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Departamento de Biotecnología-Biología Vegetal

Molybdenum Metabolism in *Azotobacter vinelandii* and its
Biotechnological Applications

PhD Thesis

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BSc in Agricultural Engineering

MSc in Agricultural and
Forestry Biotechnology

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A mis padres

“La medida de la inteligencia es la capacidad de cambiar”

Albert Einstein

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Como cada viaje, tiene su fin, y el mío aquí termina con una gran etapa de mucho esfuerzo y dedicación. Sin embargo, estos 8 años no hubieran sido iguales sin todas esas personas que han marcado momentos inolvidables.

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Abbreviations

Abs	Absorbance
Amp	Ampicillin
AOH	Arms of homology
APS	Ammonium persulfate
APSC1	Adenosine 5'-phosphosulfate carrier
ATP/ADP	Adenosine triphosphate / Adenosine diphosphate
Av NifQ	<i>Azotobacter vinelandii</i> NifQ
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BNF	Biological nitrogen fixation
BSA	Bovine serum albumin
CDS	Coding sequence
CI	Competitive index
cPMP	Cyclic pyranopterin monophosphate
DIC	Differential interference contrast
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphate
DTH	Sodium dithionite
DTT	1,4-dithiothreitol
DUNN buffer	Carbonate buffer
DW	Dry weight
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced Green fluorescent protein
FeMo-co	Iron molybdenum cofactor
FID	Flame ionization detector
FPLC	Fast protein liquid chromatography

GB	Golden Braid
GC-2014	Gas Chromatograph 2014
gDNA/cDNA	Genomic/Complementary DNA
GTP	Guanosine triphosphate
HEPES	N-(2-hydroxyethyl)-piperazine-N-(2-ethanesulfonic acid)
HphMX	Hygromycin selection marker (Hygro)
HRP	Horseradish peroxidase
HSP60	Heat shock protein 60
IAA	Indole-3-acetic acid
ICP-MS	Inductively coupled plasma mass spectrometry
ICP-OES	Inductively coupled plasma optical emission spectroscopy
IPTG	Isopropyl- β -D-thiogalactopyranoside
KanMX	Kanamycin A selection marker (G418)
kb/bp	Kilobase pair /pair of bases
kDa	kiloDalton
Km	Michaelis constant
l/ml/ μ l	Liter / milliliter / microliter
LB	Luria-Bertani
LiAc	Lithium acetate
LTR	Long terminal repeat
MALDI/TOF	Matrix Assisted Laser Desorption Ionization - Time of Flight
mM/ μ M/nM	milliMolar / microMolar / nanoMolar
Mo	Molybdenum
Mo-co	Molybdenum cofactor
MOPS	3-(N-Morpholino)propane sulfonic acid
MoSto	Molybdenum storage protein
MOT1/MOT2	Molybdate transporter type 1 / type 2
MPT	Molybdopterin
MTS	Mitochondrial target signal
N	Nitrogen
NBT	Nitro blue tetrazolium
OAC	Oxaloacetate-sulfate carrier
OD _{600nm}	Optical density at 600 nm

OPD buffer	o-Phenylenediamine dihydrochloride
PBS	Phosphate buffer saline
PEG 4000	Polyethylene glycol 4000
PES	Polyethersulfone
PMSF	Phenylmethanesulfonyl fluoride
POM	Polyoxomolybdate
PVDF	Polyvinylidene difluoride
PYC1	Pyruvate carboxylase 1
rMoSto	Recombinant MoSto expressed in <i>Escherichia coli</i>
RNA	Ribonucleic acid
rpm	Revolutions per minute
SAM	S-adenosylmethionine
Sc NifQ	<i>Saccharomyces cerevisiae</i> NifQ
SD	Synthetic defined
SDS	Sodium dodecylsulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHST1	<i>Stylosanthes hamata</i> sulfate transporter type 1
Sp	Spectinomycin
STAS	Sulfate transporter anti-sigma domain
SULTR	Sulfate transporter
Tet	Tetracycline
TEV	Tobacco Etch Virus
TOF/TOF	Dual reflectron tandem time-of-flight
TU	Transcriptional unit
Tup	Tungstate uptake protein
U	Units
UCP1/UCP2	Uncoupling protein 1 / Uncoupling protein 2
UMP/UDP	Uridine monophosphate / Uridine diphosphate
UV-visible	Ultraviolet-visible
V	Vanadium
v/v	Volume / volume
Vf	Final volume
Vmax	Maximum velocity

W	Tungstate
w/v	Weight / volume
Wtp	Tungstate transport protein
X-Gal	5-bromo-4-cloro-3-indolil- β -D-galactopiranoside
yMoSto	Yeast MoSto
YNB	Yeast nitrogen base
yNifQ	Yeast expressed NifQ
YPAUD/Gal/Glu	Yeast peptone adenine uracil dextrose / galactose / glucose

Abstract

Biological nitrogen fixation carried out by a group of microorganisms termed diazotrophs is key to sustainable agricultural practices. Diazotrophs use nitrogenases to reduce N_2 into NH_3 . Depending on the metal composition of their active-site cofactors, nitrogenases are classified as Mo, V or Fe-only nitrogenases, which carry FeMo-co, FeV-co or FeFe-co, respectively. *Azotobacter vinelandii* harbors the three types of nitrogenases but it preferentially expresses the Mo nitrogenase as the presence of molybdate in the medium represses the other two. In this thesis we have performed a genetic and biochemical analysis of the *A. vinelandii* unique molybdenum storage protein (MoSto) encoded in *mosA* and *mosB* genes. Because MoSto enables the accumulation of enormous amounts of Mo inside the cell, we have also investigated its application in the development of engineered N_2 -fixing yeast strains. We show that MoSto confers *A. vinelandii* a competing edge in situations of transient Mo deprivation. It also provides resistance against W, a metal that impairs the activity of Mo-enzymes such as nitrogenase. Moreover, MoSto buffers the regulatory effects of transient Mo step-down, preventing early derepression of alternative nitrogenase genes. Mo stored at MoSto is biologically active as purified MoSto replaces molybdate in the *in vitro* FeMo-co synthesis assay. The properties of MoSto make it a desirable component for an engineered Mo pathway to nitrogenase in *S. cerevisiae*. Indeed, we show here that expression and targeting of MoSto to the mitochondrial matrix protected *S. cerevisiae* from Mo toxicity and additionally permitted the maturation of co-expressed NifQ into a fully active form. As NifQ is the physiological Mo donor to FeMo-co in *A. vinelandii* and other diazotrophs, achieving functional NifQ completed the Mo pathway in *S. cerevisiae* mitochondria. Thus, the engineered Mo pathway required, at a minimum, the incorporation of *nifQ*, *mosA* and *mosB* genes into the chromosome and proper targeting of their products to mitochondria. Genes encoding molybdate transporters might be required under certain yeast genetic backgrounds or growth conditions.

Resumen

La fijación biológica de nitrógeno realizada por un grupo de microorganismos denominados diazotrofos es clave para las prácticas agrícolas sostenibles. Los diazotrofos usan nitrogenasas para reducir el N_2 en NH_3 . Dependiendo de la composición metálica de sus cofactores en el sitio activo, las nitrogenasas se clasifican como Mo, V o Fe- nitrogenasas, que transportan FeMo-co, FeV-co o FeFe-co, respectivamente. *Azotobacter vinelandii* alberga los tres tipos de nitrogenasas, pero preferentemente expresa la Mo nitrogenasa ya que la presencia de molibdato en el medio reprime las otras dos. En esta tesis hemos realizado un análisis genético y bioquímico de la proteína de almacenamiento de molibdeno (MoSto) única en *A. vinelandii* que es codificada por los genes *mosA* y *mosB*. Debido a que MoSto permite la acumulación de enormes cantidades de Mo dentro de la célula, también hemos investigado su aplicación en el desarrollo de cepas de levadura fijadoras de N_2 . Mostramos que MoSto confiere a *A. vinelandii* una ventaja competitiva en situaciones de privación transitoria de Mo. También proporciona resistencia contra W, un metal que deteriora la actividad de las enzimas Mo, como la nitrogenasa. Además, MoSto amortigua los efectos reguladores de la reducción transitoria de Mo, evitando la desrepresión temprana de genes de las nitrogenasas alternativas. El Mo almacenado en MoSto es biológicamente activo ya que MoSto purificado reemplaza al molibdato en el ensayo de síntesis *in vitro* de FeMo-co. Las propiedades de MoSto lo convierten en un componente deseable para una ruta de ingeniería genética de Mo a la nitrogenasa en *S. cerevisiae*. De hecho, mostramos aquí que la expresión y el direccionamiento de MoSto a la matriz mitocondrial protegieron a *S. cerevisiae* de la toxicidad de Mo y además permitieron la maduración de NifQ coexpresado en una forma completamente activa. Como NifQ es el donante fisiológico de Mo para FeMo-co en *A. vinelandii* y otros diazotrofos, el logro de NifQ funcional completó la vía de Mo en las mitocondrias de *S. cerevisiae*. Por lo tanto, la vía Mo diseñada requirió, como mínimo, la incorporación de genes *nifQ*, *mosA* y *mosB* en el cromosoma. Los genes que codifican los transportadores de molibdato podrían ser necesarios bajo ciertos antecedentes genéticos de levadura o condiciones de crecimiento.

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Introduction

1. BIOLOGICAL IMPORTANCE OF MOLYBDENUM

Molybdenum (Mo) is an essential micronutrient for most organisms. Both, Prokaryotes (Archaea and Bacteria) and Eukaryotes (including plants, fungi and animals) typically harbor a number of enzymes involved in Mo uptake, storage, homeostasis, regulation, and Mo cofactor biosynthesis (Hernandez *et al.*, 2009; Hille *et al.*, 2014).

Mo is not biologically active by itself, but it is rather found coordinated by organic pterin molecules in the so-called molybdenum cofactors (Mo-co), which play key roles in the catalytic centers of a wide range of molybdoenzymes relevant to global carbon, sulfur and nitrogen cycles (Zhang *et al.*, 2011; Peng *et al.*, 2018). Hitherto more than 50 Mo-containing enzymes have been characterized, and many more protein sequences have been annotated as putative molybdoproteins. Most of them have been found in bacteria while only a restricted number were identified in eukaryotes (Hille *et al.*, 2011). The Mo atom of Mo-co is coordinated to the chemical backbone of an organic molybdopterin moiety (MPT; Schwarz, 2005; Schwarz and Mendel, 2006; Mendel, 2007). Mo-co biosynthetic pathways of prokaryotes and eukaryotes present many similarities albeit in higher organisms this process is compartmentalized and some steps take place in mitochondria and others in the cytosol (Leimkühler, 2017).

Mo-co synthesis starts from 5'GTP (guanosine triphosphate) and produces the Mo-co intermediate cPMP (cyclic pyranopterin monophosphate). The conversion of cPMP through specific proteins and the addition of two sulfur atoms result in the MPT molecule. Finally, an adenylation reaction followed by molybdate insertion in the dithiolene produces the Mo-co molecule (Yokoyama and Leimkühler, 2015).

Bacteria molybdoenzymes contain different forms of Mo-MPT. For example: guanosine can be added to the phosphate group of Mo-co to form the MGD cofactor, from which two equivalents will be bound to a single Mo atom to form the bis-molybdopterin-guanine-dinucleotide cofactor (bis-MGD). Mo-co can also be modified by the addition of cytosine to form the molybdopterin-cytosine-dinucleotide (MCD) (Magalon *et al.*, 2011). Prokaryotic Mo enzymes are grouped into four families based on their cofactor type and

domain arrangement: (i) the dimethyl sulfoxide reductase family which contains the bis-MGD variant cofactor, (ii) the sulfite oxidase family that possesses the prototypic Mo-MPT cofactor also known from the eukaryotes, (ii) the xanthine oxidase family which has the MCD cofactor, and (iv) the W-containing aldehyde oxidoreductase family with a bis-MPT cofactor with a coordinated W ligand (Johnson *et al.*, 1990*a,b*; Schwarz *et al.*, 2007).

In eukaryotes, the Mo-co biosynthesis pathway can be divided in three distinct stages involving the activities of a wide range of proteins. The biosynthetic enzymes involved in this process, which are highly conserved, are designated as Cnx (cofactor for nitrate reductase and xanthine dehydrogenase). The first stage entails the conversion of 5'-GTP into cyclic pyranopterin monophosphate (cPMP) within the mitochondrial matrix, and is carried out by the Cnx2 and Cnx3 enzymes (Hoff *et al.*, 1995). This stable intermediate, which is exported to the cytosol by the mitochondrial inner membrane transporter ATM3, will incorporate two sulfur atoms to form the dithiolene of MPT (Teschner *et al.*, 2010). This reaction is catalyzed by the MPT synthase, a heterotetramer with two large Cnx6 and two small Cnx7 subunits. After the transfer of sulfur, the Cnx7 subunits need to be re-sulfurated via the MPT synthase sulfurase Cnx5 (Matthies *et al.*, 2004). Finally, Mo insertion into the MPT takes place after a preparatory step of MPT adenylation carried out by the Mo insertase Cnx1, which consists of a larger N-terminal Cnx1E domain and a smaller C-terminal Cnx1G domain. The MPT molecule, which is bound to the Cnx1G domain, is activated through adenylation forming the MPT-AMP. This intermediate is transferred to the Cnx1E domain, which releases AMP and inserts the Mo atom derived from molybdate (Llamas *et al.*, 2006, 2007).

Eukaryotic Mo-enzymes are classified according to the coordination chemistry of the Mo atom bound to the pterin. Two different types of Mo centers have been described: (i) the di-oxo Mo-enzymes from the sulfite oxidase family such as the sulfite oxidase (SO), nitrate reductase (NR) and mARC enzymes and, (ii) the mono-oxo Mo-enzymes from the xanthine oxidase family such as the xanthine dehydrogenase (XDH) and aldehyde oxidase (AO) enzymes (Mendel and Kruse, 2012).

There is another type of Mo cofactor called iron-molybdenum cofactor (FeMo-co) that is exclusively found in the Mo-nitrogenase (see title 4.1; see Figure 1) The FeMo-co is composed of an inorganic [Fe₇-S₉-C-Mo] moiety in which the Mo atom is additionally coordinated by the organic acid *R*-homocitrate (Rubio and Ludden, 2008).

Figure 1. Mo-co and FeMo-co biosynthesis pathways. (A) Biosynthesis Mo-co and FeMo-co pathways in bacteria. Left panel shows Mo-co synthesis from the 5'-GTP molecule to the final molybdenum cofactor. Bacterial proteins involved in each step are detailed by each arrow. Right panel shows the simplified pathway for active-site metallocluster biosynthesis of the Mo-nitrogenase, V-nitrogenase and Fe-only nitrogenase. Bacterial proteins are detailed by each arrow. (B) Mo-co (left panel) and FeMo-co (right panel) chemical structures. Molybdenum atom is highlighted in red. (Demtröder et al., 2018)

The biosyntheses of Mo-co and FeMo-co do not share a common pathway (see Figure 1). While Mo-co biosynthesis proceeds in the three steps outline before, which are widely distributed and highly conserved in bacteria, archaea and eukaryotes (Mendel, 2013; Leimkühler, 2017), FeMo-co biosynthesis is found exclusively in a restricted group of N₂-fixing prokaryotes and requires multiple steps that are carried out by proteins encoded in the nitrogen fixation (*nif*) gene clusters (Rubio and Ludden, 2008). The FeMo-co biosynthetic pathway is described in detail in section 4.1.

2. MOLYBDATE UPTAKE, TRANSPORT AND STORAGE

Despite being a minor component in the Earth's crust, in the present-day, Mo is highly available in aquatic environments as molybdate (Mendel and Bittner, 2006; Anbar, 2008). In soils, Mo is more soluble in alkaline than in acidic (pH<5.5) ones. In general Mo availability decreases depending on the pH, reactive oxide species, and water drainage (Gupta, 1997; Kaiser *et al.*, 2005). In molecules, the Mo atom can exit in several oxidation states ranging from -II to +VI, being the latter the most commonly found in most agricultural soils in its oxoanionic form molybdate (MoO₄²⁻). Molybdate is the most accessible Mo species for living organisms, which have developed discriminating systems to distinguish molybdate from competing oxyanions thus preventing toxic effects. Organisms have developed specific chelating and transport systems to scavenge molybdate in the presence of tungstate (WO₄²⁻). Due to the chemical and physical similarities of Mo and W, the latter can replace Mo yielding inactive catalytic centers at the corresponding molybdoenzymes. This introduction will focus on Mo metabolism and Mo-enzymes. However, it should be noted that tungstate is biologically active as part of the enzymes of some microorganisms (mostly thermophilic archaea) where in coordination with pyranopterins (W-co) catalyzes low potential redox reactions of carbon substrates (Schmitz *et al.*, 1992).

2.1. *Molybdenum metabolism in bacteria*

The uptake, transport and storage of molybdenum have been well studied for many years in bacteria. Cellular uptake systems must be able to distinguish molybdate from other tetrahedral oxyanions including sulfate, phosphate and tungstate (Pflugrath and Quioco, 1985; Luecke and Quioco, 1990; Rech *et al.*, 1996). In the well-studied systems of *Escherichia coli* and *Clostridium pasteurianum* it has been found that tungstate and sulfate are competitive inhibitors of molybdate uptake (Elliott and Mortenson, 1976; Aguilar-Barajas *et al.*, 2011).

One of the most studied systems that control the transport of specific substrates across the membrane are the ABC-type transporters, which are multi-subunit transporters that contain an essential cytoplasmic subunit with ATP hydrolysis activity. The ATP-binding domain of these transporters is highly conserved (Higgins *et al.*, 1986) and the family was termed as ABC transporters in recognition of the “cassette-like” nature of the ATP-binding subunit.

In bacteria, molybdate scavenging from the environment and transport into the cell is carried out by high-affinity ModABC transport systems (Maupin-Furlow *et al.*, 1995; Grunden *et al.*, 1997). In *E. coli*, the *mod* gene cluster consists of two bi-directionally divergent operons: the first comprises the *modA*, *modB*, and *modC* genes, while the second one comprises the *modE* and *modF* genes. The *modA* gene encodes for the high-affinity ModA protein located in the periplasm, which is responsible of binding molybdate and presenting it to the channel protein. However, it can bind molybdate and tungstate with similar K_D values, suggesting that this protein cannot discriminate between these two oxyanions (Bever *et al.*, 2011). The *modB* gene encodes an integral cytoplasmic membrane protein that provides the transport channel. The *modC* gene encodes the so-called conserved component, which is a typical ATPase that provides the energy for molybdate transport. The molybdate-responsive ModE protein, which is a member of the LysR family of transcriptional regulators, negatively controls the transcription of the *modABC* operon at high molybdate concentrations (Mouncey *et al.*, 1995; Grunden *et al.*, 1996). ModE also controls transcription of genes involved in further Mo metabolism and in biosynthesis of Mo-co (Anderson *et al.*, 1997).

Unlike most microorganisms, *Azotobacter vinelandii* harbors three genetically distinct *modABC* operons, underlying the importance and complexity of Mo metabolism in this microorganism (Mouncey *et al.*, 1995). The *modA₁B₁C₁* genes encoding a molybdate transport system located between Avin50670 to Avin50650, and the *modE* and *modG* genes encoding a Mo homeostasis regulator cluster, are positioned upstream of the minor *nif* gene cluster. A second locus containing molybdate transport genes (*modA₂B₂C₂*) is located between Avin01300 to Avin01280, and a third putative molybdate transport system encoded by the *modA₃B₃C₃* genes is found between Avin50730 and Avin50700 (see Figure 4; (Setubal *et al.*, 2009). In addition, *A. vinelandii* secretes, under extreme metal limiting conditions, metal chelators termed catechol siderophores (Cornish and Page, 1995; Kraepiel *et al.*, 2009). At low metal concentrations, specific siderophores are secreted from the bacteria to form metal complexes to further deliver them to the bacteria (Bellenger *et al.*, 2008).

Bacteria can use other membrane transport systems that operate as non-specific carriers as low affinity molybdate transporters depending on the metal concentration. Thus, bacterial cells can use low-affinity transporters when high molybdate concentrations repress the expression of the high-affinity transport systems. This is the case of the high-affinity sulfate transport system in *E. coli*, which has been suggested to transport molybdate albeit ten-fold less efficiently than the Mod transporter (Lee *et al.*, 1990; Rosentel *et al.*, 1995).

In addition to the Mod system, two tungstate transport systems have been described to date over the bacteria and archaeal kingdoms: the Wtp and the Tup (Aguilar-Barajas *et al.*, 2011). Both systems are composed of a periplasmic protein (component A), a transmembrane protein which forms the pore (component B) and a cytoplasmic protein (component C), which hydrolyzes ATP to generate the energy necessary to transport the oxyanion into the cell cytoplasm (Grunden *et al.*, 1997; Hagen, 2011). Indeed, while the ModABC system do not discriminate between molybdate and tungstate, the TupABC and WtpABC systems are strongly selective in favor of tungstate (Makdessi *et al.*, 2001; Bevers *et al.*, 2006).

Cellular storage systems provide the cell with essential elements such as transition metals by binding or releasing metals under specific conditions. Two types of microbial Mo storage systems have been discovered until now. The first group is the molbindin family present in archaea and bacteria, proteins that contain one or two molybdate-binding (Mop) domains (Hinton and Mortenson, 1985; M. Lawson *et al.*, 1997; Wagner *et al.*, 2000). One molbindin containing protein not related to Mo storage is the transcriptional regulator ModE functions as a homodimer that displays a high affinity for molybdate and tungstate. The intracellular availability of the metal determines the conformation of ModE, that will regulate gene transcription of the metal transport system operon, and thereby, controlling the acquisition and subsequent use of the element (Anderson *et al.*, 1997).

The second type consists of a unique protein termed the Mo storage protein or MoSto. This protein has been described in the N₂-fixing bacterium *A. vinelandii* and underlies its ability to accumulate 25 times more of Mo than it requires for maximum nitrogenase activity (Pienkos and Brill, 1981). MoSto quaternary structure is an $\alpha_3\beta_3$ hexamer with a molecular mass of 172 kDa (α : 29.1 kDa; β : 28.2 kDa) encoded by the *mosA* (Avin 43200) and the *mosB* (Avin 43210) gene products. MoSto can store up to 100 Mo atoms per hexamer. Metal storage within MoSto occurs in the form of a variety of compact polynuclear oxyanions (POM cluster) that are embedded into specific pockets locked inside the cage-shaped $\alpha_3\beta_3$ protein (see Figure 2; (Kowalewski *et al.*, 2012; Poppe *et al.*, 2014). Fully loaded, the MoSto cage contains around 15 discrete POM clusters with a nuclearity between 3 and 14 MoOx atoms and very diverse geometry; e.g. Mo₃Ox triangles, Mo₆Ox hexagons, Mo₅Ox pentamers and three-layered Mo₈Ox units (Brünle *et al.*, 2018). These POM clusters are linked to the polypeptides by covalent bonds, hydrogen bonds and/or by multiple van der Waals contacts that encapsulate them (Kowalewski *et al.*, 2012; Poppe *et al.*, 2014) It is noteworthy that, due to the similar chemical properties of Mo and W, MoSto can also bind about the same number of W atoms *in vitro* (Fenske *et al.*, 2005).

Figure 2. MoSto 3D atomic structure. PDB number 6H6W. Resolution 1.9 Å. (A) hexameric 3D structure of the molybdenum storage protein (MoSto). Chain α (in red) and chain β (in blue) are indicated. (B) shows the containing molecules of MoSto. POM (Polyoxometalate) clusters and ATP (Adenosine-5'-Triphosphate) molecules are indicated with arrows. (Brünle et al., 2018)

MoSto shows no structural or amino acid sequence similarities with other Mo-containing proteins, including molbindins, Mo-co containing enzymes, or the FeMo-co containing Mo-nitrogenase (Shah and Brill, 1977; Hille, 2002). However, protein database comparisons revealed that MoSto genes, *mosA* and *mosB*, are also present in *Rhodopseudomonas palustris*, *Xanthobacter autotrophicus*, *Bradyrhizobium sp.* and two non-diazotrophic *Nitrobacter* species. Amino acid sequence comparisons with different protein databases revealed certain similarities with uridine monophosphate kinases, which catalyze the reaction $\text{ATP} + \text{UMP} \rightarrow \text{ADP} + \text{UDP}$ (Meyer et al., 2008).

Although MoSto expression is not repressed by ammonium, as it is the case of the *nif* genes, the level of Mo incorporation into MoSto increases in N_2 -fixing cells (Pienkos and Brill, 1981; Allen et al., 1999). Regardless of the nitrogen source, the protein can accumulate substantial amounts of Mo into the cell and supply, among others, the Mo-nitrogenase under extremely impoverished molybdate conditions. The combination of MoSto with the high-affinity molybdate transport systems and the synthesis and

excretion of high-affinity metallophores, gives a selective advantage to *A. vinelandii* when competing for molybdate in natural habitats.

On the other hand, regulation of MoSto expression by Mo has not been clearly stated. There are discrepancies in the literature as to whether or not MoSto expression is regulated by Mo. Constitutive expression was reported by Pienkos and Brill in 1981, while Mo-regulation was reported by Fenske et al. 2005, which showed MoSto expression in *A. vinelandii* cells grown in medium with molybdate concentrations as low as 1 nM but not in molybdate-free medium (Pienkos and Brill, 1981; Fenske *et al.*, 2005).

The metal storage mechanism within MoSto follows physiological changes related to the pH and ATP. Binding of molybdate into the apo-protein, which involves the biosynthesis and insertion of the compact polynuclear oxyanions, is a nucleotide-dependent and pH-independent process that requires the hydrolysis of ATP to ADP + Pi (Fenske *et al.*, 2005). This mechanism is presumably carried out in the α -subunits due to their ability to bind Mg^{2+} , which is catalytically essential for ATP hydrolysis. The X-ray structure revealed higher occupancy of the ATP-binding sites at the outer surface of the α -subunits than those at the β -subunit sites. Even though the function of the β -ATP sites remains open due to the lack of Mg^{2+} , its involvement in Mo process cannot be discarded. In fact, ATP, ADP, AMP and adenosine composition analyzed by HPLC suggested that β -ATP hydrolysis was triggered by α -ATP hydrolysis during turnover (Poppe *et al.*, 2018). *In vitro*, the metal release from MoSto appears to be pH-dependent and step-wise occurring from weakly acidic to weakly alkaline pH (6.8-7.8). Molybdate release starts occurring at pH above 6.8. The increase of pH (increasing OH^-) plays a key role in POM degradation and in the deprotonation of the His residues involved in binding the negatively charged POM clusters (Schemberg *et al.*, 2007). However, experimental *in vitro* conditions such as temperature, protein concentration and incubation time, seem to be also important for the equilibrium of molybdate binding and release (Schemberg *et al.*, 2008).

2.2. Molybdenum uptake and metabolism in eukaryotes

In contrast to bacteria, molybdate transport in eukaryotes is poorly understood. For many years it was believed that molybdate was transported nonspecifically by other anion carriers (Heuwinkel *et al.*, 1992; Mendel and Hänsch, 2002; Kaiser *et al.*, 2005). However, a decade ago, specific plant-type molybdate transporters were identified in two simultaneous studies carried out with the green algae *Chlamydomonas reinhardtii* (Tejada-Jiménez *et al.*, 2007) and the higher plant *Arabidopsis thaliana* (Tomatsu *et al.*, 2007), which defined a molybdate transporter type 1 (MOT1) family. MOT1 proteins had previously been related to the sulfate transporter family, SULTR. However, amino acid sequence comparisons showed that MOT1 proteins were significantly different from SULTR members, including the lack of the STAS (sulfate transporter and anti-sigma factor antagonist) domain (Tejada-Jiménez *et al.*, 2007). Metal affinity studies from *C. reinhardtii* CrMOT1 showed the specificity of this system for molybdate uptake since competitors such as sulfate did not affect Mo transport (Tejada-Jiménez *et al.*, 2007). MOT1 shows a K_m of 7 nM for molybdate, indicating very high affinity, to ensure the provision of this essential micronutrient even if it is not abundant in the environment. This K_m value is similar to that reported for the *ModABC* transport system in *E. coli* (see Table 1; López Corcuera *et al.*, 1993).

In *A. thaliana*, two members of the MOT1 family (*AtMOT1.1* and *AtMOT1.2*) have been reported to be involved in Mo transport (Tomatsu *et al.*, 2007; Gasber *et al.*, 2011). *AtMOT1.1* is a high-affinity and specific molybdate transporter with a reported K_m of 20 nM, similar to the CrMOT1 protein. The physiological role of *AtMOT1.1* has been controversial as two different subcellular localizations had been reported for it. First, a GFP-MOT1.1 fusion was localized to the plasma membrane and the endomembrane system of tobacco cells (Tomatsu *et al.*, 2007). This localization disagrees with a latter report in which a mitochondrial targeting sequence was predicted at the N-terminus of MOT1.1 and a C-terminal a fusion of GFP to MOT1.1 was then generated and expressed in leaf protoplast and roots of *A. thaliana*, both showing mitochondrial localization (Baxter *et al.*, 2008). One explanation to this difference is that the N-terminal GFP fusion impeded the function of the mitochondrial targeting signal of MOT1.1. On the other hand, the *AtMOT1.2* has been proposed to function as vacuolar molybdate transporter mostly involved in Mo homeostasis and Mo storage in the cytosol (Gasber *et al.*, 2011).

The mechanism for actual transport of Mo into mitochondria of yeast is crucial to the work of this thesis. Albeit MOT1 transporters are related to the sulfate transporter superfamily they do not appear to transport sulfate (Tejada-Jiménez *et al.*, 2007). However, sulfate transporters such as the SHST1 are known to enhance uptake of molybdate at nM concentrations into yeast cells (Fitzpatrick *et al.*, 2008). The dicarboxylate (DIC) carrier has been described to transport sulfate, among other anions, into mitochondria both in yeast and humans (Crompton *et al.*, 1974; Palmieri *et al.*, 1996). In addition, eukaryotic UCP1/UCP2 proteins (Uncoupling proteins 1 and 2), which transport C4 metabolites out of mitochondria by an antiport mechanism, or the yeast mitochondrial transporters OAC and APSC1, are able to carry sulfate into the mitochondrial matrix (Palmieri *et al.*, 1999; Todisco *et al.*, 2014; Vozza *et al.*, 2014; Monné *et al.*, 2018). Molybdate can be also transported nonspecifically by sulfate transporters since both oxyanions have similar ionic radius, charge and geometry.

More recently, two new transporters of the MOT1 family related to symbiotic N₂ fixation (SNF) have been described in *Medicago truncatula* (Tejada-Jiménez *et al.*, 2017; Gil-Díez *et al.*, 2018) and designated as *MtMOT1.2* and *MtMOT1.3*. Immunolocalization of *MtMOT1.2* showed its association to the plasma membrane and the intracellular membrane systems. Its position in the endodermis of the root indicated its role in the transfer of molybdate from the nodule vessels into endodermal cells. However, the transport system involved in molybdate release into the apoplast has not yet been identified. Once metal delivery is produced in the apoplast, *MtMOT1.3* will introduce this metal into the nodules, either infected or not infected cells (Tejada-Jiménez *et al.*, 2017).

Phylogenetic analysis of the MOT1-type proteins showed significant conservation to proteins of unknown function in bacteria, algae, fungi, and plants but not in animal genomes. A second family of eukaryotic molybdate transporters, termed MOT2, has been identified in humans and in *C. reinhardtii* (Tejada-Jiménez *et al.*, 2011). Molybdate transport capability of the *CrMOT2* and *Homo sapiens* *HsMOT2* expressed in *S. cerevisiae* cells, showed a K_m of 550 nM, which indicates significantly lower affinity for this metal than the *CrMOT1* transport protein (see Table 1). While in this organism *CrMOT1* seems to be connected to the molybdoenzyme nitrate reductase, *CrMOT2* might be responsible

of ensuring the molybdate availability under environmental scarceness. Reduced expression of *MOT1* in *C. reinhardtii* impaired molybdate transport and nitrate reductase activity in the cells (Tejada-Jiménez *et al.*, 2007), while reduced expression of *MOT2* gene resulted in diminished molybdate uptake (Tejada-Jiménez *et al.*, 2011).

Table 1. Transport systems capable of transporting molybdate and their biochemical properties. (Tejada-Jiménez and Schwarz, 2014)

<i>Transport system</i>	<i>Specific anion</i>	<i>K_m for MoO₄²⁻</i>	<i>Other substrates</i>	<i>Organisms</i>
ModABC	MoO ₄ ²⁻	50 nM	WO ₄ ²⁻ SO ₄ ²⁻	Bacteria
Non-specific system	-	-	SeO ₃ ²⁻ MoO ₄ ²⁻	Bacteria
MOT1	MoO ₄ ²⁻	7 – 20 nM	WO ₄ ²⁻	Algae, plants, fungi, bacteria
MOT2	MoO ₄ ²⁻	≈ 500 nM	WO ₄ ²⁻	Algae, plants, animals
SHST1	SO ₄ ²⁻	10 μM	MoO ₄ ²⁻	Plants

Interestingly, comparative genomics of molybdate uptake and Mo utilization in Mo-co biosynthetic pathways and molybdoenzyme in eukaryotes showed that most alveolata (such as *Ciliophora* and *Apicomplexa*), yeast (*Saccharomycotina* and *Schizosaccharomycetes*), and a number of parasitic protists have lost the ability to utilize Mo (Peng *et al.*, 2018).

3. NITROGEN IN NATURE

Nitrogen is an essential occurring nutrient for living beings, which is found in fundamental biomolecules such as amino acids and nucleic acids. It makes up approximately 78% of the gas in the Earth's atmosphere. N₂ is an extremely stable molecule (N≡N) and very high energy (941 kJ/mol) is required to break the triple bond that joins the two N atoms (Kalescky *et al.*, 2013). Despite its abundance N₂ is not

assimilable by most organisms, importantly including plants, fungi and animals, which makes it a limiting nutrient for current extensive agricultural practices. Only a few microorganisms can use N_2 while plants, for instance, need to assimilate it in the form of ammonium or nitrate from the soil. One of the main challenges for the 21st century is to obtain sustainable agricultural production and supply with food a human population that is expected to increase from 7 to 9 billion. The current production methods in agriculture are sustained by the use of chemical fertilizers that sharply increase yields but generate serious environmental and health problems (Erisman *et al.*, 2008; White and Brown, 2010). Nitrogen fertilizers are mainly obtained by the Haber-Bosch process, in which the atmospheric N_2 is mixed with H_2 or methane at very high pressure and temperature to produce ammonia or urea. About the 80% of the total nitrogen manufactured by this process is used for agricultural purposes. However, about 60% of the N fertilizer applied to agricultural soils is lost to the environment by physical or biochemical processes such as leaching, surface run-off, denitrification, volatilization, and microbial consumption (Kant *et al.*, 2011). Importantly, denitrification of the N fertilizers produces N_2O , a “green-house gas” 300 fold more potent than CO_2 that, if release in great quantities, might have powerful impact on climate change (Crutzen *et al.*, 2008). In addition, N leaching contaminates surface, underground and coastal waters leading to microbial blooms that damage ecosystems (Erisman *et al.*, 2008).

Despite the awareness of the environmental problems and other safety concerns associated with the Haber-Bosch process, the fact is that abundance of reactive nitrogen species resulting from this process (nitrous oxide , nitrate , nitrite , ammonia) and downstream microbial metabolism is indispensable for modern agriculture. Albeit much needed, the cost of these N fertilizers is out of reach for poor farmers, which is an enormous problem underlying poverty, especially in Sub-Saharan Africa (Sanchez and Swaminathan, 2005; Bodirsky *et al.*, 2014). Scientists deal with different approaches to reduce N fertilizer demand while sustaining cereal crop productivity. There is a group of microorganisms within bacteria and archaea that can reduce the N_2 molecule to NH_4^+ , an assimilable N form for most organisms (Hillel, 2008). This biological process, called biological nitrogen fixation (BNF), is key to solve the N fertilization problem. Some of these “ N_2 eaters” (called diazotrophs) engage in symbiotic associations with eukaryotes while others fix N_2 in their free-living forms. The main purpose of current research

programs in this field of science is to engineer new associations of diazotrophic microbes with crops, or to directly engineer this function into plants so that crops can be self-supported by BNF. Cereals are the main engineering target as wheat, rice and maize products constitute 75% of the world's total caloric uptake (Sands *et al.*, 2009). Three general bioengineering approaches have been recently proposed: (i) to express nitrogenase heterologously in plant cells, (ii) to engineer some kind of symbiotic nodule into non-nodulating plants, and (iii) to use plant-associative diazotrophs to fix N_2 for the benefit of their non-legume host plant (Santi *et al.*, 2013; Curatti and Rubio, 2014; Oldroyd and Dixon, 2014; Geddes *et al.*, 2015).

BNF is an essential part of the nitrogen cycle that accounts for about two-thirds of the total fixed N_2 (Curatti and Rubio, 2014). A quite conserved metalloenzyme called nitrogenase, which is formed by a complex of proteins, is the catalyst responsible of BNF. This enzyme catalyzes, in a high-energy demanding reaction, the reduction of one N_2 molecule to NH_3 requiring a minimum of 8 electrons and the hydrolysis of 16 ATP molecules. The resulting reaction yields two molecules of NH_3 (protonated to NH_4^+ in aqueous environment) and one molecule of H_2 . However, previous data showed that H_2 can behave as a competitive inhibitor *in vivo* of N_2 (Wilson and Umbreit, 1937; Guth and Burris, 1983). Thus, most N_2 fixing organisms possess an uptake hydrogenase capable of recycling the by-product H_2 thus improving the energetic balance of the reaction. In addition, it has been proposed that hydrogenase protects nitrogenase from oxygen damage (Tamagnini *et al.*, 2007; Zhang *et al.*, 2014). As nitrogenase is rapidly destroyed by O_2 , microorganisms also face the problem of protecting the enzyme by developing O_2 barriers to avoid irreversible nitrogenase inactivation even at low O_2 concentrations (Gallon, 1992; Soto-Urzúa and Baca, 2001). Different protection mechanisms exist: (i) high respiratory rates that remove O_2 at the cell membrane, (ii) reversible conversion of nitrogenase into an inactivated state by binding of protective proteins (e. g. Sethna protein), and (iii) increasing the cell size by the production of polymers of alginate as a barrier to O_2 diffusion (Linkerhagner and Oelze, 1995; Sabra *et al.*, 2000).

In addition to reducing N_2 and protons, nitrogenase can reduce several small, non-physiological substrates. Among these alternative substrates are included a wide range of carbon containing compounds such as acetylene. The reduction of acetylene into

ethylene is the most notable activity assay since it allows very easy *in vivo* and *in vitro* determination of nitrogenase activity (Seefeldt *et al.*, 2013).

4. MOLYBDENUM AND N₂ FIXATION

The most abundant and extensively studied nitrogenase is the molybdenum (Mo)-dependent enzyme. Mo-nitrogenase complex has two protein components encoded by the *nifD*, *nifK* and *nifH* genes (Bulen and Lecomte, 1966). Component I or dinitrogenase, also referred to as the MoFe protein, is a 230 kDa heterotetrametric (NifD₂K₂; $\alpha_2\beta_2$) protein formed by two $\alpha\beta$ dimers related by a two-fold rotational symmetry. In each α -subunit (NifD) the iron-molybdenum cofactor FeMo-co [7Fe-9S-Mo-C-homocitrate] comprises the active site of the enzyme while one P-cluster [8Fe-7S] is located at the interface of the α - and β -subunits in each $\alpha\beta$ pair (Lee *et al.*, 2009; Spatzal *et al.*, 2011). Component II or NifH, also referred to as the Fe protein, is the obligate electron donor to NifDK and is therefore it is also known as dinitrogenase reductase. NifH is a 60 kDa homodimer of the *nifH* gene product. At the dimer interface within each monomer is located one nucleotide binding site (Mg²⁺-ATP/ Mg²⁺-ADP). A single surface-exposed [4Fe-4S]^{+1/+2} cluster bridges the two subunits (Georgiadis *et al.*, 1992). The transfer of one electron transfer from the [4Fe-4S]¹⁺ cluster of NifH to the oxidized P-cluster of the NifDK component is couple to downstream conformational changes promoted by the hydrolysis of Mg²⁺-ATP to Mg²⁺-ADP (Hageman and Burris, 1978; Seefeldt *et al.*, 2018). Electrons are subsequently transferred from the reduced P-cluster to FeMo-co. When enough electrons (and H⁺) are accumulated in FeMo-co the reduction of N₂ takes place. Importantly, nitrogenase can reduce additional substrates such as H⁺ to H₂ and acetylene (C₂H₂) to ethylene (C₂H₄). The reduction of acetylene is normally used as proxy for nitrogenase activity because it is easy and fast to determine by gas chromatography.

4.1. Biosynthesis of nitrogenase metalloclusters

The complexity of FeMo-co and the P-cluster requires the activity of a wide range of nitrogen fixation (Nif) proteins. The biosynthesis of the P-cluster involves the synthesis of two [4Fe-4S] cubanes sharing a sulfide atom in between to form the [8Fe-7S] cluster. The first step of P-cluster synthesis involves the activities of the NifU and NifS proteins, which are needed for the formation of two pairs of [4Fe-4S] cubanes in NifDK. These clusters will serve as precursors for the P-clusters, and the action of the NifH and NifZ proteins will complete the biosynthesis of the [8Fe-7S] clusters. Previous studies reported that the lack of any of those proteins, NifH or NifZ, leads to incomplete P-cluster maturation (Hu *et al.*, 2007; Jimenez-Vicente *et al.*, 2019).

The biosynthesis of FeMo-co is a highly complex process that requires, at least, the participation of the *nifS*, *nifU*, *nifB*, *nifE*, *nifN*, *nifV*, *nifQ*, and *nifH* gene products (Imperial *et al.*, 1984; Shah *et al.*, 1986; Hoover *et al.*, 1987; Paustian *et al.*, 1989; Zhao *et al.*, 2007). As it is the case of P-cluster synthesis, FeMo-co biosynthesis starts by NifU and NifS mobilizing Fe and S for the biogenesis of simple Fe-S clusters. It should be noted that mutations in either *nifU* or *nifS* resulted in a large decrease of activity in both nitrogenase components, not only in NifDK, indicating that they are also essential to the synthesis of NifH [4Fe-4S] cluster (Roberts *et al.*, 1978; Jacobson *et al.*, 1989b). Homologs of these proteins, IscU and IscS, have been later identified and shown to be involved in supplying [Fe-S] clusters for general cellular functions (Johnson *et al.*, 2005; Dos-Santos *et al.*, 2007).

This early step is followed by the synthesis of NifB-co, a [8Fe-9S-C] cluster with a central carbide atom symmetrically coordinated by 6 Fe atoms that is generated by the SAM-radical protein NifB (Shah *et al.*, 1994; Curatti *et al.*, 2006; George *et al.*, 2008; Guo *et al.*, 2016). NifB-co is not only a biosynthetic intermediate of FeMo-co but also of FeV-co and FeFe-co, which are the catalytic clusters of the alternative nitrogenases present in some organisms (Bishop *et al.*, 1980; McRose *et al.*, 2017; Sippel *et al.*, 2017). NifB-co is then transferred to the NifEN scaffold in which it is immediately converted into the VK-cluster, a cluster with the same atomic composition as NifB-co but with different EPR properties (Hernandez *et al.*, 2007). In some diazotrophs, the transfer of NifB-co

from NifB to NifEN is mediated by the metallocluster carrier NifX that is homologous to the C-terminal domain of the most common type of NifB proteins (Hernandez *et al.*, 2007; Arragain *et al.*, 2017). The late steps of FeMo-co synthesis are catalyzed by NifEN and NifH. The VK-cluster is converted into FeMo-co by replacing one Fe atom with Mo and incorporating homocitrate (Curatti *et al.*, 2006; Hu *et al.*, 2006). Homocitrate produced by NifV (Hoover *et al.*, 1987) is incorporated into the VK-cluster precursor after the insertion of Mo (Fay *et al.*, 2010), which has been previously donated by NifQ (Hernandez *et al.*, 2008). Once FeMo-co is formed in NifEN, the NafY carrier protein, a non-*nif* gene that also stabilizes the FeMo-co deficient apo-NifDK, binds it and inserts it into the apo-NifDK to reconstitute the holoenzyme that will be competent for N₂ reduction (see Figure 3; (Homer *et al.*, 1995; Hernandez *et al.*, 2011).

NifQ is a monomer of 22.4 kDa that carries two redox-responsive clusters in the form of [3Fe-4S]⁺ and [MoFe₃S₄]³⁺ species. These clusters have been proposed to serve in the uptake and release Mo in an interconvertible way (Hernandez *et al.*, 2008, 2009). Further studies revealed the existence of an additional Mo environment in NifQ that is also relevant to the FeMo-co synthesis pathway. Chemical chelation and Mo displacement from pure NifQ protein treated with α-α'-bipyridyl and CuCl₂ components impaired NifQ function as Mo donor to the NifEN/NifH complex (George *et al.*, 2016). The two Mo environments were identified by Mo EXAFS and corresponded to the previously reported [MoFe₃S₄] cluster, which was chelated by the α-α'-bipyridyl component, and to a second NifQ-bound Mo species in the form of dioxodithiomolybdate (MoS₂O₂), which was displaced by CuCl₂. In addition, Fe EXAFS and EPR spectra showed the existence of a Mo-free [3Fe-4S] cluster that was suggested to be the site for reductive incorporation of Mo, yielding a [MoFe₃S₄] cluster. The role of MoS₂O₂ has not been well characterized, albeit it was shown to be active in FeMo-co synthesis and two plausible roles were suggested: (i) it would serve as intermediate to readily incorporate Mo into the [3Fe-4S] cluster of NifQ, or (ii) it would be the form that is delivered by NifQ to the NifEN/NifH complex for FeMo-co biosynthesis (George *et al.*, 2016).

The phenotypic characterization of *nifQ* mutants preceded its biochemical analysis and clearly indicated its role in Mo trafficking towards nitrogenase. Results showed that *K.*

pneumoniae nifQ mutants accumulated little Mo and were severely affected in Mo-dependent N_2 fixation. The *nifQ* mutant phenotype could be reverted by adding mM levels of molybdate to the culture medium (Imperial *et al.*, 1984). Suppression of the *nifQ* phenotype was also achieved by supplementing cultures with cystine as a S source (Ugalde *et al.*, 1985). The phenotype of *nifQ* mutants have also been described in *A. vinelandii* and *R. capsulatus* (Moreno-Vivian *et al.*, 1989; Rodriguez-Quinones *et al.*, 1993). As *nifQ* mutants were not defective in activity of the Mo-co-containing enzyme nitrate reductase, it was concluded that its activity was only essential to FeMo-co biosynthesis (Imperial *et al.*, 1984).

Figure 3. Schematic model of the FeMo-co biosynthetic pathway. Fe-S precursor clusters from the NifU/NifS scaffold are required for the NifB-co synthesis. The NifB-co biosynthetic pathway requires the activity of NifS, NifU, NifB and NifX; the Mo source involves NifQ activity, and the homocitrate is synthesized by NifV. The NifEN protein serves as scaffold for the biosynthesis of FeMo-co in the presence of NifH by the incorporation of Mo and homocitrate into NifB-co. Completed FeMo-co is then transferred by NafY to the apo-NifDK protein to generate active Mo-nitrogenase. Orange squares indicate the VK-cluster. Green squares indicate FeMo-co cluster. Nif proteins are represented by circles: NifH, purple; NifE, light green; NifN, dark green; NifD, light blue; and NifK dark blue.

4.2. *A. vinelandii* alternative nitrogenases and their nitrogen fixation gene clusters

The bacterium *A. vinelandii* has been one of the models to study Mo metabolism and biological N₂ fixation in prokaryotes. *A. vinelandii* has developed highly efficient metabolism of Mo uptake, storage and usage towards the Mo-nitrogenase enzyme (see title 2.1). In addition, to the Mo-nitrogenase, this bacterium has the peculiarity to synthesize two alternative Mo-independent nitrogenases containing Fe and V or only-Fe in their active sites (Luque and Pau, 1991). All three nitrogenases require for function low-potential electron donors, MgATP, a source of Fe, and the absence of O₂. However, they differ in other metal requirements. Expression of alternative nitrogenases is regulated by the metal availability in the environment. While the presence of molybdate in the culture medium is absolutely required for the expression of Mo-nitrogenase structural genes, it represses expression of both alternative nitrogenases (Luque and Pau, 1991). Under Mo-deficient conditions, but sufficient V and Fe in the medium, the V-nitrogenase is expressed. Vanadate in the culture medium represses transcription of the Fe-only nitrogenase genes when molybdate is absent (Pau *et al.*, 1989). However, when both molybdate and vanadate are lacking, metal repression of the Fe-only nitrogenase disappears.

In *A. vinelandii*, the structural genes of the three nitrogenases are located in different genomic locations (Jacobson *et al.*, 1989a; Joerger *et al.*, 1989; Pau *et al.*, 1989). The Mo-nitrogenase *nif* genes are distributed as two clusters in the chromosome: a major *nif* cluster that contains *nifHDKTYENX*, *iscA^{nif}*, *nifUSV*, *cysE*, *nifWZM*, *clpX*, and *nifF*, among others; and a minor *nif* cluster that contains the *nifLAB*, *fdxN*, *nifOQ*, *rhDN*, and *grx5* genes in one transcriptional direction and the *rnfABCDGEH*, *nafY* genes on the opposite transcriptional direction (see Figure 4; (Setubal *et al.*, 2009)). The V- and Fe-only nitrogenases are the result of the gene products of the *vnf* and *anf* gene clusters, respectively (Joerger *et al.*, 1988; Eady, 1989). In contrast to the Mo-nitrogenase and the Fe-only nitrogenase, *vnf* genes encoding the nitrogenase component proteins are separated in two cistrons, one comprising the *vnfDGGK* genes and the second having a standalone *vnfH* gene (see Figure 4; (Joerger *et al.*, 1989, 1990). Although there is little amino acid sequence similarity between NifD, VnfD and AnfD, and between NifK, VnfK, and AnfK, the residues that coordinate their P-clusters and active-site cofactors (FeMo-

co, FeV-co and FeFe-co) are conserved across all nitrogenases. In addition, X-ray atomic structures of Mo-nitrogenase and V-nitrogenase showed they are structurally very similar (Einsle *et al.*, 2002; Sippel *et al.*, 2017). The nitrogenase component of alternative nitrogenases retain the structural core of the MoFe protein but require the presence of an extra δ -subunit encoded by the *vnfG* and *anfG* genes, respectively. The G subunit is essential for N₂ reducing activity as its removal renders nitrogenase with altered catalytic properties (Chatterjee *et al.*, 1997). The G subunits were also suggested to play a role in transferring the active-site cofactors, FeV-co or FeFe-co, to their respective apoenzymes, similar to the role of NafY in Mo-nitrogenase. This hypothesis was based on the fact that both proteins interact with their respective cofactor, although they do not exhibit similar amino acid sequence (Waugh *et al.*, 1995; Dyer *et al.*, 2003).

Other gene products required for the biosynthesis of FeV-co are encoded in the *vnfENX* operon located downstream of *vnfH*. VnfEN is homologous to NifEN and is supposed to play a similar role in the incorporation of V into FeV-co (Eady, 1996).

In *A. vinelandii*, the minimal set of *nif* genes that appear to be required for the biosynthesis of cofactors for all three nitrogenases consists of *nifU*, *nifS*, *nifB*, *nifV* and *nifM*. NifU and NifS function as providers of [4Fe-4S] cluster precursors to NifB-co, the product of NifB catalysis that serves as obligate intermediate for FeMo-co, FeV-co and FeFe-co biosyntheses (Shah *et al.*, 1994). Differences in cofactor biosynthesis appear to occur after NifB-co synthesis, which pinpoints them to its specific EN scaffold protein. Since Mo- and V- nitrogenases have their own EN scaffolds, while the Fe-only nitrogenase does not, heterometal discrimination appears to have place at the EN scaffold. The participation of NifV is required to synthesize the organic moiety homocitrate, required by all nitrogenases for N₂ reduction (Hawkes *et al.*, 1984; Jacobson *et al.*, 1989b; Kennedy and Dean, 1992). Finally, a role for NifM in maturation of VnfH and AnfH proteins has been proposed, since diazotrophic growth of *nifM* mutants medium was also affected in Mo-deficient (Kennedy *et al.*, 1986). Nevertheless, more recent work has shown that NifM was not essential for the functional expression of Fe-only nitrogenase in engineered *E. coli* strains (Yang *et al.*, 2014).

Figure 4. Organization of Mo, V and Fe nitrogenase gene clusters in *A. vinelandii*. Chromosomal position of genes for the Mo-nitrogenase (*nif*, orange code), V-nitrogenase (*vnf*, green code), Fe-only nitrogenase (*anf*, blue code), and molybdate transport (*mod*, pink to purple codes) are shown in a circular *A. vinelandii* genome. Relevant genes within each gene cluster are labeled. Black arrows indicate promoter regions.

In *A. vinelandii*, nitrogenase expression is also regulated by the presence of ammonium which signal is mediated by the cellular C/N balance (Kennedy and Toukdarian, 1987). Repression by ammonium differs between nitrogenases. While regulation of the V-nitrogenase is produced at the post-transcriptional level, the Mo- and Fe-only nitrogenases are repressed at the transcriptional level (Jacobitz and Bishop, 1992). The regulatory elements NifA, VnfA and AnfA are required for the transcription of the *nif*, *vnf* and *anf* operons, respectively. For Mo-nitrogenase expression, *A. vinelandii* requires the NifA activator and the presence of Mo. NifA is an enhancer-binding protein that binds to specific DNA sequences upstream of the *nif* genes and interacts with the sigma subunit (α^{54}) of the RNA polymerase to initiate the transcription (Dixon and Kahn, 2004). NifA is activated under nitrogen-limiting conditions (i.e. lack of ammonium and high C/N ratio), whereas the NifL anti-activator senses the cellular redox (oxidation) status and binds to NifA to block its activity when conditions are not optimal for nitrogenase

activity (Martinez-Argudo *et al.*, 2004). Unlike the Mo-nitrogenase, expression of the alternative systems is not repressed by an anti-activator, such as NifL (Walmsley *et al.*, 1994). Regulation of nitrogenases by metal ion availability is particularly interesting. Transcription of *vnfA* is repressed by Mo but not by ammonium, in contrast to *anfA* transcription, which is repressed by ammonium, V and Mo. As mentioned above the V-nitrogenase structural genes are split into two operons (see Figure 4) so that VnfD₂GK and VnfH can be expressed independently (Mus *et al.*, 2018). Early studies reported that favorable conditions in which the Fe-only nitrogenase was expressed, also requires the presence of the *vnfH* product (Joerger *et al.*, 1991). Mutant strains lacking *vnfH* showed growth impairment in a Mo- and V-deficient diazotrophic growth medium. In addition, VnfA is involved in repressing the expression of *nifHDK* operon in the absence of Mo and that of the *anfHDK* operon in the presence of V (Walmsley *et al.*, 1994).

Despite the similar features shared by all three nitrogenases, they show some differences in catalytic activities. The Mo-nitrogenase is the most efficient enzyme in terms of catalytic N₂ reduction and only 27% of the total electron flux is directed towards H₂ evolution (Simpson and Burris, 1984). The Fe-only nitrogenase diverts more electrons to H₂ production having reported ratios of 7 H₂ produced by 1 N₂ reduced in *A. vinelandii* (Harris *et al.*, 2018). Early studies found that nitrogenases could also reduce other substrates such as acetylene (C₂H₂). The Mo-nitrogenase yields ethylene (C₂H₄) by the consumption of two protons and two electrons in a one-step reaction, whereas alternative nitrogenases yield a mixture of ethylene (C₂H₄) and ethane (C₂H₆), the latter requiring the consumption of four protons and four electrons (Dilworth *et al.*, 1987). About 1.5% of total acetylene reduced by alternative nitrogenases produce ethane. This feature allow scientists to measure and distinguish by gas chromatography the *in vivo* and *in vitro* activities of the Mo-nitrogenase from those of the alternative nitrogenases (Seefeldt *et al.*, 2013).

5. ENGINEERING NITROGENASE IN EUKARYOTES

Successful expression and maturation of nitrogenase in eukaryotes is one of the proposed approaches to develop crops self-supported by BNF (see title 3). As the Nif proteins are from bacterial or archaeal origin, it was proposed that expression of the nitrogenase components in plant cells should be carried out in cell organelles such as plastids or mitochondria as they arose evolutionary from prokaryotic systems (Schwartz and Dayhoff, 1978). However, there are two main barriers believed to impair expression of functional nitrogenase in eukaryotes, especially in plants: the sensitivity of nitrogenase to O₂ exposure and the complexity of emulating the finely tuned process of nitrogenase biosynthesis (Goldberg *et al.*, 1987; Curatti and Rubio, 2014; Poza-Carrión *et al.*, 2014).

Expressing Nif proteins into the mitochondria is an attractive strategy with several advantages (Curatti and Rubio, 2014). This organelle has a high rate of O₂ consumption, and plentiful ATP and reducing power generated by respiration. This organelle also harbors a [Fe-S] cluster assembly machinery that is not only required for their own set of Fe-S proteins, but it is also involved in the biogenesis of metalloproteins located in the cytosol and nucleus (Lill *et al.*, 2012). Mitochondrial Isu1/Isu2 and Nfs1 are homologues to bacterial IscU/IscS and Nif-specific NifU/NifS, which are all involved in Fe-S cluster assembly (Zheng *et al.*, 1998; Agar *et al.*, 2000; Ramazzotti *et al.*, 2004). All these advantages favor using the mitochondria matrix for the biosynthesis of FeMo-co and the complete Mo-nitrogenase system.

To address the expression of functional nitrogenase in a eukaryotic cell, the simpler organism *S. cerevisiae* has been used as model because of its genetic amenability and easiness to culture under different oxygenation regimes. Successful attempts have been recently reported in which the O₂ sensitivity problem was greatly ameliorated by respiratory protection. First, the O₂-labile NifH component has been functionally expressed in the matrix of *S. cerevisiae* mitochondria, providing proof of concept that O₂-sensitive proteins can be assembled in this organelle (López-Torrejón *et al.*, 2016). Furthermore, expression of 9 *nif* genes (*nifU*, *nifS*, *nifM*, *nifH*, *nifB*, *nifE*, *nifN*, *nifD*, and *nifK*) resulted in proper target to the mitochondria matrix, protein processing, and

NifDK tetramer formation (Burén *et al.*, 2017b). In addition, the GoldenBraid 2.0 toolkit was adapted to independently express 14 *nif* genes (*nifU*, *nifS*, *nifM*, *nifH*, *nifB*, *nifE*, *nifN*, *nifD*, *nifK*, *nifV*, *nifX*, *nifQ*, *nifF*, and *nifJ*) in *S. cerevisiae* using promoters of different strength and regulation and a set of several mitochondria targeting signals (Pérez-González *et al.*, 2017). Proper expression and mitochondrial processing of the 14 Nif proteins was reported, opening the possibility of rapid combination and testing of these elements. Hence, research towards expression of active assemblies of nitrogenase components in yeast mitochondria is being established for further transfer it into plants.

However, appropriate supply of Fe and Mo is necessary for Mo-nitrogenase. In this thesis we engineered a rudimentary pathway in *S. cerevisiae* that transfers Mo from the extracellular environment into the mitochondrial matrix and that converts it into a form usable for FeMo-co synthesis. In this regard, the pathway must ensure that Mo-loaded active NifQ is present in the mitochondrial matrix. Since *S. cerevisiae* is one of the organisms that have lost the ability to utilize Mo, this biological system is a relatively clean model to establish a Mo trafficking pathway towards N₂ fixation in eukaryotes.

Material and methods

6. ORGANISMS

6.1. *A. vinelandii*

A. vinelandii DJ, a highly transformable variant of OP, was obtained from Prof. Dennis Dean (Virginia Tech, USA) and used as wild-type organism. All transformants were obtained by double recombination events in the DJ background summarized in Table 2 (see section 9.1). Strain DJ33 is mutant for the alpha and beta subunits of the MoFe protein (Robinson *et al.*, 1986). Strain UW356 is a Nif⁻ mutant with a substitution in the transcriptional regulator *nifA* gene with an spectinomycin cassette (Poza-Carrión *et al.*, 2014). Storage at -80°C was carried out in cryo-cultures of 1.5 ml by adding glycerol to a fresh culture to a final concentration of 20% v/v and freezing in liquid N₂.

Table 2. Strains of *A. vinelandii*.

<i>Strain</i>	<i>Genotype</i>	<i>Source</i>
DJ	<i>Wild type</i> . Highly transformable variant of OP	Dennis Dean
DJ33	$\Delta nifDK$	Dennis Dean
UW300	$P_{nifH}::9His-nifQ$	This lab
UW356	$\Delta nifA::spc$	This lab
UW394	$\Delta mosBA::spc$	This thesis
UW463	$\Delta mosBA::spc; \Delta vnfDGK::tet_{\rightarrow}; \Delta anfDGK::kan_{\rightarrow}$	This thesis
UW466	$\Delta vnfDGK::tet_{\rightarrow}; \Delta anfDGK::kan_{\rightarrow}$	This thesis

6.2. *E. coli*

The strains used in this thesis are summarized in Table 3. Transformants were obtained as detailed in section 9.2. Storage at -80°C was carried out in cryo-cultures by adding glycerol to a fresh culture to a final concentration of 20% v/v and freezing in liquid N₂.

Table 3. *E. coli* strains used.

<i>Strain</i>	<i>Genotype</i>	<i>Selection</i>	<i>Source</i>
DH5 α	F'/endA1 hsdR17 (rK- mK+) supE44 thi-1 recA1 gyrA (NaIR) rel A1 Δ (lacZIYA -argF) U169 deoR ($\emptyset$ 80 dlac Δ (lacZ) M15)	lacZ interruption using 5-bromo-4-chloro-3-indolyl- β -galactopyranoside	(Sambrook and Russell, 2001)
BL21(DE3) pLysS	F'- ompT gal[dcmlon] hsdSB (rB- mB-; an <i>E. Coli</i> B strain) with DE3 and pLysS	DE3, a λ prophage carrying the T7 RNA polymerase gene. Also carries the polymerase lysozyme in plasmid pLysS	Promega

6.3. *S. cerevisiae*

The wild type strains and mutant strains used in this thesis are summarized in Table 4 and Table 5. Transformants harboring expression vectors or integrative modules were obtained as detailed in section 10.3. Some strains were bought from EURO-SCARF or were kindly donated by Prof. Manuel Gonzalez Guerrero (UPM, Spain). Storage at -80°C was carried out in cryo-cultures of 1.5 ml by adding glycerol to a fresh culture to a final concentration of 20% v/v and freezing in liquid N₂.

Table 4. Collection strains and vector-transformed strains of *S. cerevisiae*.

<i>Strain</i>	<i>Genotype</i>	<i>Source</i>
ATCC W303-1A	MATa ura3-1; trp1-1; leu2-3,112; his3-11; ade2-1; can1-100	EURO-SCARF
BY4741	MATa his3 Δ 1; leu2 Δ 0; met15 Δ 0; ura3 Δ 0	Gonzalez's lab
CEN.PK2-1C	MATa ura3-52; trp1-289; leu2-3,112; his3 Δ 1; MAL2-8C; SUC2	EURO-SCARF

<i>Strain</i>	<i>Genotype</i>	<i>Source</i>
YLR58	BY4741 a his3 Δ 1; leu2 Δ 0; met15 Δ 0; pDR196	Gonzalez 's lab
YLR59	W303-1A a trp1-1; leu2-3,112; his3-11; ade2-1; can1-100; pDR196	This thesis
YLR260	CEN.PK2-1C a trp1-289; leu2-3,112; his3 Δ 1; MAL2-8C; SUC2; pDR196	This thesis
YLR254	BY4741 a his3 Δ 1; leu2 Δ 0; met15 Δ 0; pDR196[MtMOT1.3]	This thesis
YLR256	W303-1A a trp1-1; leu2-3,112; his3-11; ade2-1; can1-100; pDR196[MtMOT1.3]	This thesis
YLR261	CEN.PK2-1C a trp1-289; leu2-3,112; his3 Δ 1; MAL2-8C; SUC2; pDR196[MtMOT1.3]	This thesis
YLR257	W303-1A a trp1-1; leu2-3,112; his3-11; ade2-1; can1-100; pDR196[AtMOT1]	This thesis
YLR258	BY4741 a his3 Δ 1; leu2 Δ 0; met15 Δ 0; pDR196[AtMOT1]	This thesis
YLR262	CEN.PK2-1C a trp1-289; leu2-3,112; his3 Δ 1; MAL2-8C; SUC2; pDR196[AtMOT1]	This thesis

Table 5. CEN.PK2-1C strains with modules integrated in its genome.

<i>Strain</i>	<i>Genotype</i>	<i>Source</i>
YLR372	ura3-52; trp1-289; leu2-3,112; his3 Δ 1; MAL2-8C; SUC2; TDH3p-FLAG-ymosA-FBA1t-TDH3p-FLAG-ymosB-FBA1t-hphMX4::YORW Δ 22	This thesis
YLR373	ura3-52; trp1-289; leu2-3,112; his3 Δ 1; MAL2-8C; SUC2; can1-100; TDH3p-MAM33-Strep-ymosA-FBA1t-TDH3p-MAM33-Strep-ymosB-FBA1t-hphMX4::YORW Δ 22	This thesis
YLR396	ura3-52; trp1-289; leu2-3,112; his3 Δ 1; MAL2-8C; SUC2; pTEF1-SU9-AtMOT1-yEGFP-tCYC1-hphMX4::YORW Δ 15	This thesis

<i>Strain</i>	<i>Genotype</i>	<i>Source</i>
YLR397	ura3-52; trp1-289; leu2-3,112; his3Δ 1; MAL2-8C; SUC2; pTEF1- <i>AtMOT1</i> -yEGFP-tCYC1-hphMX4::YORWΔ15	This thesis
YLR418	ura3-52; trp1-289; leu2-3,112; his3Δ 1; MAL2-8C; SUC2; pGal1-SU9(MTS)-Strep tag- <i>ynifQ</i> -tTDH2- KanMX4::YORWΔ22	This thesis
YLR565	ura3-52; trp1-289; leu2-3,112; his3Δ 1; MAL2-8C; SUC2; pGal1-ATPA-Strep tag- <i>ynifQ</i> -tTDH2-KanMX4::YORWΔ22; TDH3p-SU9-8His- <i>ymosA</i> -FBA1t-TDH3p-SU9-8His- <i>ymosB</i> -FBA1t-hphMX4-pTEF1- <i>AtMOT1</i> -yEGFP- tCYC1::YORWΔ15	This thesis
YLR574	ura3-52; trp1-289; leu2-3,112; his3Δ 1; MAL2-8C; SUC2; TDH3p-SU9-8His- <i>ymosA</i> -FBA1t-TDH3p-SU9-8His- <i>ymosB</i> -FBA1t-hphMX4-pTEF1- <i>AtMOT1</i> -yEGFP- tCYC1::YORWΔ15	This thesis
YLR659	ura3-52; trp1-289; leu2-3,112; his3Δ 1; MAL2-8C; SUC2; pGal1-ATPA-Strep tag- <i>ynifQ</i> -tTDH2-KanMX4::YORWΔ22; TDH3p-SU9-8His- <i>ymosA</i> -FBA1t-TDH3p-SU9-8His- <i>ymosB</i> -FBA1t-hphMX4::YORWΔ15	This thesis

7. MEDIA AND REAGENTS

All chemicals were purchased from Sigma-Aldrich, VWR, New England Biolabs, IBIAN Technologies, Merck or Invitrogen unless specifically stated. All reagents purchased were of appropriate quality for their use.

7.1. Culture media for bacteria

7.1.1. Burk's medium

Burk's medium was used for cultivation of *A. vinelandii* (Strandberg and Wilson, 1968). Non-diazotrophic Burk's medium contains ammonium (NH₄⁺) as nitrogen source, while

diazotrophic Burk's medium will not contain any combined nitrogen source. Antibiotics were added as needed.

Liquid Burk's medium

H ₂ O MilliQ	35 ml
BB (5x)	10 ml
BS (10x)	5 ml
NH ₄ Acetate 22%	0.5 ml

Agar Burk's medium

H ₂ O MilliQ	15 ml
BB (5x)	10 ml
BS (10x)	5 ml
NH ₄ Acetate 22%	0.5 ml
Agar 3%	20 ml

BB (5x) = 0.2% (w/v) KH₂PO₄ + 0.8% (w/v) K₂HPO₄.

BS (10X) = 0.05% (v/v) 21 mM Na₂MoO₄ + 0.2% (w/v) MgSO₄·7H₂O + 0.09% (w/v) CaCl₂·2H₂O + 0.1% (v/v) 179 mM FeCl₃·6H₂O + 20% (w/v) sucrose.

Note: Na₂MoO₄ concentration was changed depending on the growth conditions. When indicated, NaVO₃ was added.

Antibiotics (Final concentration)

Spectinomycin (10 µg/ml)

Kanamycin (0.5 µg/ml)

Tetracycline (2.5 µg/ml)

Rifampicin (5 µg/ml)

Ampicillin (50 µg/ml)

7.1.2.LB (Luria-Bertani)

LB medium was used for cultivation of *E. coli* strains (Bertani, 1951). Antibiotics were added as needed.

1% (w/v) Tryptone

1% (w/v) Yeast extract

1% (w/v) NaCl

Adjust pH to 7.0. For agar plates, add 15g/l agar.

Antibiotics and visual indicators (Final concentrations)

Ampicillin (150 µg/ml)

Carbenicillin (100 µg/ml)

Spectinomycin (50 µg/ml)

Kanamycin (50 µg/ml)

Tetracycline (12.5 µg/ml)

Chloramphenicol (50 µg/ml)

IPTG (1 mM)

X-Gal (20 µg/ml)

7.2. Culture media for *S. cerevisiae*

7.2.1. YPAU (Dextrose/Glycerol/Galactose)

The YPAUD and YPAUGly are rich media used for *S. cerevisiae* growth (Sherman *et al.*, 1979). The YPAUGal is a rich medium containing galactose as inductor for GAL promoters. When indicated, YPD without adenine and uracil was used.

<u>Final concentration</u>		1X	2X
		<u>500ml</u>	<u>500ml</u>
Yeast Extract	1%	5	10 g
Bacto Peptone	2%	10	20 g
(Agar)	2%	10	20 g
Adenine Hemi sulfate	0.2 mg/ml	0.1	0.2 g
Uracil	0.27 mg/ml	0.135	0.27 g
(Dextrose)	2%	25	50 ml of 40% stock
(Glycerol)	3%	18.75	37.5 ml of 80% stock
(Galactose)	2%	25	50 ml of 40% stock
(G418)	200 µg/ml		
(Hygro)	200 µg/ml		

Note: Always add dextrose, glycerol or galactose separately after autoclaving base medium.

7.2.2.SD (*Synthetic defined medium*)

SD is a selective growth medium for *S. cerevisiae* (Sherman *et al.*, 1979). SD was used with glucose or galactose when required. Amino acids were added when auxotrophic strains were grown.

0.19% (w/v) YNB (Yeast Nitrogen Base without amino acids and $(\text{NH}_4)_2\text{SO}_4$)

0.5% (w/v) $(\text{NH}_4)_2\text{SO}_4$

2% (w/v) Glucose or Galactose

0.147% (w/v) Dropout (without Leucine, Adenine, Uracil, Tryptophan, Histidine)

For agar plates, add 15 g/l agar.

Amino acids (Final concentration)

Uracil 0.2% (w/v)

Adenine Hemi sulfate 0.22% (w/v)

Tryptophan 0.3% (w/v)

Leucine 0.8% (w/v)

Histidine 0.2% (w/v)

8. CULTURE CONDITIONS AND PROTEIN INDUCTIONS

8.1. *A. vinelandii*

A. vinelandii strains were cultured at 30°C in baffled flasks with shaking at 200 rpm. Culture volume was $\frac{1}{4}$ of the flask volume to favor oxygenation. Conventional cultures were carried out in liquid Burk's medium (see section 7.1.1). For growth curves, three biological replicates were assayed, and OD measurement standard error for each time is represented.

8.1.1. Time-course experiments

Experimental procedure for Mo-limited conditions consisted in acid-wash treatment of all glassware and the use of polypropylene plastic material. Culture medium for those conditions was made with special reagents containing less than 0.0005% heavy metals. Sterile medium was obtained by medium filtration in polypropylene bottles containing a PES membrane of 0.2 μm (Thermo Fisher Scientific, Waltham, Massachusetts, USA). When time-course experiments required molybdate in the medium, then sodium molybdate (Na_2MoO_4) was added at 0.2 μM , 1 μM or 1 mM. Sodium vanadate (NaVO_3) was added at 1 μM in all media to allow derepression of the V-nitrogenase and growth in the absence of Mo.

A. vinelandii cells were grown in non-diazotrophic Burk's agar plates (ammonia-containing medium; see section 7.1.1) with or without molybdate for at least 3 days. One colony was inoculated in non-diazotrophic liquid Burk's medium (see section 7.1.1) and grown overnight to an $\text{OD}_{600\text{nm}} \approx 1.2$. When Mo-free cells were required, pre-cultures of *A. vinelandii* strains were grown for at least three days by changing the Mo-limited medium each day. In all cases, cells from the exponential phase were centrifuged at 5,000 rpm for 5 minutes, washed with Burk's medium without ammonium (with or without molybdate) and inoculated to an initial $\text{OD}_{600\text{nm}}$ of 0.3 in 1-liter flasks containing 250 ml of diazotrophic Burk's medium (ammonium-free medium) with the indicated molybdate conditions. Samples from the time-course induction were taken for further analysis.

8.1.2. Small-scale cultures

Growth curves were performed at small scale to determine the growth rates of *A. vinelandii* strains grown under different media conditions. Strains were grown in non-diazotrophic Burk's agar plates (see section 7.1.1) with or without molybdate for at least 3 days. One colony from each strain was inoculated into non-diazotrophic liquid Burk's medium (see section 7.1.1) and grown overnight to an $\text{OD}_{600\text{nm}} \approx 2$. When Mo-free cells were required, pre-cultures of *A. vinelandii* strains were grown for at least three days by changing the Mo-limited medium each day. The metal content of the diazotrophic Burk's liquid medium (see section 7.1.1) was modified depending on the

growth condition being tested: 0 μM Na_2MoO_4 + 1 μM NaVO_3 ; 1 μM Na_2MoO_4 + 1 μM NaVO_3 ; 0 μM Na_2MoO_4 + 1 μM NaVO_3 + 1 μM Na_2WO_4 , and 1 μM Na_2MoO_4 + 1 μM NaVO_3 + 1 μM Na_2WO_4 . In addition, non-diazotrophic growth conditions were tested. Cells were inoculated to an initial $\text{OD}_{600\text{nm}}$ of 0.1 in 300 μl of medium. Bioscreen C was used with the following growth conditions:

Temperature: 30°C	Speed: intensive
Measure length: 2 days	Continuous shaking
Measure interval: 60 minutes	Step: 80 mini movements per second
$\text{OD}_{600\text{nm}}$	

8.1.3. Competitive index assay

Competitive index (CI) was defined as the mutant to *wild-type* ratio within the output sample of a co-culture, divided by the corresponding ratio in the co-culture inoculum (Macho *et al.*, 2007). Pre-cultures of *wild-type* and UW394 (see section 7.1.1) were grown till log phase was reached. Inocula of both strains were mixed at $\text{OD}_{600\text{nm}}$ of 0.1 each in the indicated Burk's medium and incubated at 30°C. Twenty μl of serial dilutions of the mixed cultures were sampled at incubation times 0 hour and 22 hours and plated onto Burk's solid medium and Burk's solid medium supplemented with spectinomycin (to inhibit growth of *wild-type* strain). Time 0-hour determination was needed to obtain the input mutant to *wild-type* ratio. The time 22 hours determination was needed to obtain the output CI values. The CI value is the mean of three independent experiments and the error bars represent standard error.

8.1.4. Mo-depleted cultures

Exhaustive Mo-deplete conditions were needed in some experiments to investigate the role of MoSto and the possible expression of hybrid nitrogenases in certain mutant strains. All glassware was acid treated and polypropylene plastic material was used to manipulate the medium. The culture medium was made with reagents with less than 0.0005% heavy metals and filtered in polypropylene bottles containing a PES membrane with pore size of 0.2 μm . Further removal of molybdate from the medium was achieved by using *A. vinelandii* scavenging capability, as follows.

A. vinelandii DJ strain was grown in non-diazotrophic Burk's agar plates without molybdate for three days. One colony was inoculated into non-diazotrophic liquid Burk's medium without molybdate and grown for at least three days by changing the medium each day. When cells were in exponential phase at around $OD_{600nm} \approx 1.2$, the culture volume required to inoculate a 4-liter bioreactor to an initial $OD_{600nm} \approx 0.4-0.5$ was centrifuged at 5,000 rpm for 5 minutes and washed with Mo-free medium. The Mo-starved cells were then inoculated into the bioreactor containing 4 liters of Mo-limited diazotrophic Burk's medium supplemented with $1 \mu M NaVO_3$. After 2 hours of growth, 2 liters of culture were removed from the bioreactor, centrifuged at 5,000 rpm for 5 minutes and the supernatant was filtered in polypropylene bottles containing a PES membrane with pore size of $0.2 \mu m$. The remaining 2 liters of culture were amended with 22 mM ammonium acetate and incubated for 1 additional hour. Finally, the rest of the culture was centrifuged at 5,000 rpm for 5 minutes and the supernatant was filtered in polypropylene bottles containing a PES membrane with pore size of $0.2 \mu m$. Both filtrates remained sterile at room temperature for months.

The Mo-deplete ammonium-containing Burk's medium was used as non-diazotrophic medium for the 3-day pre-cultures of further experiments. The Mo-deplete ammonium-free Burk's medium was used as diazotrophic medium for time-course and protein induction experiments. Time-course growth experiments in Mo-deplete medium were carried out as described in section 8.1.2.

8.1.5. Growth conditions to purify MoSto from wild-type cells

One colony of *A. vinelandii* DJ was used to inoculate 250 ml of non-diazotrophic Burk's medium and the culture was grown overnight to an $OD_{600nm} \approx 2$. As MoSto protein is also expressed under non-diazotrophic conditions, 100 liters of Burk's medium (see section 7.1.1) supplemented with 12.8 mM urea and $10 \mu M Na_2MoO_4$ were inoculated with the pre-culture at an OD_{600nm} of 0.015 and cultured in a 300-liter bioreactor. Cells were grown at $30^\circ C$ maintaining 3% dissolved O_2 and pH 7.2 for 22 h. Cells were collected by passing through a hollow fiber membrane, which concentrated the culture down to 6 liters, and then by centrifugation at 5,000 rpm for 5 minutes. The cell pellet was collected in liquid N_2 and stored at $-80^\circ C$.

8.1.6. Growth conditions to purify Mo-nitrogenase components from UW463

For NifDK/NifH purification, derepression of UW463 strain (see Table 2) was carried out in a 4-liter bioreactor. First, UW463 was cultured in Mo-depleted agar plates for at least 3 days. Then, one colony was inoculated in non-diazotrophic Mo-deplete medium for at least three days changing the medium each day. Cells in the exponential growth phase ($OD_{600nm} \approx 1.2$) were collected and used to inoculate a 4-liter bioreactor containing diazotrophic Mo-deplete medium. Cells were grown at 30°C with 200 rpm agitation and injection of 2 l/min of air for 4 days. Cells were collected by centrifugation at 5,000 rpm for 5 minutes and the collected pellet was frozen in liquid N₂ and stored at -80°C. Nitrogenase derepression under Mo-deplete conditions was extremely slow. Therefore, the required time for observing nitrogenase activity was much longer than the standard 4 hours. Before collection, cells were checked for production of ethane by the *in vivo* nitrogenase activity (indicative of alternative nitrogenase expression) (see section 11.8.1)

8.2. *E. coli*

E. coli strains were cultured in flask at 37°C with a shaking speed of 200 rpm. When required for proper protein induction, culture temperature was changed. Conventional growth was carried out in LB medium (see section 11.8.1).

8.2.1. Induction of MoSto protein in *E. coli*

One colony from a plate of BL21(DE3) pLysS *E. coli* transformants (see section 9.2) was grown in 250 ml of LB medium to an $OD_{600nm} \approx 2$. An aliquot was used to inoculate, at OD_{600nm} of 0.022, a bioreactor containing 4 liters of LB medium supplemented with 1% peptone and 0.05% glucose. To induce MoSto expression, the medium was supplemented with 1 mM Na₂MoO₄, 0.3 mM L-cysteine and 0.3 mM ferric ammonium citrate. Growth was monitored for 20 hours by maintaining a temperature of 30°C, an agitation speed of 300 rpm, and the injection of 2.5 l/min of air. Cells were collected by centrifugation at 5,000 rpm for 5 minutes and the cell pellet was frozen in liquid N₂ and stored at -80°C.

8.3. *S. cerevisiae*

S. cerevisiae strains were cultured in flasks at 28°C with a shaking speed of 200 rpm. When required for proper expression of recombinant proteins, culture temperature was changed. Conventional growth was carried out in YPAU (see section 7.2.1) or SD medium (see section 7.2.2). For growth curves, three biological replicates were assayed, and OD measurement standard error for each time is represented.

8.3.1. *Time-course experiments*

Time-course experiments were carried out to test growth and molybdenum accumulation in the presence of increasing Na₂MoO₄ concentrations. Molybdate was added to the medium at final concentrations of 25 μM or 50 μM. Control growth conditions in the absence of Na₂MoO₄ were also assayed.

Yeast strains were cultured in SD glucose (2%) agar plates. To start liquid cultures, one colony was used to inoculate SD glucose (2%) liquid medium and grown was carried out overnight until an OD_{600nm} ≈ 2 was reached. Cells were then collected by centrifugation at 5,000 rpm for 5 minutes and used to inoculate, to an initial OD_{600nm} of 0.4, 250-ml flasks containing 50 ml of SD glucose (2%) liquid medium supplemented with different concentrations of molybdate. Yeast were cultured for 6 hours, during which samples were taken at times 0 hours, 30 minutes, 2 hours and 6 hours for further analysis (see section 8.3.1).

For molybdenum test toxicity, Na₂MoO₄ was added at 50 μM, either in liquid or solid minimal medium. A control condition without the addition of Na₂MoO₄ was also assayed. Solid plates containing dilutions of fresh cultured yeast and small-scale cultures (see section 8.3.2) were grown for 2 days.

8.3.2. *Small-scale cultures*

Growth curves of parental and engineered yeast strains expressing heterologous proteins were obtained from small-scale cultures in which molybdate toxicity was tested. Strains were first plated in SD glucose (2%). Then, one colony was used to inoculate SD glucose (2%) liquid medium or YPAU glucose (2%) liquid medium and

growth proceeded overnight until an $OD_{600nm} \approx 2$. Cells were collected by centrifugation at 5,000rpm for 5 minutes and use to inoculate 300 μ l of medium in 96-well plates to an initial OD_{600nm} of 0.1 . The Na_2MoO_4 concentrations tested in SD glucose (2%) or YPAU glucose (2%) liquid medium were: 0 μ M; 25 μ M; 50 μ M ; 100 μ M. Bioscreen C was used with the following growth conditions:

Temperature: 28°C	Speed: medium
Measure length: 2 days	Continuous shaking
Measure interval: 60 minutes	Step: 4 mini movements per second
OD_{600nm}	

8.3.3. Protein induction

Yeast strains were grown in YPD plates for two days. One colony was used to inoculate a 1-liter flask containing 250 ml of YPAUGly (3%) supplemented with carbenicillin (100 μ g/l; to avoid bacteria contamination), and this pre-culture was grown overnight. Pre-culture cells were collected by centrifugation at 5,000 rpm for 5 minutes after which the pellet was re-suspended in YPAUGal (2%) and used to inoculate a 4-liter bioreactor containing YPAUGal (2%) medium supplemented with ampicillin (100 μ g/ml) and kanamycin (50 μ g/ml) to an initial OD_{600nm} of 0.2. Growth proceeded for at least 16 hours at a temperature of 30°C with agitation at 200 rpm and injection of 2.5 l/min of air. Cells were then collected by centrifugation at 5,000 rpm for 5 minutes and reintroduced in the bioreactor filled with 3 l of SD minimal medium with dropout supplements, 2% galactose, Na_2MoO_4 , 0.6 mM L-cysteine and 0.9 mM ferric ammonium citrate. The culture was incubated for 2-3 h at 30°C with 200 rpm and injection of 2.5 ml/min of air. Cells were collected by centrifugation at 5,000 rpm for 5 minutes and the pellet was stored in liquid N_2 .

9. TRANSFORMATION

9.1. *A. vinelandii*

Protocols for *A. vinelandii* transformation and gene replacement were carried out according to (Page and Igerstrom, 1979; Dos-Santos, 2011).

- Competent cells preparation:
 - *A. vinelandii* cells were grown on Burk's Mo-limited solid medium for 2-3 days at 30°C.
 - Three-four loops of plated cells were used to inoculate 1 ml of Burk's-liquid medium lacking Mo and Fe.
 - Three different cell-suspension dilutions were prepared by inoculating 10 ml of Mo-free, Fe-free Burk's liquid in 50-ml Falcon tubes with 500 µl, 100 µl, or 20 µl of the cell suspension prepared in the second step. Cells were incubated overnight at 30°C with shaking at 200 rpm.
 - The culture showing OD_{600nm} of 1.0 by the time of carrying out the transformation was used. Cells cultured this way develop the greenish pigment azotobactin, which is an iron siderophore.
- Transformation:
 - 200 µl of the chosen cell suspension was combined in a sterile 1.5 ml centrifuge tube with 200 µl of a buffer containing 20 mM MOPS, pH 7.4 and 16 mM MgSO₄.
 - 1 µg of DNA was added to the cell mixture and incubated at 30°C with shaking at 200 rpm for 45min.
 - The transformation mixture was transferred to 12.5 ml of Burk's liquid medium in a 100 ml flask and incubated overnight at 30°C with shaking at 200 rpm.
- Selection:
 - Recombinant strains were selected on solid medium containing the appropriate antibiotic and growth conditions. Three different plates were inoculated with 1000 µl, 100 µl and 10 µl of the 12.5 ml culture prepared in the previous step.
 - Positive colonies were streaked at least once on a fresh plate and then cultured in liquid medium.
- Genotypic analysis of the putative mutant strains was performed by PCR (see section 10.1). One-ml samples taken from the previous cultures were used for gDNA purification (see section 10.6.1).

- Strains conservation:
 - After positive genotypic analysis, cultured cells were collected by centrifugation at 5,000 rpm for 5 minutes and then re-suspended in 1 ml Burk's medium mixed with ¼ volumes of sterile 80% glycerol. Cells were stored at -80°C.

9.2. *E. coli*

Protocol for *E. coli* transformation were carried out according to (Sambrook and Russell, 2001).

- Competent cells preparation (quimiocompetent):
 - *E. coli* cells were streaked on LB solid medium and incubated at 37°C for 1 day.
 - Plated cells were used to inoculate a 3-ml pre-culture of LB liquid medium that was grown overnight at 37°C with shaking.
 - 1 ml of the pre-culture was used to inoculate 200 ml of LB in a 1-l flask and was grown at 37°C until the culture reached an OD_{600nm} around 0.4 - 0.45.
 - Cells were collected by centrifugation at 4°C for 7 minutes at 3,000 rpm.
 - Pellet was re-suspended in 40 ml of cold MgCl₂ 0.1 M. The suspension was kept on ice for 15-20 minutes.
 - Cells were then centrifuged at 4°C for 7 minutes at 3,000 rpm.
 - Pellet was re-suspended in 8 ml of cold CaCl₂ 0.1 M. The suspension was kept on ice for 1 hour.
 - 3 ml of 60% glycerol were added to the cell suspension.
 - Aliquots of 100 µl were made and frozen in liquid N₂. Aliquots were stored at -80°C until use.
- Transformation:
 - 100 µl of competent cells were thawed for 5 minutes on ice.
 - Cells were mixed with 100-200 ng of plasmid DNA and the mixture was incubated on ice for 20 minutes.
 - The mixture was subjected to a heat shock at 42°C for 1 minute.
 - 1 ml of LB was added and the transformation mixture was incubated for 1 hour at 37°C with shaking at 200 rpm.

- Selection:
 - The transformation mixture was centrifuged at maximum speed (13,000 rpm in a microfuge) for 1 minute. Pellet was re-suspended in 50 μ l of LB and inoculated plated on LB medium with the appropriate antibiotic for the selection of positive strains. LB plates were incubated at 37°C overnight.
 - Positive colonies were streaked on fresh LB plates containing the appropriate antibiotic and after growth they were reinoculated in liquid LB medium.
 - Genotypic analysis of putative mutant strains was carried out by PCR (see section 10.1). One ml samples from previous cultures were used for plasmid extraction (see section 10.5).
 - When necessary, DNA extracted from positive colonies were sent for DNA sequencing to Macrogen (Geumcheon-gu, Seoul, Korea).
- Strains conservation:
 - After positive genotypic analysis (and DNA sequencing), cultured cells were collected by centrifugation at 5,000 rpm for 5 minutes and then re-suspended in 1 ml of LB medium mixed with $\frac{1}{4}$ volumes of sterile 80% glycerol. Cells were stored at -80°C.

9.3. *S. cerevisiae*

The protocol for transformation of *S. cerevisiae* was carried out according to (Gietz and Schiest, 1990)

- Competent cells preparation:
 - *S. cerevisiae* cells were streaked on YPAUD plates and grown for 2 days at 28°C.
 - One colony was used to inoculate a 5-ml pre-culture of YPAUD medium in a 50-ml Falcon tube. Cells were grown at 28°C with 200 rpm shaking overnight.
 - When the pre-culture reached an OD_{600nm} 0.2, cells were removed and used to inoculate 25 ml of 2xYPAUD medium in a 250-ml flask.
 - When the culture reached an OD_{600nm}~1.2, cells were collected by centrifugation at 5,000 rpm for 5 minutes, washed with 25 ml of sterile MilliQ water and pelleted again.

- The pellet was re-suspended in 2 ml of 100 mM LiAc and 500- μ l aliquots were transferred to 1.5 ml tubes for each transformation.
- Cells from the previous aliquots were pelleted by centrifugation at 5,000 rpm for 3 minutes and then re-suspended in 100 μ l of 100 mM LiAc. Cell suspensions were incubated at 28°C for 30 min in a water bath.
- Transformation:
 - 5 μ l of salmon sperm DNA (10 mg/ml) was denatured by incubation at 98°C for 5 minutes and placed on ice. Components of the transformation mixture were added to denatured salmon sperm DNA in the following order:
 - x μ l DNA to be transformed (DNA vector: 1-2 μ g; Linearized DNA for genomic integration: 5 μ g)
 - 50 μ l competent cell solution in 100 mM LiAc
 - 240 μ l 50% PEG 4000 (w/w)
 - 30 μ l 1 M LiAc
 - Up to 350- μ l H₂O
 - Components were mixed by vortexing and then were incubated at 28°C with shaking of 200 rpm for 30 minutes.
 - Heat shock of the cell mixture was carried out at 42°C for 20 minutes.
 - The transformation mixture was incubated again at 28°C with shaking of 200 rpm for 30 minutes.
- Selection:
 - Cells were collected by centrifugation at 5,000 rpm for 3 minutes and re-suspended in 100 μ l of sterile MilliQ water.
 - Cell suspension was used to plate the appropriate solid medium. YPAUD plates were used when antibiotics were needed; and SD Glu (2%) plates were used when auxotrophic selection was needed.
 - Plates were kept at 28°C for 3-5 days until colonies were observed.
 - Positive colonies were streaked on a fresh YPAUD or SD Glu (2%) auxotrophic plate and then cultured in the corresponding liquid medium.
 - Genotypic analysis of the putative mutant strains was performed by PCR (see section 10.1). A portion from each positive colony from the previous step was used for gDNA purification (see section 10.6.2).

- Strain conservation.
 - After positive genotypic analysis, cultured cells were collected by centrifugation at 5,000 rpm for 5 minutes and then re-suspended in 1 ml of liquid medium mixed with ¼ volumes of sterile 80% glycerol. Cells were stored at -80°C.

10. DNA TECHNIQUES

10.1. *Polymerase chain reaction (PCR)*

This DNA technique was used to amplify fragments and to verify DNA fragments from generated clones.

10.1.1. *PCR for DNA amplification*

The DNA fragments for further cloning were amplified with Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Waltham, Massachusetts, USA). This polymerase is characterized for its ability to amplify large fragments, proofreading activity and generate blunt ends in the amplified products. Table 6 shows the concentration of the components for each reaction. Table 7 shows the standard PCR conditions used in this thesis. Template products for DNA amplification in this thesis were: genomic DNA from *A. vinelandii*, cDNA from *Arabidopsis thaliana Col-0* or synthetic DNA codon-optimized for yeast generated by GenScript (Piscataway, USA). The annealing temperature from each primer was calculated by the bioinformatics tool available in Thermo Fisher Scientific website. When the annealing temperature reached more than 72°C, a 2-step protocol is used. Primers are shown in section Table 10. DNA amplified by PCR was subjected to agarose gel electrophoresis (see section 10.7) and extracted as detailed in section 10.8.

Table 6. Concentration of PCR components.

<i>Components</i>	<i>Final concentration</i>
5x Phusion buffer	1X
dNTPs	200 μ M
Primer forward	0.5 μ M
Primer reverse	0.5 μ M
DMSO (for GC-rich amplicons)	3%
gDNA/cDNA/plasmid DNA	maximum 250 ng/ 10% V _f / 10 ng
Phusion Hot Start II Polymerase	0.5 U

Table 7. Standard PCR conditions.

<i>Cycle step</i>	<i>2-step protocol</i>		<i>3-step protocol</i>		<i>cycles</i>
	Temperature	Time	Temperature	Time	
Initial denaturation	98°C	30 ''	98°C	30 ''	1
Denaturation	98°C	10 ''	98°C	10 ''	35
Annealing	-	-	X°C	20 ''	
Extension	72°C	15 ''/ kb	72°C	15 ''/ kb	
Final extension	72°C	7 '	72°C	5 '	1
	4°C	hold	4°C	hold	

10.1.2. PCR to verify DNA fragments

DNA fragments cloned into vectors or integrated into the genome were verified by amplification with KAPA2G Fast HotStart ReadyMix PCR Kit (Sigma-Aldrich, St. Louis,

Missouri, USA). In some cases, colony PCR was carried out to find positive colonies. For this, PCR conditions were changed by increasing the initial denaturation step up to 13 minutes. Table 8 shows the concentration of the components for each reaction. Table 9 shows the standard PCR conditions used. The annealing temperature from each primer was calculated by the bioinformatics tool available in Thermo Fisher Scientific website. Primers are shown in section 0. DNA amplified by PCR was subjected to agarose gel electrophoresis (see section 10.7).

Table 8. Concentration of PCR components.

<i>Components</i>	<i>Final concentration</i>
KAPA2G Fast HotStart ReadyMix	1X
Primer forward	0.5 μ M
Primer reverse	0.5 μ M
gDNA/plasmid DNA	maximum 100 ng/ 1 ng

Table 9. Standard PCR conditions.

<i>Cycle step</i>	<i>3-step protocol</i>		<i>cycles</i>
	Temperature	Time	
Initial denaturation	95°C	3 ′	1
Denaturation	95°C	15 ″	35
Annealing	X°C	20 ″	
Extension	72°C	15 ″/ kb	
Final extension	72°C 4°C	7 ′ hold	1

10.1.3. Primers

The following tables show primers used in this thesis to amplify DNA fragments and to verify DNA sequences. Table 10 shows the primers used for traditional cloning (see section 10.2) with the restriction sites typed in bold, and Table 11 shows primers used for GoldenBraid 2.0 cloning (see section 10.3).

Table 10. Primers used for traditional cloning.

<i>Name</i>	<i>Sequence</i>	<i>Purpose</i>
506	CGCTATTACCTAAGATTCG	check <i>mosAB</i> wild-type copies
511	CACCGATCTTGATCACCG	check <i>mosAB</i> wild-type copies
1243	ATCGGCGAGCAGGAAGGCAC	check direction of Sp cassette
1244	CGCGGATCTGCCGGAAGTA	check direction of Sp cassette
1344	GCTTAGTAAAGCCCTCGCTA	check direction of Sp cassette
1345	GCTCTCGGGTAACATCAAGG	check direction of Sp cassette
1590	GGTTT CTCGAG ATGACTCGTAAAGTAGCCAT	amplify AOH <i>anf</i> region
1591	GAT GGATCC GACACATCTCCTTTAGAGTGA	amplify AOH <i>anf</i> region
1628	ACCT GGATCC GAAATGGACATCGAAGCCA	amplify AOH <i>anf</i> region
1629	TAC CTCTAGAT GAGGACCCATTCCTTGTTTC	amplify AOH <i>anf</i> region
1594	AGGC CTCGAGT GCATGACCGATGGGAC	amplify AOH <i>vnf</i> region
1595	CCAT GGATCC GATTGAAGTCTCCTCGGCTCT	amplify AOH <i>vnf</i> region
1596	GTGGT GGATCC AGGTGCCGAGCGGTTTCC	amplify AOH <i>vnf</i> region
1597	GGGT CTAGA AGTCCAGGCGGACATGGC	amplify AOH <i>vnf</i> region
1718	CCG GGATCC CTCATGTTTGACAGCTTATCAT	amplify TetR cassette/check <i>vnf</i> region
1719	CCG GGATCC GGAGTGGTGAATCCGTTAGC	amplify TetR cassette/check <i>vnf</i> region

<i>Name</i>	<i>Sequence</i>	<i>Purpose</i>
1601	TGAATGTTGTACTCGCCCAC	check presence of <i>anf</i> wild-type copies
1609	GAAGAAAACGGCGCCTATAC	check <i>anf</i> wild-type copies
1300	AATTAAGCTTGGGAAAGCCACGTTGTGTCTC	check <i>anf</i> region
1301	AATTAAGCTTCTTTTGCTTTGCCACGGAACG G	check <i>anf</i> region
1598	ATCGTCTCGACTTTCTCGTTG	check presence of <i>vnf</i> wild-type copies
1599	ATCGCCCTCGACGCCCTG	check presence of <i>vnf</i> wild-type copies
2012	ACGCGTCGACGAATTCACAATGGAGTCTCAG TCTCAGAGA	amplify <i>AtMOT1;1</i>
2042	CCGGTCTGACAAGCATGTTACCGGATTGCGG GGG	amplify <i>AtMOT1;1</i>
2057	GCGCGAATTCGCCAACTCGACAGCG	amplify <i>mosBA</i>
2058	GCGCGCGGCGGCTCAGGCCGGACGCACA	amplify <i>mosBA</i>

Table 11. Primers used for GoldenBraid 2.0 cloning.

<i>Name</i>	<i>Sequence</i>	<i>Purpose</i>
1848	GCGCCGTCTCGCTCGCCATGGATTACAAGGATGACGACG ATAAGGCAGCCCGAGCGAGACGGCGC	GB amplify Flag tag 12 -13/check TU
1849	GCGCCGTCTCGCTCGGGCTGCCTTATCGTCGTCATCCTTG TAATCCATGGCGAGCGAGACGGCGC	GB amplify Flag tag 12 -13/check TU
1856	GCGCCGTCTCGCTCGAGCCATGACCGATACTACAAACTC C	GB amplify <i>y_{mosA}</i> 14-16/check TU
1857	GCGCCGTCTCGCTCGAAGCTTAAGCAGGTCTGACACCGG	GB amplify <i>y_{mosA}</i> 14-16/check TU
1904	GCGCCGTCTCGCTCGAGCCATGGCTAATAGTACAGCGGA A	GB amplify <i>y_{mosB}</i> 14-16/check TU

<i>Name</i>	<i>Sequence</i>	<i>Purpose</i>
1859	GCGCCGTCTCGGCCTCATCCATAACTTATAAGG	GB amplify <i>ymosB</i> 14-16/check TU
1860	GCGCCGTCTCGAGGCCAGCTGCAGAAGGTGT	GB amplify <i>ymosB</i> 14-16/check TU
1861	GCGCCGTCTCGCTCGAAGCTTAAGAAGCTGTTATAATAG TACCAA	GB amplify <i>ymosB</i> 14-16/check TU
1862	GCGCCGTCTCGCTCGAGCCATGGGTTCGCTGCCGCACA	GB amplify <i>ynifQ</i> 14-16/check TU
1863	GCGCCGTCTCGCATCTCTTCTCATTTCCAACAAAT	GB amplify <i>ynifQ</i> 14-16/check TU
1864	GCGCCGTCTCGGATGAATGGGAAGAATTGAGAG	GB amplify <i>ynifQ</i> 14-16/check TU
1865	GCGCCGTCTCGCTCGAAGCTCAAATTTCTGCACCGAAAC AATC	GB amplify <i>ynifQ</i> 14-16/check TU
2061	GCGCCGTCTCGCTCGAGCCATGGAGTCTCAGTCTCAGAG A	GB amplify <i>AtMOT1</i> 14-15/check TU
2062	GCGCCGTCTCGCTCACTGCTGTCCCCGCGGCGGCAGCATG TTCACCGGATTG	GB amplify <i>AtMOT1</i> 14-15/check TU
2065	GCGCCGTCTCGCTCGGCAGATGTCAAAGGGTGAAGAATT GTTT	GB amplify yEGFP 16/ check TU
2066	GCGCCGTCTCGTGATCTCTCTTTTCATTTGGATCT	GB amplify yEGFP 16/ check TU
2067	GCGCCGTCTCGATCATATGGTCTTGTTAGAATTTGTT	GB amplify yEGFP 16/ check TU
2068	GCGCCGTCTCGCTCAAAGCTCATTTATACAATTCATCCA TACCG	GB amplify yEGFP 16/ check TU
2117	GCGCCGTCTCGCTCGGGAGATAGCTTCAAAATGTTTCTA CTCC	GB amplify TEF1p 01-14/check TU
2118	GCGCCGTCTCGGAGATGCGCGGAGTCCGAGA	GB amplify TEF1p 01-14/check TU
2119	GCGCCGTCTCGTCTCCGTACCACTTCAAAACA	GB amplify TEF1p 01-14/check TU

<i>Name</i>	<i>Sequence</i>	<i>Purpose</i>
2120	GCGCCGTCTCGCGATCTCTTTTTCTTTTCCAAAC	GB amplify TEF1p 01-14/check TU
2121	GCGCCGTCTCGATCGCCTCGTTTCTTTTCTTC	GB amplify TEF1p 01-14/check TU
2122	GCGCCGTCTCGCTCAGGCTTTTGTAATTAAACTTAGAT TAGATTGCT	GB amplify TEF1p 01-14/check TU
1829	TTTTTTCACGTCAGAAGTTAAGGC	GB check TU/ <i>YORWΔ22</i>
1832	ATCTTGATTTGATGGGACTTCCTT	GB check TU/ <i>YORWΔ22</i>
1833	AGGTGATTGAGTCTCTTGAAGTAC	GB check TU/ <i>YPRCΔ15</i>
1836	GGTTGTTTGGAGTCTATCTCTGAT	GB check TU/ <i>YPRCΔ15</i>
1557	GCGCCGTCTCGCTTCGACCTGATGCAGCTCT	GB check TU/Hygro
1558	GCGCCGTCTCGCGTCGCCTCGCTCCAGTCAA	GB check TU/Hygro
1517	GCGCCGTCTCGACCGCTTCGCTGATTAATTAC	GB check TU/Gal1p
1548	GCGCCGTCTCGCTCGGGAGCAGCTGAAGCTTCGTACGCT	GB check TU/G418
1549	GCGCCGTCTCGCTCGAGCGGCATAGGCCACTAGTGGATC	GB check TU/ G418
1499	GCGCCGTCTCGCTCGATGGTTTGTGTTTATGTGTGTT TATTCG	GB check TU/ TDH3p
1528	GCGCCGTCTCGCTCGGCTTATCCGCTCTAACCGAAAAGG	GB check TU/ CYC1t

GB: GoldenBraid; TU: Transcriptional Unit. Code used corresponds to the relative position desired for the amplified DNA in the final genetic construction (see Figure 5).

10.2. Traditional cloning

This method relies on the amplification of the DNA sequence of interest from a template for later insertion into a target vector. Restriction enzymes were used to cut both sides of the sequence of interest and the vector. Ligation of the fragments was carried out by a

DNA ligase from sticky or blunt ends made by the restriction enzymes. The primers used for the amplification of the inserts by PCR contained the restriction sites that are unique in both the amplified DNA and the vector (see section 0). After amplification and purification of the DNA, digestion of the insert and the vector was carried out by restriction enzymes (New England Biolabs, Ipswich, Massachusetts, USA) according to the conditions delivered by the bioinformatics tools of the NEB website. Digested DNA was purified from agarose gel (see section 10.8) or cleaned up as detailed in section 10.9.

Ligation of the digested DNA fragments and the target vector was carried out by the T4 DNA ligase (Promega, Madison, Wisconsin, USA). Different ratios of insert/vector, temperature and times from each cloning were set by following the protocols available in the website of Promega company.

The ligation mixtures were used to transform *E. coli* DH5 α strain (see section 9.2). When the DNA construct was intended for heterologous protein expression, BL21(DE3) pLysS strain was transformed (see section 9.2). Positive colonies were selected by antibiotic resistance and, when available, by the blue/white *lacZ* selection. The fidelity of DNA constructs was verified by PCR (see section 10.1.2), restriction enzyme digestion, and DNA sequencing. Sequencing data was analyzed using the bioinformatics software Geneious.

Plasmids generated for expression in *S. cerevisiae* were used for transformation protocols as described in section 9.3.

10.3. GoldenBraid cloning

The basis of this method, originally developed by the Plant Genomics and Biotechnology laboratory (IBMCP-CSIC-UPV in Valencia, Spain), was used in this thesis. Modular DNA cloning is a strategy for complex genetic devices that combines prefabricated standard modules. GoldenBraid (GB) cloning relies on an assembly method in which large constructs (more than 10 kb) containing several transcriptional units composed of individual manufactured modules (GB parts) can be constructed in few days. The GB

parts are DNA pieces (promoters, CDSs, organelle or membrane target signals, terminators, etc.), which are cloned into a special circular vector called pup (“GB domesticator”). Pup contains 4-bp overhangs that are produced after cleavage with a Type IIS restriction enzyme, either *BsaI* or *BtgZI*. The 4-bp flanking sites define each part’s relative position into the later transcriptional unit generation. For this reason, the GB tool has a universal “grammar” (see Figure 5) and primers are designed according to the relative position desired for the amplified DNA in the final genetic construction.

Figure 5. GB cloning positions used in this thesis. MTS; mitochondrial target signal; Tag: peptide sequences that are attached to proteins to facilitate easy detection and purification; CDS: coding sequence.

For modular DNA constructs there are destination vectors that contain arms of homology (AOH) identical to the DNA sequences where the exogenous DNA will be integrated. In this laboratory, the destination vectors of the GB 2.0 version have been modified to generate new synthetic GoldenBraid destination vectors adapted for *S. cerevisiae* genome. Two different loci (*YPRCΔ15* and *YORWΔ22*) were selected to synthesize the AOH at the solo long terminal repeat (LTR) loci compatible with the W303 and the CEN.PK2-1C genomes (Pérez-González *et al.*, 2017). There are four vectors from each locus designated as type Ω (1 and 2) and type α (1 and 2), which play a key role in the generation of constructs with multiple transcriptional units by allowing piling-up the genetic modules through a digestion/ligation reaction. Vectors 1 and 2 of the same type can be used to pile-up two transcriptional units in one vector of the other type. This is repeated until all the desired transcriptional units are in the same modular DNA construct.

PCR and reaction conditions were set up by the bioinformatics tools available in the GB 2.0 website (nowadays GB 3.0). GB plasmids were introduced into DH5 α strain by transformation (see section 6.2; see section 9.2). Positive colonies were selected by

antibiotic resistance and the blue/white *lacZ* selection. DNA constructs were verified by PCR (see section 10.1.2) and DNA sequencing by MacroGen (Geumcheon-gu, Seoul, Korea). DNA sequencing data was analyzed by the bioinformatics software Geneious.

Plasmids generated for integration into *S. cerevisiae* genome were transformed as described in section 9.3. GB vector containing the DNA required for the transformation, which contains I-SceI restriction sites flanking the AOH, was first linearized. For this, 10 µg of the plasmid was digested with the I-SceI enzyme following the conditions of the NEB website tools. The restriction enzyme was inactivated by incubation at 65°C for 20 minutes. Digestion was checked by agarose gel electrophoresis (see section 10.7).

10.4. Vectors

The following tables show plasmids generated in this thesis. Plasmid extraction from DH5α was carried out as described in section 10.5. Table 12 shows genetic constructs generated by traditional cloning. Table 13 shows GB parts and modular DNA constructs.

Table 12. Plasmids obtained by traditional cloning.

<i>Plasmid</i>	<i>Description</i>	<i>Resistance</i>	<i>Source</i>
	pDR196 expression vector for yeast	Amp	(Tejada-Jiménez <i>et al.</i> , 2017)
	pDR196 [<i>Medicago truncatula</i> MOT1.3]	Amp	(Tejada-Jiménez <i>et al.</i> , 2017)
pN2MN14	pBS KS(+) carrying anfDGK flanking regions and kan cassette	Amp Km	This thesis
pN2MN18	pBS KS(+) carrying vnfDGK flanking regions and tet cassette	Amp Tet	This thesis
pN2MN63	pDR196 [<i>Arabidopsis thaliana</i> MOT1;1]	Amp	This thesis
pRHB152	pET28a(+) incorporating TEV site	Km	This lab
pN2MN72	pRHB152 [<i>mosBA</i>]	Km	This thesis

Table 13. Plasmids obtained by GoldenBraid 2.0 cloning.

<i>Plasmid</i>	<i>Description</i>	<i>Vector</i>	<i>Source</i>
pRHB734	YORWdelta22 AOH alpha1 adapted in pDGB2c_alpha_Kan	$\Delta 22\alpha 1$	(Pérez-González <i>et al.</i> , 2017)
pRHB735	YORWdelta22 AOH alpha2 adapted in pDGB2c_alpha_Kan	$\Delta 22\alpha 2$	(Pérez-González <i>et al.</i> , 2017)
pRHB736	YORWdelta22 AOH omega1 adapted in pDGB2c_omega_Spm	$\Delta 22\Omega 1$	(Pérez-González <i>et al.</i> , 2017)
pRHB737	YORWdelta22 AOH omega2 adapted in pDGB2c_omega_Spm	$\Delta 22\Omega 2$	(Pérez-González <i>et al.</i> , 2017)
pRHB738	YPRCdelta15 AOH alpha1 adapted pDGB2c_alpha1-Kan	$\Delta 15\alpha 1$	(Pérez-González <i>et al.</i> , 2017)
pRHB739	YPRCdelta15 AOH alpha2 adapted pDGB2c_alpha2-Kan	$\Delta 15\alpha 2$	(Pérez-González <i>et al.</i> , 2017)
pRHB740	YPRCdelta15 AOH omega1 adapted in pDGB2c_omega1-Spm	$\Delta 15\Omega 1$	(Pérez-González <i>et al.</i> , 2017)
pRHB741	YPRCdelta15 AOH omega2 adapted in pDGB2c_omega2-Spm	$\Delta 15\Omega 2$	(Pérez-González <i>et al.</i> , 2017)
	pUC57- <i>ynifQ</i>	pUC57	GenScript
pRHB815	pUPD-KanMX (01-21) (G418R)	pUPD	(Pérez-González <i>et al.</i> , 2017)
pRHB819	pUPD-HphMX (01-21) (HygroR)	pUPD	(Pérez-González <i>et al.</i> , 2017)
pRHB820	pUPD-FBA1t (17-21)	pUPD	This lab
pRHB821	pUPD-CYC1t (17-21)	pUPD	(Pérez-González <i>et al.</i> , 2017)
pRHB822	pUPD-TDH2t (17-21)	pUPD	(Pérez-González <i>et al.</i> , 2017)
pRHB826	pUPD-TDH3p (01-11)	pUPD	(Pérez-González <i>et al.</i> , 2017)
pRHB827	pUPD-GAL1p (01-11)	pUPD	(Pérez-González <i>et al.</i> , 2017)

<i>Plasmid</i>	<i>Description</i>	<i>Vector</i>	<i>Source</i>
pRHB831	pUPD-TEF1p (01-11)	pUPD	This lab
pRHB844	pUPD-SU9 (12)	pUPD	(Pérez-González <i>et al.</i> , 2017)
pRHB845	pUPD-MAM33 (12)	pUPD	(Pérez-González <i>et al.</i> , 2017)
pUC57-ymosA	pUC57-ymosA	pUC57	GenScript
pUC57-ymosB	pUC57-ymosB	pUC57	GenScript
pRHB931	pUPD-ynifQ (14-16)	pUPD	This thesis
pRHB937	pUPD-ymosA (14-16)	pUPD	This thesis
pN2MN28	pUPD-ymosB (14-16)	pUPD	This thesis
pN2MN124	pUPD2-TEF1p (01-14)	pUPD2	This thesis
pN2MN31	pUPD-FLAG (12-13)	pUPD	This thesis
pUC57-8His	BtgZI BsaI 13 8xHis BsaI BtgZI	pUC57	GenScript
pN2MN74	pUPD2-yEGFP (16)	pUPD2	This thesis
pN2MN73	pUPD2-At MOT1;1 (14-15)	pUPD2	This thesis
pN2AP43	pUPD2-ATPA (12)	pUPD2	(Pérez-González <i>et al.</i> , 2017)
pN2MN79	G418R ($\Delta 15\alpha 2$)	$\Delta 15\alpha 2$	This thesis
pN2MN83	HygroR ($\Delta 15\alpha 2$)	$\Delta 15\alpha 2$	This thesis
pN2MN85	HygroR ($\Delta 15\Omega 2$)	$\Delta 15\Omega 2$	This thesis
pN2MN38	TDH3p-FLAG-ymosA-FBA1t	$\Delta 22\alpha 1$	This thesis
pN2MN39	TDH3p-FLAG-ymosB-FBA1t	$\Delta 22\alpha 2$	This thesis
pN2MN65	TDH3p-MAM33-Strep-ymosA-FBA1t	$\Delta 22\alpha 1$	This thesis
pN2MN66	TDH3p-MAM33-Strep-ymosB-FBA1t	$\Delta 22\alpha 2$	This thesis
pN2MN126	TEF1p-At MOT1;1-yEGFP-CYC1t	$\Delta 15\alpha 1$	This thesis
pN2MN127	TEF1p-SU9-At MOT1;1-yEGFP-CYC1t	$\Delta 15\alpha 1$	This thesis
pN2MN141	Gal1p-SU9-Strep-ynifQ-TDH2t	$\Delta 22\alpha 1$	This thesis
pN2MN145	TDH3p-SU9-8xHis-ymosA-FBA1t	$\Delta 22\alpha 1$	This thesis
pN2MN146	TDH3p-SU9-8xHis-ymosB-FBA1t	$\Delta 22\alpha 2$	This thesis
pN2MN147	Gal1p-ATPA-Strep-ynifQ-TDH2t	$\Delta 22\alpha 1$	This thesis
pN2MN42	[TDH3p-FLAG-ymosA-FBA1t] - [TDH3p-FLAG-ymosB-FBA1t]	$\Delta 22\Omega 1$	This thesis

<i>Plasmid</i>	<i>Description</i>	<i>Vector</i>	<i>Source</i>
pN2MN67	[TDH3p-MAM33-Strep- <i>ymosA</i> -FBA1t] - [TDH3p-MAM33-Strep- <i>ymosB</i> -FBA1t]	$\Delta 22\Omega 1$	This thesis
pN2MN113	[TDH3p-FLAG- <i>ymosA</i> -FBA1t] - [TDH3p-FLAG- <i>ymosB</i> -FBA1t] - [HphMX]	$\Delta 22\alpha 1$	This thesis
pN2MN114	[TDH3p-MAM33-Strep- <i>ymosA</i> -FBA1t] - [TDH3p-MAM33-Strep- <i>ymosB</i> -FBA1t] - [HphMX]	$\Delta 22\alpha 1$	This thesis
pN2MN128	[TEF1p-SU9- <i>At MOT1;1</i> -yEGFP-CYC1t] - [HphMX]	$\Delta 15\Omega 1$	This thesis
pN2MN130	[TEF1p- <i>At MOT1;1</i> -yEGFP-CYC1t] - [HphMX]	$\Delta 15\Omega 1$	This thesis
pN2MN142	[Gal1p-SU9-Strep- <i>ynifQ</i> -TDH2t] - [KanMX]	$\Delta 22\Omega 2$	This thesis
pN2MN148	[Gal1p-ATPA-Strep- <i>ynifQ</i> -TDH2t] - [KanMX]	$\Delta 22\Omega 1$	This thesis
pN2MN149	[TDH3p-SU9-8xHis- <i>ymosA</i> -FBA1t] - [TDH3p-SU9-8xHis- <i>ymosB</i> -FBA1t]	$\Delta 22\Omega 1$	This thesis
pN2MN150	[TDH3p-SU9-8xHis- <i>ymosA</i> -FBA1t] - [TDH3p-SU9-8xHis- <i>ymosB</i> -FBA1t] - [HphMX]	$\Delta 15\alpha 2$	This thesis
pN2MN151	[TDH3p-SU9-8xHis- <i>ymosA</i> -FBA1t] - [TDH3p-SU9-8xHis- <i>ymosB</i> -FBA1t] - [HphMX] - [TEF1p- <i>At MOT1;1</i> -yEGFP- CYC1t]	$\Delta 15\Omega 1$	This thesis

ynifQ: yeast optimized *nifQ*; *ymosA*: yeast optimized *mosA*; *ymosB* yeast optimized *mosB* TU: Transcriptional Unit. Numerical codes used correspond to the relative position of the amplified DNA in the final genetic construction (see Figure 5). See also Supplemental Table 1.

10.5. Plasmid DNA extraction

QIAGEN Plasmid Mini Kit and QIAGEN Plasmid Maxi Kit were used for plasmid extraction from *E. coli* strains. The Miniprep procedure was as follows (Maxiprep variations indicated in parenthesis along the protocol):

- *E. coli* cells were grown overnight in 5 ml (200 ml) of LB medium supplemented with the appropriate antibiotic.
- Cultured cells were collected by centrifugation at 5,000 rpm for 5 minutes at 4°C.
- Bacterial pellet was re-suspended in 0.3 ml (10 ml) of P1 buffer.
- 0.3 ml (10 ml) of P2 buffer was added to the previous solution and mixed thoroughly by inverting 4-6 times. The mixture was incubated at room temperature for 5 minutes.
- 0.3 ml (10 ml) of pre-chilled P3 (N3) buffer was added to the previous solution, mixed thoroughly and incubated on ice for 5 minutes (20 minutes).
- The sample was centrifuged at 18,000 x *g* for 10 minutes (20,000 x *g* for 30 minutes) at 4°C.
- For a Maxiprep, the QIAGEN gravity column was equilibrated with 10 ml of QBT buffer. Supernatant from previous centrifugation was added and allowed to enter the resin by gravity flow. Column was washed with 60 ml of QC buffer and DNA was eluted with 15 ml of QF buffer pre-warmed at 65°C. DNA was precipitated by adding 10.5 ml of room temperature isopropanol and centrifuged at 15,000 x *g* for 30 minutes at 4°C. The DNA pellet was air-dried for 10 minutes and re-suspended in TE buffer.
- For a Miniprep, the supernatant was added to a 2-ml QIAprep column and the column was centrifuged at 13,000 rpm for 1 minute. Flow-through was discarded. Column was washed by adding 750 µl of PE buffer and centrifuged at 13,000 rpm for 1 minute. Flow-through was discarded. DNA was eluted with 50 µl of TE buffer in a 1.5 ml microcentrifuge tube by centrifugation at 13,000 rpm for 1 minute.
- Eluted DNA was quantified in a NanoDrop® ND-1000 Spectrophotometer.

TE buffer: 10 mM Tris-HCl pH 8.0, 1 mM EDTA.

10.6. Genomic DNA extraction

10.6.1. *A. vinelandii*

Genomic DNA was extracted from *A. vinelandii* cells using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). The protocol followed was:

- Cells were harvested (about 1.5 ml of culture) in a microcentrifuge tube and centrifuged for 10 minutes at 7,500 rpm. Supernatant was discarded.
- Pellet was re-suspended in 180 µl of ATL buffer
- 20 µl of proteinase K was added to the previous step. Sample was mixed thoroughly by vortexing and incubated at 56°C in a thermomixer block until complete cells lysis (about 1 hour).
- 200 µl of AL buffer was added to the sample and mixed thoroughly by vortexing. Then 200 µl of 100% ethanol was added and mixed again by vortexing.
- Mixture was pipetted into a DNeasy Mini spin column that was placed in a 2 ml collection tube. The tube was centrifuged at 8,000 rpm for 1 minute and the flow-through was discarded.
- 500 µl of AW1 buffer was added to the column and this was centrifuged at 8,000 rpm for 1 minute. Flow-through was discarded.
- 500 µl of AW2 buffer was added to the column and this was centrifuged at 13,000 rpm for 3 min. The flow-through was discarded.
- The DNeasy Mini spin column was placed into a clean 1.5 ml microcentrifuge tube, after which 200 µl of AE buffer was pipetted onto the DNeasy membrane and incubated at room temperature for 1 minute. The column was centrifuged at 8,000 rpm for 1 minute and the eluate containing genomic DNA was withdrawn.
- Eluted DNA was quantified in a NanoDrop® ND-1000 Spectrophotometer.

AE buffer: 10 mM Tris-HCl, 0.5 mM EDTA pH 9.0

10.6.2. *S. cerevisiae*

Genomic DNA was extracted from *S. cerevisiae* cells following the protocol published by (Harju *et al.*, 2004). Extraction was carried out as follows:

- About 10 colonies worth of cells were transferred from the agar plate into a 1.5 ml microcentrifuge tube.

- 200 μ l of lysis buffer were added to the yeast.
- Tubes were immersed in liquid N₂ for 2 minutes and rapidly transferred to a 100°C water bath for 1 minute. This step was repeated once, and finally tubes were vortexed for 30 seconds.
- 200 μ l of chloroform was added to the sample and the mixture was vortexed for 2 minutes.
- Samples were subject to centrifugation at 20,000 x *g* for 3 minutes.
- The upper aqueous phase was transferred into a 1.5 ml microcentrifuge tube containing 400 μ l of ice-cold 100% ethanol. Sample was mixed by inversion and incubated at -20°C for at least 2 hours.
- Sample was centrifuged at 20,000 x *g* for 5 minutes and the supernatant was removed carefully to avoid mixing with pellet.
- Pellet was resuspended in 0.5 ml of 70% ethanol and centrifuged at 20,000 x *g* for 5 minutes.
- Supernatant was removed and pellet was air-dried for 15 minutes.
- Pellet was re-suspended in 100 μ l of TE buffer.
- Eluted DNA was quantified in a NanoDrop® ND-1000 Spectrophotometer.

Lysis buffer: 2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA.

TE buffer: 10 mM Tris-HCl pH 8.0, 1 mM EDTA.

10.7. DNA gel electrophoresis

Gel electrophoresis was carried out to visualize DNA products from PCR amplifications or from restriction enzyme digestion. DNA samples were mixed with 6x loading dye buffer. Gels of 3% agarose were used to visualize PCR fragments while 0.8% agarose gels were used to visualize and cut DNA fragments for cloning. DNA fragments of commercial 100 bp or 1 kb ladders (Nippon Genetics, Dueren, Germany) were used as molecular mass markers. TAE buffer was used as running buffer for gel electrophoresis.

6 x Loading dye buffer: 0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol.

TAE: 40 mM Tris-HCl pH 8, 20 mM acetic acid, 1 mM EDTA.

10.8. DNA extraction from agarose gels

DNA extraction from agarose gel was carried out using the QIAEX® II Gel Extraction Kit (Qiagen, Hilden, Germany). The protocol followed was:

- A DNA band was excised from the agarose gel with a clean sharp scalpel and placed into a 1.5 ml microcentrifuge tube.
- The gel slice was weighted to estimate volume, and 3 volumes of QX1 buffer were added to 1 volume of the gel (for extracting DNA fragments of sizes between 100 bp and 4 kb).
- 10 µl of QIAEX II resin was added to the previous mixture and the sample was incubated at 50°C for 10 minutes in a thermomixer block.
- Sample was centrifuged at 13,000 rpm for 30 seconds and supernatant was carefully removed with a pipette.
- Pellet was washed with 500 µl of QX1 buffer and centrifuged at 13,000 rpm for 30 seconds. Supernatant was carefully removed with a pipette.
- Pellet was washed twice with 500 µl of PE buffer and centrifuged at 13,000 rpm for 30 seconds.
- Pellet was air-dried for 15 minutes until it becomes white.
- DNA was resuspended by adding 20 µl of TE buffer and vortex. The DNA solution was incubated at 50°C for 5 minutes.
- The DNA solution was centrifuged at 13,000 rpm for 30 seconds to remove debris and the supernatant was pipetted into a new tube.
- Isolated DNA was quantified in a NanoDrop® ND-1000 Spectrophotometer.

TE buffer: 10 mM Tris-HCl pH 8.0, 1 mM EDTA.

10.9. Purification of DNA from PCR amplifications

DNA purification (single or double-stranded DNA fragments) from PCR amplifications and other enzymatic reactions such as restriction enzyme digestion was carried out using the QIAquick PCR purification Kit (Qiagen, Hilden, Germany). DNA fragments ranging from 100 bp to 10 kb can be purified from primers, nucleotides, polymerases and salts. The protocol followed was:

- Five volumes of PB buffer were added to 1 volume of the DNA sample.
- Mixture was placed onto a QIAquick spin column and centrifuged at 13,000 rpm for 30 seconds.
- Flow-through was discarded and column was washed with 0.75 ml of PE buffer. QIAquick column was centrifuged at 13,000 rpm for 30 seconds.
- Flow-through was discarded and DNA was eluted with 30 µl of EB buffer
- Eluted DNA was quantified in a NanoDrop® ND-1000 Spectrophotometer.

EB buffer: 10 mM Tris-HCl pH 8.5.

11. PROTEIN TECHNIQUES

Reagents used in this work are from VWR, Sigma-Aldrich and IBIAN Technologies.

11.1. Protein extraction

Protein extraction from cultured cells is the first step for many biochemical and analytical techniques (SDS-PAGE, Western blotting, etc.) or protein purification. After cell lysis, total cellular protein is separated into two fractions, the soluble fraction containing hydrophilic proteins and an insoluble fraction containing mostly membrane associated proteins. The following protocols describe extraction of proteins destined to denaturing SDS gel electrophoresis.

11.1.1. Cytoplasmic proteins

Cytoplasmatic proteins are easily solubilized, especially in bacteria. Pelleted *A. vinelandii* or *E. coli* cells were subjected to whole-cell protein extraction by the addition of 4 x Laemmli buffer and heating for 3 minutes at 95°C. Lysed cells were centrifuged at 15,000 rpm for 1 minute at 4°C and supernatant (soluble part) was pipetted into a new 1.5 ml microcentrifuge tube for further analysis.

S. cerevisiae cells were subjected to whole cell protein extraction following the alkali extraction protocol described by (Kushnirov, 2000). Pelleted cells were re-suspended in 100 µl of PBS supplemented with 100 µl of 0.2 M NaOH and 50 µl of 4 x Laemmli buffer.

Sample was heated for 3 minutes at 95°C. Sample was centrifuged at 15,000 rpm for 1 minute at 4°C and supernatant (soluble part) was pipetted into a new 1.5 ml microcentrifuge tube for further analysis.

PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.47 mM KH₂PO₄ pH 7.4.

4x Laemmli buffer: 200 mM Tris-HCl pH 6.8, 40% glycerol, 8% SDS, 10% 2-mercaptoethanol, 0.008% bromophenol blue, 100 mM DTT.

11.1.2. *Membrane proteins*

Membrane bound proteins were subjected to a different protein extraction protocol in which detergents play a key role in solubilization. Pelleted cells or liquid samples from *S. cerevisiae* cultures were mixed in a 1:1 ratio with RIPA buffer containing 1 mM PMSF. Samples were kept cold during protein extraction. Zirconia beads (Thermo Fisher Scientific, Waltham, Massachusetts, USA) were added to the mixture and cell lysis was carried out in a homogenizer with an intense of 26 s⁻¹ for 3 minutes. This shaking was repeated twice and then samples were centrifuged at 1,500 x *g* for 10 minutes at 4°C. Supernatant was pipetted into a new 1.5 ml microcentrifuge tube with 4x Laemmli buffer and sample was heated for 3 minutes at 95°C. Sample was kept at 4°C for further analysis.

PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.47 mM KH₂PO₄ pH 7.4.

4x Laemmli buffer: 200 mM Tris-HCl pH 6.8, 40% glycerol, 8% SDS, 10% 2-mercaptoethanol, 0.008% bromophenol blue, 100 mM DTT.

RIPA buffer: 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl pH 8.0.

11.2. *Denaturing gel electrophoresis*

The progress of protein purification protocols and the protein profiles of cells induce for expression of a protein were checked denaturing gel electrophoresis and immunodetection techniques. Acrylamide gels were prepared depending on the molecular weight of the target protein. Table 14 shows the percentage of acrylamide

used for a linear range of protein separation. Table 15 shows the standard protocol to prepare denaturing acrylamide gels.

Table 14. Linear range of separation from each percentage of acrylamide.

Acrylamide concentration (%)	Linear Range of separation (kDa)
15	10 – 43
12	12 – 60
10	20 – 80
7.5	36 – 94

Table 15. Composition of acrylamide gel resolving and stacking mixtures.

<i>Resolving</i>	<i>Gel X %</i>
Acrylamide 40% (37.5:1)	X ml (depending on final %)
1.5 M Tris-HCl pH 8.8	4 ml
10% SDS	160 µl
APS 10%	80 µl
TEMED	8 µl
H ₂ O	To complete 16 ml
<i>Stacking</i>	<i>Gel 4.5%</i>
Acrylamide 40% (37.5:1)	562.5 µl
0.5 M Tris-HCl pH 6.8	1.25 ml
10% SDS	60 µl
APS 10%	30 µl
TEMED	4 µl
H ₂ O	To complete 5 ml

When analysis was focus on small polypeptides (1 to 20 kDa), a protocol for small proteins was used as detailed in Table 16.

Table 16. Composition of resolving and stacking mixtures in acrylamide gels for small proteins.

<i>Resolving</i>	<i>Gel 18%</i>
Acrylamide 40% (19:1)	3.6 ml
3 M Tris-HCl pH 8.45, 0.4% SDS	2 ml
Ethylene glycol	2.4 µl
APS 40%	8 µl
TEMED	12 µl
H ₂ O	-

<i>Stacking</i>	<i>Gel 5%</i>
Acrylamide 40% (29:1)	500 µl
3 M Tris-HCl pH 8.45, 0.4% SDS	1 ml
APS 40%	4 µl
TEMED	16 µl
H ₂ O	2.5 ml

When using Bio-Rad Mini-protean devices, gel electrophoresis was run at 200 V for 40 minutes. Running buffer used were: 250 mM Glycine, 25 mM Tris-HCl pH 8.3, 0.1% SDS or, for small proteins, 100 mM Tris-HCl pH 8.3, 100 mM Tricine, 0.1% SDS.

11.2.1. *Coomassie blue staining*

Gel staining was carried out overnight in orbital shakers while gel destaining was carried out in controlled conditions until the blue background was removed.

Coomassie blue staining solution: 0.1% Coomassie brilliant blue R-250, 40% ethanol, 10% glacial acetic acid.

Destaining solution: 40% ethanol, 10% glacial acetic acid.

11.2.2. Immunodetection (Western blots)

Protein immunodetection requires the transfer of polypeptides from the electrophoresis acrylamide gel to a membrane that can be nitrocellulose or polyvinylidene difluoride (PVDF). The semi-dry transfer device (Bio-Rad, Hercules, California, USA) was used in this thesis to transfer proteins to nitrocellulose or PVDF membranes for immunodetection with antibodies. Nitrocellulose membrane of 0.45 μm was used to detect all proteins of this work except for the yEGFP-AtMOT1 fusion protein, which was transferred to a 0,2 μm pore size PVDF membrane.

The nitrocellulose membrane was soaked in Transfer buffer together with the Extra-thick blot paper of the transfer device (Bio-Rad, Hercules, California, USA) and then placed in the semi-dry transfer system in the following order: wet blot paper, wet nitrocellulose membrane, acrylamide gel and wet blot paper. Transfer of proteins was carried out at 21 V for 45 minutes.

The PVDF membrane was first activated for 3 minutes by soaking in methanol. Extra-thick blot paper (Bio-Rad, Hercules, California, USA) and activated PVDF membrane were soaked in DUNN buffer (required to transfer proteins with high pI values) and then placed in the semi-dry system in the order indicated above. Transfer of the proteins was carried out at 24 V for 45 minutes. Afterwards, the PVDF membrane was soaked in methanol for 3 minutes and dried at 30°C.

Immunodetection was carried out as follows:

- The membrane was blocked in 5% milk for 1 hour.
- Membrane was washed 3 times with PBS-Tween for 5 minutes each time.
- Membrane was blocked for 3 hours, or overnight, at 4°C at the same time it was incubated with the primary antibody in 2% BSA supplemented with 0.01% azide (antibodies and dilutions are shown in Table 17).
- Membrane was washed 3 times with PBS-Tween for 5 minutes each time.
- Membrane was incubated with secondary antibody for 45 minutes in 1% milk
- Membrane was washed 3 times with PBS-Tween for 5 minutes each time.
- Membrane was developed using the alkaline phosphatase or the ECL protocol.

The alkaline phosphatase protocol consisted of the mixture of 10 ml of developing solution, 33 μ l of BCIP and 66 μ l of NBT. The mixture was added to the membrane until precipitated bands appeared. The reaction was stopped by the addition of dH₂O and extensive washing. When required, ImageJ software was used to quantify the precipitate stains in immunoblot membranes.

The ECL protocol consisted in the addition to the membrane of a mixture of ECL solution 1 and ECL solution 2 (ratio 1:1) and incubation for 2 minutes. Membrane was then exposed to an X-ray film (Innovagen, Madrid, Spain) at various times depending on the expected signal. Film was subjected to a developer solution, fixer solution and then washed with dH₂O.

Table 17. Antibodies used in this thesis.

	<i>Name</i>	<i>dilution</i>	<i>Source</i>
Primary antibody	α – NifDK rabbit	1 : 2,500	Rubio's lab
	α – NifH rabbit	1 : 2,500	Rubio's lab
	α – VnfK rabbit	1 : 4,000	Rubio's lab
	α – VnfG rabbit	1 : 2,000	Rubio's lab
	α – NifQ rabbit	1 : 2,500	Rubio's lab
	α – MosAB rabbit	1 : 5,000	This thesis
	α –Tubulin rat	1 : 500	Santa Cruz Biotechnology
	α – GFP mouse	1 : 500	Santa Cruz Biotechnology
	α – HSP60 rabbit	1 : 2,000	Abcam
Secondary antibody	α – rabbit HRP	1 : 50,000	Invitrogen
	α – rabbit AP	1 : 5,000	Sigma-Aldrich
	α – mouse HRP	1 : 20,000	Santa Cruz Biotechnology
	α – rat HRP	1 : 10,000	Amersham

Transfer buffer: 25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3.

DUNN carbonate buffer: 10 mM NaHCO₃, 3 mM Na₂CO₃, 20% methanol, pH 9.9.

PBS-Tween: PBS, 0.01% Tween 20.

ECL developing solution: 6.66 mM Tris pH 9.5, 45 mM NaCl, 0.05 mM MgCl₂.

BCIP: 0.5 g in 10 ml of 100% DMF.

NBT: 0.5 g in 10 ml 70% DMF in water.

ECL solution 1: 2.5 mM Luminol, 0.4 mM p-coumaric acid, 100 mM Tris-HCl pH 8.5.

ECL solution 2: 0.04% H₂O₂, 100 mM Tris-HCl pH 8.5.

11.3. Protein purifications

FPLC AKTAprime plus was used for protein purifications (GE Healthcare, Chicago, USA). 5-ml HiTrap IMAC HP, 5-ml HiTrap Q HP, PD10 desalting columns, DEAE-Sephacel resin and HiLoad 16/60 Superdex 200 resin (all from GE Healthcare, Chicago, USA) were used, when detailed, in the purifications. 1-ml Strep-Tactin®XT Superflow® high capacity (IBA Lifesciences, Göttingen, Germany) was used, when detailed, in purifications.

11.3.1. MoSto from *A. vinelandii*

A. vinelandii DJ cells were induced as detailed in section 8.1.5. Aerobic MoSto purification was performed as described previously (Fenske *et al.*, 2005) with some slight modifications. Cells were re-suspended in a ratio 1:2 in buffer A supplemented with 2 mM PMSF, 1 mg/ml leupeptin and 5 mg/ml DNase I. Lysate was obtained in a French Press at 1,500 p.s.i and 4°C. Cell debris was sedimented by centrifugation at 24,000 rpm and 4°C during 1 hour in an Avanti J-26XP centrifuge with a JA25.50 rotor rpm. The cell-free extract was filtered through a 0.2 µm pore size membrane. DEAE-Sephacel resin packed in a XK26/20 column with a cooling jacket set at 10°C was pre-equilibrated with buffer A. Cell-free extract was loaded at a flow of 2 ml/min. Wash A was performed with buffer A at 2 ml/min until absorbance was stabilized. Wash B was performed with 80% buffer A and 20% of buffer B until absorbance was stabilized. Elution was performed by applying 360 ml of 20% to 100% buffer B in a linear gradient. Eluted fractions (10 ml each) were analyzed to check for the presence of MosAB by using SDS-PAGE and Coomassie staining (see section 11.2.1). Mo quantification of eluted fractions was also performed (see section 11.10.2). MosAB enriched fractions were selected, pooled and subjected to ammonium sulfate precipitations (Isenberg, 1995). The first precipitation was achieved by incubating with 38% ammonium sulfate under stirring for 4 hours at 4°C. Sample was centrifuged at 24,000 rpm at 4°C for 30

minutes in rotor JA25.50 and the pellet was stored at 4°C for further analysis. The supernatant was subjected to a second precipitation by incubating with 50% ammonium sulfate under stirring overnight at 4°C. Sample was centrifuged at 24,000 rpm at 4°C for 30 minutes in rotor JA25.50 and the pellet was re-suspended in 5 ml of buffer C and filtered through a Ultrafree MC filter of 0.2 µm pore size (Millipore, Burlington, Massachusetts, USA) at 12,000 x *g* during 4 minutes at 4°C. Size-exclusion chromatography was performed in a HiLoad 16/60 Superdex 200 column. Sample was loaded from a 5-ml superloop into the Superdex 200 column at a flow of 1 ml/min. Buffer C was used in this chromatography. Eluted fractions were analyzed to check for the presence of MosAB by using SDS-PAGE and Coomassie staining (see section 11.2.1) and Mo quantification of eluted fractions was also performed (see section 11.10.2). Selected fractions were pooled and concentrated in a 100-kDa Amicon Ultra-15 device (Millipore, Burlington, Massachusetts, USA). Purified MoSto protein was stored in buffer C supplemented with 5% glycerol in liquid N₂ until used.

Buffer A: 50 mM MOPS, pH 6.5.

Buffer B: 50 mM MOPS, pH 6.5, 250 mM NaCl.

Buffer C: 50 mM MOPS, pH 6.5, 50 mM NaCl.

11.3.2. *NifDK and NifH proteins from A. vinelandii*

A. vinelandii UW463 cells were induced as detailed in section 8.1.4. Anaerobic purification of nitrogenase NifDK and NifH components was performed as described previously (Shah and Brill, 1973) with some slight modifications. Anaerobic purification was carried out inside a glove box (MBraun, Garching, Germany). *A. vinelandii* cell pellets were re-suspended in a ratio 1:5 in lysis buffer supplemented with 2 mM PMSF, 1 mg/ml leupeptin and 5 mg/ml DNase I inside a glove box. The lysate was obtained using an EmulsiFlex-C5 High Pressure Homogenizer (Avestin, Ottawa, Canada) at 15,000 p.s.i and 4°C. Cell debris was sedimented by centrifugation at 24,000 rpm at 4°C for 1 hour in an Avanti J-26XP centrifuge equipped with a JA25.50 rotor rpm. Cell-free extract was loaded into a 5-ml HiTrap Q HP column pre-equilibrated with a mixture of 80% buffer A and 20% buffer B. Elution was performed by applying a 250-ml linear gradient of 20-100% buffer B. Eluted fractions (5 ml) were analyzed to check for the

presence of NifDK and NifH by using SDS-PAGE and Coomassie staining (see section 11.2.1) and immunodetection (see section 11.2.2).

Eluted fractions containing NifH were pooled and concentrated in a 30-kDa size cut-off Amicon Ultra-15 device (Millipore, Burlington, Massachusetts, USA). Protein was stored in liquid N₂ until used.

Eluted fractions containing NifDK were pooled, and the salt concentration of the pool was calculated from the position of the fractions in the eluting gradient. The NaCl concentration for optimal crystallization is 42 mM. The mixture was incubated at 52°C for 20 minutes and centrifuged at 5,000 x *g* for 10 minutes at 4°C in rotor JA25.50. The supernatant was concentrated at 4°C in a 100-kDa Amicon Ultra-15 device. NifDK crystallization was carried out overnight at 38°C after which the sample was centrifuged at 14,000 rpm for 4 hours. The pellet containing crystallized NifDK was re-suspended in buffer C and stored in liquid N₂ until used.

Lysis buffer: 25 mM Tris-HCl pH 7.5, 1.7 mM DTH, 100 mM NaCl, 10% glycerol.

Buffer A: 25 mM Tris-HCl pH 7.5, 1.7 mM DTH, 10% glycerol.

Buffer B: 25 mM Tris-HCl pH 7.5, 1.7 mM DTH, 500 mM NaCl, 10% glycerol.

Buffer C: 25 mM Tris-HCl pH 7.5, 1.7 mM DTH, 250 mM NaCl, 10% glycerol.

11.3.3. *rMoSto* from *E. coli*

E. coli BL21 cells carrying the *A. vinelandii* *mosA* and *mosB* genes were grown and *mos* genes were induced as detailed in section 8.2.1. Anaerobic purification of the recombinant molybdenum storage protein (*rMoSto*) was carried out inside a glove box (MBraun, Garching, Germany). Metal affinity chromatography to purify the His-tagged *rMoSto* was performed using a 5-ml HiTrap IMAC column pre-loaded with NiCl₂. A pellet of induced *E. coli* cells was re-suspended in a ratio 1:2 with binding buffer supplemented with 2 mM PMSF, 1 mg/ml leupeptin and 5 mg/ml DNase I inside a glove box for 30 minutes. The lysate was obtained using an EmulsiFlex-C5 High Pressure Homogenizer (Avestin, Ottawa, Canada) 15,000 p.s.i at 4°C. Cell debris was sedimented by centrifugation at 24,000 rpm at 4°C for 1 hour in an Avanti J-26XP centrifuge

equipped with rotor JA25.50rpm. The cell-free extract was loaded into the HiTrap Ni column pre-equilibrated with buffer A at a flow of 2 ml/min. Wash A was performed with a mixture of 99% buffer A and 1% buffer B at 2 ml/min until absorbance of eluate was stable. Wash B was performed with a mix of 97% buffer A and 3% buffer B until absorbance of eluate was stable. Wash C was performed with a mix of 94% buffer A and 6% buffer B until absorbance of eluate was stable. Elution was carried out with a mix of 75% buffer A and 25% buffer B, and elution fractions showing absorbance at 280 nm were collected. Fractions were then analyzed to check for the presence of rMoSto by using SDS-PAGE and Coomassie staining (see section 11.2.1). Selected fractions were pooled and concentrated to 2.5 ml in a 100-kDa size cut-off Amicon Ultra-15 device (Millipore, Burlington, Massachusetts, USA). rMoSto was desalted in a PD10 column by loading 2.5 ml of sample and eluting with 3.5 ml of desalting buffer. Desalted rMoSto was concentrated in a 100-kDa Amicon Ultra-15 and stored in liquid N₂ until used.

Binding buffer: 50 mM sodium phosphate pH 7.2, 500 mM NaCl, 10 mM imidazole.

Buffer A: 50 mM sodium phosphate pH 7.2, 500 mM NaCl.

Buffer B: 50 mM sodium phosphate pH 7.2, 500 mM NaCl, 1 M imidazole.

Desalting buffer: 50 mM sodium phosphate pH 7.2, 500 mM NaCl, 10% glycerol.

11.3.4. *Yeast molybdenum storage protein (yMoSto) from S. cerevisiae*

Recombinant *S. cerevisiae* cells carrying the *A. vinelandii* *mosA* and *mosB* genes were induced for yMoSto expression as detailed in section 8.3.3. Anaerobic yMoSto purification was carried out inside a glove box (MBraun, Garching, Germany). Metal affinity chromatography to purify the His-tagged yMoSto was performed using a 5-ml HiTrap IMAC column pre-loaded with NiCl₂. Pelleted yeast cells were re-suspended in a ratio 1:2 with lysis buffer supplemented with 2 mM PMSF, 1 mg/ml leupeptin and 5 mg/ml DNase I inside a glove box for 30 minutes. Lysate was obtained in an EmulsiFlex-C5 High Pressure Homogenizer (Avestin, Ottawa, Canada) at 20,000 p.s.i at 4°C. Cell debris was sedimented by centrifugation at 24,000 rpm and 4°C for 1 hour in Avanti J-26XP centrifuge with a rotor JA25.50rpm. The soluble cell-free extract was loaded into the HiTrap Ni column pre-equilibrated with buffer A at 2 ml/min. The column was washed with buffer A (Wash A) followed by 10% buffer B in buffer A (Wash B). yMoSto

was eluted by applying a 100-ml linear gradient from 10% to 100% of buffer B. Eluted fractions were analyzed for the presence of MosAB by using SDS-PAGE and Coomassie staining (see section 11.2.1). Selected fractions were pooled and concentrated to 2.5 ml in a 100-kDa Amicon Ultra-15 (Millipore, Burlington, Massachusetts, USA). yMoSto was desalted in a PD10 column by loading 2.5 ml of sample and eluting with 3.5 ml of desalting buffer. Desalted rMoSto was concentrated in a 100-kDa Amicon Ultra-15 and stored in liquid N₂ until used.

Lysis buffer: 100 mM Tris-HCl pH 8.5, 300 mM NaCl, 10% glycerol.

Buffer A: 100 mM Tris-HCl pH 8, 300 mM NaCl, 10% glycerol.

Buffer B: 100 mM Tris-HCl pH 8, 150 mM NaCl, 400 mM imidazole, 10% glycerol.

Desalting buffer: 100 mM Tris-HCl pH 8, 150 mM NaCl, 5% glycerol.

11.3.5. *yNifQ* from *S. cerevisiae*

Recombinant *S. cerevisiae* cells carrying the *A. vinelandii nifQ* gene were induced for “yeast” NifQ (yNifQ) expression as detailed in section 8.3.3. Anaerobic yNifQ purification was carried out inside a glove box (MBraun, Garching, Germany). Strep-tag affinity chromatography was performed using a 1-ml Strep-Tactin XT column. Pelleted yeast cells were re-suspended in a ratio 1:2 with lysis buffer supplemented with 2 mM PMSF, 1 mg/ml leupeptin and 5 mg/ml DNase I inside a glove box for 30 minutes. Lysate was obtained in an EmulsiFlex-C5 High Pressure Homogenizer (Avestin, Ottawa, Canada) operated at 20,000 p.s.i and 4°C. Cell debris was sediment by centrifugation at 24,000 rpm at 4°C for 1 hour in an Avanti J-26XP centrifuge with JA25.50 rotor. The cell-free extract was loaded into the Strep-Tactin XT column pre-equilibrated with buffer A at 2 ml/min (pH of the cell-free extract should be above 8 for optimal binding). Wash A was performed with buffer A until no changes of absorbance were observed. yNifQ was eluted with 15 ml of buffer E and concentrated in a 10-kDa size cut-off Amicon Ultra-15 (Millipore, Burlington, Massachusetts, USA). Buffer was exchanged to desalting buffer in a PD10 column as described above. The presence of yNifQ was checked by SDS-PAGE and Coomassie staining (see section 11.2.1). The yNifQ protein was stored in liquid N₂ until used.

Lysis buffer: 100 mM Tris-HCl pH 8.5, 300 mM NaCl, 10% glycerol.

Buffer A: 100 mM Tris-HCl pH 8, 300 mM NaCl, 10% glycerol.

Buffer E: 100 mM Tris-HCl pH 8, 300 mM NaCl, 10.% glycerol, 50 mM biotin.

Desalting buffer: 100 mM Tris-HCl pH 8, 150 mM NaCl, 5% glycerol.

11.4. Prediction of mitochondrial targeting sequence

The bioinformatic tool from <http://mitf.cbrc.jp/MitoFates/cgi-bin/top.cgi> was used to predict the mitochondrial pre-sequence in the N-terminal of the target protein and its cleaved position

11.5. Ultraviolet-visible spectroscopy

UV-visible absorption spectra were recorded under anaerobic conditions in septum-sealed cuvettes inside an MBraun glove box using a Shimadzu UV-2600 spectrophotometer equipped with optical fibers. The samples were subjected to different treatments before UV-visible measurements: oxidation by air exposure, chemical oxidation by hydrogen peroxide, or exchange in 100 mM Tris-HCl pH 8.0, 250 mM NaCl by desalting in a PD-10 column (Landry and Ding, 2014).

11.6. Protein Mass spectrometry

Protein mass spectrometry was carried out with a 4800 Proteomics Analyzer equipped with TOF/TOF mass spectrometer at the Proteomics from Pharmacy faculty of Universidad Complutense de Madrid (Madrid, Spain).

11.7. Antibody generation

Polyclonal MosAB antibody was generated in rabbit at the CIB-CSIC (Madrid, Spain). Purified rMoSto was first sent to MALDI-TOF analysis at the Department of Proteomics of the Pharmacy School of Universidad Complutense de Madrid. After identity confirmation, 1 mg of rMoSto was sent to the CIB-CSIC for antibody generation.

MosAB antibody was tested by ELISA following the protocol:

- MaxiSorp™ ELISA plate (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was inoculated with 5 µg/ml of target protein (MosAB) and kept overnight at 4°C.
- Plate was washed with PBS-Tween three times.
- 100 µl of BSA 1% were added and incubated for 1h.
- The plate was washed with PBS-Tween three times.
- 100 µl of PBS-Tween with different dilutions of the MosAB antibody were added and incubated for 1h.
- The plate was washed with PBS-Tween three times.
- 100 µl of PBS-Tween with different dilutions of secondary antibody anti-rabbit-HRP were added and incubated for 45 minutes.
- The plate was washed with PBS-Tween three times.
- The plate was developed with 50 µl of OPD buffer and the reaction was stopped adding 50 µl of 2 N HCl.
- The plate was measured at 492 nm in a Varioskan LUX multimode microplate reader (Thermo Fisher Scientific, Waltham, Massachusetts, USA).
- The antibody working dilution was also checked by immunodetection (see section 11.2.2 and Supplemental Figure 1).

PBS-Tween: PBS, 0.01% Tween 20.

OPD buffer: 1 OPD pill in 10 ml of MilliQ H₂O with 10 µl of H₂O₂.

11.8. Enzymatic assays

Nitrogenase activity was determined by the acetylene reduction assay. Ethylene and, in those cases in which the alternative nitrogenase systems were expressed, ethane formation was quantified by gas chromatography. A Shimadzu GC-2014 was used to separate and quantify the reaction products. Nitrogen gas at a flow of 25 ml/min was used as carrier gas and a hydrogen flame ionization detector (FID) was used. A PoraPak N 80/100 packed column (Agilent technologies, Santa Clara, California, USA) was used to detect acetylene and ethylene. A PoraPak N 50/80 packed column (Agilent technologies, Santa Clara, California, USA) was used to detect acetylene, ethylene and ethane. Table 18 shows the GC conditions used for each PoraPak column.

Table 18. GC conditions.

	PoraPak N 80/100	PoraPak N 50/80
Injector temperature	150°C	180°C
Column temperature	90°C	60°C
FID temperature	150°C	200°C
Volume of gas phase injected	50 µl	500 µl
Chromatogram gas peak order	C ₂ H ₄ /C ₂ H ₂	C ₂ H ₄ /C ₂ H ₆ /C ₂ H ₂

11.8.1. *In vivo* nitrogenase activity

In vivo nitrogenase activity was performed as described previously (Stewart *et al.*, 1967). Acetylene reduction assay on whole cells de-repressed for Mo-nitrogenase (detection of ethylene) was carried out in 1 ml samples of cultured cells withdrawn from a time-course experiment (see section 8.1.1) and placed into a 9-ml serum vial sealed with a rubber stopper. Half ml of acetylene gas (Contse S.A., Madrid, Spain) was added to the sealed vial followed by incubation at 30°C for 30 min with shaking. Reaction was stopped adding 0.1 ml of 8 M NaOH.

Acetylene reduction assay on whole cells de-repressed for alternative nitrogenase system (detection of ethylene and ethane) was carried out by the addition of 5 ml samples of cultured cells withdrawn from a time-course experiment (see section 8.1.1; see section 8.1.4) and placed into a 26-ml vial sealed with rubber stopper. After injection of 1.5 ml of acetylene, the mixture was incubated for 60 min at 30°C with shaking. Reaction was stopped by addition of 0.1 ml of 8 M NaOH. The specific activity of each sample is defined as nmol of ethylene or ethane formed per min per ml per OD_{600nm}.

11.8.2. *In vitro* nitrogenase activity

In vitro FeMo-co synthesis assays were carried out as described (Curatti *et al.*, 2007) with some modifications. Acid-treated 9-ml serum vials sealed with serum stoppers under argon/acetylene (93%/7%) atmosphere were used. For FeMo-co synthesis assay, serum vials contained 2.5 μ M holo-NifEN, 3.0 μ M NifH and 0.44 μ M apo-NifDK, 0.175 mM *R*-homocitrate and an ATP-regenerating mixture (1.8 mM ATP, 25 mM phosphocreatine, 3 mM MgCl₂, and 40 μ g of creatine phosphokinase) in 25 mM Tris-HCl buffer (pH 7.5). When indicated, MoSto or NifQ purified from *A. vinelandii*, *E. coli* or *S. cerevisiae* was added to the *in vitro* FeMo-co synthesis reactions as sole Mo source. In some assays, the Mo clusters of NifQ and MoSto were previously reconstituted by incubation with 5 mM DTH, 1 mM Na₂MoO₄ and 1 mM Na₂S at 30°C for 2 hours followed by desalting with a PD10 column. Positive control reactions lack NifQ and MoSto but contain 7.5 μ M of Na₂MoO₄ as Mo source. All reactions were incubated for 35 min at 30°C. Activity of reconstituted apo-NifDK was analyzed by the acetylene reduction assay after addition of excess NifH and ATP-regenerating mixture. The specific activity of each protein is defined as nmol of ethylene formed per min per mg of NifDK protein in the assay.

Protein-protein interaction requirement for Mo donation by MoSto purified from *A. vinelandii* or *E. coli* was addressed by inserting a 3-kDa pore-size cut-off dialysis membrane between a MoSto solution on one side and the mixture of FeMo-co biosynthetic proteins and apo-NifDK on the other side. FeMo-co synthesis and nitrogenase activation reactions were carried out at 30°C for 2 min, 10 min, or 30 min. Activity of reconstituted NifDK was analyzed by the acetylene reduction assay after addition of excess NifH. The specific activity of each protein is defined as nanomoles of ethylene formed per minute per mg of NifDK protein.

Purified holo-NifEN, apo-NifDK, holo-NifDK and NifH were obtained from the laboratory common stocks.

11.9. Yeast techniques

All reagents used were from Sigma-Aldrich or VWR.

11.9.1. Mitochondria isolation

For mitochondria isolation, *S. cerevisiae* cells were grown in a 4-l bioreactor (see section 8.3.3) or in 4-liter flasks. Flask inductions were carried out by growing cells in 2 liters of YPAUGly (3%) or SD Glu (2%) in 4-liter flasks supplemented with 30 μ M of Na₂MoO₄ when indicated. Cells were inoculated at an OD_{600nm} around 0.3 and cultured overnight at 30°C with shaking at 200 rpm. The procedure for mitochondria isolation has been described previously (Diekert *et al.*, 2001). Six grams of cells were mixed with 15 ml of TD buffer and incubated at 30°C for 5 minutes under shaking. Sample was centrifuged at 2,500 x *g* for 5 minutes at 4°C and the pellet was re-suspended in 20 ml of SP buffer. This procedure was repeated twice and finally 25 ml of SP buffer containing 10 mg of Zymolyase 20T were added. The sample was incubated at 30°C for 1 hour under gentle shaking to obtain spheroplasts. All subsequent operations were conducted at 2°C. Spheroplasts were collected by centrifugation at 2,500 x *g* for 5 minutes. Pellet was carefully washed twice with SP buffer and then re-suspended in 15 ml of ice-cold 2xSHP buffer. Homogenization was carried out in a 50-ml glass homogenizer Potter-Elvehjem. Cell debris were removed by centrifugation at 2,500 x *g* for 5 minutes. The mitochondria from this supernatant were pelleted by centrifugation at 12,000 x *g* for 10 minutes. Mitochondria pellet was re-suspended in SH buffer and stored at - 80°C until used.

The quality of the mitochondria isolation process was analyzed by immunodetection (see section 11.2.2) using specific antibodies against the mitochondria matrix protein HSP60 (α -HSP60) and against the cytosolic located tubulin (α -tubulin) detailed in Table 17.

TD buffer: 100 mM Tris-SO₄, pH 9.4, 10 mM DTT.

SP buffer: 1.2 M sorbitol, 20 mM potassium phosphate pH 7.4.

2xSHP buffer: 1.2 M sorbitol, 40 mM HEPES- KOH, pH 7.4, 1 mM PMSF.

SH buffer: 0.6 M sorbitol, 20 mM HEPES- KOH, pH 7.4.

11.9.2. *Mitochondria treatment*

Localization of proteins in mitochondria was analyzed as described previously (Diekert *et al.*, 2001). The assay involved the treatment of mitochondria with proteinase K (0.5 mg/ml) in the absence or presence of 2 mM CaCl_2 and 0.2% Triton X-100 by incubation for 30 minutes at 30°C. Reaction was stopped by adding 1 mM PMSF and mixing with 4x Laemmli buffer without SDS, 2-mercaptoethanol and DTT. The proteolysis of recombinant proteins targeted to the mitochondria membrane or matrix was analyzed by immunoblot developed with antibodies against the protein of interest (see section 11.2.2).

Proteinase K: 20 mg/ml stock.

CaCl₂: 1 M stock.

Triton X-100: 20% stock.

PMSF: 100 mM stock.

11.10. *Quantifications*

All reagents are from Sigma-Aldrich, ThermoFisher scientific or VWR.

11.10.1. *Protein quantification*

Proteins were quantified by the BCA method (Smith *et al.*, 1985). The principle of this method is that proteins can reduce Cu^{+2} to Cu^{+1} in an alkaline solution (mainly caused by four amino acid residues including cysteine or cystine, tyrosine, and tryptophan) and result in a purple color formation by bicinchoninic acid.

BSA protein was used as a standard to extrapolate the concentration of the target proteins. When DTH was present in a target protein, IAA at 1 mg/ml was added to the samples and incubated at 37°C for 15 minutes. Colorimetric process was carried out by the addition of reagent A and reagent B in a ratio 49:1 and incubated for 30 minutes at 37°C. Sample absorbance were measured in a spectrophotometer at 562 nm.

BSA: 2 mg/ml stock.

Reagent A (Thermo Fisher Scientific, Waltham, Massachusetts, USA)

Reagent B (Thermo Fisher Scientific, Waltham, Massachusetts, USA)

11.10.2. Colorimetric molybdenum quantification

To quantify Mo present in the MoSto purification fractions (see section 8.3.1 and 11.3.1) the colorimetric method of (Cardenas and Mortenson, 1974) was used with some modifications.

- Digestion of the sample:
 - 300 µl of supernatant was mixed with 110 µl of 36 N H₂SO₄
 - The pellet was washed twice with MilliQ H₂O, re-suspended in 30 µl of 36 N H₂SO₄ and boiled at 100°C for 15 minutes. Afterwards, 300 µl of MilliQ H₂O mixed with 80 µl of 36 N H₂SO₄ were added.
- A standard curve with different concentrations of Mo was prepared using a Mo stock of ultrapure ammonium heptamolybdate (CertiPUR®) at 1,000 mg Mo/l in water.
- 200 µl of dithiol reagent were added to the samples and the mixtures were vortexed for few seconds.
- Samples were kept at room temperature for 20 minutes (in this step the formation of Mo-dithiol complex occurs)
- 220 µl of isoamylacetate were added to the samples and vortexed for 1 minute.
- Separation of the organic and aqueous phases was complete within 20 minutes.
- The upper phase was carefully pipetted out and its absorbance at 680 nm measured in a Varioskan LUX multimode microplate reader (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Dithiol reagent: 1 g of toluene-3,4-dithiol in 500 ml 1% NaOH is mixed for 1 hour and few drops of mercaptoacetic acid were added until a faint permanent opalescent turbidity developed.

11.10.3. ICP Mo quantification

For sensitive and accurate Mo measurements, ICP-OES or ICP-MS techniques were used. ICP-OES technique was used for samples containing Mo in the ppm range while ICP-MS was used in samples containing Mo in the ppb range. ICP-OES was carried out at the Ionomic Department of the CSIC-CEBAS (Murcia, Spain). ICP-MS samples were measured in the CCiTUB (Barcelona, Spain).

11.11. Fluorescence detection

The *S. cerevisiae* strain carrying the AtMOT1 transporter fused to the yEGFP fluorescent protein was subjected to fluorescence detection. *S. cerevisiae* cells were used to inoculate a 5 ml YPAUGly (3%) medium in a 50 ml Falcon tube with cap ajar for airing. Cells were cultured at 28°C with shaking 200 rpm overnight. Culture was scaled-up to 50 ml in 250 ml flask and grown for few hours until the log phase was reached (OD_{600nm} around 2).

First, the GFP fluorescence of cells was analyzed in microplates. Confirmed samples were treated with an organelle dye marker to determine the localization of the fusion protein using laser confocal scanning microscopy.

11.11.1. Microplate fluorescence detection

Measurement of GFP fluorescence was accomplished by using a Molecular Imager Pharos FX Plus (Bio-Rad Laboratories) with an excitation wavelength of 488 nm and an emission wavelength of 520 nm. Ten ml of cells from a log phase culture were centrifuged at 5,000 rpm for 5 minutes. The pellet was re-suspended in 1 ml of fixing buffer and 200 µl from each sample were pipetted into a microplate (UltraCruz® Plates, 96 well, PS) and measured in the Pharos FX Plus.

Fixing buffer: 50 mM Tris-HCl pH 7.5, 5 mM EDTA, 10% glycerol, 1 mM PMSF.

11.11.2. Confocal microscopy

Two 10-ml aliquots of cells from log phase cultures were placed in two 50-ml Falcon tubes. For organelle localization, 25 nM of MitoTracker Deep Red (Thermo Fisher

Scientific, Waltham, Massachusetts, USA) was added to one of the tubes. Both samples were covered with opaque film to protect them from the light and incubated at 30°C for 30 minutes. Samples were then centrifuged at 5,000 rpm for 3 minutes, washed twice with PBS buffer, and pelleted again. Pellets were re-suspended in 1 ml of a 1:1 mixture of YPAUGly (3%) and glycerol and were kept on ice. Whole-cell fluorescence was carried out by using a Leica TCS SP8 confocal microscope equipped with a PL APO 40x/1.1 water immersion objective. Excitation beam splitters DD 488/561 were used to capture detailed localization images of AtMOT1-yEGFP fusion protein and MitoTracker Deep Red.

PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.47 mM KH₂PO₄ pH 7.4.

Results

12. Genetic and biochemical analysis of the *A. vinelandii* molybdenum storage protein

A version of this chapter has been published in *Frontiers in Microbiology* (Navarro-Rodríguez *et al.*, 2019).

12.1. The absence of MoSto impairs cellular Mo accumulation and *in vivo* activity of the Mo-nitrogenase.

The *A. vinelandii* strain UW394 having an in-frame deletion of MoSto-encoding genes ($\Delta mosBA$) was generated to investigate cellular Mo levels and the effects of MoSto absence on the *in vivo* activity of the Mo-dependent nitrogenase when cells were exposed to changes in Mo availability. Four culture conditions reflecting various levels and lengths of Mo starvation were tested. On one hand, pre-cultures obtained by sub-culturing at least three times in molybdate-limited Burk's medium (hereinafter called Mo Starved) were inoculated into ammonium-free molybdate-limited medium (-Mo) or into ammonium-free molybdate-standard medium (+Mo) (see Figure 6; panel A). On the other hand, pre-cultures grown in Burk's medium (hereinafter called Mo Standard) were inoculated into ammonium-free molybdate-limited medium (-Mo) or into ammonium-free molybdate-standard medium (+Mo) (see Figure 6, panel B). To minimize nitrogen stress all testing growth media contained 1 μ M vanadate to induce expression of the V-nitrogenase in the presence of molybdate traces in the medium.

This experiment resulted in the following observations: (i) In the *A. vinelandii* strain that carries MoSto (*wild type*), the uptake of molybdate by Mo-starved cells occurred much faster than in the strain lacking MoSto (UW394); (ii) maximum intracellular Mo accumulation was 100-fold higher in the wild type than in cells lacking MoSto (UW394); (iii) The Mo content in UW394 did not always correlate with the levels of nitrogenase activity (compare panels A and B of Figure 6) suggesting the presence of alternative sinks or reservoirs for Mo; (iv) MoSto was not required for maximum nitrogenase activity as long as molybdate was provided in the growth medium indicating that its role in Mo-nitrogenase (i.e. as donor of Mo for FeMo-co synthesis) was not essential; (v)

However, under transient Mo starvation, tested by derepressing standard precultures in molybdate-limited medium, the MoSto mutant was impaired in nitrogenase activity while the *wild type* was not (compare activity open circles in panels A and B of Figure 6). This indicates that the metal accumulated at MoSto is readily accessible for FeMo-co synthesis allowing maximum nitrogenase levels under transient molybdate-limiting conditions. (vi) Prolonged Mo starvation equally affected intracellular Mo accumulation and *in vivo* nitrogenase activity in the *wild-type* and MoSto mutant strains; (vii) 30% nitrogenase activity remained in both strains after prolonged Mo starvation (Figure 6, panel A) suggesting that the V-nitrogenase was being expressed in these conditions.

Despite its pronounced impairment in Mo accumulation and *in vivo* nitrogenase activity, the $\Delta mosBA$ mutant strain was only mildly affected in diazotrophic growth when cultured in molybdate-limiting or even in molybdate-depleted medium (prepared as described in section 8.1.4). This apparent lack of diazotrophic phenotype was consistent with the Mo requirements for maximum nitrogenase activity being much lower than the capacity of *A. vinelandii* to scavenge and accumulate Mo in MoSto. Surprisingly, the $\Delta mosBA$ mutant strain showed slightly better growth than the *wild-type* strain in the presence of 1 μ M molybdate, both under diazotrophic and non-diazotrophic conditions (see Figure 7), probably as result of the above-mentioned ATP expenditure related to MoSto activity.

Figure 6. Effect of MoSto on Mo accumulation and *in vivo* nitrogenase activity in *A. vinelandii*. Mo-Starved (A) or Standard grown (B) cells of *A. vinelandii* DJ (wild type; black symbols) or UW394 ($\Delta mosBA$, red symbols) were inoculated into Burk's ammonium-free medium containing (close symbols) or lacking (open symbols) 1 μ M molybdate. All media contained 1 μ M vanadate. Data represent mean $\pm$ standard deviation of three independent experiments.

Competitive index (CI) assays were carried out to analyze the $\Delta mosBA$ phenotype in situations of competition for limiting Mo that are of environmental importance (see section 1). In these assays, growth interference takes place between the *wild type* and mutant strain. A CI > 1 indicates that the mutant is more competitive and grows faster than *wild type* whereas a CI < 1 indicates the mutant is less competitive. Figure 8 shows

that the MoSto mutant is less competitive than the *wild type* in diazotrophic growth under Mo starvation. CI was lowest under severe Mo starvation imposed by continuous growing in molybdate-depleted medium. This result was consistent with a role for MoSto as Mo reservoir. Interestingly the MoSto mutant was more competitive than *wild type* in the presence of 1 μM molybdate in the medium. Because MoSto is present at similar levels in both conditions (see section 12.3; Figure 10 panel A and B) this result suggests that the energetic burden of spending ATP to load molybdate and synthesize the POM clusters into MoSto is not negligible for the cell.

Figure 7. Diazotrophic growth of the *A. vinelandii* MoSto mutant strain. *A. vinelandii* DJ (wild type; black symbols) and UW394 (ΔmosBA , red symbols) were cultured in Burk's ammonium-free medium supplemented (close symbols) or not supplemented (open symbols) with 1 μM molybdate. Mo-limited (4 nM molybdate), Mo-depleted (no detectable Mo) and Mo standard (1 μM molybdate) conditions were tested. All ammonium-free media contained 1 μM vanadate. Growth in Burk's medium containing ammonium acetate (non-diazotrophic) was carried out as control.

Figure 8. Competitive Index of the *A. vinelandii* MoSto mutant strain.

(A) CI values were obtained from mixing cultures (1:1 inoculum ratio) of DJ and UW394 strains (see section 8.1.3.) Data represent the mean and standard deviation of three or four independent experiments. *** indicates unpaired t-test P value < 0.001. (B) Plates showing growth on solid medium of mixed cultures that were pre-cultured in liquid medium for 22 hours. Four 20- μ l drops with different dilutions (10^3 , 10^4 , 10^5 and 10^6) of the mixed culture were plated on each quarter of the plate. Arrows show the direction of the dilutions. Upper plates contain medium lacking spectinomycin (to allow DJ and UW394 growth) whereas lower plates contain medium supplemented with spectinomycin (to allow only UW394 growth).

12.2. MoSto protects the *A. vinelandii* Mo-nitrogenase system from tungstate toxicity.

W is a well-known competitive inhibitor of Mo functions having negative effect on *A. vinelandii* growth (Keeler and Varner, 1957). Importantly, accumulation of tungstate in MoSto has been shown to occur *in vivo* (Pienkos and Brill, 1981) and *in vitro* (Schemberg *et al.*, 2007). The possible role of MoSto in protection against the toxicity of W present in the environment was investigated by comparing the diazotrophic growth of the $\Delta mosBA$ mutant strain to that of the wild type in medium containing environmentally relevant concentrations of molybdate and tungstate (see Figure 9). Mo starved pre-cultures of both wild type and the MoSto mutant strains were highly sensitive to inhibition by 1 μ M tungstate (see Figure 9, panel A). Determination of the Mo content of routinely prepared molybdate-limiting medium as 4 nM molybdate results in a calculated tungstate to molybdate ratio of 250, an amount that could exceed the W discriminating or trapping capacity of MoSto. In contrast, when *A. vinelandii* cells grown in Mo standard conditions were transferred to molybdate-limited medium in the presence of 1 μ M tungstate, the wild-type strain grew normally while growth of the MoSto mutant was clearly inhibited (see Figure 9, panel B). The differential behavior of wild type and the $\Delta mosBA$ mutant under the transient Mo starving conditions established by transferring cells from Mo standard to Mo-limited can be rationalized if MoSto functioned as a reservoir that continuously provides Mo for FeMo-co synthesis thus preventing tungstate toxicity effects. In all cases, co-presence of 1 μ M molybdate in the medium protected from 1 μ M tungstate inhibition whereas 1 μ M vanadate did not. The lack of vanadate protection was not surprising since vanadate and molybdate (or tungstate) have different uptake mechanisms and different metabolic fates.

Figure 9. Effect of tungstate on the diazotrophic growth of an *A. vinelandii* $\Delta mosBA$ strain. Mo starved (A) or Mo standard (B) pre-cultures of *A. vinelandii* DJ (wild type; black symbols) or UW394 ($\Delta mosBA$, red symbols) were inoculated into Burk's ammonium-free medium containing 1 μ M molybdate (close symbols) or 4 nM molybdate (open symbols). One μ M tungstate was included when indicated and all media contained 1 μ M vanadate. Data represent mean $\pm$ standard deviation of three independent experiments.

12.3. Deletion of *mosAB* genes affects V-nitrogenase accumulation under Mo deficiency.

The expression of V-nitrogenase is tightly repressed by the presence of molybdate in the culture medium (Bishop and Joerger, 1990). However, it has been shown that the transcripts of V-nitrogenase and Mo-nitrogenase structural genes co-exist in the cell when molybdate levels in the medium are in the range of 10-50 nM (Jacobson *et al.*, 1986), i. e. just above the levels of molybdate (4 nM) considered as limiting in this thesis. Because the presence or absence of MoSto largely determines the intracellular concentration of Mo and therefore its physiological effects, the effect of $\Delta mosBA$ mutation on the accumulation of Mo-nitrogenase and V-nitrogenase structural components was investigated in relation to changes in molybdate availability. On one hand, Mo starved cells were transferred to the same Mo-limiting conditions (4 nM molybdate) or to Mo standard medium (1 μ M molybdate). In the absence of ammonium, the Mo standard medium allowed maximum NifDK accumulation and completely repressed VnfDGK synthesis, as expected (see Figure 10; panel A). Under Mo-limiting conditions (4 nM molybdate), Nif polypeptides were present at much lower levels and co-existed with Vnf polypeptides, both in the wild type and in the $\Delta mosBA$ strain. On the other hand, transferring Mo-sufficient cells to molybdate-limited medium (Mo step down) readily derepressed *vnfDGK* expression in the $\Delta mosAB$ strain but not in the wild type, which in contrast accumulated more NifDK polypeptides than the mutant (see Figure 10, panel B). Importantly, MoSto was accumulated to similar levels under all Mo concentrations tested in this study, in agreement with previous observations (Pienkos and Brill, 1981; Fenske *et al.*, 2005). These results indicate that the buffering effect that MoSto has in Mo homeostasis (see Figure 6, panel B) softens the regulatory response to transient Mo limitation repressing early *vnf* expression and maintaining *nif* expression levels to sustain Mo-dependent nitrogenase activity.

Figure 10. Detection of Mo-nitrogenase and V-nitrogenase structural polypeptides in *A. vinelandii*. Mo-starved (A,C) or Mo standard (B) precultures of *A. vinelandii* DJ (wild type), UW394 ($\Delta mosBA$), UW466 ($\Delta vnf \Delta anf$), UW463 ($\Delta vnf \Delta anf \Delta mosBA$), UW356 ($\Delta nifA$) or DJ33 ($\Delta nifDK$) were inoculated into Burk's ammonium-free medium containing either 0 nM, 4 nM or 1 μ M molybdate to allow nitrogenase derepression. NifDK, VnfK, VnfG, and MosAB polypeptides accumulated in the cells were detected by Western blot analysis against specific antibodies (see section 11.2.2).

12.4. Deleting the *mosBA* genes has no have any apparent effect on the activity of alternative nitrogenases.

The $\Delta mosBA$ mutation was also introduced into the $\Delta vnfDGK \Delta anfDGK$ mutant strain UW466, lacking the structural genes of V-dependent and Fe-only nitrogenases, to generate strain UW463. Strain UW463 was used to investigate a possible role of MoSto in these alternative nitrogenases. *A. vinelandii* cells cultured in Mo-depleted medium were derepressed for nitrogenase in either Mo-depleted or Mo-standard medium supplemented with 1 μ M vanadate. The presence of molybdate in the medium would repress *vnf* gene expression. However, in the absence of molybdate and presence of vanadate, the V-dependent nitrogenase is expressed while the Fe-only nitrogenase is repressed by vanadate. Strain DJ33, which lacks Mo-nitrogenase *nifDK* structural genes, and UW356 ($\Delta nifA$) unable to activate *nif* gene expression were used as control strains to ensure the absence of Mo-nitrogenase polypeptides.

The *in vivo* activity of expressed nitrogenases was estimated by measuring acetylene reduction into ethylene but also into ethane, an exclusive property of the alternative nitrogenases that is missing in the Mo-nitrogenase (Dilworth *et al.*, 1987). In addition, the presence of the catalytic components of Mo- or V-nitrogenase under Mo-depleted culturing conditions was determined by immunoblot analysis (see Figure 10, panel C). Nitrogenase activities and the presence or absence of nitrogenases polypeptides were determined after 8 hours of derepression. Importantly, all Mo-depleted cultures contained less than 0.7 pmol Mo per ml of culture at OD₆₀₀ of 1 at 8 h of derepression, as determined by ICP-MS analysis (see section 11.10.3).

Strains with intact Mo-nitrogenase structural genes produced maximum levels of ethylene and showed repression of alternative nitrogenase structural genes in medium containing 1 μ M molybdate and 1 μ M vanadate, as expected. Under Mo-depleted conditions (but 1 μ M vanadate), the *wild type* strain, as well as DJ33 ($\Delta nifDK$) and UW356 ($\Delta nifA$, encoding the *nif* gene transcriptional activator), exhibited concomitant production of ethylene and ethane diagnostic of alternative nitrogenase activity (see Table 19). No effect of the *mosAB* mutation on nitrogenase activity was detected under Mo-depleted conditions in any of the tested genetic backgrounds. This lack of mutant

phenotype could be explained if the MoSto protein was completely unloaded under sustained growth in Mo-depleted medium.

Surprisingly, the $\Delta vnfDGK \Delta anfDGK$ strains UW466 and UW463 exhibited similar ethylene and ethane production levels than $Vnf^+ Anf^+$ strains despite the absence of Vnf proteins (see Figure 10, panel C). However, these strains accumulated elevated levels of NifDK. This observation suggested the plausible insertion of FeV-co into NifDK under Mo-depleted conditions, which would be responsible for the observed residual nitrogenase activities.

Table 19. Ratio of ethane to ethylene production in MoSto deficient strains derepressed in medium with different metal availability.

<i>Strain^a</i>	<i>Metal</i>	<i>C₂H₄^b</i>	<i>C₂H₆ / C₂H₄ (%)^c</i>
DJ	V	1.58 ± 0.07	0.63
	Mo, V	13.65 ± 0.55	0
UW394	V	1.57 ± 0.01	0.71
	Mo, V	15.24 ± 1.04	0
UW466	V	1.83 ± 0.28	0.65
	Mo, V	13.94 ± 1.03	0
UW463	V	1.92 ± 0.18	0.60
	Mo, V	14.95 ± 1.04	0
UW356	V	0.47 ± 0.01	1.00
	Mo, V	0	0
DJ33	V	1.64 ± 0.59	1.22
	Mo, V	0	0

^a Precultures grown in ammonium-containing Mo-depleted medium were transferred to ammonium-free medium supplemented with 1 μ M of the indicated metal.

^b Activities are in nmol ethylene per min per ml at an OD₆₀₀ of 1. Data represent mean and standard deviations of 3 independent experiments.

^c C₂H₆ production shown as % of C₂H₄.

12.5. Hypothetical formation of a hybrid NifDK dinitrogenase harboring FeV-co in *A. vinelandii*.

The phenotypic analysis of UW463 and UW466 strains, lacking both alternative nitrogenases, suggested the formation of a hybrid NifDK/FeV-co nitrogenase in Mo-depleted culturing conditions (see section 12.4). To test this hypothesis, nitrogenase component proteins were purified from UW463 cells derepressed for 4 days in Burk's Mo-deplete medium as detailed in sections 8.1.6 and 8.1.4. *In vivo* acetylene reduction activity of UW463 culture was determined before collecting the cells resulting in 6.9×10^{-1} nmol $C_2H_4 \cdot min^{-1} \cdot ml^{-1} \cdot OD^{-1}$ and 1.3×10^{-3} nmol $C_2H_6 \cdot min^{-1} \cdot ml^{-1} \cdot OD^{-1}$ (5% of total product was ethane).

Figure 11 shows the result of an anion exchange chromatography in which UW463 proteins were separated by a NaCl gradient on a HiTrap Q HP column. SDS-PAGE and immunoblot analysis of protein fractions eluted from the column show clear separation of the NifDK (fractions E13-E19) and NifH (E26-E30) components although Nif protein levels were much lower than those typically purified from the wild-type strain. Fractions containing NifDK were pooled and subjected to a crystallization procedure for further purification, as described in section 8.1.6. (Shah and Brill, 1973). However, as the total volume of the sample was low, high-speed centrifugation was not possible and complete purification failed. The three different NifDK fractions (see Figure 11, panel B) were stored frozen in liquid N_2 . The fraction labeled as "2nd DK supernatant" was used for further analysis. NifH-enriched fractions were concentrated in a 30-kDa pore size Amicon and frozen in liquid N_2 . Partly purified UW463 nitrogenase components were referred to as UWNifDK and UWNifH.

Figure 11. SDS-PAGE and Western blot analysis of UWNifH and UWNifDK purification from UW463. 10% acrylamide SDS-gels and nitrocellulose membranes were used for PAGE and Western blot, respectively. Immunodetection was carried out with specific anti-NifDK and anti-NifH antibodies. (A) Analysis of anion exchange chromatography of UW463 cell-free extract. Each lane of the SDS-gels was loaded with 10 μ l sample from each chromatography fraction. CFE, cell free extract; FT, flow through; E5 to E46, fractions collected during NaCl gradient. (B) Partly purified UWNifH and UWNifDK. Each SDS-gel lane was loaded with 1.5 μ l of sample.

Because UWNifDK or UWNifH preparations were not completely pure, their quantification by routine colorimetric methods would not be accurate. Therefore, UWNifDK or UWNifH were subjected to immunodetection developed with alkaline phosphatase conjugates and their signals quantified against the signal from known amounts of pure wild-type NifDK and NifH proteins (see section 11.2.2; see Figure 12). Calibrating membranes obtained by immunodetection of known amounts of NifDK and NifH are shown in Figure 12 panel A while panel B shows immunodetection of UWNifH and UWNifDK 2nd supernatant samples. Concentration of UWNifDK and UWNifH in the samples were 1.55 mg/ml and 0.73 mg/ml, respectively.

Figure 12. Quantification of UWNifH and UWNifDK by immunoblot analysis. (A) Calibration standards obtained with known amounts of purified NifH and NifDK proteins. (B) Immunoblot analysis of membranes with increasing amounts of UWNifH and UWNifDK developed with anti-NifH and anti-NifDK antibodies. Calibration standards and sample proteins were transferred to the same membrane and developed together.

UWNifDK and UWNifH samples were assayed for *in vitro* nitrogenase activity (see section 11.8.2) after addition of wild-type complementary component (i.e. NifH to UWNifDK and NifDK to UWNifH). Titration of *wild-type* NifH and NifDK activities were

carried out as calibrating standards (see Figure 13, panel A). UWNifH appeared to be fully active, producing 600 nmol of $C_2H_4 \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ NifDK at a 9:1 UWNifH:NifDK molar ratio. On the other hand, activity from UWNifDK (ratio 1.6) was like *wild type* NifDK (see Figure 13, panel A ratio 2). Surprisingly, partly purified UWNifDK produced extremely low amounts of ethane compared to ethylene (0.05%) (see Figure 13, panel B), 100-fold less than observed for UW463 cells in the *in vivo* nitrogenase activity assays. As control reactions, it was observed that neither UWNifDK nor UWNifH samples reduced acetylene separately, demonstrating lack of cross-contamination.

Figure 13. *In vitro* activities of UWNifH and UWNifDK components. (A) Positive control was performed at a 40:1 molar ratio of NifH to NifDK. Wild type NifDK and NifH were titrated with the complementary wild-type component to serve as calibrating standards for UWNifH and UWNifDK activity determinations. UWNifH and UWNifDK activities were determined by addition of 3 μg NifDK or by increasing amounts of NifH, respectively. The red box indicates the reaction conditions in which ethane production was also measured. (B) Ethylene and ethane values from the reaction containing 23 μg UWNifDK and 10 μg AvNifH. Data is the average of three technical replicates with SD error.

12.6. MoSto serves as Mo donor for *in vitro* FeMo-co synthesis.

The *A. vinelandii* NifQ protein has been demonstrated to participate in Mo mobilization to the NifEN/NifH complex in the *in vitro* FeMo-co synthesis assay (Hernandez *et al.*, 2008). However, nothing is known about Mo pathway steps before inclusion of Mo into the [3Fe-4S] cluster of NifQ or whether Mo released from the MoSto POM clusters is in chemical forms that are suitable for FeMo-co synthesis. In this thesis, two versions of MoSto were purified to test their capacity to serve as Mo donor in the *in vitro* FeMo-co synthesis assay (see section 11.8.2). A non-tagged version of MoSto was expressed and purified from *A. vinelandii* DJ cells (see Figure 15) as detailed in section 8.1.5 and 11.3.1, respectively. In addition, a histidine-tagged version (named rMoSto) was cloned, overexpressed, and purified from recombinant *E. coli* cells (see Figure 17) as detailed in section 8.2.1 and 11.3.3.

MoSto purification from *A. vinelandii* cells required two sequential chromatographic steps. First, proteins of cell-free extracts were separated by anion exchange chromatography in DEAE-Sephacel applying an elution gradient with increasing concentrations of NaCl (Figure 14, panel A). Colorimetric Mo determination (see 11.10.2) and SDS-PAGE analysis of the eluted fractions (see 11.2.1) were decisive to select and pool fractions 3 to 13 for the next purification step. Selected fractions were subjected to ammonium sulfate precipitation as described in section 11.3.1. The first fractionation was carried out at 38% ammonium sulfate and it eliminated most of the contaminants (see Figure 15). A second fractionation was carried out at 50% ammonium sulfate and the resulting pellet was re-suspended and filtered before loading into a gel filtration column. The result of a size-exclusion chromatography is shown in Figure 14 panel B. Positions of MosA (29.2 KDa) and MosB (28.2 KDa) polypeptides in the SDS-gel are indicated. Fractions 3 to 7, which contained high Mo levels, were pooled and concentrated to 1 ml. SDS-PAGE analysis of the complete purification process is shown in Figure 15.

Figure 14. MoSto purification process from *A. vinelandii*. (A) The upper panel shows an SDS-PAGE analysis of protein fractions after DEAE-Sephacel chromatography of *A. vinelandii* cell-free extracts. The lower panel shows amounts of Mo present in eluted fractions and its corresponding Mo calibration curve. Selected fractions are highlighted in orange. (B) The upper panel shows an SDS-PAGE analysis of protein fractions after size-exclusion chromatography. The lower panel shows amounts of Mo present in eluted fractions and its corresponding Mo calibration curve. Selected fractions are highlighted in green.

Figure 15. SDS-PAGE analysis of MoSto purification from *A. vinelandii*. CFE, cell free extract; FT, flow through; WA, wash; P1, pellet from the 38% ammonium sulfate precipitation; S1, supernatant from the 38% ammonium sulfate precipitation; P2, pellet from 50% ammonium sulfate precipitation; S2, supernatant from the 50% ammonium sulfate precipitation. Five μ l from each sample were loaded per lane, except for the purified MoSto sample that contains 1 μ l of protein.

Figure 16. Quantification of partially purified MosA and MosB polypeptides by immunoblot analysis. (A) Calibration curve obtained with known amounts of pure MoSto expressed in *E. coli* (B) Immunoblot analysis of membranes with increasing amounts of partially purified MoSto developed with anti-MosAB antibody. Calibration standards and sample proteins were transferred to the same membrane and developed together.

As *A. vinelandii* MoSto preparations were not completely pure, quantification of MosA and MosB polypeptides was performed using a calibration curve obtained with known amounts of the same protein in a purified state (see section 11.2.2; see Figure 16, panel A). Results showed that the 10% of the final elution were MosAB polypeptides (see Figure 16, panel B).

rMoSto purification process from *E. coli* cells was performed by affinity chromatography as described in section 11.3.3 (see Figure 17). In contrast to the *A. vinelandii*, rMoSto was purified from *E. coli* induced cells grown in absence or presence of molybdate. rMoSto purified in absence of molybdate contained very little Mo, consistent with BL21 deficiency in high-affinity molybdate transport (Pinske *et al.*, 2011), and were subsequently used as negative control in the *in vitro* FeMo-co biosynthesis assay. rMoSto purified from cells grown in presence of 1 mM molybdate carried 25-fold less Mo than MoSto (see Table 20).

Figure 17. SDS-PAGE analysis of rMoSto purification from recombinant *E. coli*. CFE, cell free extract; FT, flow through; WA, wash A; WB, wash B; WC, wash C. Five μ l from each fraction were loaded per lane, except for purified rMoSto lanes that contain 0.5 μ l, 1 μ l and 2 μ l sample.

Both MoSto and rMoSto functioned as sole Mo source for *in vitro* FeMo-co synthesis (see Table 20). Apparent pronounced differences in activity between rMoSto and MoSto

were due to their different Mo content as both versions were equally efficient when reactions were normalized by Mo content.

Table 20. Activity of nitrogenase reconstituted with MoSto as Mo donor in the *in vitro* FeMo-co synthesis assay.

MoSto type	Mo in growth medium (mM) ^a	Mo atoms in MoSto ^b	μg MoSto used (μM Mo in reaction)	% Activity ^c
rMoSto	-	0.05 ± 0.00	10 (<0.01)	2.3 ± 0.8
			40 (0.02)	4.5 ± 0.3
rMoSto	1	4.12 ± 1.18	10 (0.48)	34.3 ± 3.2
			40 (1.94)	78.7 ± 4.6
MoSto	0.01	104.14 ± 19.8	0.5 (0.99)	55.3 ± 4.9
			1.0 (1.98)	63.1 ± 6.3

^a Cells used to purify MoSto were grown in medium containing the indicated amount of molybdate.

^b Per purified MoSto hexamer.

^c Percentage of activity compared to control reactions containing 7.5 μM molybdate as Mo source. Molybdate containing reactions formed 475 nmol of C₂H₄ per minute per mg of MoFe protein. Data represent mean and standard deviations of 3 independent experiments.

Direct protein-protein interaction of MoSto with the FeMo-co biosynthetic components was tested in a time-course FeMo-co synthesis experiment (see Figure 18, panel A). MoSto (or rMoSto) were separated from the remaining components by 3-kDa membranes (see Figure 18, panel B). The interaction of MoSto with FeMo-co biosynthetic proteins stimulated three-fold the cofactor synthesis. Reconstitution levels when MoSto was separated from the Nif proteins were probably due to Mo release from MoSto at pH 7.5 of the reaction mixture (Schemberg *et al.*, 2008). Interestingly, reconstitution due to unspecific Mo release did not increase over time. These biochemical assays established a role for MoSto as possible direct Mo donor for FeMo-co synthesis.

Figure 18. *In vitro* FeMo-co synthesis and nitrogenase reconstitution time-course assays using MoSto as source of Mo. (A) Reactions in which the molybdenum source (molybdate or MoSto) is mixed with the FeMo-co biosynthetic proteins. Molybdenum sources used were: 7.5 μ M molybdate (white bars) or MoSto protein equivalent to 4.5 μ M Mo (black bars). (B) Reaction mixtures in which the molybdenum source: molybdate (white bars), MoSto (black bars), or rMoSto (red bars) is separated from FeMo-co biosynthetic proteins by a dialysis membrane. Molybdenum sources used were: 7.5 μ M molybdate, 22.8 μ g MoSto (4.5 μ M Mo), or 229 μ g rMoSto (11 μ M Mo). Data represent mean $\pm$ standard deviation of three independent experiments.

13. A Mo pathway to nitrogenase in mitochondria of *S. cerevisiae*

A version of this chapter is being prepared for publication.

13.1. Selection of the *S. cerevisiae* strain to host the molybdenum pathway.

Three widely used *S. cerevisiae* strains (W303-1A, BY4741 and CEN.PK2-1C) were tested as host for the expression of MOT1 transporters of *A. thaliana* (hereinafter called *AtMOT1* instead of *AtMOT1;1*) and *M. truncatula* (*MtMOT1.3*). For each host strain, three variants were generated that harbored either the empty expression vector pDR196 (control strain), the *AtMOT1* or the *MtMOT1.3* transporters (see Figure 19). Strains were subjected to Mo toxicity tests by culturing for 2 days in SD medium supplemented with 50 μ M molybdate. Control yeast cultures in SD medium lacking molybdate were carried out in parallel (see section 8.3.2). Mo toxicity was evident in CEN.PK2-1C carrying the empty pDR196 and more pronounced in the CEN.PK2-1C strains carrying either one of the MOT1 transporters (see Figure 19, panel A). This response suggested that CEN.PK2-1C was able to take up molybdate, probably using the transport system of another anion, and that expression of a MOT1 transporter enhanced uptake activity. In contrast, the growth of W303-1A and BY4741 strains was not affected by the presence of 50 μ M molybdate in the medium. Whether W303-1A and BY4741 were unable to uptake molybdate or whether they are more resistant than CEN.PK2-1C to Mo toxicity was not further investigated. These Mo effects (or lack of them) were identical when tested in solid SD medium supplemented with 50 μ M molybdate (see Figure 19, panel B). Strain CEN.PK2-1C was therefore selected as host to express the components of the Mo trafficking pathway.

Figure 19. Growth of W303-1A, BY4741 and CEN.PK2-1C strains in SD medium containing 50 μM molybdate. (A) Growth curves in SD glucose minimal medium lacking (left) or containing 50 μM molybdate (right). Strain legend is common to panels A and B. Red circles represent W303-1A variants. Purple triangles represent BY4741 variants. Blue squares represent CEN.PK2-1C variants. Media was supplemented with uracil to select for vector maintenance. Average of three biological replicates with SD error is represented. **(B)** Growth in solid SD glucose minimal medium lacking (left) or containing 50 μM of molybdate (right). Five μl of 1:1, 1:10 and 1:100 dilutions of pre-cultures grown in medium without molybdate were plated in each spot.

13.2. Selection of a *MOT1* transporter gene.

The experiments showed in Figure 19 suggested that expression of *AtMOT1* enhanced Mo sensitivity in CEN.PK2-1C compared to *MtMOT1.3*. To focus on this difference without affecting growth of the CEN.PK2-1C control, Mo toxicity was investigated in a range of lower molybdate concentrations. Thus, the three CEN.PK2-1C derived strains were grown in SD medium supplemented with either 15 μM or 25 μM molybdate. Growth of the CEN.PK2-1C strain carrying *AtMOT1* was affected at 25 μM molybdate while CEN.PK2-1C strain carrying *MtMOT1.3* or empty pDR196 was not (see Figure 20). As the strain with *AtMOT1* was more responsive to molybdate, this gene was selected for further Mo pathway construction. Importantly, *MtMOT1.3* has been reported to locate in the cytoplasmic membrane (Tejada-Jiménez *et al.*, 2017) whereas location of *AtMOT1* is controversial and could either be in the cytoplasmic membrane or in mitochondria (Tomatsu *et al.*, 2007; Baxter *et al.*, 2008).

Figure 20. Growth of CEN.PK2-1C derived strains in SD medium supplemented with molybdate. Growth curves in SD glucose minimal medium lacking (left) or containing increasing amounts of molybdate. Blue squares represent CEN.PK2-1C variants. Average of three biological replicates with SD error is represented.

13.3. Effect of *AtMOT1* gene on molybdate uptake by CEN.PK2-1C.

Molybdate uptake assays were carried out by measuring concomitant changes in intracellular and extracellular Mo levels over a 6-hour time course culture experiment (see section 8.3.1). Strain growth and Mo toxicity of the same cultures were monitored for 24 hours. The CEN.PK2-1C strain carrying *AtMOT1* in expression vector pDR196 was compared to the strain carrying the empty vector. Consistent with previous results CEN.PK2-1C / *AtMOT1* was more sensitive to molybdate than the strain lacking the *AtMOT1* transporter (Figure 19; panel A). However, carrying *AtMOT1* did neither result in significantly higher intracellular Mo nor in faster molybdate consumption from the medium compared to the strain carrying the empty vector, as data statistical analysis rendered no significant differences between these strains (see Figure 21; panel B and C). The increase of intracellular Mo levels during the time course of the experiment demonstrated that CEN.PK2-1C was able to take up molybdate from the medium independently of carrying the *AtMOT1* gene. It was therefore concluded that activity of a heterologous molybdate-specific transporter, such as *AtMOT1*, would not be essential to enhance molybdate uptake but rather to diminish the toxic effects produced by Mo accumulation, which was otherwise required to engineer nitrogenase. It also pinpointed the need to clarify the cellular location of *AtMOT1* and the underlying nature of Mo toxicity.

Figure 21. Molybdate uptake by CENPK2-1C harboring or lacking *AtMOT1*. (A) Growth curves in SD glucose minimal medium lacking (left) or containing increasing amounts of molybdate. Blue squares represent CEN.PK2-1C variants. Intracellular Mo accumulation (B) and molybdate levels (C) in the growth medium of samples from the cultures shown in panel A during the first 6 hours of the time course. Average of three biological replicates with SD error is shown.

13.4. Localization of *AtMOT1* to the external membrane of mitochondria.

Two different *AtMOT1*-yEGFP fusion proteins having or lacking an N-terminal Su9 mitochondria targeting signal were constructed, placed under the control of a TEF1 promoter, and integrated into the chromosome of CEN.PK2-1C to generate strains YLR396 and YLR397, respectively (see section 10.3 and Table 5). yEGFP was fused to the C-terminal end of *AtMOT1* via flexible amino acid linker to facilitate folding of the chimeric protein. Fusion to the C-terminal end was chosen because bioinformatic tools

predicted (see section 11.4) the presence of a mitochondrial targeting signal at the N-terminus of wild-type *AtMOT1*, in agreement with Baxter *et al.*, 2008.

CEN.PK2-1C and the mutant strains YLR396 and YLR397 were cultured in SD medium and subjected to a molybdate toxicity experiment (see section 8.3.2). In contrast to the molybdate sensitive phenotype of wild type, the YLR396 and YLR397 strains were equally resistant to 50 μ M molybdate in the medium (see Figure 22; panel A). We noted that Mo toxicity was much more pronounced in strains carrying high copy number plasmids than in those with *AtMOT1*-yEGFP chromosomal integrations (compare panel A from Figure 24 and Figure 27). In addition, fluorescence of YLR396 and YLR397 cells was determined using a Molecular Imager Pharos FX Plus apparatus. Panel B from Figure 22 shows that strain YLR397 displayed more intense fluorescence than YLR396. Thus, strain YLR397 (genetic construct without Su9 mitochondria targeting signal) was selected for further analysis.

Figure 22. Phenotype of CEN.PK2-1C strains expressing *AtMOT1*-yEGFP variants. (A) Growth curves in SD glucose minimal medium lacking (left) or containing 50 μ M molybdate (right). Growth of CEN.PK2-1C *wild-type* and YLR396 and YLR397 strains expressing the *AtMOT1*-yEGFP variants is shown. Results are the average of three biological replicates with SD error. (B) yEGFP fluorescence emitted by CEN.PK2-1C, YLR396 and YLR397 cells grown in YPAU glycerol rich medium.

To visually locate the *AtMOT1* protein, YLR397 cells were grown on YPAU rich medium (see section 11.11) and confocal microscopy was performed as detailed in section 11.11.2. The medium contained glycerol as carbon source because its respiration favors mitochondria biogenesis. Fluorescence of yEGFP and the mitochondrial marker

MitoTracker Deep Red overlapped in discrete regions of the cell suggesting *AtMOT1* mitochondrial localization (see Figure 23; panel A).

Figure 23. Localization of *AtMOT1* in mitochondria. (A) Confocal fluorescence microscopy of a YLR397 cell expressing *AtMOT1*-yEGFP. Mitochondria were stained with MitoTracker Deep Red. Scale bar, 5 μ m. (B) YLR397 fractionation and mitochondria isolation. The mitochondria matrix protein HSP60 and the cytosolic protein tubulin were used as markers. *AtMOT1*-yEGFP was detected with anti-GFP antibodies. The amount of sample loaded was 5 μ l for HSP60 and tubulin detection and 50 μ l for yEGFP detection. (C) Immunodetection of full-length and proteolyzed *AtMOT1*-yEGFP protein in isolated mitochondria treated with proteinase K in the absence or presence of Triton X-100. Strain YLR397 was grown in YPAU glycerol medium in all cases. 50 μ l of sample was loaded in each lane.

Independent proof of *AtMOT1* mitochondrial localization was obtained by immunodetecting yEGFP in preparations of isolated mitochondria (see Figure 23; panel B). Quality of the mitochondrial isolation process was controlled by immunodetecting HSP60 as mitochondria matrix marker and tubulin a cytosolic marker. *AtMOT1* was immunodetected in the mitochondrial but not in the post mitochondrial (PM) supernatant fraction that includes cytosolic proteins, confirming the functionality of its natural mitochondrial targeting signal. In addition, YLR397 mitochondria preparations

were subjected to proteolytic treatments as detailed in section 11.9.2. Immunodetection of yEGFP was carried out in isolated mitochondria treated with proteinase K in the absence of detergent (to remove outer membrane proteins) or in the presence of Triton X-100 (to permeabilize the organelle and additionally proteolyze matrix proteins). Non-treated mitochondria isolated from CEN.PK2-1C and YLR397 were analyzed as controls. Proteinase K readily degraded *AtMOT1*-yEGFP independently of the presence of detergent, suggesting a localization in the outer membrane of the organelle (see Figure 23; panel C).

AtMOT1-yEGFP appeared to be active in translocating molybdate from the mitochondria matrix to the cytosol since Mo levels in mitochondria preparations of YLR397 were 5-fold lower than in CEN.PK2-1C (see Figure 24; panel A). Interestingly, decrease of mitochondrial Mo was observed in YLR397 after 16 hours of culture in SD medium supplemented with 30 μ M molybdate. Correlation between mitochondrial Mo levels and molybdate sensitivity (CEN.PK2-1C) or resistance (YLR397) could be inferred (compare panels A from Figure 22 and Figure 24), suggesting that at least one target of Mo toxicity was located in the mitochondria matrix.

Figure 24. Mitochondrial Mo determinations. (A) Mitochondria were isolated from CEN.PK2-1C (YLR3) or YLR397 cells grown for 2 hours in SD glucose medium (A) or in YPAU glycerol medium (B) lacking (-) or containing (+) 30 μ M molybdate. (C) Comparison of HSP60 protein levels in mitochondria isolated from cells cultured in SD glucose or in YPAU glycerol medium. One hundred micrograms of isolated mitochondria were loaded in each lane of the gel. HSP60 was immunodetected with specific anti-HSP60 antibodies.

As shown below in section 13.6, Mo toxicity was inexistent when culturing in YPAU rich medium. In those conditions with glycerol as carbon source very little Mo accumulated in mitochondria was shown (see Figure 24, panel B), even though immunodetection of mitochondrial matrix protein HSP60 indicated that this medium favored biogenesis of mitochondria compared to the SD glucose medium (see Figure 24, panel C).

13.5. Expression of MoSto protects from Mo toxicity in *S. cerevisiae*.

The *A. vinelandii* *mosA* and *mosB* genes encoding the subunits of MoSto were integrated into the chromosome of *S. cerevisiae* CEN.PK2-1C under the control of the TDH3 promoter. Two different yeast strains expressing MoSto with (YLR373) or without (YLR372) a mitochondrial targeting signal were generated (see section 10.3 and Table 5). We hypothesized that expression of MoSto could protect *S. cerevisiae* against Mo toxicity due to its high capacity for Mo accumulation (Pienkos and Brill, 1981; Navarro-Rodríguez *et al.*, 2019). For Mo toxicity assays, strains were cultured in SD glucose medium supplemented with increasing amounts of molybdate (see section 8.3.2). Growth curves showed that 25 μ M molybdate started to be toxic to the CEN.PK2-1C strain and 50 μ M molybdate strongly impaired CEN.PK2-1C growth. Both YLR372 and YLR373 strains showed resistance to molybdate in the medium although this was much higher in the YLR373 strain, expressing mitochondria targeted MoSto (see Figure 25). This difference could be explained by the large amount of ATP that MoSto requires for molybdate incorporation in the form of POM clusters (Fenske *et al.*, 2005; Poppe *et al.*, 2018), which is more available by mitochondria. It could also reflect different sensitivity to Mo in the cytosolic and mitochondria compartments. In any case, targeting MoSto to mitochondria appeared to be advantageous because it could play a detoxifier role in addition to its role as Mo reservoir.

Figure 25. Resistance to Mo toxicity in strains harboring MoSto at the cytosol or mitochondria. Growth curves in SD glucose minimal medium lacking (left) or containing increasing amounts of molybdate. Growth of CEN.PK2-1C (orange circles) and the strains targeting MoSto to the cytosol (YLR372, dark green squares) or mitochondrial matrix (YLR373, light green triangles) is shown. Results are the average of three biological replicates with SD error.

13.6. Co-expression and targeting to mitochondria of *AtMOT1*, *MoSto*, and *NifQ*

An engineered Mo trafficking pathway should contain *NifQ*, the physiological Mo donor for the biosynthesis of nitrogenase FeMo-co. Four additional *S. cerevisiae* CEN.PK2-1C derivative strains were generated with chromosomal insertions to express *NifQ* alone (YLR418) or the following combinations of *NifQ*, *MoSto* and *AtMOT1*-yEGFP (see Table 5): *MoSto* + *AtMOT1*-yEGFP (YLR574), *NifQ* + *MoSto* (YLR659), and *NifQ* + *AtMOT1*-yEGFP + *MoSto* (YLR565).

Mitochondrial localization of *NifQ*, *AtMOT1*-yEGFP and *MoSto* was demonstrated by immunoblot analysis of mitochondria isolated from the corresponding strains grown in bioreactors at large scale, as detailed in sections 8.3.3 and 11.9.1. Quality of the mitochondrial preparations were assessed by determining the presence or absence of mitochondria-specific HSP60 and cytosol-specific tubulin in the different fractions (see Figure 26).

Figure 26. Immunodetection of NifQ, MoSto and AtMOT1-yEGFP in mitochondria. Mitochondria were isolated from strains YLR418 (NifQ), YLR574 (AtMOT1-yEGFP and MoSto) and YLR565 (NifQ, AtMOT1-yEGFP, and MoSto). HSP60 and tubulin were used as markers. Pellet: unbroken cells; CFE, cell-free extract; Cytosol: cytosolic fraction; Mitochondria: isolated mitochondria. AtMOT1-yEGFP was detected using anti-GFP antibodies. The amount of sample loaded to detect each protein was HSP60 (5 μ l), tubulin (5 μ l), AtMOT1-yEGFP (50 μ l), MosAB, (2 μ l) and NifQ (5 μ l).

In all analyzed samples, isolated mitochondria were free of tubulin contamination. However, the cytosolic fractions shown in Figure 26 were slightly contaminated with HSP60, probably due to breakage of some mitochondria during the high-pressure homogenization step of the isolation process. Importantly, NifQ, MoSto and AtMOT1-yEGFP were detected in the mitochondrial fractions of the corresponding strains, demonstrating correct targeting and co-localization (see Figure 26).

The Mo sensitivity/resistance phenotype of YLR397, YLR418, YLR373, YLR574 and YLR565 strains was analyzed by culturing in SD glucose medium supplemented with increasing amounts of molybdate. The NifQ expressing strain YLR418 was as sensitive to molybdate as the *wild-type* strain, while the AtMOT1-yEGFP expressing strain YLR397 showed more resistance at 25-50 μ M molybdate but was equally affected at 100 μ M molybdate (see Figure 27, panel A). In contrast, all *S. cerevisiae* strains expressing in mitochondria MoSto alone (Figure 27) or in combination with NifQ and AtMOT1-yEGFP were very resistant to molybdate toxicity indicating a dominant role of MoSto in this phenotype (see Figure 27, panel B).

As genes encoding the proteins of interest were integrated into the chromosome of *S. cerevisiae* and no auxotrophic marker was required, the use of YPD rich medium was considered to increase biomass of the culture permitting large-scale protein purifications. YLR418, YLR574 and YLR565 strains were cultured in YPD or SD media (see section 8.3.2) and the toxic effect of increasing molybdate concentrations in each medium was compared.

Figure 27. Growth of *S. cerevisiae* engineered strains expressing different combinations of NifQ, MoSto and AtMOT1-yEGFP. (A) Growth curves of CEN.PK2-1C (wild type, orange circles), YLR418 (NifQ, red squares) and YLR397 (AtMOT1-yEGFP, purple triangles). (B) Growth curves of CEN.PK2-1C (wild type, orange circles), YLR373 (MoSto, green squares), YLR574 (MoSto+AtMOT1-yEGFP, blue triangles) and YLR565 (MoSto+AtMOT1-yEGFP+NifQ, brown inversed triangles). SD glucose minimal medium lacking or containing the indicated amount of molybdate was used. Results are the average of three biological replicates with SD errors.

Figure 28 shows data that confirm the sensitivity of *wild-type* CEN.PK2-1C and YLR418 to molybdate, as well as the resistance of strains expressing MoSto (see Figure 28; panel A). In contrast, no molybdate toxicity was observed for any strain cultured in YPD medium (see Figure 28; panel B). In CEN.PK2-1C molybdate is probably transported

into the cytoplasm by promiscuous transporter(s) and the lack of Mo toxicity indicated this non-specific molybdate transport was repressed by components of YPD rich medium. Sulfate transporters, such as SHST1, have been shown to enhance molybdate uptake into yeast cells even when present in the medium at nM concentrations (Fitzpatrick et al., 2008). Lack of Mo toxicity in YLR565 was consistent with *AtMOT1* being located at mitochondria and not at the cytoplasmic membrane as the latter location would probably catalyze molybdate uptake.

Figure 28. Comparison of molybdate toxicity in minimal and rich media. Strains YLR418 (red squares), YLR574 (blue triangles), YLR565 (brown inversed triangles) and the *wild-type* CEN.PK2-1C (orange circles) were grown in media containing increasing amounts of molybdate. (A) Growth curves of strains in SD glucose minimal medium. (B) Growth curves of strains in YPAU glycerol rich medium. Results are the average of three biological replicates with SD errors.

For large-scale mitochondria isolations, YLR418, YLR565 and YLR574 strains were fermented in a two-stage process. First, strains were cultured in rich YPAU galactose medium to produce large biomass and favour mitochondria biogenesis. Cells were then collected, resuspended in SD galactose minimal medium to derepress non-specific

molybdate uptake, and then cultured during 3 additional hours. The Mo content of isolated mitochondria was quantified by ICP-MS. Strain YLR565, expressing the entire pathway accumulated 5-fold more Mo than YLR574 (MoSto + AtMOT1-yEGFP) and 22-fold more Mo than YLR418 (expressing NifQ alone) (see Table 21). Altogether, the results shown in this section support that the elements allowing non-toxic delivery of Mo to mitochondrial NifQ were in place in strain YLR565.

Table 21. Mitochondrial molybdenum determination from strains YLR418, YLR574 and YLR565 grown in medium supplemented with 30 μ M of molybdate

YLR418		YLR574		YKR565	
Mean ^a	SD ^b	Mean ^a	SD ^b	Mean ^a	SD ^b
0.007	6 x 10 ⁻⁴	0.034	2.1 x 10 ⁻³	0.157	1x 10 ⁻²

^a: Three biological replicates. Values are represented in nmol of Mo per μ g of mitochondria.

^b: standard deviation from three biological replicates.

13.7. Purification and characterization of NifQ and MoSto from mitochondria of *S.cerevisiae* engineered strains.

For protein purification experiments, strains YLR418, YLR574 and YLR565 were grown in fermentors at large scale. Three biological replicates of each strain were fermented, and protein purifications were carried out as detailed in sections 11.3.4 and 11.3.5. NifQ was purified both from YLR418 and YLR565 while MoSto was purified from YLR574 and YLR565 strains.

Pure MoSto preparations from strains were obtained by IMAC after applying an imidazole gradient to elute MoSto from the column. MoSto purification yields were 10-fold higher than those of NifQ (see below). However, as isolated from yeast, MoSto was unstable and precipitated during the final concentration step impairing accurate Mo determination and *in vitro* activity assays (see Table 23). This behavior could be due to purification conditions that were not optimal for MoSto extracted from yeast. Attempts

to optimize MoSto purification by using several buffers and different pH failed (data not shown). Purified MoSto protein was colorless.

NifQ preparations from YLR418 were contaminated by a polypeptide with molecular weight of 130 kDa (see Figure 29; panel B). Mass spectrometry determined that the polypeptide was the PYC1 pyruvate carboxylase (see methods in section 11.6). NifQ preparations from YLR565 were additionally contaminated by MoSto as confirmed by immunodetection with anti-MosAB antibody. This result suggested the interaction between the NifQ and MoSto in the mitochondrial matrix of YLR565 and was further investigated as described in section 13.8. Pure NifQ was yellowish to reddish depending on the strain background and the concentration of molybdate used in the culture medium (see Figure 29; panel A).

Figure 29. Purification of NifQ and MoSto from engineered *S. cerevisiae* strains. (A) NifQ preparations purified from YLR418 or YLR565 cells grown at the indicated molybdate concentrations. (C) SDS-PAGE analysis of NifQ and MoSto affinity purification processes from YLR418, YLR574 and YLR565 cells. Pellet, unbroken cells; CFE, cell-free extract; FT, flow through; WA, wash A (first); WB, wash B (second). Purification from YLR565 cells involved sequential IMAC and Strep-tag affinity chromatography. Five μL from each sample were loaded in each lane, except for purified MoSto and NifQ samples. Triangles above NifQ and MoSto lanes indicate increasing loaded amounts. Red arrows point to NifQ protein. Green arrows point to MoSto protein.

Since growth of YLR565 was robust and very resistant to molybdate, growth in medium supplemented with 30, 60 or 120 μM molybdate was tested and NifQ purified preparations were obtained from the corresponding cells (see Table 22). These preparations contained only 0.039 Mo atoms per NifQ monomer (see Table 22) and were inactive as Mo donors in the *in vitro* FeMo-co synthesis and nitrogenase reconstitution assays (see Table 23).

Table 22. Determination of Mo content in purified NifQ and MoSto preparations. Mo was determined by ICP-MS determination (see section 11.10.3)

			Mean ^b	SD ^c
NifQ	YLR418	30 μM^a	0.016	1.5×10^{-3}
	YLR565	30 μM^a	0.039	2.6×10^{-3}
		60 μM^a	0.039	1.1×10^{-2}
		120 μM^a	0.038	7.1×10^{-3}
		1.2 mM ^a	0.068	3.6×10^{-3}
MosAB	YLR565	30 μM^a	0.001	1.7×10^{-4}
	YLR574	30 μM^a	0.001	6.9×10^{-5}

^a: Na_2Mo_4 added to the growth media

^b: Mo values corresponds to Mo atoms per protein from three biological replicates

^c: standard deviation from three biological replicates.

These assays contained, in purified forms, the FeMo-co deficient apo-NifDK protein, NifH, the FeMo-co biosynthetic protein apo-NifEN, the FeMo-co precursor NifB-co, and *R*-homocitrate (see Figure 30). Pure NifQ was added as Mo donor for its incorporation into NifB-co within a NifEN/NifH complex during FeMo-co synthesis. To investigate whether co-expression of MoSto in YLR565 would result in Mo competition and depletion of Mo from NifQ, additional NifQ preparations were isolated from the YLR418

strain. This hypothesis was rejected by the observation that YLR418 NifQ contained less Mo than YLR565 NifQ and was also inactive as Mo donor for FeMo-co (see Table 22 and Table 23). Importantly, NifQ purified preparations could be activated by incubating with 1 mM Na₂MoO₄ in the presence of 5 mM DTH, and 1 mM Na₂S (see Table 23), conditions that have been shown to increase the content of its [3Fe-4S-Mo] cluster (Hernandez *et al.*, 2008).

Figure 30. SDS-PAGE analysis of purified components from the FeMo-co biosynthesis pathway.

Molybdate in the culture medium of YLR565 was then increased to 1.2 mM to enhance the incorporation of Mo into NifQ *in vivo*. NifQ purified from these cells showed deep red color (see Figure 29; panel A) and contained 0.068 Mo atoms per NifQ monomer (see Table 22). However, these NifQ preparations were surprisingly active as Mo donors in the *in vitro* FeMo-co synthesis and nitrogenase reconstitution assays showing 80% activity compared to NifQ purified from the N₂-fixing bacterium *A. vinelandii* (see Table 23). We note that purified YLR565 NifQ preparations were contaminated by PYC1 and MoSto. Because MoSto preparations from yeast were inactive in the *in vitro* FeMo-co synthesis assay (see Table 23) it was unlikely that MoSto was responsible of the Mo donation activity of YLR565 NifQ preparations.

UV-vis absorption spectra of YLR565 NifQ in the oxidized and reduced states were recorded and compared to those of the *A. vinelandii* NifQ UV-spectrum (see Figure 31). As isolated (oxidized) YLR565 NifQ exhibited the typical of [Fe-S] proteins with a

smooth peak at 340 nm and a broad peak at 410 nm. Comparison of absorption intensities indicated similar [Fe-S] cluster content in the yeast and the *A. vinelandii* preparations. Incubation with DTH eliminated those features as expected for a redox responsive [Fe-S] cluster.

Figure 31. UV-visible spectra of NifQ purified from *A. vinelandii* or *S. cerevisiae* YLR565. Left graph, *A. vinelandii* NifQ in the as-isolated (solid line) or DTH-reduced (dotted line) states. Right graph, *S. cerevisiae* YLR565 NifQ in the as-isolated (solid line) or DTH-reduced (dotted line) states. The inset shows the color of as isolated YLR565 NifQ preparation. All spectra were normalized by their absorbance at 280 nm.

YLR565 co-expresses NifQ with MoSto and AtMOT1. The location of AtMOT1 transporter and the phenotype of strains expressing it suggested that AtMOT1 could transport molybdate from mitochondria to cytosol, in which case it would lower the amount of Mo available to NifQ. A *S. cerevisiae* strain identical to YLR565 but lacking the AtMOT1-yEGFP transcriptional unit was constructed and named YLR659. NifQ preparations were obtained from YLR659 (see Figure 32).

Importantly, YLR659 NifQ contained 0.1 Mo atoms per NifQ monomer and was as active as *A. vinelandii* NifQ in the *in vitro* nitrogenase activity assay (see Table 23), demonstrating that AtMOT1 was not an essential component to engineer a Mo pathway to nitrogenase in mitochondria of *S. cerevisiae*.

Figure 32. Purification of NifQ from engineered *S. cerevisiae* strain. SDS-PAGE analysis of NifQ affinity purification process from YLR659 cells. Pellet, unbroken cells; CFE, cell-free extract; FT, flow through; WA, wash A. Five μ l from each sample were loaded in each lane, except for purified NifQ sample. Triangles above NifQ lanes indicate increasing loaded amounts. Red arrows point to NifQ protein. Green arrows point to MoSto protein.

Table 23. Activity of purified NifQ preparations in the *in vitro* FeMo-co synthesis and nitrogenase reconstitution assay.

Protein	Strain	Mo in growth medium (μ M) ^a	Mo atoms per protein ^b	nmol protein used (μ M Mo in reaction)	% Activity ^c
NifQ	YLR418	30	0.016 ± 0.002	0.21 (0.1)	0
	YLR418*	30	2.5 ± 0.002	0.27 (1.88)	64.5 ± 0.01
NifQ	YLR565	30	0.039 ± 0.003	0.4 (0.05)	0
	YLR565*	30	1.48 ± 0.25	1.73 (6.04)	59.4 ± 0.01
NifQ	YLR565	1200	0.068 ± 0.003	10.2 (2.11)	80.7 ± 0.01
NifQ	YLR659	1200	0.098 ± 0.005	3.45 (1.01)	98 ± 0.01
MoSto	YLR565	30	$0.001 \pm 2 \times 10^{-4}$	4.65 (0.018)	0
	YLR574	30	$0.1 \times 10^{-2} \pm 0.6 \times 10^{-4}$	4.65 (0.015)	0
NifQ	UW300	1	0.41 ± 0.014	4.9 (5.57)	100

^a Cells used to purify NifQ or MoSto were grown in fermentors containing the indicated amount of molybdate.

^b Per purified NifQ monomer or MoSto hexamer.

^c Percentage of activity compared to UW300 activity.

Molybdate containing reactions, carried out as controls, produced 478 ± 15 nmol of C_2H_4 per minute per mg of apo-NifDK. Data represent mean and standard deviations of 3 independent experiments.

* Samples previously reconstituted with 5 mM DTH, 1 mM Na_2MoO_4 and 1 mM Na_2S

13.8. Interaction of MoSto and NifQ in mitochondria of *S. cerevisiae* YLR565

Purified preparations of NifQ from YLR565 cells were slightly contaminated with MoSto (see Figure 29, panel B) suggesting *in vivo* interaction of these two proteins. This interaction was further investigated by carrying out pull-down assays using NifQ immobilized in a Strep-Tactin®XT column. A cell-free extract of strain YLR574, expressing MoSto and AtMOT1-yEGFP but not NifQ was loaded into the immobilized NifQ column. After three washes to remove contaminants weakly bound to the column, NifQ was eluted using biotin. Samples from the eluate and the collected washes were analyzed by immunoblot with anti-MosAB and anti-NifQ antibodies (see Figure 33). A fraction of MoSto present in YLR574 extracts was trapped by the column and co-eluted with NifQ after applying biotin. Control experiments carried out by loading the cell-free extract of strain YLR418 expressing only NifQ showed the specificity of the NifQ-MoSto interaction.

Figure 33. Pull-down experiments with column immobilized NifQ. YLR418 or YLR574 cell-free extracts were loaded into the column. CFE, cell-free extract; FT, flow through; WA1, wash1; WA2, wash 2; WA3, wash 3; Elution, eluted proteins after applying biotin. Left panel shows the immunoblot membrane developed with anti-MosAB antibodies. Right panel shows the immunoblot membrane developed with anti-NifQ antibodies. Ten μ l of sample were loaded in each lane, except for WA lanes that contained 15 μ l of sample.

Discussion

Molybdoenzymes are widely spread among living organisms which, to mature them into active forms need to take up, store and use molybdate for cofactor biosynthesis. All these processes are genetically regulated by molybdate. *A. vinelandii* has complex Mo genetics and metabolism, including three genetically distinct molybdate transport systems, a unique Mo storage protein (MoSto), and co-existing biosynthetic pathways for molybdopterin-based Mo-cofactors and the iron-molybdenum cofactor of nitrogenase. MoSto is rather unique both in function and in amino acid sequence having no known homologs in protein databases. MoSto exhibits several outstanding properties that differ from those of all other known Mo-containing proteins, such as the type of metal clusters or the number of atoms that can be stored (Fenske *et al.*, 2005; Kowalewski *et al.*, 2012). MoSto has two types of subunits encoded by the *mosA* and *mosB* genes which expression is neither repressed by ammonium nor depends on the NifA transcriptional activator (Pienkos and Brill, 1981). Therefore, MoSto is not a Nif protein. However, as this study shows, MoSto is important for correct functioning of the Mo-nitrogenase and regulation of expression of alternative nitrogenases in environmentally relevant conditions, such as transient molybdate scarcity or elevated levels of competing tungstate.

The main function of MoSto has been related to its capability to supply Mo to molybdoenzymes and to protect the cell from toxicity of the competing metal W, favorable traits to add to an engineered N₂-fixing yeast or plant (Curatti and Rubio, 2014). Under environmental Mo limiting conditions, MoSto endowed *A. vinelandii* with the ability to maintain high Mo-dependent diazotrophic growth rates increasing species competitiveness. In terrestrial environments, in which the average concentration of molybdate is in the range of 50 nM and its distribution is irregular (Hernandez *et al.*, 2008), MoSto would confer enormous advantage. Under laboratory growth conditions in which large excess of molybdate is present in the standard medium, this situation is obscured. Thus, presence or absence of MoSto did not affect nitrogenase activity unless the cells were subjected to transient molybdate starvation (Navarro-Rodríguez *et al.*, 2019).

MoSto also confers certain degree of protection against W, a Mo antagonist that renders inactive molybdoenzymes. Previous studies showed *in vivo* (Pienkos and Brill, 1981) and *in vitro* binding of W to MoSto (Schemberg *et al.*, 2007). W toxicity for N₂-fixing cells of *wild-type A. vinelandii* is evident only when large excess of W over Mo (i.e., for W/Mo ratios higher than 150) is present in the culture medium (Keeler and Varner, 1957). This was not the case of the MoSto mutant, which exhibited high sensitivity to equimolar concentrations of Mo and W. Similar W sensitive phenotypes have been observed in mutants deficient in catechol siderophore production (Wichard *et al.*, 2008). Both, Mo fluctuating conditions, including severe Mo limitation, and the presence of W at concentrations equal or higher than Mo, are environmentally relevant conditions (Xu *et al.*, 2006).

A. vinelandii accumulates enormous amounts of Mo both under diazotrophic and non-diazotrophic growth (Pienkos and Brill, 1981). This ability depends on the ability of the MoSto hexamer to accumulate around 100 Mo atoms (Fenske *et al.*, 2005). Thus, most Mo accumulated by *A. vinelandii* is bound to MoSto although Mo storage is not essential to achieve maximum nitrogenase activity. The notable capacity of *A. vinelandii* to accumulate such levels indicated that high-affinity molybdate transport was independent of the storage process (Mouncey *et al.*, 1995; Navarro-Rodríguez *et al.*, 2019). However, the necessity of both systems to rely on ATPase activity to carry out the release or uptake of molybdate entail an energy burden to the cell (Allen *et al.*, 1999; Schemberg *et al.*, 2008). This fact would explain why the mutant strain lacking MoSto is more competitive than the wild type under non-diazotrophic Mo-sufficient growth conditions (Navarro-Rodríguez *et al.*, 2019). There are discrepancies in the literature as to whether MoSto expression is regulated by Mo. First, it was reported that MoSto was constitutively expressed even in cells grown in absence of Mo. This was evidenced by the ability of *A. vinelandii* to exhaust Mo from the growth medium after cell treatment with chloramphenicol, an inhibitor of protein synthesis (Pienkos and Brill, 1981). Later, it was found that MoSto was expressed at molybdate concentrations as low as 1 nM but not in Mo-free medium. Determination of MoSto content, which was quantitatively assayed by immunoblot analysis, was constant at concentrations higher than 50 nM of molybdate (Fenske *et al.*, 2005). Our results are in line with constitutive expression

since we were able to detect MoSto in cells grown in medium with Mo levels below an ICP-MS detection limit of 0.05 ppb. It is however possible that molybdate traces below our experimental detection limit are enough to induce MoSto expression (Navarro-Rodríguez *et al.*, 2019).

A. vinelandii has the ability to synthesize three different nitrogenases, which depend mostly on the intracellular metal content (Joerger *et al.*, 1988). MoSto acts indirectly as part of a repressing system for the alternative nitrogenases, since it is responsible of the elevated levels of intracellular Mo. In the absence of MoSto, Vnf polypeptides were readily derepressed upon Mo step down, indicating that the buffering effect of MoSto may also be important to maintain tight regulation of nitrogenase with different metal specificities (Navarro-Rodríguez *et al.*, 2019).

Wild-type cells grown under severe Mo-starved or Mo-depleted conditions, exhibited co-existence of structural polypeptides of Mo-nitrogenase (NifDK) and V-nitrogenase (VnfDGK) although the levels of NifDK were much lower than those accumulated in standard Mo conditions (Navarro-Rodríguez *et al.*, 2019). The low levels of NifDK are consistent with the Mo requirement for full expression of *nif* genes (Premakumar *et al.*, 1998). Previous studies reported the co-existence of *nif* and *vnf* transcripts at Mo concentrations between 10 nM and 50 nM (Jacobson *et al.*, 1986) but not at the 4 nM and Mo-depleted concentrations of our work (Navarro-Rodríguez *et al.*, 2019). It is likely that these discrepancies among reports are due to the different sensitivity of Mo determination or product detection methods.

On the other hand, strains lacking the alternative nitrogenase systems appear to compensate with higher amounts of NifDK polypeptides when cultured under Mo-deplete conditions. It is known that the alternative nitrogenases catalyze the reduction of acetylene by either two or four electrons to yield ethylene or ethane, respectively (Dilworth *et al.*, 1988). Surprisingly, all strains exhibited activity with features typical of alternative nitrogenases under Mo-depleted conditions regardless of the presence of VnfDGK. Thus, simultaneous contributions from both systems cannot be ruled out.

Activity of a hypothetical *in vivo* generated NifDK/FeV-co hybrid nitrogenase was different to previous published data in which C₂H₆ production by an *in vitro* generated hybrid was 4 fold-lower than C₂H₄ (Ribbe *et al.*, 2018). This discrepancy could be due to the source of hybrid nitrogenase (*in vivo* vs. *in vitro* generated) but it could also indicate that our experimental conditions to obtain the hybrid nitrogenase are not highly reproducible due to the difficulty to achieve Mo-depleted medium at large scale fermentation.

Other hybrid Mo-nitrogenases carrying the cofactor of V-nitrogenase or even biosynthetic precursor to FeMo-co have been generated *in vitro* and were shown to exhibit altered substrate specificities and product formation (Moore *et al.*, 1994; Soboh *et al.*, 2010). Although in standard derepressing conditions the NafY protein seems to have a discriminating role in the insertion of the metal cofactor in the Mo-nitrogenase active site (Rubio *et al.*, 2004), the situation under extremely Mo-deficient conditions has not been tested.

Although MoSto has been biochemically characterized (Brünle *et al.*, 2018), there were no reports of MoSto activity in the *in vitro* FeMo-co synthesis assays. This thesis shows that Mo stored at MoSto is directly available for FeMo-co synthesis *in vitro* and that interaction of MoSto with FeMo-co biosynthetic proteins stimulates cofactor synthesis (Navarro-Rodríguez *et al.*, 2019). The exact mechanism by which this interaction operates is unclear. Direct transfer of Mo from NifQ to the NifEN/NifH complex during FeMo-co synthesis has also been reported (Hernandez *et al.*, 2008). The similarities between the [Mo-Fe-S] clusters found in NifQ and in NifEN support such direct connection (Soboh *et al.*, 2006; George *et al.*, 2007). In addition, Mo-EXAFS revealed a second Mo environments in NifQ containing Mo-O and Mo-S bonds (George *et al.*, 2016). In contrast MoSto had been described to contain POM clusters in the form of Mo-O bonds (Kowalewski *et al.*, 2012), i.e. full oxygen coordination similar to molybdate and very different from the coordination found at the FeMo-cofactor.

The expected primary path for Mo incorporation into FeMo-co would include molybdate transport, storage at MoSto, transfer to NifQ, and finally to the NifEN/NifH complex. However, the results shown here indicate some degree of NifQ function replacement by

MoSto in the *in vitro* system. Mo release from MoSto occurs spontaneously *in vitro* above pH 6.8 and perhaps an interaction with NifEN facilitated Mo transfer (Schemberg *et al.*, 2008). In this context, it is interesting to note that a NifH-independent pathway for Mo transfer into NifEN was previously postulated (Soboh *et al.*, 2006). Thus, the ability of MoSto to donate Mo for FeMo-co synthesis might also be relevant *in vivo* and could underlie the phenotypic reversal of *nifQ*⁻ by excess of molybdate (Joerger and Bishop, 1988; Rodriguez-Quinones *et al.*, 1993)

In vitro Mo release from MoSto and its utilization for FeMo-co synthesis occurred at pH 7.5 in the presence of 1.23 mM ATP and an ATP regenerating enzyme present in the reaction mixtures. It is known that MoSto catalyzed ATP-hydrolysis promotes formation of POM clusters inside the MoSto cage while molybdate release from MoSto is favored at pH 7.5, but only after ATP is consumed (Poppe *et al.*, 2018). Therefore, the buffer composition of the *in vitro* FeMo-co synthesis assay should preclude Mo release from MoSto. This discrepancy can be explained if FeMo-co biosynthesis and nitrogenase reconstitution alter the equilibrium of Mo binding/release by removing Mo from the available pool.

In recent years much effort has been dedicated to successful expression and maturation of nitrogenase components in eukaryotes with the final objective of developing cereal crops that can be self-supported by BNF. *S. cerevisiae* and *Nicotiana benthamiana* have been used as models to establish the required tools before engineering much more challenging cereals. First achieved attempts involved expressing functional NifH and NifB proteins key to the Mo-nitrogenase (López-Torrejón *et al.*, 2016; Burén *et al.*, 2017a).

Engineering Mo-nitrogenase in yeast and plants would also require building specific Mo pathways, albeit the exact requirements might differ among hosts. In this thesis I have used *S. cerevisiae* as model to build the Mo pathway to nitrogenase. Contrary to plants, *S. cerevisiae* does not contain molybdoenzymes and, in this regard, it is a cleaner system to engineer. Plants carry various molybdoenzymes that catalyze important metabolic processes (Tejada-Jiménez *et al.*, 2013). In contrast to the situation with the well-studied bacterial Mo⁰ transport systems, eukaryotic molybdate transporter were

unknown until the MOT1 protein family was described (Tejada-Jiménez *et al.*, 2007, 2017; Baxter *et al.*, 2008). As shown in previous report, expression of MOT1 transporters in yeast altered molybdate toxicity profiles. Both *MtMOT1.3* and *AtMOT1* have been described to transport molybdate to the cytosol. However, they are not located in the same compartment membrane (Baxter *et al.*, 2008; Tejada-Jiménez *et al.*, 2017). Subcellular localization of *AtMOT1* has been controversial for years. By indicating localization in the outer membrane of mitochondria, our results are in agreement with those of Baxter (Baxter *et al.*, 2008). These results, in combination with the need to express functional nitrogenase in a low O₂ organelle, led to design a Mo pathway from the extracellular medium to the physiological Mo donor NifQ located at the matrix of mitochondria. Such pathway should also protect the yeast from Mo toxicity.

Defining Mo toxicity is difficult since its mechanism of action, if any, is still unknown. In plants, it has been shown that excess Mo results in its accumulation in specific parts, which will initiate other responses (Pasricha *et al.*, 1977). Toxicity in other organisms that do not carry Mo-containing enzymes, such as *S. cerevisiae*, remains possible if molybdate is transported across the plasma membrane through promiscuous transporter(s) with broad ion specificity, as shown in *E. coli* (Self *et al.*, 2001) and *S. cerevisiae*. Both sulfate and phosphate starvation have been shown to increase Mo accumulation in yeast (Heuwinkel *et al.*, 1992; Fitzpatrick *et al.*, 2008). A *S. cerevisiae* strain heterologously expressing the *A. thaliana* MOT1 transporter did not differ from the *wild type* in terms of cellular Mo accumulation but did alleviate Mo toxicity. The Mo toxicity problem was completely solved by expressing *mosAB* genes and targeting the MoSto protein to mitochondria of engineered strains. It was expected that MoSto would chelate free molybdate molecules available in the matrix. Such success could be due to the plentiful ATP and reducing power generated by respiration in this organelle. Molybdate binding to MoSto has been described to be dependent of ATP hydrolysis (Schemberg *et al.*, 2008). In addition, pH conditions in the mitochondrial matrix would favor continuous release of the metal that will then be available for FeMo-cofactor biosynthesis (at this stage of engineering, this Mo would be available to NifQ) (Santo-Domingo and Demareux, 2012).

At least one target of Mo toxicity lied in the mitochondrial matrix. This conclusion is supported by comparing the phenotypes of strains targeting MoSto to mitochondria or to the cytosol, the former being much more resistant. In addition, strains expressing *AtMOT1* showed lower mitochondrial Mo levels which correlated with certain level of resistance to Mo toxicity. Combining MoSto and *AtMOT1* expression notably increased resistance to Mo. Kinetics analysis of the K_m and V_{max} values for molybdate uptake of yeast expressing *AtMOT1* are in the range of 21 nM and 0.5 $\mu\text{g}\cdot\text{g}^{-1}\text{DW}\cdot\text{min}^{-1}$, respectively (Tomatsu *et al.*, 2007). Therefore, *AtMOT1* saturation could be happening at those Mo levels.

A previous study from our laboratory showed that the O_2 -sensitive NifH protein could be assembled in the mitochondrial matrix of *S. cerevisiae* (López-Torrejón *et al.*, 2016; Burén *et al.*, 2017b). O_2 consumption by respiration in this organelle is critical to overcome the known sensitivity of nitrogenase [Fe-S] clusters to O_2 (Imlay, 2006). The NifQ protein, which has been characterized as the physiological Mo donor to NifEN in FeMo-co synthesis (Hernandez *et al.*, 2008), was also targeted to this organelle. As it was shown in the preceding results, cell toxicity was produced unless *AtMOT1* was expressed, or in the extreme case, expression of MoSto acted as a protective sink from high intracellular Mo levels. On the other hand, medium composition was decisive for molybdate transport. While no Mo toxic effects were observed in rich medium, increasing amounts of molybdate in minimal medium clearly affected those strains that did not carry any protective mechanism. Mo accumulation levels in mitochondria isolated from cells grown in rich medium or minimal medium were in accordance with the corresponding Mo toxicity assays. Despite the fact that glycerol was added as carbon source in the rich medium in order to increase mitochondria biogenesis (Roberts and Hudson, 2006; Swinnen *et al.*, 2013), Mo accumulation was impaired in comparison to strains grown in minimal medium. This could be due to the expression of promiscuous transport systems in minimal medium and repression of those systems in rich medium.

Mo levels in isolated mitochondria were consistent with the genetic background of each strain. While Mo levels were quite low in a strain expressing NifQ alone, the strains containing MoSto showed higher Mo levels. Mitochondria Mo levels were highest in the YLR565 strain expressing the three components of the pathway. In fact, NifQ purified

from this strain exhibited a colorful pattern from yellow to reddish in agreement with the increasing amounts of molybdate added to the growth medium. Previous reports of NifQ purified from *A. vinelandii* cells also reported a reddish color, albeit, 4-fold higher Mo content than found in yeast NifQ (Hernandez *et al.*, 2008). Despite the lower Mo content, both proteins displayed very similar UV-visible spectra typical of [Fe-S] proteins with peaks at 340 nm and 410 nm that were reduced after DTH treatment.

This thesis proves that purified yeast NifQ functions as direct Mo source for FeMo-co synthesis *in vitro*. It also established the need to complete the Mo trafficking pathway with MoSto to avoid cellular Mo toxicity. Initial results showed that yeast NifQ as isolated from cells lacking the Mo pathway was completely inactive. Furthermore, attempts to grow those cells in increasing amounts of molybdate showed cellular toxic effects that hindered activity improvement. Inactive yeast NifQ could be activated by Mo reconstitution *in vitro* and it then worked in the *in vitro* FeMo-co synthesis assay. On the other hand, having the complete Mo pathway in place (including MoSto and AtMOT1) permitted culturing the engineered yeast in medium with high molybdate concentration. In these conditions, purified NifQ was as active as the *A. vinelandii* NifQ *in vitro*. These results highlight the importance of constructing the complete Mo pathway: MoSto detoxifies intracellular Mo and additionally functions as Mo sink to supply NifQ, while AtMOT1 provides extra protection by removing free molybdate from mitochondria into the cytoplasm. Consistently, a strain carrying MoSto and NifQ but lacking AtMOT1 showed more sensitivity to molybdate and required shorter fermentation times but produced NifQ that was 10% more active than the strain carrying the complete pathway.

Conclusions

Conclusions from the genetic and biochemical analysis of MoSto in A. vinelandii:

- More than 90% of intracellular molybdenum in *A. vinelandii* is bound to the MoSto protein.
- MoSto confers *A. vinelandii* resilience to transient molybdenum deprivation and resistance against tungsten toxicity.
- MoSto buffers the regulatory effects of transient molybdenum step-down, preventing early derepression of alternative nitrogenase genes.
- Purified MoSto functions as Mo donor in the *in vitro* FeMo-co synthesis assay. Direct interaction of MoSto with FeMo-co biosynthetic proteins stimulates Mo donation.

Conclusions from engineering a Mo pathway in mitochondria of S. cerevisiae:

- In *S. cerevisiae*, the heterologous AtMOT1 molybdate transporter is targeted to the outer membrane of mitochondria.
- Expression and targeting of MoSto to the mitochondrial matrix protect *S. cerevisiae* from molybdenum toxicity.
- Co-expression and mitochondria targeting of NifQ and MoSto results in maturation of NifQ into a [Mo-Fe-S] cluster-containing protein that functions as Mo source in the *in vitro* FeMo-co synthesis assay.
- NifQ and MoSto interaction in mitochondria of *S. cerevisiae* has been demonstrated.

- The engineered molybdenum pathway to nitrogenase requires, at a minimum, the incorporation of *nifQ*, *mosA* and *mosB* genes into the chromosome. Genes encoding molybdate transporters might be required under certain yeast genetic backgrounds or growth conditions.

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Supplemental information

Supplemental Table 1

Modular piece	Type	Template	Strenght	Description
TDH3p	Constitutive promoter	<i>S. cerevisiae</i>	Strong ²	Glyceraldehyde-3-phosphate dehydrogenase(GAPDH),isozyme 3
TEF1p	Constitutive promoter	<i>S. cerevisiae</i>	Strong ²	Translational elongation factor EF-1 alpha
GAL1p	Inducible promoter	<i>S. cerevisiae</i>	Moderate ²	Galactokinase
FBA1t	Constitutive terminator	<i>S. cerevisiae</i>	Moderate ³	Fructose 1,6-bisphosphate aldolase
CYC1t	Constitutive terminator	<i>S. cerevisiae</i>	Moderate ³	Cytochrome c, isoform 1
TDH2t	Constitutive terminator	<i>S. cerevisiae</i>	Moderate ³	Glyceraldehyde-3-phosphate dehydrogenase(GAPDH),isozyme 2
MAM33	MTS ¹	<i>S. cerevisiae</i>		Translational activator for the mitochondrial COX1 mRNA
SU9	MTS ¹	<i>Neurospora crassa</i>		Subunit 9 of mitochondrial ATPase
ATPA	MTS ¹	<i>S. cerevisiae</i>		ATP synthase subunit 9
yEGFP	Fluorescence tag	<i>Aequorea victoria</i>		Yeast enhanced green fluorescence protein

¹MTS: mitochondrial targeting signal

²(Sun *et al.*, 2012)

³(Curran *et al.*, 2013; Gao *et al.*, 2017)

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Supplemental Figure 1. MosAB antibody generation. Purification of pure MosAB polypeptides expressed in *E.coli* cells were subjected for antibody generation. (see section 11.7; (Navarro-Rodríguez *et al.*, 2019))

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