v) glycerol, pH 7.4 (adjusted at 4 °C with HCl)
6. Digitonin (Sigma Aldrich, St. Louis, MO, US)
7. Solubilization buffer with digitonin: 30 mM HEPES, 150 mM potassium acetate, 10 % (v/v) glycerol, 5 % (w/v) digitonin, pH 7.4 (adjusted at 4 °C with HCl)
8. Incubation buffer: 1 % (w/v) SDS, 1 % (v/v) 2-mercaptoethanol
9. Tricine gel buffer: 3 M Tris, 0.3 % (w/v) SDS, pH 8.45
10. Tricine anode buffer: 200 mM Tris, pH 8.9
11. Tricine cathode buffer: 100 mM Tris, 100 mM Tricine, 0.1 % (w/v) SDS, pH 8.25
12. Overlay solution: 1 M Tris, 0,1 % (w/v) SDS, pH 8.45
13. 10 % (w/v) ammonium persulfate (APS)
14. Tetramethylethylenediamine (TEMED)
15. Gradient former: BioRad 485 Gradient Former (BioRad, Richmond, CA, US)
16. Peristaltic pump: BioRad EP-1 Econo Pump (BioRad, Richmond, CA, US)
17. Hose attached to hypodermic needle (18 gauge / 1.2 mm diameter, length 40 mm)
18. BioRad Protean II gel unit (BioRad, Richmond, CA, US)

2.7 Tryptic digestion of samples from gel-based proteomics for MS

1. 100 mM ammonium bicarbonate (Ambic, NH_4HCO_3)
2. Trypsin solution: Promega Sequencing Grade Modified Trypsin (Promega, Madison, MI, US), prepared according to manufacturer (for example, Promega Sequencing Grade Modified Trypsin, Porcine, 20µg, reconstituted in 100µl Promega Trypsin Resuspension Buffer and kept at RT for 30 min). Before use the buffer is diluted in 900µl 100mM Ambic to yield a final concentration of 0.02 µg/µl.

3. Low-binding reaction tubes: Eppendorf Protein LoBind Tube 1.5 ml (Eppendorf, Hamburg, Germany)
4. Vacuum centrifuge: Eppendorf Concentrator plus (Eppendorf, Hamburg, Germany)

3. Methods

3.1. Mitochondria isolation from Arabidopsis cell suspension cultures

For the in-depth analysis of mitochondrial membrane proteomes organelles have to be isolated first. From these, either total membranes (3.2) or the OMM (3.3) and IMM (3.4) can be prepared. For the isolation of OMM and IMM it is mandatory to use freshly prepared mitochondria.

1. For each 50 g fresh weight used produce one discontinuous Percoll gradient (see Note 1). Casting the gradients into ultracentrifuge tubes is done as follows: underlay the lightest Percoll solution with the two heavier solutions by the help of a 20 ml glass syringe attached to a big-diameter (~ 3 mm) injection needle. First, 10 ml of the 18 % Percoll solution are transferred into a centrifuge tube. Then, 10 ml of the 23 % Percoll solution are injected below the 18 % solution. Finally, 10 ml of the 40 % Percoll solution are placed below the 23 % Percoll solution.
2. Harvest the cells by filtration through muslin cotton gauze and determine the fresh weight.

The following steps must be performed at 4 °C or on ice.

3. Add chilled disruption buffer to the cells at a ratio of 2 ml of buffer per gram fresh weight (min. 100 ml). Homogenize the cells in a blender. The first homogenization step is done at high speed (15 seconds), followed by two steps at low speed (2 x 15 seconds). Leave 30 second intervals in between the three blending steps (see Note 2).
4. Filter the homogenate through 2 to 4 layers of Miracloth.
5. Centrifuge the filtrate for 5 minutes at 2700 xg and 4 °C.
6. The supernatant is centrifuged at 4 °C three times (see Note 3):
    a. 5 minutes at 2700 xg (discard pellet).
    b. 5 minutes at 8300 xg (discard pellet).
    c. 10 minutes at 17000 xg (retain pellet, discard supernatant).

7. Mitochondria (and other organelles) are pelletized in the last centrifugation step. Carefully suspend the mitochondria pellet in 3 to 5 ml of washing buffer using a fine, soft paintbrush. Transfer the mitochondria solution into a Dounce homogenizer and adjust the volume [ml] with washing buffer to a maximum of 3 ml per Percoll gradient. Homogenize the solution performing two careful strokes.
8. Carefully lay the homogenized suspension on top of the Percoll gradients and ultra-centrifuge for 90 minutes at 70000 xg and 4 °C.
9. Mitochondria are located in the area of the interphase between the 23 % and 40 % Percoll phase. Remove the 18 % phase and part of the 23 % phase with a vacuum water pump without disturbing the mitochondrial band. Carefully collect the mitochondria using a Pasteur pipette (see Note 4).
10. Remove Percoll by diluting the mitochondria in resuspension buffer and centrifuge for 10 minutes at 14500 xg and 4 °C. Repeat until the mitochondrial pellet becomes stable (usually three to four times). Before each centrifugation step gently suspend organelles using a pipette.
11. Determine the weight of the mitochondria pellet after the last wash step by determining the weight of the dry tube before the last centrifugation step and after pelletizing the mitochondria and removing the supernatant.
12. Resuspend the mitochondria in 0.2 to 1.0 ml of resuspension buffer (depending on yield) using a pipette. Adjust final mitochondria concentration to 0.1 g mitochondria per ml (see Note 5). Use a chilled glass pipette to measure the volume of the mitochondria solution and to fill up with resuspension buffer.
13. Split the mitochondria suspension into 100 µl aliquots or continue with 3.2 or 3.3.
14. Centrifuge aliquots for 10 minutes at 14300 xg and 4 °C. Discard supernatant and store pellets at -80 °C.

3.2. Subfractionation of intact mitochondria into mitochondrial membranes and matrix

1. Using a pipette resuspend the mitochondrial pellet (from 3.1) in resuspension buffer without mannitol by adding 1 ml of resuspension buffer to 100 mg of mitochondria.
2. Sonicate the solution four times using a probe-type instrument with one minute intervals in between.
3. Centrifuge the suspension for 7 minutes at 5000 xg and 4 °C. Retain the supernatant and store the pellet at -80 °C (see Note 6).
4. Ultra-centrifuge the supernatant for 90 minutes at 150000 xg and 4 °C. The supernatant includes the matrix fraction and the pellet represents the membrane fraction. Store pellet at -80 °C.

3.3 Subfractionation of intact mitochondria into outer mitochondrial membrane and mitoplasts

1. Calculate the number of required gradients by first estimating the final volume of the mitochondria suspension to be loaded on the gradients as outlined in steps 3 and 4. Each gradient will be loaded with 3 ml of this suspension.
2. Prepare the appropriate number of discontinuous sucrose gradients (15/32/60 % sucrose by transfering 1 ml of the 60 % sucrose solution into a centrifugation tube. Overlay this solution with 4 ml of the 32 % sucrose solution, then overlay with 1.5 ml of the 15 % sucrose solution.
3. Suspend the freshly isolated mitochondria (from 3.1) in 6 ml of swelling buffer and incubate for 6 minutes (see Note 7).
4. Add the equivalent volume of swelling buffer by referring to the pellet weight as determined in step 11 of chapter 3.1 and Table 3.
5. Incubate on ice for additional 4 minutes.
6. Transfer the solution to a Dounce homogenizer and release OMM fragments by 20 careful strokes.
7. Layer 3 ml of the solution on top of each sucrose gradient and ultra-centrifuge for 60 minutes at 92000 xg and 4 °C.

8. The outer mitochondrial membranes are located at the interphase between 15 % and 32 % sucrose. Mitoplasts form a band at the interphase between 32 % and 60 % sucrose. Collect the OMM and mitoplasts using a Pasteur pipette (see Note 8). Store mitoplast fraction at -80°C.
9. Continue with the OMM fraction and determine the total volume. The concentration of sucrose is approximately 25 %. Adjust to a final sucrose concentration of 50 % by adding the 70 % sucrose solution (Table 2). Determine the volume of the solution. Below 5 ml one gradient is prepared in the next step; between 5 ml and 10 ml two of them are required.
10. Prepare the discontinuous sucrose gradients (0/32/50 %) by first transferring 5 ml of the 50 % sucrose solution into a centrifugation tube. Overlay the solution with 5 ml of the 32 % sucrose solution and then overlay with 1.5 ml of the 0 % sucrose solution (see Note 9).
11. Transfer the OMM fraction to the top of the sucrose gradient and ultra-centrifuge for 5 hours at 170000 xg and 4 °C (overnight).
12. To sharpen the bands ultra-centrifuge for 30 minutes at 170000 xg at 4 °C next morning.
13. The outer mitochondrial membranes are located at the interphase between 0 % and 32 % sucrose. Collect the OMM using a Pasteur pipette. Dilute OMM in gradient buffer containing 0 % sucrose by factor 4 and ultra-centrifuge for 90 minutes at 140000 xg and 4 °C to pelletize the OMM.
14. Remove supernatant and store pellet at -80°C.

3.4 Subfractionation into inner mitochondrial membrane and matrix

1. Carefully thaw the frozen mitoplasts (from 3.3) and determine the total volume (about 2.5 ml). Transfer the mitoplasts into a fresh (>20 ml) centrifugation tube.
2. Add swelling buffer in the intervals described in Table 4. The added volume is proportional to the initial volume; values given in Table 4 are for 2.5 ml of mitoplast fraction.
3. Centrifuge for 15 minutes at 12000 xg and 4 °C.
4. Carefully remove the supernatant (see Note 10).
5. Resuspend the pellet in 10 ml swelling buffer and sonicate for 3 seconds. Repeat sonication step twice leaving 2 minute intervals between each repetition.

6. Centrifuge for 7 minutes at 5100 xg and 4 °C.
7. Intact mitoplasts are found in the pellet. The inner membrane remains in the supernatant.
8. Ultra-centrifuge the supernatant for 90 minutes at 140000 xg and 4 °C to pelletize the IMM.
9. Remove supernatant and store IMM pellet at -80 °C.

3.5 Sample preparation for shotgun-MS

3.5.1 Sample preparation

Important: Tris-glycine SDS gel must be ready before commencing with the sample preparation. For best quality this should be done one day in advance.

1. Add 2 x sample buffer to your mitochondrial membranes (both membranes: 100 µl, OMM or IMM pellet: 50 µl). Incubate for 5 minutes at 60 °C under mild shaking. If necessary, resuspend pellet with pipette and incubate for another 5 minutes.
2. Add equal amount of H_2O.
3. Centrifuge samples for 10 minutes at maximum speed in your benchtop centrifuge (> 18000 xg).
4. Determine protein concentration using a SDS-compatible protein assay (for example the BCA protein assay kit by Pierce). Produce aliquots equivalent to 50 µg of protein (see Note 11).
5. If more than one sample is to be loaded on a gel, adjust sample volumes taking the highest one as the reference. Keep final volume below 150 µl.
6. Add bromophenol blue in 2-mercaptoethanol mix to a final concentration of 5 % 2-mercaptoethanol (see Note 12).

3.5.2 Tris-glycine SDS-PAGE

1. Set up gel caster and prepare solution for the separating gel (14 % acrylamide, 1.5 mm x 200 mm x 200 mm, see Table 5).
2. Cast the separating gel and overlay with iso-butanol (under the fume hood). To minimize disturbance of the gel phase when applying the iso-butanol tilt the assembly backwards to a

near horizontal orientation and let the iso-butanol slowly run down on inside edge of the spacer.

3. Remove the iso-butanol soon after polymerization, shortly rinse gel with H_2O and indicate the top of the separation gel with a waterproof marker pen on the outside of the larger gel plate.
4. Prepare solution for the stacking gel (4 % acrylamide, see Table 5).
5. For proper polymerization and well-formed pockets warm gel stand, solution, comb and pipette to 37 °C, cast the gel and leave it at 37 °C until setting is complete (see Note 13). Cover the gel with cling film and store at RT until further use.
6. Prepare 1 x Tris-glycine SDS buffer from 10x buffer and load sample.
7. Perform the gel run at 30 mA (max. 500 V) at room temperature (see Note 14). Gel run is finished when the bromophenol blue front reaches the separation gel (as indicated by the marker).
8. Stain the gel (for Coomassie colloidal see ***17***) and cut bands. Dice bands to yield cubes with edge lengths of approximately 1.5 to 2 mm. Dry cubes in a vacuum centrifuge and store at -20 °C until further use.

3.6 2D BN/SDS-PAGE

3.6.1 First dimension: Blue Native PAGE

1. Prepare the gradient mixer (close all valves) as well as 4.5 % and 16 % acrylamide gel solutions (see Table 6). Pre cool the solutions in the gradient mixer for 10 minutes at -20 °C.
2. Prepare gel casting assembly using 1.5 mm spacers. Pre cool the gel caster at 4 °C.
3. Insert injection needle (attached to pump hose) in between gel glass plates of the gel assembly by piercing the lower rubber gasket (**Figure 3**).
4. Inject a small volume of water (~ 5 ml) using the pump. This will act as an overlay solution.
5. Let the pump draw some air to create diffusion barrier between the water and the gel solutions.
6. Connect gradient mixer to pump tubing. Add APS and TEMED to both chambers and mix thoroughly while keeping both valves of the gradient mixer closed. Remove stirring rod in the second chamber and open outlet valve of the first chamber. Start the pump with a speed of

~1.3 ml/min until the 4.5 % solution reaches the gel caster. Lower the speed to ~ 0.6 ml/min and scan the needle tip for small residual air bubbles. If these persist stop pump and carefully tilt the gel assembly sideways until the needle tip is not covered in fluid anymore, then slowly tilt back to horizontal position. Continue pumping until volumes in both chambers are equal. Open the tap connecting both chambers and ensure that no air bubbles are trapped in the connection of the two chambers by sealing the top of the first chamber with Parafilm, then applying light pressure on the middle of the sealed opening with a fingertip (wear gloves!).

7. Slowly increase pump speed to ~ 1.1 ml/min in 0.1 ml/min intervals of 30 seconds each (see Note 15).
8. Remove the overlay solution (H_2O) and add comb (for 10 or 15 sample pockets).
9. Cast the stacking gel: 11 ml H_2O, 2.5 ml 6 x BN – gel buffer, 1.5 ml 40 % (w/v) acrylamide solution, 65 µl 10 % (w/v) APS, 6.5 µl TEMED (see Note 16).
10. Prepare 1 x BN - cathode and 1 x BN - anode buffer. Store at 4°C until further use.

3.6.2 Sample preparation

The protocol below gives directions for the analysis of mitochondrial fractions. Sub-mitochondrial fractions may be subjected to BN-PAGE as well but the solubilization procedure may benefit from optimization.

Important: BN gel must be ready before commencing with sample preparation. For best quality this should be done one day in advance.

1. Suspend mitochondria pellet in solubilization buffer containing 5 % digitonin and incubate 10 to 20 minutes on ice. Pellet originating from 100 µl of mitochondria solution (corresponding to 500 µg protein, please refer to step 12 chapter 3.1 and Note 5) is resolved in 100 µl of solubilization buffer (see Notes 17 and 18).
2. Centrifuge for 10 minutes at 18300 xg and 4 °C.
3. Solubilized proteins and protein complexes are in the supernatant. Transfer the supernatant to a new tube and add 5% Serva blue G (1 µl buffer/ 20 µl supernatant).

4. Load samples, perform a two-step gel run at 4 °C: first step at constant voltage (100 V for 45 minutes) and second step at constant current (15 mA for 11 hours).

3.6.3 Second dimension: Tricine SDS-PAGE

1. Cut a lane from the BN-PAGE, remove the stacking gel and incubate for 30 minutes in incubation buffer (1 % (w/v) SDS, 1 % (v/v) 2-mercaptoethanol) and rinse in H_2O afterwards.
2. Place the gel strip horizontally on a glass plate at the height of a stacking gel. Make sure to leave a gap of approximately 1cm to both spacers. If the gel strip is too long, cut it on the least important end (usually the low molecular weight end) and continue assembling the gel caster.
3. Prepare solutions for a Tricine-SDS-PAGE (1.0 mm x 200 mm x 200 mm, see Table 7).
4. Transfer 25 ml of separation gel solution in the gel caster by using a pipette and letting the solution run down on the inner side of the spacer without it touching the BN gel strip. To overlay the spacer gel, tilt the gel caster backwards to a near-horizontal position and let 5 ml of spacer gel solution slowly run down on the inner edge of the gel spacers. Make sure that the spacer gel does not touch the BN gel strip. Repeat procedure with 1 ml of overlay solution. Polymerization takes up to 45 minutes and setting of the gel is indicated by a sharp phase border between spacer gel and overlay solution.
5. Decant the overlay solution and rinse with H_2O. Remove as much H_2O as possible by inserting strip of suitably thick Whatman paper between the glass plates.
6. Cast the stacking gel by tilting the gel caster sideways left by approximately 30°. Cast the gel by pouring the solution along the inner edge of the lower spacer and slowly move the gel caster back to its horizontal position while continuing to add the gel solution. This will avoid air bubbles getting trapped under the gel strip (see Note 19). The stacking gel should now fully enclose the BN gel strip. Polymerization takes up to 60 minutes.
7. Run the gel at 30 mA (max. 500 V) at room temperature for 18 to 20 hours.
8. Stain the gel (for Coomassie colloidal see *17*) and cut out spots. Dry in vacuum centrifuge.

3.7 Tryptic digestion of samples for MS

Stated volumes are for gel spots. For bands volumes are given in brackets

1. Reduce cysteine residues by adding 40 µl (150 µl) of reduction solution (20 mM DTT [3.1 mg/mL] in 0.1 M NH_4HCO_3) to the dried gel piece(s).
2. Incubate for 30 minutes at 56 °C. Discard supernatant.
3. Dehydrate by adding 200 µl ACN. Discard supernatant.
4. Alkylate by adding 40µl (150 µl) 55 mM iodoacetamide (10.2 mg/ml) in 0.1 M NH_4HCO_3.
5. Incubate 30 minutes at RT in the dark. Discard supernatant.
6. Dehydrate by adding 200 µl ACN. Discard supernatant.
7. Wash gel pieces in 200 µl (500 µl) 0.1M NH_4HCO_3.
8. Incubate for 15 min at RT. Discard supernatant.
9. Dehydrate by adding 200 µl ACN. Discard supernatant.
10. Dry gel pieces using a vacuum centrifuge for 5 minutes.
11. Add 20 µl (70 µl) of trypsin solution equaling 0.4µg (1.4µg).
12. Incubate for 10 minutes, then check that the gel pieces are fully rehydrated and that there are only minute amounts of liquid left. If all liquid has been taken up by the gel pieces add more trypsin solution (see Note 20).
13. Incubate over night at 37 °C.
14. For peptide extraction add 20 µl (70µl) 5 % (v/v) formic acid (FA) in 50 % (v/v) ACN.
15. Incubate for 20 minutes at 37 °C under mild shaking.
16. Collect supernatant in new Eppendorf tubes. The supernatant already contains extracted peptides. For the following steps it is advisable to start drying down the peptides in a vacuum centrifuge. This will reduce final fluid level in the tubes therefore reducing unspecific binding of peptides to the tube wall.
17. Repeat steps 14 to 16 twice with the exception of using 1 % (v/v) FA, 50 % (v/v) ACN. Add supernatant to the corresponding Eppendorf tubes already containing the first extracts.
18. Add 20µl (70 µl) of ACN, incubate for 20 minutes. Gel pieces will turn white.
19. Add supernatants to the corresponding Eppendorf tubes containing the previous extracts.

20. Dry extracts in a vacuum centrifuge and store at -20 °C until further use.

4. Notes

1. For maximal yield use about 300 g of fresh material and 6 Percoll gradients.
2. The homogenization steps in the blender should be interrupted to prevent the solution from heating and to allow the undisrupted material to sediment again.
3. For all centrifugation steps it is advantageous to choose a low brake setting in order to avoid perturbations of pellets or gradients.
4. To collect mitochondrial bands from an interphase it is important to expel the air from the pipette before it is immersed in gradient solution and carefully collect the mitochondria by moving the pipette tip in smooth circles at the interphase.
5. The protein content of this mitochondria diluted to 0.1 g/ml typically is about 5 µg/µl (according to Bradford protein concentration determination). If further fractionation of mitochondria is required, stop here and continue with 3.2 or 3.3.
6. Disruption of mitochondria is usually not quantitative and the pellet often contains residual intact mitochondria. To increase the yield of membrane and matrix fraction, sonication and centrifugation can be repeated using the pellets of step 3 in chapter 3.2.
7. For isolation of outer membranes it is mandatory to use fresh mitochondria directly after isolation. Rapid freezing of mitochondria in liquid nitrogen is not sufficient to keep the outer membrane intact.
8. For higher purity OMM fractions continue the protocol, if the purity at this step is deemed sufficient and higher yields are desired skip further steps.
9. When using ultracentrifuge tubes with thin walls (e.g. Beckman Coulter Ultra-Clear Centrifuge Tubes, 14 x 95 mm) it is important to fill them up completely to prevent the upper tube walls from collapsing into the solution which results in the complete loss of the sample. For the tubes described here at least 10 ml must be used.
10. The mitoplast pellet is typically very soft so handle carefully. Heating all components increases the polymerization and results in well-defined pockets. This increases the maximum

loading volume and sharpens the lanes. The gel should be prepared the day before usage to ensure complete polymerization and absence of residual persulfate radicals.

11. The gel run has to be stopped as soon as the bromophenol blue running front reaches the interphase between stacking and separation gel. Initially, check every 15 minutes, more often during the final stages.
12. The gradient gel is cast from the bottom. With increasing amount of solution from the second chamber the amount of glycerol (and acrylamide) concentration increases. This increases density of the solution in the first chamber and allows layering the new (heavier) solution underneath the lighter gel solution. To circumvent mixing, injection speed is slow at the beginning and raised only moderately. Gel casting takes 20 to 25 minutes and polymerization of the acrylamide (at 37 °C) additionally requires 45 to 60 minutes.
13. Prepare the gel the day before usage to guarantee optimal polymerization and reduce the presence of residual persulfate radicals. Store at 4 °C overnight.
14. If protein concentration is checked using a standard assay (e.g. Bradford) it should be done prior to addition of digitonin because it will disturb quantification. The protein amount in mitochondria isolated from cell culture (0.1 g mitochondria per ml) is approximately 5 µg/µl.
15. Preparing the solubilization buffer with digitonin requires heating close to the boiling point to dissolve the digitonin. Cool down to 4° C before use.
16. It is important to carefully cast the stacking gel avoiding any air becoming trapped underneath the gel strip since migration of proteins into the second dimension gel is disturbed at these sites. Avoid creating sharp edges on the gel strip when cutting it from the first dimension gel because air bubbles preferentially get stuck in these places.
17. It is important to add just enough trypsin solution to ensure full rehydration. Adding more trypsin than necessary may compromise analytical sensitivity.

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Table 1: Casting Percoll gradients (3/4/6 gradients) for mitochondria isolation

	3 gradients			4 gradients			6 gradients		
	18 %	**23 %**	**40 %**	**18 %**	**23 %**	**40 %**	**18 %**	**23 %**	**40 %**
Percoll [ml]	5.4	6.9	12	8.3	10.5	18	10.8	13.8	24
H_2O [ml]	18.6	17.1	12	27.8	25.5	18	37.2	34.2	24
5× gradient buffer [ml]	6	6	6	9	9	9	12	12	12

Table 2: Composition of sucrose gradient solutions for OMM isolation

Sucrose concentration	**Sucrose**	**10 x Gradient buffer**	**PMSF [200 mM]**	**Total volume**
[%]	**[g]**	**[ml]**	**[µl]**	**[ml]**
0	0	10	100	100
15	7.5	5	50	50
32	32.0	10	100	100
60	30.0	5	50	50
70	35.0	5	50	50

Table 3: Swelling buffer volume for OMM isolation

Mitochondria pellet [g]	**Total volume [ml]**
< 0.3	6
< 0.45	9
< 0.6	12
< 0.8	15
≥ 0.8	18

Table 4: Addition of swelling buffer to Mitoplast

t [min]	**V [ml]**
0	0.5
2	1
4	2
6	4
8	8
15	centrifugation

Table 5: Composition of Tris-glycine SDS-PAGE (1.5 mm x 200 mm x 200 mm)

	Separation gel (14 %)	**Stacking gel (4 %)**
1.5 M Tris-HCl, pH 8.8	12.5 ml	-
0.5 M Tris-HCl, pH 6.8	-	4 ml
10 % SDS (w/v)	0.5 ml	0.16 ml
40 % acrylamide	17.5 ml	1.6 ml
H_2O	19.5 ml	10.2 ml
Σ	**50 ml**	**16 ml**
10 % APS*	171.5 µl	100 µl
TEMED*	17.1 µl	10 µl

*add prior to use

Table 6: Composition of gel solutions for BN-PAGE (1.5 mm x 200 mm x 200 mm)

	Chamber one 4.5 %	**Chamber two 16 %**
H_2O	15.1 ml	4.6 ml
6 x BN - gel buffer	3.5 ml	3 ml
acrylamide (40 %)	2.4 ml	7.4 ml
glycerol (100 %)	-	3.5 ml
Σ	21 ml	18.5 ml
APS (10 %)*	95 µl	61 µl
TEMED*	9.5 µl	6.1 µl

*add prior to use

Table 7: Composition of Tricine SDS gel (1.0 mm x 200 mm x 200 mm)

	Separation gel (16.5 %)	**Spacer gel (10 %)**	**Stacking gel (10 %)**
H_2O	3.6 ml	4.1 ml	2.9 ml
Tricine - gel buffer	10 ml	3.4 ml	-
6 x BN – gel buffer	-	-	3.4 ml
SDS (10 %)	-	-	100 µl
glycerol	4 ml (87 %)	-	1 ml (100 %)
acrylamide (40 %)	12.4 ml	2.5 ml	2.5 ml
Σ	**30 ml**	**10 ml**	**10 ml**
APS (10 %)*	100 µl	34 µl	83 µl
TEMED*	10 µl	3.4 µl	8.3 µl

*add prior to use

Figure Captions

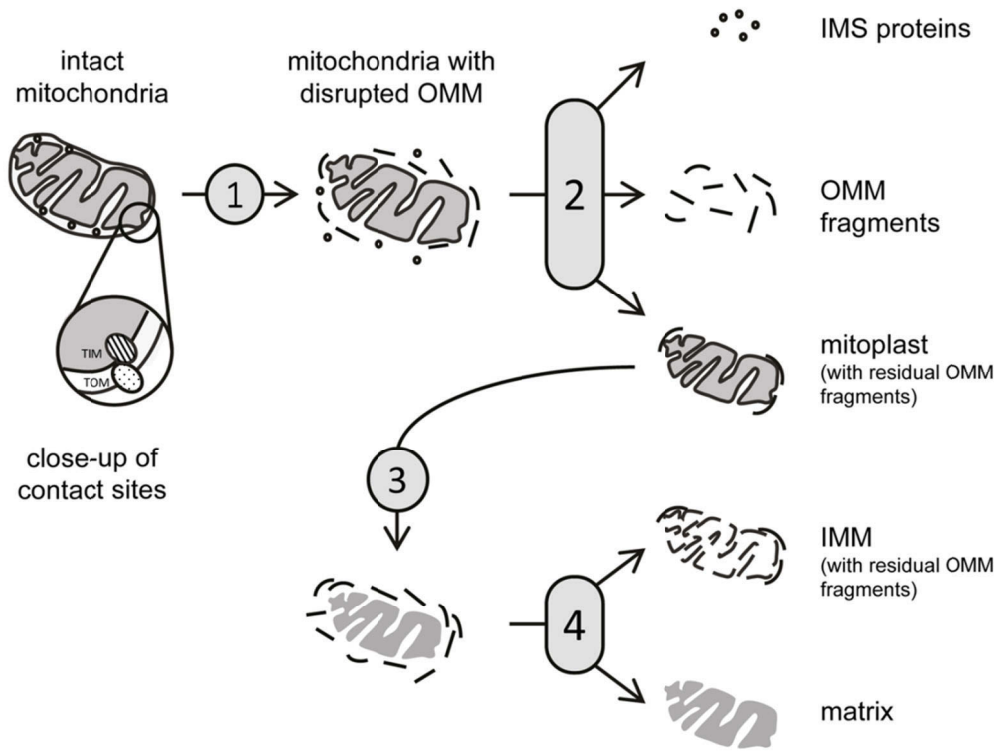

Figure 1: Subfractionation of isolated mitochondria. Rupture of the outer mitochondrial membrane (OMM) caused by osmotic swelling (1) releases OMM fragments as well as proteins of the intermembrane space (IMS, small circles). However, removal of the OMM is not quantitative since contact sites between the inner mitochondrial membrane (IMM) and the OMM tether OMM fragments to the IMM, which is mediated by the protein import pore consisting of the translocases of the inner and outer mitochondrial membranes (TIM and TOM). OMM fragments (most likely forming vesicles) can be removed from mitoplasts by centrifugation in a sucrose density gradient (2), which also produces a top phase enriched in IMS. Mitoplasts (mitochondrial matrix surrounded by IMM) can be sub-fractionated by further swelling and sonication (3) and centrifugation (4). IMM fractions will, however, be contaminated with OMM to some degree.

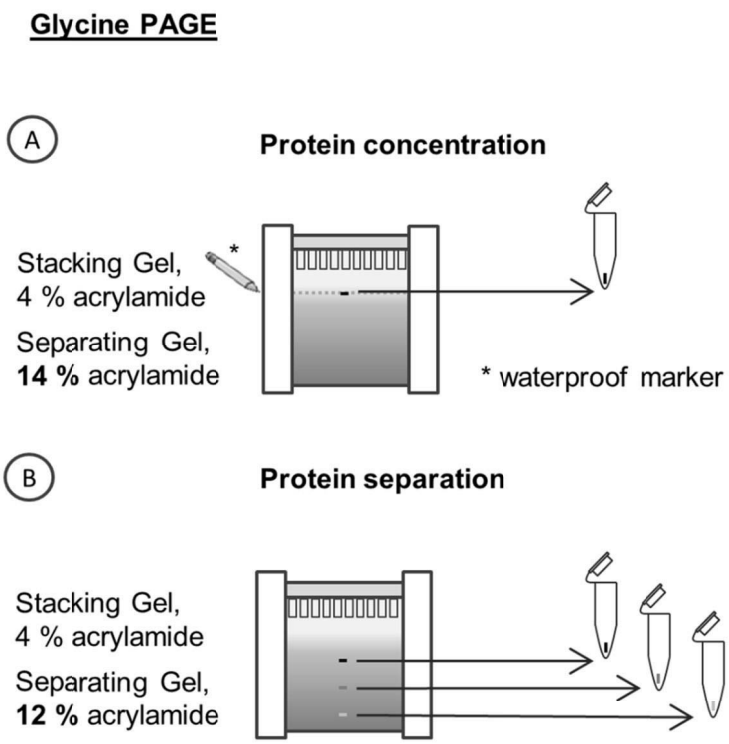

Figure 1: Utilization of glycine PAGE for preparation of shotgun mass spectrometry samples. Glycine gels consisting of the separating gel (dark grey) and the overlying stacking gel (light grey) can be used to deplete detergent form SDS-solubilized proteins and prepare them for mass spectrometry in two ways. Proteins can be concentrated at the border between stacking and separation gel (A). For this, a separation gel with a high acrylamide concentration is used (14 % instead of 12 %). It is recommended to highlight the border between both gel phases with a waterproof marker on the glass plate. When the running front reaches this line the gel run is stopped and a gel slab containing both, stacking and separating gel is cut from the gel and submitted to in gel tryptic digestion. For pre-fractionation prior to MS analysis proteins are separated according to size by means of a standard glycine/SDS PAGE procedure (B). The gel lane is then cut out and sliced. Each gel slab is then dried, digested and analyzed individually.

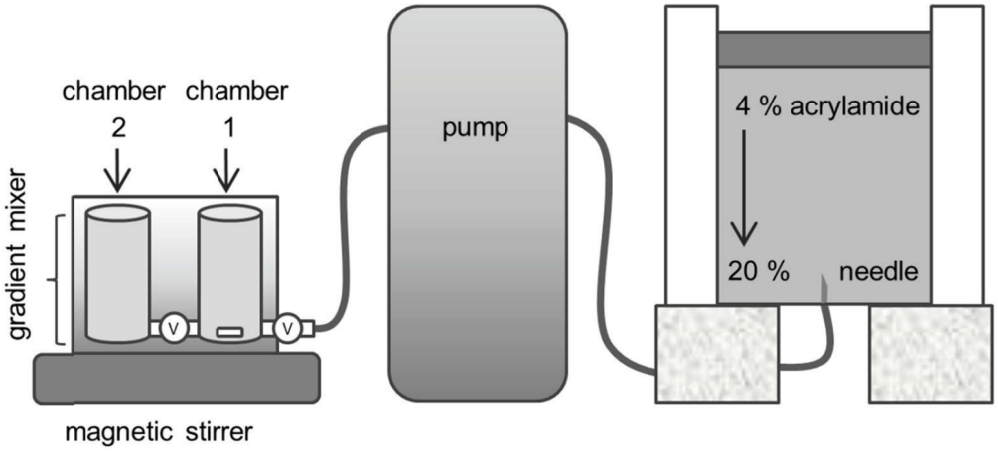

Figure 3: Casting a Blue Native electrophoresis gel. The gradient mixer consists of two chambers linked via a valve (v). Chamber one contains a magnetic stirring bar (white bar). Chambers are filled with the gel solutions given in Table 6. A flexible hose (dark grey line) connects the gradient mixer via the pump with the gel casting device. The hose ends in a hypodermic needle which is pierced between the glass plates from below. For this purpose the gel casting device is placed on two Styrofoam blocks (or similar). Due to the pump continuously removing the light phase (low acrylamide concentration) from the first chamber, the difference in hydrostatic pressure between the chambers is forcing heavy solution (high acrylamide concentration containing glycerol) from the second chamber into chamber 1 where it mixes with the light solution. As such, acrylamide and glycerol concentrations are increasing constantly in the first chamber and the light solution initially pumped between the glass plates is continuously underlaid with heavier gel solution.

7.3 Publication 1

The proteome of higher plant mitochondria

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The proteome of higher plant mitochondria

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ABSTRACT

Plant mitochondria perform a wide range of functions in the plant cell ranging from providing energy and metabolic intermediates, via coenzyme biosynthesis and their own biogenesis to retrograde signaling and programmed cell death. To perform these functions, they contain a proteome of >2000 different proteins expressed in some cells under some conditions. The vast majority of these proteins are imported, in many cases by a dedicated protein import machinery. Recent proteomic studies have identified about 1000 different proteins in both Arabidopsis and potato mitochondria, but even for energy-related proteins, the most well-studied functional protein group in mitochondria, <75% of the proteins are recognized as mitochondrial by even one of six of the most widely used prediction algorithms. The mitochondrial proteomes contain proteins representing a wide range of different functions. Some protein groups, like energy-related proteins, membrane transporters, and de novo fatty acid synthesis, appear to be well covered by the proteome, while others like RNA metabolism appear to be poorly covered possibly because of low abundance. The proteomic studies have improved our understanding of basic mitochondrial functions, have led to the discovery of new mitochondrial metabolic pathways and are helping us towards appreciating the dynamic role of the mitochondria in the responses of the plant cell to biotic and abiotic stress.

1. Introduction

Mitochondria have a central position in the metabolism of plant cells. Not only do they provide energy and metabolic intermediates for the cell, but they also catalyze often terminal steps in the biosynthesis of several coenzymes and cofactors as well as lipids. Their metabolism is fully integrated into cellular metabolism via a range of transmembrane transporters and a number of regulatory mechanisms, which permit them to adjust their metabolism to developmental and environmental clues. Additionally, mitochondria are semi-autonomous and grow and divide. They therefore contain their own DNA as well as their own machinery for DNA replication, DNA transcription, RNA translation and protein synthesis. Since the mitochondrial genome contains only 20–40 protein-coding genes (Kubo and Newton, 2008), the vast majority of the mitochondrial proteins are encoded in the nuclear DNA, synthesized in the cytosol and imported into the mitochondria using a specialized import machinery. Finally, the mitochondria are involved in retrograde signaling and programmed cell death in response to external stimuli like abiotic and biotic stress (Welchen et al., 2014).

Proteomics is the large-scale study of proteins particularly their localization, function and abundance. Since 2001 >20 papers have been published describing various aspects of the plant mitochondrial proteome and we now have extensive lists of proteins identified in isolated plant mitochondria from several different species. These studies have led to a deeper understanding of the structure and function of the classical mitochondrial proteins, e.g. those involved in the tricarboxylic acid (TCA) cycle and oxidative phosphorylation, but also to the discovery of new mitochondrial functions. In this review, we will first describe the methods by which plant mitochondria are isolated and the proteome analyzed using both wet laboratory methods and dry bioinfomatic methods, including coverage of the relevant databases and prediction algorithms. We will then describe the general properties of the mitochondrial proteome before we discuss each of the most important functional groups of proteins in more details with an emphasis on new knowledge gained through the proteomic approach. Finally, we will

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attempt to draw some general conclusions about the mitochondrial proteome and outline the perspectives for plant mitochondrial proteomics in the future.

2. Isolation of mitochondria

Crude mitochondria obtained using differential centrifugation of tissue homogenates are heavily contaminated. Initially (starting in the 1950's), crude mitochondria from non-green tissues, such as etiolated seedlings or tubers, were used for biochemical characterization, probably because they looked relatively uncontaminated, in contrast to mitochondria from green tissues, which were dark green and obviously contaminated by thylakoids to such an extent that they produced, rather than consumed, oxygen when their respiration was measured on a sunny lab bench! (Møller, unpublished observation 1975). Since around 1980, contaminating membrane systems, mainly peroxisomes, membrane vesicles from plastid envelope or thylakoids, have been removed using density gradients. Most density gradients have used Percoll consisting of polyvinylpyrrolidone-coated colloidal silica particles of 10–30 nm in diameter, which means that it is inert and has low osmolarity and low viscosity (Pertoft et al., 1978; Pertoft, 2000). In the first application of Percoll gradient purification of plant mitochondria, a step gradient was used (Jackson et al., 1979), but most subsequent methods have used continuous gradients. A continuous self-generated Percoll gradient can separate mitochondria from other organelles differing in density by only 0.02 g/ml and recognize sub-populations of mitochondria differing in density by even less (Schwitzguébel et al., 1981; Struglics et al., 1993). In this way highly purified mitochondria containing <1% contamination by peroxisomes, plastids and plasma membranes can be isolated from potato tubers. At the same time, damaged mitochondria are removed, which have lost (part of) their matrix content and are lighter as a consequence (Neuburger et al., 1982; Struglics et al., 1993; Considine et al., 2003).

Isolating intact and uncontaminated mitochondria from green tissues has presented unique problems, requiring special solutions to remove the thylakoid vesicles. Bergman et al. (1980) combined phase partitioning, which separates according to surface properties, with a step Percoll gradient to produce chlorophyll-free mitochondria from spinach leaves, while Day et al. (1985) used a combined PVP-25 and Percoll gradient to produce chlorophyll-free mitochondria from pea leaves.

It has been particularly difficult to isolate pure and functional mitochondria from the leaves of the model plant Arabidopsis and here yet another separation technique, free-flow electrophoresis (FFE) has been applied (Eubel et al., 2007). FFE separates particles according to their charge (the zeta potential at the plane of shear) and all plant membrane surfaces including mitochondria have a net negative charge under physiological conditions (Møller et al., 1981; Kinraide and Wang, 2010).

In all the above studies, the purity of the mitochondria was assessed using biochemical markers for the mitochondria and for various potential contaminants. In a few cases, purity was also documented using electron microscopy (e.g., Neuburger et al., 1982). While some of the markers used are considered absolute, e.g., chlorophyll for the thylakoid membrane and cytochrome c oxidase for the inner mitochondrial membrane, others such as catalase are open to question. The presence of dually targeted proteins, the list of which is expanding rapidly, is also making it very difficult to assess purity not least because many of the dually targeted proteins are located both in mitochondria as well as in one of the most persistent contaminants in purified mitochondria, the plastids.

Once the proteins in a preparation of purified mitochondria have been identified (and possibly quantified) by the techniques described in the following sections, it is desirable to establish their mitochondrial localization in an independent way. An established technique is to attach a fluorescent tag to the protein and use fluorescence microscopy to localize the protein in living cells (e.g., Duncan et al., 2011; Salvato et al., 2014). Unfortunately, that method is not suitable for high through-put, so that the long lists of mitochondrial proteins presented later have generally only been verified by in silico techniques, which have severe limitations, as we shall see.

3. Methods to characterize the mitochondrial proteome

3.1. Experimental approaches

Some of the earliest plant proteomic investigations were performed on isolated mitochondria or fractions therein. As such the evolution of proteomic techniques and best practices mirrors the chronological characterization of plant mitochondrial proteomes. The earliest studies of plant mitochondria proteomes primarily employed two-dimensional gel electrophoresis (2-DGE) as a means to resolve proteins prior to protein sequencing and mass spectrometry (Kruft et al., 2001; Millar et al., 2001). From these studies 800 (Kruft et al., 2001) and 350 (Millar et al., 2001) protein spots were resolved and detected prior to identification (Table 1, Table S1). Up to 90 proteins were identified using primarily a peptide mass fingerprinting approach from these investigations. The field advanced to the use of three-dimensional separation employing either blue native (BN) PAGE (Werhahn and Braun, 2002) or size-exclusion chromatography (Bardel et al., 2002) as the third dimension in tandem to 2-DGE. Fractionation of mitochondrial proteins by native size proved to be quite fruitful as a means to resolve the various complexes of the respiratory chain and discovery of novel protein associations that could not be predicted solely using bioinformatics. Many larger-scale proteomic studies of plant mitochondria ensued employing either 2-DGE or BN PAGE in combination with 2D gels, but the number of resolved proteins did not greatly exceed initial investigations.

Millar and Heazlewood (2003) noted that 2-DGE methodology was biased against membrane proteins in a targeted proteomic study of mitochondrial carrier proteins. To better represent hydrophobic proteins the authors employed SDS-PAGE coupled to liquid chromatography

Table 1
The higher plant mitochondrial proteomes – the numbers of experimentally identified mitochondrial proteins.

Species	Mitochondrial proteome	Proteome	References
Solanum tuberosum (potato)	1060	52,760 (PGSC[a])	Salvato et al. (2014)
Arabidopsis thaliana	1005	35,386 (TAIR10)	Lee et al. (2013a) Tan et al. (2012) Duncan et al. (2011) Carrie et al. (2009) Huang et al. (2009b) Heazlewood et al. (2004) Meyer et al. (2011) Kruft et al. (2001)
Oryza sativa (rice)	322	66,338 (RGAP)	Huang et al. (2009a)
Triticum aestivum (wheat)	140	112,496 (IWGSC)	Wang et al. (2015) Kim et al. (2014) Jacoby et al. (2010)
Medicago truncatula	84	62,319 (MTGP)	Dubinin et al. (2011)
Pisum sativum (pea)	49	NA	Bardel et al. (2002)
Kalanchoë pinnata	15	NA	Hong and Nose (2012)
Ananas comosus (pineapple)	12	27,024 (CoGe)	Hong and Nose (2012)

[a] PGSC – The Potato Genome Sequencing Consortium, TAIR10 – The Arabidopsis Information Resource (release 10), RGAP – Rice Genome Annotation Project (release 7), MTGP – *Medicago truncatula* Genome Project v4.0, IWGSC – International Wheat Genome Sequencing Consortium, NA – not available/sequenced, CoGe – Comparative Genomics (PMID 26523774).

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tandem mass spectrometry (LC-MS/MS) as a means to resolve such recalcitrant proteins. This tandem combination of SDS-PAGE coupled to in-gel digestion and LC-MS/MS became popular between 1996 and 2005 due to its simplicity and unbiased representation of all cellular proteins. Around 2001, the term "GeLC-MS" was used to describe this approach though it is unclear who first coined this term. Despite early indications that the GeLC-MS technique could resolve all classes of mitochondrial proteins with limited bias, 2-DGE remained pervasive as an approach for whole mitochondria prefractionation for the next 10 years. During this time the size of the experimental plant mitochondrial proteome swelled to nearly 450 unique proteins. In 2014, Salvato et al. (2014) reintroduced GeLC-MS as a technique to pre-fractionate whole plant mitochondria. In this investigation 1060 unique mitochondrial proteins were identified from potato tuber (Table 1 and Table S1), a system that yields highly pure mitochondria. When compared against the aggregate Arabidopsis mitochondrial proteome accumulated over 12 years and as many publications, over twice as many proteins were identified with less bias against "extreme" proteins (i.e. hydrophic, basic/acidic, or high/low mass). With the recent development of more sensitive mass spectrometers, notably the Q-Exactive line of instrumentation, it is possible to attain near complete coverage of the plant mitochondrial proteome without SDS-PAGE prefractionation, i.e. "gel-free MS" (Møller et al., 2015; Thal et al., 2015). This is currently the state of the art for experimental methods to characterize plant mitochondrial proteomes. Both GeLC-MS and gel-free MS were shown to be quantitative approaches when coupled to either spectral counting or peak integration.

3.2. Bioinformatic approaches

Mitochondrial proteins are targeted through a "conservative" or a "non-conservative" pathway from the cytoplasm. The proteins from the former group have cleavable mitochondrial (matrix) targeting peptide signals (mTPs) in their N-terminus (Claros and Vincens, 1996; Emanuelsson et al., 2000; Bannai et al., 2002), while the proteins from the latter group hardly have any recognizable N-terminal signals. Non-conservative pathways include different mechanisms and may have disparate internal signals (Emanuelsson et al., 2000; Calvo and Mootha,

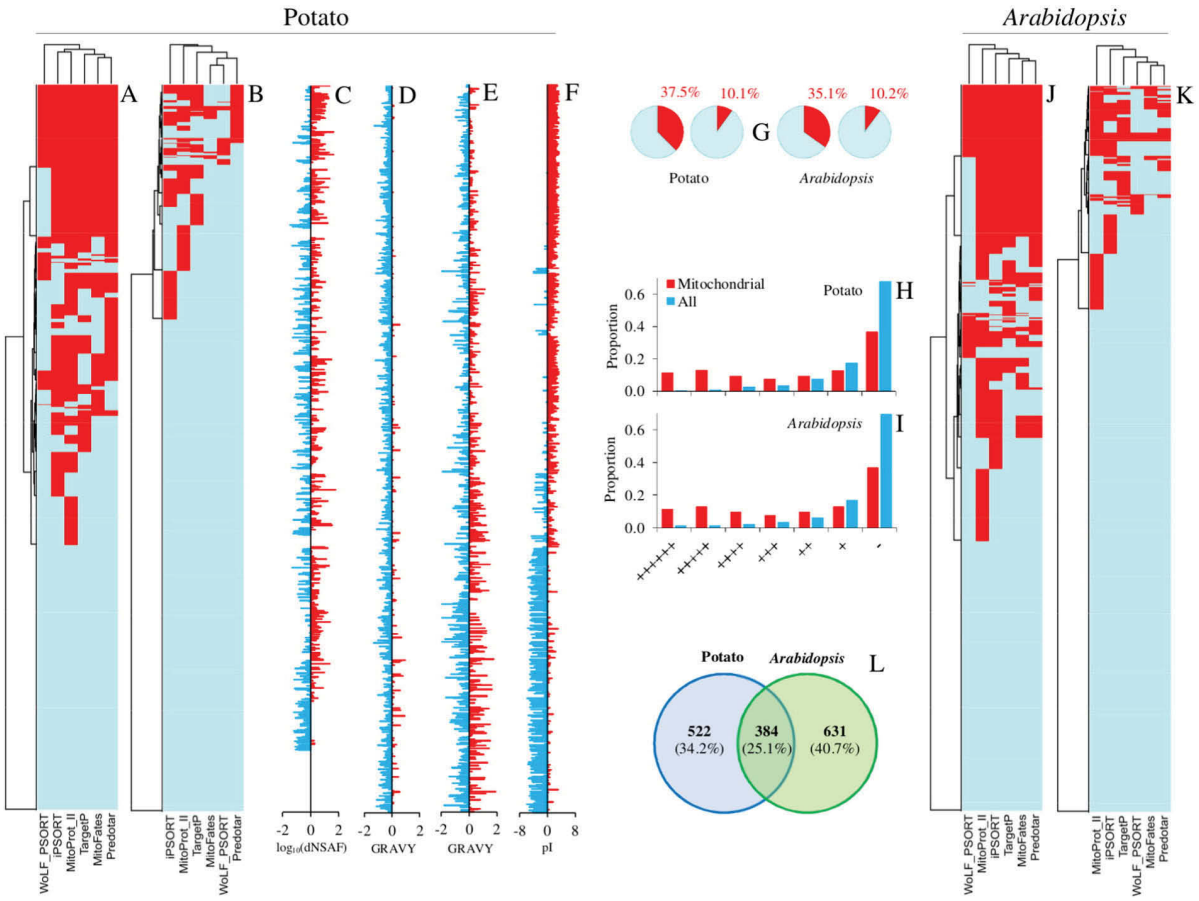

Fig. 1. Identifying mitochondrially targeted proteins. (A, B) Heat map and clustering of mitochondrial targeting prediction results using the six most widely used programs (positive prediction is in red and negative is in light blue). (A) 1060 experimentally identified mitochondrial proteins from potato and (B) an equivalent number (1000) of randomly selected proteins from the potato proteome. (C) Experimentally identified mitochondrial proteins that are also predicted to be mitochondrially targeted by most programs (top/red part of the heat map in A) appear to be the more abundant based on distributed normalized spectral average factor, $\log_{10}$(dNSAF), which ranges from −4.82 to −1.57 (1778 fold difference). Red bars in graphs C to F indicates positive value and blue for negative of mean centered data. Proteins IDs in A, and C to F are in same order. (D) Prediction programs have strong bias against proteins that have positive or strongly negative grand average hydropathy (GRAVY) scores, and (E) this is even more dramatic for N-terminal 30-residues which most prediction programs consider for mitochondrial targeting. (F) Any experimentally identified mitochondrial protein with low isoelectric point (pI, ranges from 3.25 to 12.7, mean centered) for N-terminal 30-residues is never predicted by any programs as they consider the presence of arginine, and the absence of aspartic/glutamic acid in the N-terminal 30-residues as the mitochondrial targeting signal. (G) On average, only 37.5% of experimentally identified potato mitochondrial proteins are predicted to be mitochondrial by prediction programs, whereas 10.1% proteins (several thousands in absolute number) in the proteome are predicted to be mitochondrially targeted. (H) Only a small proportion of experimentally identified mitochondrial proteins are predicted to be mitochondrially targeted by all six/most programs ("All" for general proteome). (J) Experimentally identified 1005 *Arabidopsis* mitochondrial proteins and (K) an equivalent number of 1000 randomly selected proteins from the proteome also show similar patterns (G and I). These results indicate that the mitochondrial targeting prediction programs at best perform poorly, and highlight the need for better experimental/bioinformatic approaches to identify the mitochondrially targeted proteins. (L) A Venn diagram showing the overlap between potato and *Arabidopsis* mitochondrial proteomes (based on BLAST and AtGI numbers).

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2010). Further, N-terminal sorting signals (also called pre-sequences) are not unique to mitochondrial proteins as secretary proteins have signal peptides (SPs), and plastid proteins have transit peptides (cTPs) (Emanuelsson et al., 2000).

Most programs that predict the localization of proteins to mitochondria try to discriminate them using sequence composition and amino acid physicochemical properties (Table S2). For example, iPSORT uses only the first 30 residues for prediction (Bannai et al., 2002), although mTPs are highly variable, and can be up to 120 residues in length (Fukasawa et al., 2015; Jacome et al., 2015). The mTPs are over-represented for amino acid R, A, and S, under-represented for D and E, and form an amphiphilic α-helix (Emanuelsson et al., 2000). In fact, proteins with negative pI for the first 30 residues are not predicted as mitochondrial and prediction tools show strong bias for mTPs with intermediate (near zero) GRAVY value (Fig. 1).

Bioinformatic characterization and prediction of mitochondrial proteins is increasingly moving from purely a sequence or amino acid composition-based method to a more complex and often biologically meaningful approach. One of the simplest, yet important, clues is the motif – such as the presence of amino acid R in −2 or −3 position relative to mTP cleavage site (Emanuelsson et al., 2000). For example, *Arabidopsis* and rice mitochondrial cleavage sites are grouped into three classes namely class I (conserved-2R), dominant class II (−3R, up to 58% of proteins with mTP) and class III (no conserved R, but often with novel motif [F/Y]|[S/A] or other complex motifs) (Fukasawa et al., 2015; Huang et al., 2009a; Savojardo et al., 2014). Other approaches such as homology with known mitochondrial proteins from same/different species using BLAST, mitochondria-specific functional domain analysis using HMMER and HMM from Pfam database, endosymbiont-ancestry comparison using *Rickettsia* homology, co-expression analysis, induction (mRNA up-regulation during mitochondrial proliferation) and information from protein interaction network are also used in in silico identification and characterization (like functional analysis or gene ontology, comparative phylogenetic or genomic/proteomic analysis) of mitochondrial proteins (Calvo et al., 2016; Cui et al., 2011; Desler et al., 2009; Gabaldón and Huynen, 2004; Kim et al., 2009). Numerous mitochondrial proteome databases and sophisticated machine-learning-based prediction tools (see subsequent sections), and other online tools (for example, http://www.expasy.org/tools/, Gasteiger et al., 2003) also assist in identifying/predicting and characterizing mitochondrial proteins. For example, a recent bioinformatic study on the compositional complexity of mitochondrial proteome of a unicellular eukaryote *Acanthamoeba* indicates that it rivals that of animals, fungi, and plants (Gawryluk et al., 2014).

Currently, there exist several challenges in predicting mitochondrial proteins. Foremost, there is a limited number of quality databases on mitochondrial proteins, and the performance of prediction tools is too poor. It is difficult to distinguish mitochondrial proteins for sub-localization (for example to mitochondrial inner membrane, intermembrane space, etc.) (Emanuelsson et al., 2000), or dual/multiple localization – proteins that exist in two or more locations, for example in mitochondria and plastids in plants (Chou and Shen, 2010a; Huang et al., 2009a).

3.3. Databases

The *Arabidopsis* mitochondrial proteome project (AMPP, http://www.genetik.uni-hannover.de/arabidopsis.html) is perhaps the first database on plant mitochondrial proteins (Table S3). Kruft et al. (2001) separated, on gel, about 800 protein spots from *Arabidopsis* mitochondria and identified 52 protein spots using mass spectrometry. The *Arabidopsis* mitochondrial protein database (AMPDB, http://www.plantenergy.uwa.edu.au/ampdb/) greatly expanded experimentally identified mitochondrial proteins. It lists 416 proteins from *Arabidopsis* mitochondria (Heazlewood and Millar, 2005), and is presently the only dedicated database on experimentally identified plant mitochondrial proteins. Databases such as SUBA3 provide consolidated/integrated information on plant-specific subcellular localization of proteins (Table S3).

While very limited information/databases are available on plant mitochondrial proteins (an exception is https://gelmap.de, Rode et al., 2011; Klodmann et al., 2011), starting with MitoDat, there are numerous databases on human or mammalian and fungal mitochondrial proteins (Table S3). However, almost all these databases present the results based on the computational prediction or inference of mitochondrial proteins. For example, MitoMiner (http://mitominer.mrc-mbu.cam.ac.uk/) is the most recent database that integrates different types of subcellular localization evidence with protein information from public resources, which include 58 mass spectrometry and GFP tagging studies, and claims to provide a comprehensive central resource for data on mitochondrial proteins from 12 species, but with only single plant species, *A. thaliana* (Smith and Robinson, 2016). As usual with most other databases, this resource is not entirely experimental-based as it includes information from computational predictions of mitochondrial targeting sequences and evidence from homology mapping. Further, while this database lists a meager 483 mitochondrial proteins for *A. thaliana*, the numbers of mitochondrial proteins for mouse (3076) and yeast (1291), for example, seem to be unrealistically high.

Although not in any database format, information on many more (experimentally identified) mitochondrial proteins from *Arabidopsis* is available. Cui et al. (2011) predicted a set of 2311 mitochondrial proteins (named ArathMitoP) in *Arabidopsis* using computational methods and together with other experimentally identified proteins they consolidated 2585 proteins (named CoreMitoP) as *Arabidopsis* mitochondrial proteins. However, the majority of these proteins are predicted and therefore do not have any experimental support for mitochondrial localization. Lee et al. (2013a) consolidated 841 experimentally identified mitochondrial proteins from *Arabidopsis*. In this review, we collated all experimentally identified mitochondrial proteins from *Arabidopsis* (Carrie et al., 2009; Duncan et al., 2011; Heazlewood et al., 2004; Huang et al., 2009b; Lee et al., 2013a; Millar et al., 2001; Tan et al., 2012), and present a list of 1005 proteins (Table 1, Table S1).

Until recently, *Arabidopsis* was the only plant species with information on mitochondrial proteins. Although information on mitochondrial proteins from many other species, especially from higher plants, is now available (Table 1 and Table S1), these are not present in the form of any database. For example, Salvato et al. (2014) identified 1060 mitochondrial proteins from potato (raw data present in ProteomeXchange as PXD000149), which is perhaps the largest set of mitochondrial proteins identified in any single study using mass spectrometry. Consolidated databases for different species will be useful to quickly retrieve the relevant information on mitochondrial proteins.

3.4. Prediction algorithms

As described above, the plant mitochondrial proteome is thought to comprise >2000 proteins and finding the complete set of proteins is a prerequisite to fully understand the mitochondrial biology. While a number of proteins identified experimentally early on led to the definition of classical mitochondrial energy metabolism cycles and pathways, identifying thousands of proteins experimentally is expensive, laborious, and cumbersome. Thus, even today, only a fraction of mitochondrial proteins of any model organism is thought to have been identified or experimentally characterized. In contrast, computational approaches are fast and inexpensive, and when combined with accuracy provide a complementary way to explore the mitochondrial proteins.

PSORT (http://psort.hgc.jp/form.html) is possibly the first computational tool to attempt the subcellular and extracellular localization of nuclear proteins (Nakai and Kanehisa, 1992). It uses an expert system of "if-then rules" knowledgebase of experimentally known sorting and localization signals in proteins from different (plant, animal, yeast, and bacterial) models, and classifies them as mitochondrial, chloroplastic, or proteins destined to any of 15 other locations. Given the then limited

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availability of proteins with localization information, the accuracy of this early classifier was very low at 59%. Regardless of its poor performance, interest in this early classifier lead to a plethora of more advanced and powerful computational tools (Table S2) for the prediction of subcellular and extracellular localization of nuclear proteins (Meinken and Min, 2012).

MitoProt and MitoProt II (https://ihg.gsf.de/ihg/mitoprot.html) that followed PSORT used a discriminant analysis (DA) approach to identify mitochondria-specific N-terminal target peptide and cleavage site using 47 parameters derived from protein sequence (Claros, 1995; Claros and Vincens, 1996). With the availability of more experimentally characterized protein sequences, PSORT was upgraded to PSORT II (http://psort.hgc.jp/form2.html), albeit only for yeast and animal sequences (Nakai and Horton, 1999). As it was not possible to find more if-then rules from the additional sequences, prediction algorithm in PSORT II was changed from expert system to K-nearest neighbors (k-NN) approach. TargetP (http://www.cbs.dtu.dk/services/TargetP/) is the most popular program (based on number of citations, Table S2) currently used for the mitochondrial localization prediction (Emanuelsson et al., 2000). It uses a neural network (NN) to predict the N-terminal pre-sequence and cleavage site. iPSORT (http://ipsort.hgc.jp/) uses a decision list to classify mitochondrial from secretory/other proteins (Bannai et al., 2002). Predotar (https://urgi.versailles.inra.fr/predotar/predotar.html) is a NN based classifier for N-terminal target sequence detection and mitochondrial localization (Small et al., 2004). Another very popular prediction tool WoLF PSORT (http://www.genscript.com/wolf-psort.html), similar to its predecessor PSORT II, uses k-NN algorithm to identify sorting signal and classifies mitochondrial from other proteins (Horton et al., 2007). There are numerous other tools (Table S2) such as MitPred, MultiLoc, pTARGET, TESTLoc, SUBAcon, etc. which use a variety of algorithms including support vector machine (SVM) for the prediction of mitochondrial proteins. Further, some tools claim to predict the localization of proteins destined to any location in the cell, for example, Euk-mPLoc 2.0 (http://www.csbio.sjtu.edu.cn/cgi-bin/EukmPLoc2.cgi) predicts as many as 22 locations (Chou and Shen, 2010a). A few tools such as Plant-mPLoc (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/) are specific for the prediction of subcellular localization of proteins in plants (Chou and Shen, 2010b) and SUBAcon is specific for Arabidopsis (Hooper et al., 2014). MitoFates (http://mitf.cbrc.jp/MitoFates/) is the latest mitochondrial prediction tool that uses SVM to identify target sequence and cleavage site, and claims to perform better than all previous popular tools such as TargetP, MitoProt II, Predotar, and TPpred2 (Fukasawa et al., 2015).

Different mitochondrial protein localization prediction tools use just as diverse algorithms ranging from expert system to DA, k-NN, NN, decision tree (DT), hidden Markov model (HMM), and SVM, although recent tools mostly use SVM. Further, some tools use a combination of different algorithms such as DT of SVMs (in BaCelLo) or ensemble of k-NN (in Euk-mPLoc 2.0). These algorithms/tools depend on the input of sequence composition (usually from N-terminal 30 residues as in iPSORT) and physico-chemical properties of amino acids. Apart from the conventional machine learning-based classifiers, mitochondrial protein localization predictions are increasingly performed on the basis of biologically meaningful information such as sequence homology and ancestry, presence of specific motifs and domains, co-expression profiles, protein interaction network, etc. (Calvo et al., 2016; Cui et al., 2011).

Currently, all prediction algorithms/tools have very poor performance as they are tuned to detect mostly the presence of mitochondria-specific N-terminal target signal or pre-sequence. However, pre-sequence based import is only one of several mechanisms by which proteins are localized to mitochondria in vivo (Calvo and Mootha, 2010). As a result, they give many false-negative predictions (low sensitivity, high type II error) when applied to experimentally identified mitochondrial proteins, and numerous false-positive proteins (low specificity, high type I error) when applied to entire proteomes. For example, when the six most popular prediction tools (iPSORT, MitoFates, MitoProt II, Predotar, TargetP, and WoLF PSORT) were run on 1060 experimentally identified mitochondrial proteins from potato and 1005 proteins from *Arabidopsis*, 47.9% (MitoProt II) to 83.7% (WoLF PSORT) of proteins (63.7% on average) were not predicted as mitochondrial (Fig. 1). Conversely, these tools predict 2.5% (MitoFates) to 18% (MitoProt II) of proteins (10.2% on average) in the total cellular proteomes as mitochondrial, and in absolute numbers this turns out to be several thousand proteins with predicted mitochondrial targeting pre-sequences. For instance, using just three prediction tools, Smith and Robinson (2016) showed that *Arabidopsis* proteome has at least 6323 proteins with pre-sequences. Given that these programs identify pre-sequences on average only in 36.3% of experimentally identified mitochondrial proteins, and the pre-sequence based import is only one of the several mechanisms of localization, extrapolation of prediction results on proteome scale would result in over 17,000 *Arabidopsis* proteins as mitochondrial! These prediction tools have similar performance problems with other proteomes (Calvo and Mootha, 2010). Thus, while this calls for better prediction algorithms, a purely computational approach for the identification of mitochondrial proteins may have limited authenticity.

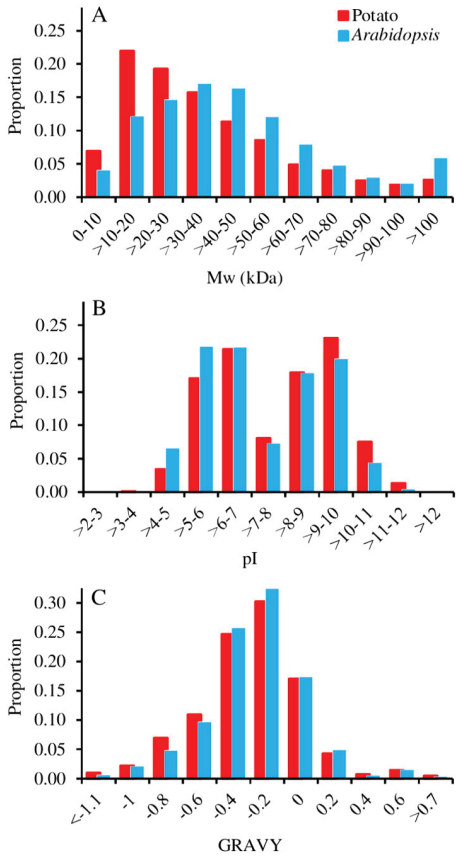

Fig. 2. Distribution of molecular mass (A), pI (B), and GRAVY (C) for potato and *Arabidopsis* mitochondrial proteomes. (A) A large number of potato mitochondrial proteins are of small molecular weight (<30 kDa) compared to *Arabidopsis*. However, this difference may be attributed to whole proteomes of these two species (Fig. S2). Mitochondrial proteins in general tend to have slightly higher molecular weight. (B) Potato mitochondrial proteome has proportionally higher pI compared to *Arabidopsis* indicating more basic residues. Further, mitochondrial proteins have higher pI (more basic) compared to whole proteomes (Fig. S2). (C) The GRAVY distributions are overall similar in two mitochondrial proteomes – which show preference for intermediate scores compared to whole proteomes (Fig. S2).

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4. The mitochondrial proteome

4.1. General properties

We have analyzed the size and isoelectric point of the experimentally identified proteins in potato and Arabidopsis mitochondria (Table S1) and compared them to each other and to the properties of the total proteome in the two species.

The potato mitochondrial proteins have a smaller median size than in Arabidopsis, while Arabidopsis mitochondria contain more proteins larger than 100 kDa (Fig. 2). Curiously, this also appears to be true for the entire proteome of the two organisms (Fig. S1). The pI for the mitochondrial proteins shows a distinct two-humped pattern with a very marked dip at pH 7–8 (Fig. 2). Again this pattern is repeated in the entire organismal proteomes. Considering that the protein concentration in the mitochondrial matrix and other cellular compartments is very high (>400 mg/ml; Srere, 1980) and that the frequency of acidic (aspartate and glutamate) and basic (lysine and arginine) amino acid sidechains is around 10%, it means that proteins by virtue of their enormous buffering capacity (> 100 mM) both towards low and high pH stabilize the matrix (and cellular) pH between pH 6 and 9. As the pH in the matrix is between 7.5 and 8.0 (Douce, 1985) very few matrix proteins will be at their pI, which might minimize protein aggregation.

The potato and Arabidopsis mitochondrial proteomes contain 31 and 27% hydrophilic proteins – proteins with a GRAVY score below −0.4 – but only 3 and 4% hydrophobic proteins – proteins with a GRAVY score above 0.2 (Fig. 2). Compared to the total organismal proteome, the mitochondrial proteomes contain significantly fewer very hydrophilic or very hydrophobic proteins (Fig. S1).

The functional categories Energy and Metabolism contain the largest number of experimentally identified proteins in both potato and Arabidopsis mitochondria, while the categories Cellular structural organization and Transcription contain the fewest (Fig. 3A). Arabidopsis mitochondria contain relatively more proteins involved in Energy and in Metabolism, while potato mitochondria contain more in RNA processing and in Unclassified proteins (Fig. 3A). The ability of six prediction programs to recognize the proteins differs very markedly between the functional categories. Proteins involved in RNA processing and Energy are particularly well recognized (but still <75% are recognized by at least one program), while proteins involved in Cellular structural organization are not recognized at all (Fig. 3B,C).

In spite of the great care taken to remove contaminants from mitochondria during isolation, the lists of identified mitochondrial proteins (Table S1) still contain proteins, such as well-established stromal enzymes, that are unlikely to be dually targeted. Similarly, well-established peroxisomal proteins, such as catalase, appear regularly hinting at a mitochondrial function.

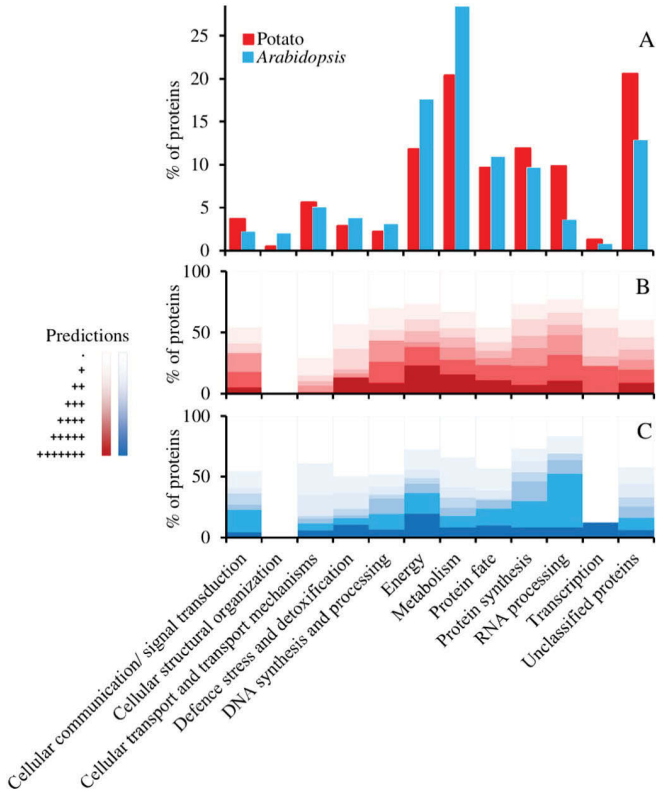

Fig. 3. Functional classification of experimentally identified mitochondrial proteins. (A) Functional classification shows metabolism followed by energy as the major classes; however, in comparison with *Arabidopsis*, potato has much lower proportion of proteins in these two classes. A large proportion of proteins (Table S1), especially in potato, are unclassified – their exact biological functions may be unknown. Mitochondrial predictions may be slightly biased (darker colour [more + signs] means positive prediction by more programs), both in (B) potato and (C) *Arabidopsis*, for more abundant functional classes. Proteins related to cellular structural organization (Table S1) are never predicted to be mitochondrial. Many transport related proteins, including TOM and TIM are also not favorably predicted.

4.2. Energy metabolism

A vast number of dehydrogenases engaged in diverse pathways are located within the mitochondrial matrix and supply the mitochondrial electron transfer chain (mETC) with reducing equivalents. These include (but are not limited to) the enzymes of the tricarboxylic acid cycle (TCA, including the pyruvate dehydrogenase complex, PDC) as well as numerous other dehydrogenases, many of which are involved in amino acid metabolism. Together, these enzymes are represented by at least 54 gene accessions in the Arabidopsis genome (Rasmusson and Møller, 2011; Schertl et al., 2014; Cavalcanti et al., 2014, Table S4). While many of the non-TCA cycle dehydrogenases contribute only little to the NADH/FADH2 pool, others become the main driving force of oxidative phosphorylation under certain conditions. Oxidation of photorespiratory glycine, for example, is the prevalent source of reducing equivalents in leaf mitochondria in the light while amino acid catabolism becomes important during a state of carbon starvation (Hildebrandt et al., 2015). Irrespectively of their source, reoxidation of reducing equivalents is performed by the entry points to a branched respiratory chain consisting of four multiprotein subunits and additional alternative enzymes (see also later). In Arabidopsis 149 accessions are associated with the transfer of electrons from NADH or FADH2 to molecular oxygen and the subsequent phosphorylation of ADP by the mitochondrial ATP synthase complex (Table S4; Millar et al., 2004a; Braun et al., 2014; Klodmann et al., 2011). In total, this accumulates to a list of 205 target Arabidopsis proteins. Of these 164 were detected in Arabidopsis while 111 of their homologs were found in potato. However, many Arabidopsis accessions appear more than once in the potato tuber list since BLAST searches of potato proteins with similar amino acid sequence are having a common best hit in Arabidopsis. Because these potato proteins have different accessions, the redundant Arabidopsis hits should also be taken into account. The potato proteome list therefore most likely contains 145 proteins involved in energy metabolism. Altogether, nearly 90% of all expected energy-related proteins are present within the two datasets. Using a set of 726 confirmed mitochondrial proteins in Arabidopsis, it was estimated that >50% (35.6% OXPHOS, 15.9% TCA cycle incl. pyruvate metabolism) of the protein content can be attributed to TCA cycle and OXPHOS components (Lee et al., 2013a). Distributed normalized spectral abundance factors (dNSAF) for the experimentally identified potato mitochondrial proteins suggest that OXPHOS and TCA cycle enzymes (including PDC) contribute 17.4% and 7.0%, respectively, to the potato mitochondrial proteome, less than half the amounts reported for the (smaller set) of confirmed

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Arabidopsis mitochondrial proteins. Despite this difference in protein abundance, the Arabidopsis and potato datasets are remarkably similar. In both sets, the energy-related proteins make up similar proportions of all identified proteins (Fig. S2). When comparing the potato and Arabidopsis OXPHOS dataset, this similarity also becomes apparent and can most likely be attributed to the finding that integrities of respiratory complexes often hinge on the presence of a full set of subunits (Kühn et al., 2011; Meyer et al., 2011; Wydro et al., 2013; Dahan et al., 2014; Fromm et al., 2016). The slightly higher coverage of OXPHOS proteins in the Arabidopsis protein list is partly due to a reduced set of alternative oxidoreductases in the potato set containing less than half of the alternative respiratory enzymes. Consistent with this observation, the capacity of alternative oxidase is rather low in potato tuber mitochondria, but is inducible by abiotic stress, for example cold (Zhou and Solomos, 1998). Differences between the potato and Arabidopsis datasets are slightly more pronounced for TCA enzymes and other matrix dehydrogenases (Fig. S2). Here, potato contained only about 80% of the proteins found in Arabidopsis. The observed differences are most likely not (only) species specific but embody the different physiological properties of the plant material used to prepare mitochondria for proteomic analyses.

4.3. Transporter proteins

Integration of mitochondrial functions into cellular metabolism requires bidirectional transport of metabolites from the matrix to the cytosol and vice versa (Wiedemann et al., 2004). The outer membrane is freely permeable for small molecules and metabolites up to 6 kDa by voltage-dependent anion channels (VDACs). Six VDAC genes have been identified in Arabidopsis of which four are expressed (Robert et al., 2012) and found in the Arabidopsis dataset. In contrast, the membrane potential across the inner mitochondrial membrane (IMM) necessitates a highly restricted transport. In total, 59 and 51 transport proteins were identified in potato and Arabidopsis, respectively, among which 33 (potato) and 21 proteins (Arabidopsis) with known ion and metabolite transporter activities are found.

4.3.1. Mitochondrial carrier family (MCF) members in plant mitochondria

MCF members contribute considerably towards plant mitochondrial metabolite transport (Haferkamp and Schmitz-Esser, 2012). The Arabidopis genome encodes 58 MCF members which are found in the IMM as well as in other, non-mitochondrial membranes of peroxisomes, chloroplasts, the endoplasmic reticulum and the plasma membrane (Picault et al., 2004; Haferkamp and Schmitz-Esser, 2012). MCF genes are highly conserved enabling good inter-species identification (Palmieri et al., 2011). To assess common and diverging features of Arabidopsis and potato mitochondria we searched for MCF members in the Arabidopsis and potato datasets (Fig. S3, Table S5). The Arabidopsis and potato datasets list 51 and 59 proteins, respectively, which are associated with 'cellular transport and transport mechanisms'. Within the list of the 58 MCF proteins eleven were found in both Arabidopsis and potato. These engage in the transport of phosphate (MPT3), adenylates (AAC2, AAC3, ANT1), di-/tricarboxylates (DIC1, DTC), carnitine/acylcarnitine (BOU), and S-adenosylmethionine (SAMC1), suggesting that the main metabolite transport requirements of plant mitochondria are covered in both species (Palmieri et al., 2011). Furthermore, the list comprises two hitherto uncharacterized MCF proteins and a plant uncoupling protein (UCP1). For each, BOU, ANT1, and MPT3, two potato accessions have a common best hit in Arabidopsis pointing towards the presence of additional transporters with similar specificities in potato. However, 32 MCF members (>50%) are not present in either dataset. Indeed, for more than half of the MCF members, a mitochondrial location is at least doubtful or can be excluded on the basis of their experimental findings summarized in the SUBA3 database (http://suba3.plantenergy.uwa.edu.au, Hooper et al., 2014, data not shown). This situation is exemplified by FOLT1, a putative plastid folate transporter found in the potato mitochondria dataset. Mitochondria, as well as plastids, contain folate-dependent enzymes, hence a need exists for folate transport into both organelles (Waller et al., 2012). GFP assays predict a plastid localization in Arabidopsis, but its knock-out did not affect plastid folate concentration (Bedhomme et al., 2005). This either means that additional folate transporters are present in Arabidopsis chloroplasts or that the folate transporter is located in non-plastidic membranes, such as the IMM. It is often difficult to establish the mitochondrial localization of identified transporters due to the difficulty of removing all contaminating membranes and the presence of dually targeted proteins.

4.3.2. Non-MCF transport proteins in plant mitochondria

Ions, like K^+, pass through the IMM via selective and regulated ion channels (Jarmuszkiewicz et al., 2010). Functional assays indicate that ATP-inhibited and -insensitive K + channels are present, but their corresponding genes are currently unknown (Pastore et al., 1999; Ruy et al., 2004). Interestingly, the potato dataset contains a putative beta subunit of K + channels. Ca2 + is considered to be an important secondary messenger, also in mitochondria. Several mitochondrial carriers and proteins with metabolic activities are Ca2 + regulated (Møller and Rasmusson, 1998; Rizzuto et al., 2000; Lorenz et al., 2015; Wagner et al., 2015). Consequently, Ca2 + transport systems in mitochondria are required, but not well characterized yet (Deryabina et al., 2004; Wagner et al., 2015). Two proteins are annotated as mitochondrial calcium carriers (MCU1, MCU2), one of which has recently been localized in mitochondria via GFP assays (Carraretto et al., 2016). However, neither was found in the two datasets. Instead, the potato proteome contains a Ca2 + antiporter/cation exchanger (Use1). Transport of water across the IMM and the subsequent swelling and contraction of the organelles impacts on mitochondrial physiology (Calamita et al., 2005; Lee and Thévenod, 2006; Casteilla et al., 2011). In plants, an aquaporin 8 homolog termed tonoplast intrinsic protein TIP5;1 with dual function in water and urea transport has been identified in pollen mitochondria (Soto et al., 2010). While this protein has not been found, aquaporin PIP2B was identified in the potato dataset. Of further interest are eight non-MCF member proteins present in both potato and Arabidopsis datasets, which indicates that there is a high probability of mitochondrial origin. Among these eight proteins are three confirmed mitochondrial proteins (VDAC1/2, and the ABC transporter ATM3). The other five are annotated as two putative cytochrome c oxidase assembly factors (HCC1/2), two outer envelope membrane proteins (ATOEP7/16), and a vacuolar ATP synthase subunit (VHA-A).

It can be concluded that the majority of mitochondrially localized MCF-members are found within the potato and Arabidopsis datasets. However, identification of currently unknown, non-MCF mitochondrial transporters is a challenging task, which is hampered by low protein abundances and often contradicting information on localization. In the future, comparisons of more and higher-coverage plant mitochondrial proteomes may help identifying new candidate transporter proteins.

4.4. Protein import, synthesis, and degradation

More than 100 proteins were identified belonging to the category protein synthesis in both potato and Arabidopsis mitochondria and the numbers were similar for the category protein fate (Fig. 3A).

4.4.1. Protein import

Given that plant mitochondria contain >2000 proteins and that fewer than 50 are encoded in the mitochondria genome, >95% of all mitochondrial proteins are nuclear-encoded, synthesized on cytosolic ribosomes and imported across both mitochondrial membranes, which therefore contain a protein import complex each (Glaser and Whelan, 2011). The mitochondrial proteomes contain a number of proteins associated with the protein import machinery in OMM and IMM: Potato/Arabidopsis mitochondrial proteomes contain 12/11 transporters

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inner membrane (TIM) and 8/9 transporters outer membrane (TOM). As a study focusing on characterizing the OMM proteome also found only eight TOM proteins (Duncan et al., 2011), we may have full coverage for this complex in potatoes and Arabidopsis.

4.4.2. Protein biosynthesis and degradation

The biosynthesis of mitochondrially encoded proteins requires the presence of ribosomes and the full complement of tRNA synthases to incorporate all 20 amino acids into the nascent polypeptide chains. The potato/Arabidopsis proteomes contain 14/7 tRNA synthetases, respectively, as well as 6/12 elongation factors and 83/60 ribosomal proteins (Table S1). This is strong evidence that protein biosynthesis is fully active in mitochondria from both species.

Protein degradation can also occur via a number of routes in mitochondria. The three classes of ATP-dependent proteases, FtsH, Clp and AAA proteases are all found in both potato and Arabidopsis mitochondria (a total of 9 and 16, respectively). Peptidases (12/16), as well as a number of protease inhibitors (10/2), are also found in potato and Arabidopsis mitochondria (Table S1). Protein degradation in plant mitochondria therefore appears to be tightly regulated.

4.5. Lipid metabolism

Plant mitochondria synthesize the eight-carbon cofactor lipoic acid through a de novo fatty acid synthesis (FAS) pathway that resembles the type II pathway of prokaryotes with some exceptions. Malonate is provided from the cytosol and activated to malonyl-CoA by a synthetase (MCS) enzyme (Chen et al., 2011) then transferred to acyl carrier protein (ACP) through a dedicated S-transferase for which a putative gene has been identified in Arabidopsis (At2g30200). A single ketoacyl-ACP synthase (KAS, At2g04540) then condenses the malonyl-ACP with acetyl-ACP to produce the nascent acyl-ACP in Arabidopsis (Yasuno et al., 2004). This KAS enzyme elongates the acyl-ACP to make the eight-carbon precursor for lipoic acid and potentially up to 14:0-ACP for other functions in the mitochondria. The remaining three enzymes of the type II fatty acid synthase, ketoacyl-ACP reductase (KAR), hydroxylacyl-ACP dehydrase (HAD), and enoyl-ACP reductase are each multigenic families in Arabidopsis. The potato tuber mitochondrial proteomic study identified nearly all of these components for de novo FAS, including four ACPs, one ACP-S-malonyltransferase, one KAS, one KAR, one HAD, and one enoyl-ACP reductase (Salvato et al., 2014, Table S1). The only missing enzyme was MCS, however, a protein annotated as "acyl-activating enzyme 10" which is homologous to At3g16170, the previously reported MCS, suggests that the potato tuber mitochondrial proteome captured the entire complement of enzymes required for synthesis of 8-14C fatty acids from malonate. Furthermore, each of the identified proteins was also predicted to be mitochondrially localized.

In addition to the enzymes of FAS, a lipoyl synthase (LS) and octanoyltransferase, were both identified in potato tuber mitochondria. The LS enzyme converts 8:0-ACP to lipoic acid-ACP using S-adenosyl L-methionine and molecular sulfur as substrates in a group transfer reaction (Yasuno and Wada, 1998). Octanoyltransferase is the acyltransferase responsible for transferring the lipoic acid moiety from lipoic acid-ACP directly to the conserved Lys on the E2 subunits of the three alpha-keto acid dehydrogenase complexes and H-protein of glycine decarboxylase.

The complete coverage of de novo FAS and the lipoic acid synthesis and transfer pathway from the single proteomic study of potato tuber mitochondria contrasts the collective proteomic studies from other plants. From the rice mitochondrial study, two ACPs and one KAS enzyme were identified and from Arabidopsis only one enoyl-ACP reductase was identified. Not a single FAS or lipoic acid enzyme was found in wheat or Medicago (Table S1).

The potato tuber mitochondrial proteome also contains other proteins associated with lipid metabolism, including many enzymes of jasmonic acid biosynthesis. Additionally mitochondria from potato tuber contain proteins annotated as acyl-coenyzme A thioesterase 9, probable cardiolipin synthase 1, acyltransferase, malonyl-CoA decarboxylase and three of the enzymes involved in the beta oxidation pathway for 2C fatty acid degradation. For some of these proteins like cardiolipin synthase the functional role is clear. However, the role of a malonyl-CoA decarboxylase in plant mitochondria is decidedly unclear and represents an opportunity to discover a new pathway or shunt. The finding of enzymes of the beta-oxidation pathway in the mitochondrial proteome may indicate that the mitochondria are contaminated by peroxisomes. On the other hand, it is possible that this pathway is dually localized in plant cells.

4.6. Amino acid turnover

Mitochondria synthesize some amino acids and are also the site of branched chain amino acid (BCAA) degradation (Hildebrandt et al., 2015). The latter pathway was particularly prominent in the various plant mitochondrial proteomic studies. BCAAs include Val, Ile, and Leu and conversion to α-ketoisovalerate, α-keto-β-methylvalerate, and α-ketoisocaproate, respectively, by a BCAA transaminase initiates their degradation. These α-keto acids are the substrate for the committed step for BCAA degradation catalyzed by the branched-chain ketoacid dehydrogenase (BCKD), a family member of the α-keto acid dehydrogenase multienzyme complexes (Mooney et al., 2002). Collectively, the three parallel pathways for conversion of BCAA to propionyl-CoA, acetyl-CoA, and acetoacetate require a minimum of 20 different proteins comprising 15 enzymatic steps as shown in Fig. 4. Within the potato tuber proteome a near complete pathway for degradation of Leu was detected. The only missing enzyme was the BCAA transaminase, though two general aminotransferases were detected that could be candidates for this step. Additionally, two enzymes of the Ile pathway were observed and one enzyme from the Val pathway. The Arabidopsis mitochondrial proteomic collection contained a BCAA transaminase, a partial BCKD and MCCase, and three additional enzymes from each of the three parallel pathways for BCAA degradation. The only other mitochondrial proteome study that identified members of the BCAA degradation pathway was from rice; two enzymes from both the Val and Leu pathway were identified.

Other enzymes involved in amino acid synthesis and degradation were also detected in the various proteomic studies. In potato, the following enzymes involved in amino acid metabolism were observed: a Pro synthase, Cys desulfurase, Asp aminotransferase, Ala aminotransferase, multiple subunits to the glycine decarboxylase complex (GDC), and two proteins annotated as β-cyanoalanine synthases previously observed to be Cys synthases (Hatzfeld et al., 2000). In Arabidopsis, the following amino acid metabolic enzymes were found: two Ala aminotransferases, two arginases, Arg biosynthesis protein (ArgJ), two Asp aminotransferases, Cys desulfhydrase, two Glu dehydrogenases, and components to the GDC.

4.7. Coenzyme biosynthesis

4.7.1. Ascorbic acid

The last enzyme in one of the two biosynthetic pathways for ascorbate, L-galactono-1,4-lactone dehydrogenase, has been found in both potato and Arabidopsis mitochondria (Table S1). It donates electrons to Complex IV via cytochrome c (Bartoli et al., 2000), but it is structurally associated with Complex I (Millar et al., 2003; Pineau et al., 2008). Although plant mitochondria contain several very important enzymes utilizing ascorbate (see below) it is unclear how ascorbate traverses the IMM and enters the matrix. One possible mechanism is that it is transported by a member of the phosphate transporter 4 family, similar to what occurs across the inner chloroplast envelope, where AtPHT4;4 is responsible for the uptake of ascorbate into the chloroplasts (Miyaji et al., 2015). Several phosphate transporters have been identified in the

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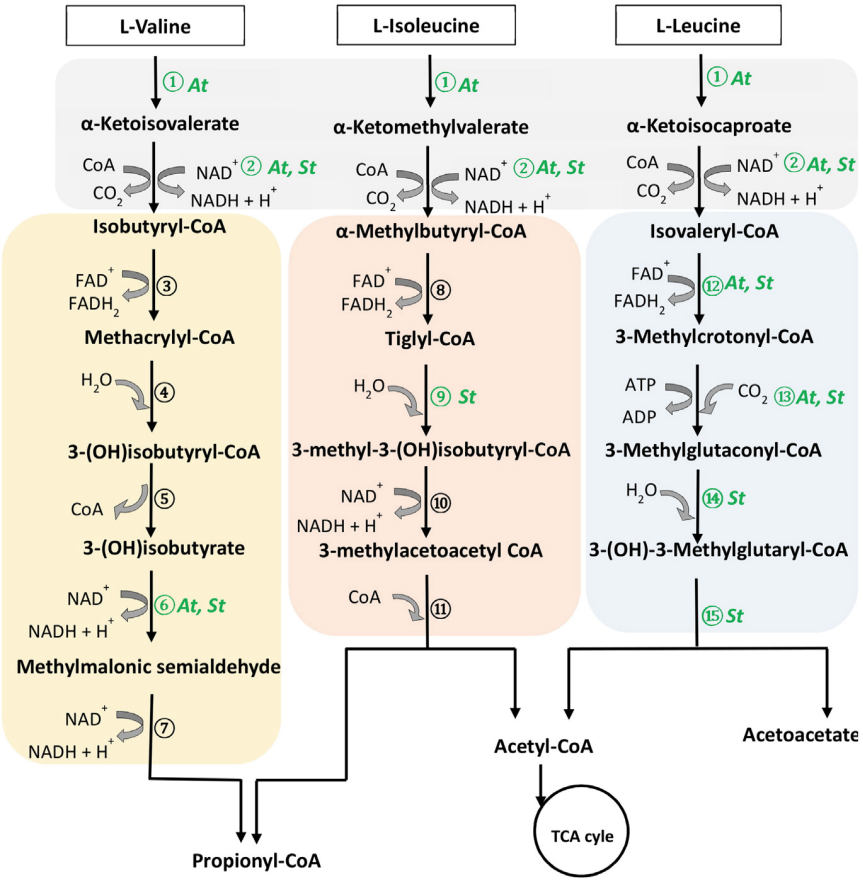

Fig. 4. Branched-chain amino acid degradation pathways. Enzymes are numbered from 1 to 15 as follows: 1:branched-chain amino acid transaminase; 2: branched-chain ketoacid dehydrogenase; 3: isobutaryl CoA dehydrogenase; 4: methacrylyl CoA hydratase; 5: hydroxyisobutyryl CoA hydrolase; 6: hydroxyisobutyryl CoA dehydrogenase; 7: methylmalonate semialdehyde dehydrogenase; 8: short-branched chain acylCoA dehydrogenase; 9: enoyl-CoA hydratase; 10: 3-hydroxylacyl-CoA dehydrogenase; 11: 3-ketoacyl CoA thiolase; 12: isovaleryl CoA dehydrogenase; 13: methylcrotonyl CoA carboxylase; 14: 3-methylglytaconyl CoA hydratase; 15: 3-(OH)-3-methylgutaryl CoA lyase. Green numbers correspond enzymes identified in Arabidopsis (At) or potato (St) mitochondrial proteomes. Grey-shaded area represents common reactions using enzymes 1 and 2 in the degradation of amino acids. Coloured-shaded areas represent independent reactions in the degradation of the three amino acids (Valine, Leucine, Isoleucine).

mitochondrial proteomes of potato, Arabidopsis and rice (Table S1), but none of them are apparent PHT4.4 homologs. Ascorbate has been reported to be taken up in the form of dehydroascorbate by a common glucose/dehydroascorbate transporter in BY2 tobacco mitochondria, since glucose and some of its derivatives inhibited dehydroascorbate uptake strongly (Szarka et al., 2004). Within the mitochondrial matrix, dehydroascorbate is then reduced to produce ascorbate by the dehydroascorbate reductase (see below). Finally, ascorbate could be transported by a Na^+-ascorbate co-transporter similar to the one in human mitochondria (Munoz-Montesino et al., 2014), but no sodium-dependent proteins have been identified in any of the proteomes (Table S1).

4.7.2. Biotin

Biotin (vitamin B8) is a coenzyme involved in carboxylation reactions, and the last steps in its biosynthesis are mitochondrial (Alban, 2011). Both potato and Arabidopsis mitochondria contain the S-adenosylmethionine carrier responsible for the import of one of the precursors. They also contain adrenodoxin reductase involved in biotin biosynthesis as well as five ferredoxin analogs, which may well be adrenodoxin, used by adrenodoxin to reduce S-S bridges. Thus, a significant part of the biotin biosynthesis pathway is expressed in plant mitochondria.

4.7.3. Folate

Tetrahydrofolate (vitamin B9) and its derivatives act as coenzymes in reactions where C1 units are added or removed (e.g. in the Gly decarboxylate reaction in the mitochondrial matrix, where a methyl group is transferred from one Gly molecule to another to form Ser). Folates consist of a pterin moiety, a p-aminobenzoate moiety, and a (poly) Glu tail, and the three parts are assembled in the mitochondria (Blancquaert et al., 2010).

Potato mitochondria contain three enzymes involved in folate biosynthesis (dihydropterin pyrophosphokinase-dihydropteroate synthase, a molybdopterin biosynthesis protein, and folylpolyglutamate synthase), five enzymes involved in the interconversion and transfer of different C1 units (5-formyltetrahydrofolate cycloligase, formyltetrahydrofolate deformylase-like, methenyltetrahydrofolate synthase domain-containing protein-like, 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase-like, and bifunctional dihydrofolate reductase-thymidylate synthase-like), as well as a folate carrier responsible for exporting folate to make the coenzyme available to the rest of the cell (Table S1). The bifunctional dihydrofolate reductase-thymidylate synthase-like enzyme is interesting because it catalyzes the conversion of a uracil base to a thymidine base. All of these proteins were present at low to medium relative abundance in potato mitochondria (Salvato et al., 2014). In

Arabidopsis only three enzymes involved in folate interconversions (dihydrofolate synthetase, tetrahydrofolate dehydrogenase/cyclohydrolase, dihydrofolate synthetase) have been found possibly because the rest were below the detection limit (Table S1).

4.7.4. NADP(H)

NAD^+ is actively imported from the cytosol across the IMM (see section on transporters) and it can be reduced to NADH by a range of NAD^+-linked dehydrogenases, e.g. those in the TCA cycle. Although NADP was traditionally seen as connected mainly with the metabolism in the cytosol and the plastids, plant mitochondria actually contain a number of enzymes requiring NADP as the coenzyme (Møller and Rasmusson, 1998) including several involved in ROS removal (see section on Defence against oxidative stress). There have been reports of $NADP^+$ uptake into plant mitochondria (Bykova and Møller, 2001), but the molecular mechanism has not been identified. An alternative is provided by the NADH kinase, which converts NADH to NADPH, found in potato mitochondria, but not yet in any other proteomic study (Table S1).

4.8. Iron-sulfur centres

Potato mitochondria contain all of the proteins necessary for the biosynthesis of iron-sulfur centres, while only a few of them have been found in Arabidopsis mitochondria to date (Table S1; Balk and Schaedler, 2014; Salvato et al., 2014). In addition, potato and Arabidopsis mitochondria both contain ABC transporters (ATM2, ATM3; Table S5) potentially responsible for the export of glutathione and glutathione trisulfide for use in the biosynthesis of iron-sulfur centres in the cytosol (Schaedler et al., 2014).

4.9. RNA metabolism

RNA processing is one of the protein categories where there is the largest difference between the number of proteins identified in potato and Arabidopsis mitochondria, 104 and 36, respectively (Fig. 3, Table S1). The prediction programs are particularly adept at recognizing mitochondrial proteins involved in RNA processing (Fig. 3B,C).

PPR proteins are one of the most prolific protein families in plants, while it is virtually absent in animals (Small and Peeters, 2000). The majority of the 450 PPR proteins in Arabidopsis are predicted to be mitochondrial, where about half are thought to be involved in RNA editing and the remainder in other types of RNA processing (Fujii and Small, 2011). A total of 71 PPR proteins were found in potato mitochondria, whereas only 18 have been found in Arabidopsis mitochondria to date (Table S1). This is remarkable since Arabidopsis cell cultures, the source of most of the mitochondria used for proteomic studies (Table 1), would be expected to contain actively growing and dividing mitochondria and hence higher protein abundances of RNA-editing proteins.

4.10. DNA metabolism

Mitochondria contain their own DNA, which need to be replicated, transcribed and repaired. DNA polymerases, DNA gyrases, RNA polymerases, RNA helicases, histones, a histone-modifying enzyme, topoisomerases, transcription factors, and transcription termination factors, representing most of the components required for DNA replication and transcription, have been found in potato and Arabidopsis mitochondria (Table S1). Several of the histones identified are of a type reported to be present in plant mitochondria (Zanin et al., 2010). Proteins involved in DNA repair processes such as an endonuclease and a dCK/dGK-like deoxyribonucleoside kinase have also been found (Table S1). Such repair systems are essential to prevent the accumulation of mtDNA damage, which can occur for instance as a result of ROS-induced oxidation (Møller et al., 2007, Boesch et al. 2009, Roldan-Arjona and Ariza 2009) and which is thought to contribute to ageing at least in animals (Maynard et al. 2015).

4.11. Defence against oxidative stress

Mitochondria are one of the main sites of production of Reactive Oxygen Species (ROS) in the plant cell (Maxwell et al., 1999; Møller, 2001; Foyer and Noctor, 2003). ROS can be used in signaling (Møller and Sweetlove, 2010; Ng et al., 2014), but their accumulation can also damage a range of cellular components, nucleic acids, proteins, lipids and carbohydrates (Møller et al., 2007). Enzymes and enzyme systems responsible for keeping the ROS level low are found many places in the cell (Mittler et al., 2004), and the mitochondria have a very extensive ROS scavenging system. The superoxide, formed mainly by the ETC (Møller, 2001; Brand, 2010), can be removed by superoxide dismutases (SOD), the classical Mn-SOD in the matrix space and a Zn,Cu-SOD in the intermembrane space, both of which have been found in potato mitochondria (Table S1). SOD converts superoxide into another ROS, H_2O_2, which also needs to be removed. Removal of H_2O_2 is done by five enzymes/enzyme systems using NADPH as reductant – ascorbate/glutathione cycle, glutathione peroxidase, thioredoxin system, peroxiredoxin system and glutaredoxin system (Mittler et al., 2004; Møller et al., 2007). They have all been found in mitochondria from potato and Arabidopsis (Table S1). Quite high catalase activity, which also removes H_2O_2, is always found in plant mitochondria even after extensive purification (e.g., Neuburger et al., 1982; Struglics et al., 1993; Eubel et al., 2007). Proteomic studies found two types of catalase in potato and three types in Arabidopsis mitochondria (Table S1). We do not know whether these catalases are located inside the mitochondria or whether they are attached to the OMM and therefore possible contaminants. The observation that catalase is found in the matrix of rat heart mitochondria (Radi et al., 1991) demonstrates that import into mitochondria can occur.

4.12. Structural proteins

Mitochondria move around in the cytosol with cytoplasmic streaming by interacting with the cytoskeleton. It is therefore not surprising that we find components of the cytoskeleton - tubulin, actin, actin-polymerizing factor, myosin – in the mitochondrial proteome. However, there are many more such proteins in Arabidopsis mitochondria than in potato mitochondria (20 vs 4 different proteins; Table S1) possibly because there is more cytoplasmic streaming in the actively growing Arabidopsis cells than in the storage cells of potato tubers? This could well be an example of organ- or tissue-dependent expression. It is striking, but perhaps not surprising given that these proteins are specifically designed to bind to OMM proteins, that the prediction programs are completely unable to predict the localization of any of these structural proteins to the mitochondria (Fig. 3, Table S1).

4.13. Communication and signaling

The pyruvate dehydrogenase kinase (PDK) is, at present, the only protein kinase in plant mitochondria that has been characterized both biochemically and at the molecular level (Thelen et al., 1998; Thelen et al., 2000). This kinase inactivates the pyruvate dehydrogenase complex (PDC) by phosphorylating a highly conserved Ser residue on the α-subunit of the pyruvate dehydrogenase component enzyme (Ahsan et al., 2012). The PDK is responsive to physiological metabolite and divalent cation concentrations to modulate PDC activity in response to demand for acetyl-CoA and reducing equivalents (Rubin and Randall, 1977; Budde and Randall, 1988). The PDK is highly specific towards the conserved Ser in the active site of the E1α subunit, as conservative single or double point mutations surrounding this residue dramatically reduces or abolished activity towards the peptide substrate (Ahsan et al., 2012). Thus it seems improbable that the PDK could have other

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protein clients that it regulates. However, phosphoproteomic studies have revealed a total of 64 phosphoproteins in plant mitochondria (Bykova et al., 2003; Ito et al., 2009; summarized in Havelund et al., 2013) and at least 10 more were identified by Salvato et al. (2014). Thus additional protein kinases must be targeted to mitochondria. Large-scale proteomics offers a glimpse into the nature of such possibilities.

The potato tuber mitochondrial proteome study (Salvato et al., 2014) revealed two PDK isoforms and four additional protein kinases, annotated as putative Ser/Thr protein kinase, probable Ser/Thr protein kinase, protein kinase, and uncharacterized aarF domain-containing protein kinase (Table S1). The latter two of these putative protein kinases were predicted to be mitochondrial. Additionally, two protein phosphatases were observed, both annotated as probable protein phosphatase 2C 55-like; both are predicted to be mitochondrial. The Arabidopsis mitochondrial proteome contains a PDK and eleven protein kinases including five leucine rich repeat receptor like kinases, three protein kinase superfamily proteins, a concanavalin A-like lectin protein kinase family, a lectin protein kinase family protein and a MAP kinase 9, none of which are predicted to be mitochondrial. No protein kinases have been found in wheat, Medicago, or rice mitochondria and no protein phosphatase was identified in any species other than potato.

Other signaling proteins detected in potato tuber mitochondrial preparations include two calcium ion binding proteins, a calcium ion channel, annotated as leucine zipper-EF-hand containing transmembrane protein, and members of the Ras GTP hydrolase proteins and Rab monomeric G proteins annotated as small GTPase Rab2, RabE1, Ras-related protein RAB8–3, Ras-related protein RABE1c-like, Rho GTPase 1. Additionally, nine GTP-binding proteins were identified. Of these proteins, only three of the GTP-binding proteins were predicted to be mitochondrial. The Arabidopsis studies also identified a calcium ion-binding protein, a calcium ion sensing receptor, and five Rab GTPases, none of which were predicted to be mitochondrial.

4.14. Proteins with posttranslational modifications

Proteins can be modified in a large number of different ways, and mitochondrial proteins are no exception. Common, and often regulatory, posttranslational modifications are oxidations (Møller et al., 2011; Nietzel et al., in this issue), acetylation (Hosp et al., in this issue) and phosphorylation (Havelund et al., 2013; Kruse and Højlund, in this issue). More than half the proteins detected in the potato mitochondrial proteome were posttranslationally modified on at least one site (Salvato et al., 2014). About 100 proteins had >10 PTM sites. The most modified protein, Gly dehydrogenase, had as many as 50 PTM events of six different kinds. Even for the most abundant proteins, the spectral counts were usually very low for each modified peptide identified. This implies that each protein is present in a large number of slightly different forms.

5. Heteroplasmy

Heteroplasmy in plant mitochondria is quite common, but it usually refers to the presence of different populations of mtDNA (Arrieta-Montiel and Mackenzie, 2011). Whether mtDNA heteroplasmy translates into heteroplasmic mitochondrial proteomes is an open question. Different subpopulations of mitochondria within a given cell type have been observed and different cell types in a tissue can contain different mitochondria (Logan, 2004). The development of photosynthesis and accompanying photorespiration in pea leaves resulted in a marked shift in mitochondrial density from 1.04 g/ml in young leaves to 1.09 g/ml in mature leaves (Vauclare et al., 1996). This was mainly due to a massive increase in the two enzymes involved in photorespiration, serine hydroxymethyl transferase and glycine decarboxylase, which ended up constituting 40% of the matrix protein. Vauclare et al. (1996) then wrote "It is interesting that cell organelles isolated from whole pea leaflets containing leaves of various ages yield a mixed population of mitochondria, leading to the impression that two distinct populations of mitochondria exist in leaf tissues." It is not clear whether individual cells in leaves of intermediate age contained both types of mitochondria.

6. Tissue- and organ-specific expression

The mitochondrial proteome is dynamic being affected by tissue/organ type and developmental and/or environmental changes. Different organs or tissues have particular metabolic needs and the mitochondrial proteome assumes different expression profiles to accommodate those needs. For example, plant mitochondria are known provide ATP for the cell, but even that core function can be modified in photosynthetic cells, where mitochondria and chloroplasts are inter-dependent (Krömer, 1995), due to the considerable energetic and metabolic exchange between the two organelles (Hoefnagel et al., 1998). When ATP demand for CO_2 fixation is high in the chloroplasts, the cytosolic ATP is provided by the mitochondria. By contrast, when ATP consumption is low in the chloroplasts, the ATP produced there will be exported to the cytosol (Gardeström and Igamberdiev, 2016).

The Arabidopsis mitochondrial proteome was first characterized for non-photosynthetic cells (Kruft et al., 2001; Millar et al., 2001; Heazlewood et al., 2004). Later studies compared the mitochondrial proteome in cell cultures vs shoots (Lee et al., 2008), roots vs shoots (Lee et al., 2011) and vegetative vs reproductive stages (Lee et al., 2012). Although non-photosynthetic cells do not represent specialized cells, in which mitochondria assume specialized metabolic functions, they can serve as a starting point to understand the core mitochondrial properties. The proteomes of Arabidopsis suspension cells (Heazlewood et al., 2004), potato tuber (Salvato et al., 2014) and etiolated rice seedlings contained many membrane carriers, components of the electron transport chain, protein import apparatus and proteins involved in coenzyme biosynthesis, etc. as described above.

Building on the improved understanding of the basal mitochondrial proteome, developmental changes in the mitochondrial proteome were compared between plant organs, tissues and cell types (Fig. 5). The first large-scale mitochondrial proteome study conducted in different plant organs was reported in peas (Bardel et al., 2002). This work revealed 433 spots from green leaf mitochondria and some proteins identified specifically in etiolated leaves, roots or seed mitochondria, demonstrating the impact of tissue differentiation at the mitochondrial level. Comparison of the mitochondrial proteome of photosynthetic and non-photosynthetic cells demonstrated high abundance of photorespiratory enzymes and a decreased abundance of many TCA cycle and respiratory enzymes in photosynthetic cells (Lee et al., 2008, 2011). Enzymes from the glycine decarboxylase complex (GDC) and serine hydroxymethyltransferase (SHMT) were up-regulated in photosynthetic shoot mitochondria compared to suspension cells (Lee et al., 2008) and roots (Lee et al., 2011) consistent with the oxidation of glycine at high rates during photorespiration in green tissues. Similarly, the increased abundance of formate dehydrogenase (FDH) in shoots compared to suspension cells and roots indicated an altered one-carbon metabolism (Lee et al., 2008, 2011). The characterization of mitochondrial proteome differences between reproductive and vegetative tissues was demonstrated among six tissues of Arabidopsis (Lee et al., 2012). Many isoforms showed differential regulation among tissues and several enzymes involved in amino acid metabolism were up-regulated in reproductive tissues. The subunit 1 of malate dehydrogenase (MDH1) was up-regulated in flowers compared to root and cell culture mitochondria, while MDH2 was more abundant in roots than in reproductive tissues. In a similar way, GDC-P1 and GDC-P2 were more abundant in leaf and reproductive tissues, respectively (Lee et al., 2012).

Interestingly, the effect of ageing on the plant mitochondrial proteome was reported for the first time during nodule senescence in common bean plants with major decreases of enzymes related to purine biosynthetic pathway and increased concentrations of oxidized proteins

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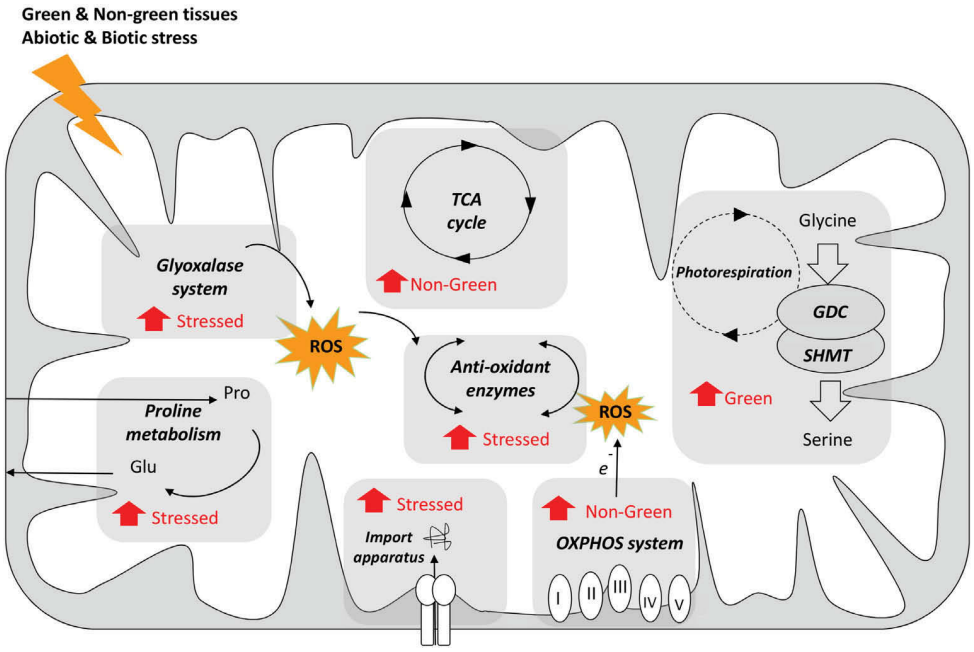

Fig. 5. General metabolic differences between photosynthetic and non-photosynthetic tissues or stressed and non-stressed tissues reported for mitochondrial proteomes. Red arrows indicate increased protein abundances for each pathway shown (Lee et al., 2008, 2011, 2012; Taylor et al., 2005; Schertl et al., 2014). Although the whole OXPHOS system is shown to be increased in non-green tissues in this figure, Peters et al. (2012) reported the opposite response for Complex I.

(Matamoros et al., 2013). These findings combined with enzymatic assays lead to the conclusion that mitochondria are early targets of oxidative modifications and an important source of redox signals in aged nodules.

The general responses of the mitochondrial proteome to various developmental (see above section) and environmental (see below section) cues are summarized in Fig. 5.

7. Response to abiotic and biotic stress

The response of the mitochondrial proteome to abiotic and biotic stress has also been studied although mainly by targeted experiments (gene overexpression or knockout mutants) rather than by large-scale proteomics. Both types of experiments brought to light new aspects of the mitochondrial proteome and its adaption to new ambient conditions.

For example, in Brassica plants, an upregulation of glyoxalase I and II activities was observed in response to Zn^{2+}, salinity, drought and ABA treatment (Veena et al., 1999; Saxena et al., 2005). These enzymes are involved in the detoxification of the cytotoxic methylglyoxal accumulated during stress and in that way help to prevent cellular damage.

In another study, pea leaves exposed to drought, cold and herbicides maintained ETC activity, while differential protein responses of the non-phosphorylating respiratory pathway and the general import pathway were observed (Taylor et al., 2005). At lower temperatures, potato leaf mitochondria showed decreased expression and lower capacity of internal rotenone-insensitive NADH oxidation, while the alternative oxidase was not affected (Svensson et al., 2002). In contrast, mild stress imposition such as cold hardening and hydrogen peroxide treatment, resulted in increased activity of the alternative oxidase, uncoupling protein and HSP70 (Lyubushkina et al., 2011).

One of the main tolerance strategies developed in plants against drought stress is the accumulation of so-called compatible solutes, such as proline, that lower the osmotic potential without being damaging to the cell (Taiz et al., 2014). During drought stress proline accumulated in the cytosol and mitochondrial ETC activity decreased (Gibon et al., 2000). Mitochondrial proline dehydrogenase activity increased in proline-treated Arabidopsis cells indicating upregulation of the proline catabolic pathway (Schertl et al., 2014). Additionally, the D-lactate dehydrogenase activity also was increased suggesting a regulatory role of the D-lactate as a competitive inhibitor of proline dehydrogenase (Schertl et al., 2014).

Large-scale comparative proteomic studies have been performed with mitochondria under different environment conditions. In the absence of oxygen, very low abundance of cytochrome b/c_1, cytochrome c oxidase and alternative oxidase was observed in rice seedling mitochondria (Millar et al., 2004b; Howell et al., 2007). In addition, the protein import capacity was correlated with the abundance of α and β subunits of mitochondrial processing peptidase, which are part of the cytochrome b/c_1 complex. In this way, the mitochondrial protein import apparatus was linked to the respiratory chain (Howell et al., 2007).

Salinity stress has also been demonstrated to affect the mitochondrial proteome in wheat (Jacoby et al., 2013). Mitochondria from roots and shoots of a salt-tolerant amphiploid wheat variety (octoploid) were compared to mitochondria from a reference wheat variety (hexaploid). Higher abundance of manganese superoxide dismutase, serine hydroxymethyltransferase, aconitase, malate dehydrogenase and β-cyanoalanine synthase were detected in the tolerant variety (Jacoby et al., 2013). Salinity stress was also used to study the role of mitochondria in programmed cell death (PCD) in rice roots (Chen et al., 2009). In this work, eight proteins were associated with early stage PCD response. It was suggested that the down-regulation of ATP synthase is an indication that this protein may not be solely involved in the ATP production in mitochondria during early stage of PCD in rice. In addition, the down-regulation of cytochrome c oxidase subunit 6b, decreased activity of complex I and reduced respiratory rates all indicated that the ETC was affected in the early stage of PCD. Furthermore, alternative oxidase (AOX) activity was kept at low level, supporting the idea that salt stress impaired the respiratory dehydrogenase and the oxidase, partially blocking the ETC.

8. Comparison to mitochondrial proteomes in non-plant organisms

With greater evolutionary distance, differences in the mitochondrial proteomes are expected to increase. Plants, animals, and fungi possess different life styles underpinned by diverging metabolic activities. In

plants photosynthesis profoundly interferes with (and depends on) plant mitochondrial functions.

Mitochondrial proteins can be divided into two subcategories: the first contains the enzymes executing mitochondrial metabolism. In terms of protein abundance this group makes up the majority of mitochondrial proteins and its members are well represented in mitochondrial proteome lists. The second group is involved in the genetic apparatus and facilitates DNA replication and gene expression. Its members are often of lower abundance, thereby making MS detection more difficult.

Mitochondrial enzymes participating in photorespiration are important hallmarks of leaf mitochondrial metabolism. Consequently, this pathway is supported by high abundances of GDC and SHMT (Oliver, 1994). However, both enzymes are also present in mitochondria of non-green plant organs and non-plant mitochondria such as those of liver and yeast (Hiraga and Kikuchi, 1980; Stover and Schirch, 1990; Piper et al., 2002; Lee et al., 2013b). Therefore, differences in mitochondrial glycine metabolism between plants, animals and fungi are a matter of protein amounts rather than their absence/presence. This also holds true for the majority of other metabolic enzymes of plant mitochondrial enzymes, for example in TCA cycle, and amino acid metabolism. One example of significant difference between plant and non-plant mitochondria is the composition of the ETC. Each plant respiratory complex contains plant-specific subunits, many of which are not involved in electron transport or proton translocation (Millar et al., 2004a; Braun et al., 2014). In many cases, functions of these additional enzymes have not yet been elucidated. Also, plant (and fungal) mitochondria contain Type II NAD(P)H dehydrogenases and alternative oxidases absent in mammalian mitochondria (Svensson and Rasmusson, 2001; Rasmusson et al., 2008). In contrast, fatty acid beta oxidation enzymes are regularly found in isolated plant mitochondria. Their presence is reduced by further organelle purification steps (see above), but it is still an open question whether they are functionally associated with plant mitochondria (Eubel et al., 2007). We conclude that the majority of the proteins involved in mitochondrial metabolism are present in the eukaryotic kingdoms of plants, animals and fungi, albeit at altered abundances.

More distinct differences can be expected for proteins involved in mitchondrial replication, transcription and translation in since the large, multi-molecular and highly complex mitochondrial genomes require a greater investment in organization and maintenance (Mackenzie and MacIntosh, 1999). Furthermore, gene expression in plant mitochondria involves *trans*-splicing and RNA editing. One particular group of proteins mostly absent in animal and fungal mitochondria are PPR proteins involved in RNA processing, editing, splicing, and translation. In addition, they also affect RNA stability (Manna, 2015). Angiosperms possess up to 600 of these proteins, the majority of them localized in mitochondria, while the rest is targeted to chloroplasts. By comparison, the numbers for human and yeast mitochondria are seven and twelve, respectively, two orders of magnitude lower (Lightowlers and Chrzanowska-Lightowlers, 2013). Furthermore, some ribosomal proteins of plant mitochondria were found to be of nuclear/plastid origin (Adams et al., 2002). Hence, proteins involved in the maintenance, organization and expression of the mitochondrial proteomes are expected to vary to a higher degree between animals, fungi, and plants than those proteins involved in mitochondrial metabolism. Detailed comparisons have not yet been made.

9. Conclusions and perspectives

The full plant mitochondrial proteome is probably in excess of 2000 different proteins. At present, the best of the predicting algorithms are able to recognize less than half of the proteins even among energy-related proteins, the most well-studied functional group. So there is a clear need for better prediction algorithms that build on the knowledge gained in recent high-coverage proteomic studies.

Not surprisingly, the prediction algorithms find it particularly difficult to recognize proteins that are not imported into the mitochondria, but have a functional association with the outer mitochondrial membrane. Examples of such proteins are cytoskeleton components, glycolytic enzymes and ER-mitochondrial contact points. We wish to include such proteins in the mitochondrial proteome to highlight the functional association.

With lists of 1000 identified mitochondrial proteins and an expected full mitochondrial proteome containing >2000 proteins in total, the best coverage in Arabidopsis and potato at present is probably not much >50%. So we can still expect to discover new mitochondrial proteins and new functions. It is highly likely that we will find many more PPR proteins, which are involved in all aspects of RNA metabolism, and we can also expect to find many proteins involved in a range of DNA repair processes.

There are at least two ways of achieving a complete coverage of the plant mitochondrial proteome: (i) Analyze mitochondria from all the organs of one plant species, from different developmental stages and under different environmental conditions, particularly different biotic and abiotic stress conditions. (ii) The ever increasing sensitivity of the analytical methods will allow us to identify more low-abundance proteins, such as DNA repair enzymes. An open question in this connection is whether the lowest possible amount of a given protein species is one molecule per mitochondrion. It has been argued that because of the stochastic nature of protein biosynthesis and import and the frequent mitochondrial fission and fusion we can expect to find that many low-abundance proteins are only present in each mitochondrion part of the time (Møller, 2016). To detect sub-stoichiometric proteins will obviously be a challenge to the sensitivity of the analytical methods.

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Abbreviations

2-DGE	Two-Dimensional Gel Electrophoresis
BN-PAGE	Blue-Native Poly Acrylamide Gel Electrophoresis
cTP	Chloroplast Transit Peptide
dNSAF	Distributed Normalized Spectral Abundance Factor
ETC	Electron Transport Chain
FFE	Free-Flow Electrophoresis
GRAVY	Grand Average Hydropathy
GeLC-MS	Gel Electrophoresis Liquid Chromatography Mass Spectrometry
IMM	Inner Mitochondrial Membrane
mETC	Mitochondrial Electron Transport Chain
mTP	Mitochondrial (Matrix) Targeting Signal/Peptide
OMM	Outer Mitochondrial Membrane
ROS	Reactive Oxygen Species
PPR	Pentatricopeptide Repeat Protein
SDS	Sodium Dodecyl Sulfate
SP	Signal Peptide
TCA	Tricarboxylic Acid

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Curriculum Vitae

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Academic education

Since Oct. 2013	PhD student at the Leibniz Universität Hannover, Institute of Plant Genetics, Department of Plant Proteomics, Prof. Dr. Hans-Peter Braun Research Training Group GRK1798 "Signaling at the Plant-Soil Interface" Thesis: “Towards the analysis of mitochondrial physiology in nitrogen fixing root nodules”
Oct. 2011 - Oct. 2013	Student at the Leibniz Universität Hannover, M. Sc. Plant Biotechnology (Grade: “with distinction”) Thesis: „Characterization of MLO- homologs from Roses“
Oct. 2008 - Sept. 2011	Student at the Leibniz Universität Hannover, B. Sc. Plant Biotechnology (grade: “good”) Thesis: „Genetic analysis of Self-Incompatibility in Roses“

School education

1999 - 2006	Secondary school „Nelly Sachs Gymnasium” in Berlin Qualification: “Abitur”
1996 – 1999	Primary school in Berlin
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Conference presentation

2015	Functional and proteomic comparison of *V. faba* root and symbiotic root nodule mitochondria, 2nd Doctoral Researchers Conference of GRK 1798, Goslar
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Publications

1. **Thal B.**, Thal B., Braun H.P. and Eubel H. (2017) Proteomic analysis shows the specific impact of nodulation and biological nitrogen fixation on *Vicia faba* root nodule physiology, to be submitted

2. Schikowsky C., **Thal B**., Braun H.P. and Eubel H. (2017) Sample preparation for analysis of the plant mitochondrial membrane proteome. Methods in Molecular Biology: Plant Membrane Proteomics, in press

3. Rao R.S.P., Salvato F., **Thal B**., Eubel H., Thelen J.J. and Moller I.M. (2016) The proteome of higher plant mitochondria. Mitochondrion, in press, doi: 10.1016/j.mito.2016.07.002

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— Isaac Newton