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protein biosynthesis in plastids**

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“What I cannot create, I do not understand”
- Richard Feynman

A mis padres

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Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
AmpR	Ampicillin resistance
At	<i>Arabidopsis thaliana</i>
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
Av	<i>Azotobacter vinelandii</i>
BNF	Biological Nitrogen Fixation
bp	Base pair(s)
BSA	Bovine serum albumin
CAD	Computer assisted design
CAI	Codon adaptation index
CaMV	Cauliflower Mosaic Virus
CDS	Coding sequence
CarbR	Carbenicillin resistance
CIB	Chloroplast Isolation Buffer
CFE	Cell-free extract
Col-0	<i>Arabidopsis thaliana</i> ecotype Columbia
COOL	Codon Optimization OnLine
CaMV	Cauliflower Mosaic Virus
DBTL	Design-build-test-learn
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTH	Sodium dithionite
DTT	1,4-dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
eGFP	Enhanced Green Fluorescent Protein
ETC	Electron-transport chain
Fe-S	Iron-sulfur
FW	Fresh weight
GB	GoldenBraid
GC	Guanine-Cytosine content
GC3	Guanine-Cytosine content in the third base of codons
h	Hour
HEPES	N-(2-hydroxyethyl)-piperazine-N-(2-ethanesulfonic acid)

Abbreviations

HPT	Hygromycin phosphotransferase
HRP	Horseradish peroxidase
ICU	Individual codon usage
IME	Intron-mediated enhancement
IPTG	Isopropyl β -D-1-thiogalactopyranoside
ISC	Iron-Sulfur Cluster
KanR	Kanamycin resistance
kb	Kilobases
kDa	Kilodalton
L	Litre
LB	Lysogeny Broth medium (also known as Luria-Bertani medium)
MARs	Matrix attachment regions
min	Minutes
M	Molar
MES	2-(N-morpholino)-ethanesulfonic acid
mL	Millilitre
mM	Millimolar
MoClo	Modular Cloning system
MOE-PCR	Multiple Overlap Extension Polymerase Chain Reaction
mRNA	Messenger RNA
MS	Murashige and Skoog
MSO	Osmoticum MS media
MSP	Callus induction MS media
MSR	Regeneration MS media
MSS	Selection MS media
MTS	Mitochondrial Target Signal
NaCl	Sodium Chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
nt	Nucleotide
Nb	<i>Nicotiana benthamiana</i>
Nt	<i>Nicotiana tabacum</i>
OD ₆₀₀	Optical Density measured at a wavelength of 600 nm
OPR	Open Reading Frame
Os	<i>Oryza sativa</i> (rice)
P	Pellet
PCR	Polymerase Chain Reaction
pDNA	Plasmid DNA

PEG	Polyethylene glycol
PIC	Protease Inhibitor Cocktail
PMSF	Phenylmethanesulfonylfluoride
PTGS	Post-transcriptional gene silencing
PVDF	Polyvinylidene difluoride
qPCR	Quantitative Polymerase Chain Reaction
RH	Relative Humidity
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
rpm	Revolutions per minute
RT	Room temperature
RT-qPCR	Reverse Transcription Quantitative Polymerase Chain Reaction
RuBisCO	Ribulose-1,5-bisphosphates carboxylase/oxygenase
S	Stroma
s	Seconds
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SOB	Super Optimal Broth medium
SOC	Super Optimal Broth medium with Catabolite repression
SPP	Stromal processing peptidase
SpecR	Spectinomycin resistance
SUF	Sulfur mobilization pathway
SynBio	Synthetic Biology
TEV	Tobacco Etch Virus
tGFP	Turbo Green Fluorescent Protein
TGS	Transcriptional gene silencing
TIC	Translocon at the inner envelope membrane of chloroplasts complex
TOC	Translocon at the outer envelope membrane of chloroplasts complex
TP	Transit Peptide
tRNA	Transfer RNA
TU	Transcriptional Unit
TMV	Tobacco mosaic virus
U	Units
UTR	Untranslated region
WB	Western Blot
WT	Wild type
X-Gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

Abbreviations

μg	Microgram
μl	Microliter
μM	Micromolar

Resumen

La biología sintética (SynBio), descrita como la aplicación de la ingeniería a la biología, tiene como objetivo desarrollar nuevos sistemas biológicos y modificar los existentes creando comportamientos predecibles.

La generación de cultivos agrícolas capaces de llevar a cabo la fijación biológica de nitrógeno, presentando bajos requerimientos de aporte de nitrógeno, se considera un desafío que podría conducir a una nueva revolución agrícola. Un posible enfoque para la generación de estos cultivos se basa en la transferencia directa de genes bacterianos *nif*, necesarios para la biogénesis de la nitrogenasa, al genoma de la planta. Los cloroplastos (plastidios) de plantas superiores son considerados compartimentos subcelulares que podrían albergar una enzima nitrogenasa activa, ya que exhiben las propiedades necesarias para acomodar el ensamblaje y la función de esta (poder reductor y abundancia de ATP) a pesar de la presencia de oxígeno. La modificación del genoma plastidial es posible en varios cultivos agrícolas, sin embargo, en cultivos de cereales esta técnica no es efectiva, lo que impone la necesidad de utilizar estrategias basadas en la expresión de transgenes codificados en el genoma nuclear, cuyas proteínas son posteriormente importadas a los plastidios.

En una primera parte, este trabajo presenta el uso de herramientas de biología sintética para optimizar la producción de la proteína de hierro de la nitrogenasa (NifH) en cloroplastos de *Nicotiana benthamiana*. Los genes de fijación de nitrógeno de *Azotobacter vinelandii* *nifH*, *M*, *U* y *S* se rediseñaron para aumentar la acumulación de sus proteínas en células de tabaco, y su importación al cloroplasto se optimizó mediante la búsqueda de péptidos de tránsito de longitud mínima que funcionaran correctamente para cada proteína Nif específica. La presencia de la proteína NifM, una supuesta peptidil-prolil cis-trans isomerasa, resultó ser necesaria para la obtención de proteína NifH soluble en el estroma de los cloroplastos. Es importante destacar que la proteína NifH purificada a partir de cloroplastos de tabaco fue activa en la reducción de acetileno a etileno, siempre que se co-expresara junto a NifU y NifS.

En una segunda parte, esta tesis describe la traslación de los conocimientos generados en experimentos con tabaco para su replicación en arroz. El mismo conjunto de genes de *A. vinelandii* se rediseñó para aumentar la acumulación de sus proteínas en *Oryza sativa* y los péptidos de tránsito, previamente seleccionados por su rendimiento en tabaco, se probaron para determinar su eficacia en la importación de una proteína marcadora a plastidios de diferentes tejidos de arroz, concretamente, callos, hojas y raíces. Los

resultados mostraron que los péptidos de tránsito de longitud mínima de *A. thaliana* seleccionados también fueron capaces de mediar la importación de las proteínas Nif a cloroplastos de hojas de arroz. NifM, similar a lo que se ha observado anteriormente en bacterias, levadura y tabaco, resultó ser necesaria para la solubilidad de NifH en arroz. Sin embargo, no pudimos obtener plantas transgénicas de arroz que acumularan proteínas Nif en cloroplastos y, por lo tanto, la actividad de la proteína de hierro en cereales sigue siendo una incógnita.

En conjunto, esta tesis presenta una prueba de concepto de la idoneidad de los cloroplastos como orgánulos para albergar nitrogenasa, y un procedimiento de optimización para asegurar la expresión de cualquier transgen codificado en el núcleo dirigido a plastidios de *N. benthamiana* y *O. sativa*, centrándose en el diseño de versiones sintéticas de genes que confieren una mayor acumulación de proteína y caracterizando un conjunto de péptidos de tránsito que dirigen de manera eficiente sus proteínas transportadas mientras minimizan los aminoácidos remanentes en la proteína madura tras la importación.

Abstract

Synthetic biology (SynBio), described as the engineering of biology, aims to develop new biological systems and to modify existing ones with predictable behaviours.

The generation of nitrogen-fixing crops with low nitrogen input requirements is considered a challenge that could lead to a new agricultural “green” revolution. A potential approach for their generation is the direct transfer of bacterial *nif* genes needed for the biogenesis of nitrogenase into the plant genome. Chloroplasts (plastids) of higher plants have been proposed as possible subcellular compartments in which to engineer active nitrogenase, as they have the necessary properties to accommodate the assembly and function of the nitrogenase enzyme (reducing power and ATP abundance), despite their oxygenic environment. Chloroplast genome engineering has been achieved in several major crops. However, cereals remain elusive to this technique, imposing the need of using strategies that rely on the expression of nucleus-encoded transgenes with proteins being subsequently imported into plastids.

The first part of this work reports the use of SynBio tools to optimize the production of nitrogenase Fe protein (NifH) in the chloroplasts of *Nicotiana benthamiana* plants. *Azotobacter vinelandii* nitrogen fixation genes *nifH*, *M*, *U*, and *S* were re-designed for protein accumulation in tobacco cells, and targeting to the chloroplast was optimized by screening minimal length transit peptides performing properly for each specific Nif protein. Putative peptidyl-prolyl cis-trans isomerase NifM proved necessary for NifH solubility in the stroma. Importantly, NifH purified from tobacco chloroplasts was active in the reduction of acetylene to ethylene, with the requirement of NifU and NifS co-expression.

In the second part, this thesis describes the translation of the lessons learned in tobacco to rice. The same set of *A. vinelandii* genes were re-designed for protein accumulation in *Oryza sativa* and the transit peptides selected for their performance in tobacco were tested for their efficiency in the import of a nuclear-encoded marker protein into plastids of different rice tissues, namely, callus, leaf, and root. Results showed that the *A. thaliana* minimal length transit peptides selected were also able to mediate Nif protein import into rice leaf chloroplasts. NifM, similar to what has been observed previously in bacteria, yeast and tobacco, proved necessary for NifH solubility in rice. However, we were unable to obtain rice transgenic plants accumulating any Nif protein in chloroplasts and thus, nitrogenase Fe protein activity in cereals remains elusive.

Altogether, this thesis presents a proof of concept of the suitability of chloroplasts as organelles to host nitrogenase, and an optimization pipeline to engineer the reliable expression of any nuclear-encoded transgenes in *N. benthamiana* and *O. sativa* plastids, focusing on designing synthetic gene versions that confer higher protein accumulation and characterizing a set of chloroplast transit peptides that efficiently target their cargo proteins while minimizing scar amino acids in the mature protein after cleavage.

Introduction

1. Synthetic Biology and plants

1.1. Introduction to Synthetic Biology

Synthetic Biology (SynBio) is a new discipline that emerged in the decade of the 2000s as a confluence of biology and engineering. SynBio can be defined as a combination of DNA technology, engineering principles, and computational tools applied to the modification of natural systems or the creation of artificial ones to exhibit predictable behaviours (Andrianantoandro *et al.*, 2006; Purnick and Weiss, 2009; Schwille, 2011).

A hallmark of SynBio is the use of the foundational engineering principles of decoupling, abstraction and standardization, to make the design of biological systems more efficient and scalable (Endy, 2005). Decoupling allows simplification of complex problems into smaller and simpler ones, that can be addressed individually. Abstraction facilitates the management of complexity by identifying and establishing hierarchies of functional levels for the design process. Standardization is used to define and characterize functionally equivalent and interchangeable (i.e. orthogonal) parts for construction and testing (Endy, 2005).

Two main approaches in the development of a SynBio process can be distinguished, consisting in either top-down or bottom-up assembly. The first is an application-driven strategy, which starts from an existing natural system that is modified with the goal to construct synthetic compositions with predictable and controllable properties. On the other hand, the second is a knowledge-driven approach, based on the *de novo* design of biological systems with properties not previously found in nature (Ausländer *et al.*, 2017; Scharff and Bock, 2014). Engineering *Azotobacter vinelandii* nitrogenase Fe protein biosynthesis in plastids, the object of study of this doctoral thesis, consists in a top-down approach, as it aims to build a new biological function into an existing organelle using *Nicotiana benthamiana* and *Oryza sativa* as chassis.

The set of steps we have taken to construct such a system are defined in the design-build-test-learn (DBTL) cycle (Figure 1). As its name states, it is a workflow that encompasses four main stages: design, build, test and learn. The first step defines the problem, objectives, expected outputs and possible constraints. Often, it is not feasible to build all the proposed solutions to the specified objective, therefore computer-aided design (CAD) tools can help to optimize the selection of parameters, choosing the most favourable design based on previous knowledge. The ‘build’ stage involves the fabrication and assembly of parts. The ‘test’ stage encompasses the integration into a host and

experimental analysis of the synthetic systems. Then, the data regarding the fitness of the solution is analysed at the ‘learn’ stage and applied back to the design stage, modifying it accordingly (Liu *et al.*, 2013; Slusarczyk *et al.*, 2012).

Figure 1. The design-build-test-learn (DBTL) cycle in synthetic biology.

Schematic representation of the cyclical synthetic biology workflow for engineering living organisms. Adapted from Boeing *et al.* (2016).

The application of the SynBio approach to microbial systems has tremendously impacted basic life science research, human health, environmental protection, and even economic growth in recent years (Meng and Ellis, 2020). Some hallmark examples of it are the rewriting of *Escherichia coli* genes essential for growth that require synthetic amino acids that do not exist in nature (Rovner *et al.*, 2015), the redesign of a minimal *Mycoplasma mycoides* genome (Hutchison *et al.*, 2016), the synthesis of five additional chromosomes in the synthetic yeast project (Sc2.0) (Richardson *et al.*, 2017), the reprogramming of the genetic code of *E. coli* to reduce the number of codons used by the host (Fredens *et al.*, 2019) or the engineering of carbon fixation into *E. coli* (Gleizer *et al.*, 2019). Furthermore, microbes, especially yeast, have become a useful chassis in metabolic engineering for the biosynthesis of several high-value compounds demanded in the pharmaceutical industry such as the antimalarial artemisinin (Paddon *et al.*, 2013), the opioids thebaine and hydrocodone (Galanie *et al.*, 2015) or the cannabinoids tetrahydrocannabinol and cannabidiol (Luo *et al.*, 2019), to cite some examples.

Microbial systems are easy and fast to engineer; however, their unicellular nature poses certain limitations, reason why synthetic biology has advanced to multicellular systems, remarkably to plants (Liu and Stewart, 2015).

1.2. Plant synthetic biology

Plants are a fundamental part of human life and society for several reasons. They are the most important source of primary metabolites (i.e. sugar, lipids and proteins) into the food chain, as well as a source of energy, raw materials and last but not least, oxygen. Their relatively slow growth, genomic complexity and lack of available tools for genetic manipulation, compared to that of bacteria and yeast, have delayed the development of plant SynBio projects (Boehm and Bock, 2019). However, from a scientific point of view, plants are an appealing chassis since they have been engineered for more than 50 years using recombinant DNA technology and also due to their ability to perform photosynthesis, their extensive secondary metabolism with products valuable for industry, and their large-scale, rapid and relatively low-cost cultivation (Boehm *et al.*, 2017).

Several species are being engineered in the present, chosen either for their ease of genetic modification or their importance in food production. Two dicotyledonous species from the first group, *Arabidopsis thaliana* and *N. benthamiana*, and one monocotyledonous from the second, *O. sativa*, are among the most popular chassis used in plant SynBio approaches and the ones used in this work. *Arabidopsis* is popular due to its little stature, short life-cycle, small well-annotated genome and amount of genetic tools available, furthermore, its research is easily transferable to *Camelina sativa* valued for oilseed production (Flavell, 2009; Holland and Jez, 2018; Stewart *et al.*, 2018). *N. benthamiana* prominence lies in the ease of its transformation and its ability to rapidly and highly express transgenes, making it the plant biofactory of choice (Bally *et al.*, 2018). In the monocotyledonous group, while *Zea mays* hoards most of the GM crop approvals (ISAAA, 2020), *O. sativa* is an appealing chassis as it is the staple crop responsible for providing one-fifth of the calories consumed worldwide (Sharif *et al.*, 2014).

Pioneering plant SynBio projects have been aimed at the generation of plants that offer solutions to our needs of food, water, materials, and energy. Engineered plants are being developed today to help us secure food yields and achieve sustainable agriculture by improving photosynthesis (Batista-Silva *et al.*, 2020), engineering disease resistance (Dong and Ronald, 2019) or enabling biological nitrogen fixation in crops (Bloch *et al.*, 2020; Burén and Rubio, 2018). Other successful projects include the biofortification of crops such as rice, maize or bananas to confront malnutrition from micronutrient deficiencies (Bouis and Saltzman, 2017; Van Der Straeten *et al.*, 2020) and the removal of plant-derived allergens in wheat, among other food crops (Sánchez-León *et al.*, 2018; Steinwand and Ronald, 2020).

The use of plants as biofactories offers an alternative for efficient and inexpensive expression of foreign proteins with low biosafety concerns. A great variety of compounds such as biopharmaceuticals proteins, antibodies or vaccine antigens are routinely manufactured using these technologies, including a candidate vaccine against the SARS-CoV-2 virus which at the moment of writing this dissertation was being evaluated in a Phase I clinical trial (Mortimer, 2019; Shanmugaraj *et al.*, 2020; Ward *et al.*, 2020). Moreover, the fibres that compose plants are a valuable element in the production of biofuels, which can be greatly improved by engineering their properties (Mortimer, 2019).

And lastly, plants are also being currently engineered to fight environmental pollution derived from human activities by augmenting the phytoremediation capabilities of certain species (Yan *et al.*, 2020) and implementing biosensors in plants to detect toxins and contaminants (Verma and Rani, 2020).

Although public opinion supports the use of microbial SynBio for industrial applications and the production of biopharmaceuticals, this is not the case for genetically engineered plants, particularly food crops (Cui and Shoemaker, 2018). Given the current situation regarding the heavy regulation of genetically engineered plants, especially in the EU, the real ability of SynBio to impact on problems such as sustainable agriculture and food security could be hampered if the regulatory framework is not changed (Guidance for risk assessment of food and feed from genetically modified plants, 2011; Nicolia *et al.*, 2014).

1.3. The DBTL process in plant SynBio

The different stages of the cycle will be discussed in more detail as follows, with attention to plant-specific SynBio idiosyncrasies.

1.3.1. Design

In the design of a synthetic system, two sets of factors need to be considered: outside features, such as the transformation method, the host organism and the location of protein accumulation, and inside features, such as those which affect the transcription of your gene(s) of interest and the translation of its(theirs) mRNA product(s) (Figures 2 and 3) (Ullrich *et al.*, 2015).

1.3.1.1. Outside parameters to optimize during design

Outside features choices such as transformation method, host system or location of protein accumulation, will depend on the final goal of the design, e.g. production of a recombinant protein for purification, biofortification of a crop or introduction of a

completely new pathway (Figure 2). The choice is to an extent bound to the requirements of the engineered trait, however, the tools used to enable those factors can be optimized.

Figure 2. Schematic overview of outside features affecting synthetic gene expression.

Outside features are those such as host system, subcellular location of the expressed protein and transformation method used. Adapted from Egelkrout *et al.* (2012) and Ullrich *et al.* (2015). Created with BioRender.com (2020).

Regarding the transformation method, foreign DNA can be introduced into plant cells by physical or biological methods. Common physical methods are particle bombardment, PEG-mediated delivery, or electroporation, which are relatively simple and can be applied to a wide range of species (Barampuram and Zhang, 2011). Biological methods mainly rely on *Agrobacterium spp.* bacteria carrying the recombinant DNA cloned into the T-region of binary plasmids. This method enables largely intact integration of a synthetic DNA into the chromosome and, depending on the strain used, the number of copies can be controlled (Tzfira and Citovsky, 2006). However, species that remain recalcitrant to *Agrobacterium* transformation still rely on physical methods (Hiei *et al.*, 2014). The choice of transformation method will influence the plasmid used for construction of the synthetic DNA since physical methods tend to prefer high-copy low-size backbones and biological methods based on *Agrobacterium* require the DNA to be integrated to be flanked by the left and right border sequences (Barampuram and Zhang, 2011).

Concerning host systems, *A. thaliana* is the most popular choice for a plant chassis, given its long laboratory history and resources available for the metabolic engineer and synthetic biologist (Holland and Jez, 2018). Other systems include dicotyledonous platforms, e.g. tobacco, which stand out for their results (Stewart *et al.*, 2018). Monocots, such as maize or rice, sometimes are the final target of the modifications engineered in model plants, but they present challenges in their manipulation that force them into a

second-place as plant chassis (Sood *et al.*, 2011). The host choice will impact on the modules, e.g. promoters, terminators or selection markers, that will be required for the expression of the recombinant DNA.

Regarding protein accumulation location, specific projects might include requirements to import recombinant proteins into certain subcellular organelles. By default, nuclear-encoded proteins synthesised by cytosolic ribosomes accumulate in the cytosol of plant cells. Only when an appropriate peptide signal is included in the transgene sequence, proteins migrate to specific organelles post-translationally (Benchabane *et al.*, 2008). A range of peptide signals exist in order to direct a protein to the desired destination organelle, including N-terminal mitochondrial signal peptides (MTP); plastid transit peptides (TP); signal peptides of the secretory pathway (SP) that direct proteins to the endoplasmic reticulum further heading to the apoplast or vacuole; the C-terminal peroxisome target sequence (SKL) and the nuclear localization signals (NLS) (Benchabane *et al.*, 2008). In all cases, the signals used need to be optimized to maximize their organelle targeting efficiency and specificity, while minimizing the residues left in the mature protein in the case of import into organelles where there is posterior signal cleavage.

1.3.1.2. Inside parameters to optimize during design

Building up new capabilities in an organism relies most of the time on the expression of newly introduced transgenes. Inside factors that can be optimized are those that affect the transcription of the genes of interest and the translation of their mRNA products (Figure 3).

The temporal and spatial expression of a transgene is controlled by the choice of its driving promoter. Promoter selection is an especially critical variable when expressing multiple transcriptional units since repeated use of the same promoter can trigger transcriptional gene silencing (TGS) (Vaucheret and Fagard, 2001). Additionally, terminators have also been described to be of high importance in the control of transgene expression level, by controlling the 3' end processing of mRNAs (Carswell and Alwine, 1989; Ingelbrecht *et al.*, 1989), and their stability as targets of post-transcriptional gene silencing (de Felippes *et al.*, 2020; Pérez-González and Caro, 2018). Fortunately, libraries of promoters originating from plants and their pathogens either constitutive, inducible or tissue-specific, as well as libraries of terminators also from plants and their pathogens, have been characterized, providing information for their selection depending on specific projects' necessities (Engler *et al.*, 2014; Feike *et al.*, 2019; Sarrion-Perdigones *et al.*, 2013; Vazquez-Vilar *et al.*, 2017).

Figure 3. Schematic overview of inside features affecting synthetic gene expression.

Inside features affecting the transcription of a gene are promoters, terminators, introns mediating enhancement (IME) in the 5' Untranslated Region (5'UTR), GC and GC3 content, motifs present in the coding sequence (CDS), and the activation of transcriptional gene silencing (TGS). Those affecting translation of its mRNA product are 5' mRNA instability, presence of RNA-instability motifs and double-stranded structures, the codon usage and the activation of post-transcriptional gene silencing (PTGS). Adapted from Egelkroun *et al.* (2012) and Ullrich *et al.* (2015).

Furthermore, transgene expression can be improved by the addition of certain introns in the 5' untranslated region (UTR) by the mechanism known as intron-mediated enhancement, i.e. the effect of an intron only when downstream of, and close to, the start of the gene, that generates an increase in mRNA accumulation (Rose, 2008, 2004). Increases of 10-fold or more in expression levels have been reported by this technique, with some introns being routinely used, e.g. the maize *ubiquitin 1* intron or the rice *TubA1* (Gallegos and Rose, 2015; Pan *et al.*, 2016).

In the case of the transgene sequence, certain level of edition is possible so that the synthetic nucleotide sequence encodes the same product as the gene of interest while optimizing its transcription and translation by adapting codon usage, tweaking GC content and removing deleterious motifs (Jackson *et al.*, 2014).

Codon usage bias refers to the frequency of use of synonymous codons in the coding DNA of a certain species. The codon usage of an organism can be measured with indexes such as the codon adaptation index (CAI), representing how often the most frequent codon for each amino acid is used in a gene (Sharp and Li, 1987), or the individual codon usage (ICU), representing how closely a sequence matches the codon usage pattern of the host (Chung and Lee, 2012). The rationale behind codon optimization is to mimic the endogenous codon usage, so the host tRNAs supply will be used in balance with the host

transcriptome, and to remove rare codons that could cause translational pausing (Makrides, 1996).

Regarding GC content, there has been some controversy as to what is the optimal value in coding sequences to be expressed in a heterologous system. It is a common practice in synthetic gene design to match the GC content of a transgene to that of the host organism's coding sequences (Gustafsson *et al.*, 2012). However, a recent study showed that, after stable transformation, synthetic genes with plant-comparable GC content failed to produce the anticipated high yield of protein production and undertook rapid and stable transposon-like TGS. On the contrary, microalgae native genes harbouring high GC content, particularly in the third position of translational triplets (GC3), displayed enhanced protein accumulation and transgenerational expression stability (Sidorenko *et al.*, 2017). This is in accordance with transposons, a major target for gene silencing, having a clear distribution preference for AT-rich sequences (Le *et al.*, 2000), but the mechanism regulating it is still unknown.

Concerning the exclusion of motifs considered to be deleterious for heterologous protein accumulation, the removal of cryptic intron splice sites and RNA instability motifs has been described to increase transcription and enhance translation in plants (Gutiérrez *et al.*, 1999; Jackson *et al.*, 2014). Furthermore, sequences causing premature polyadenylation sites have previously been implicated in poor expression (Haffani *et al.*, 2000; Mathew *et al.*, 2011).

Translation of mRNA into a mature protein is the second key step for protein accumulation. An important factor that can be optimized here to increase heterologous protein production is 5' mRNA stability. The secondary structure of mRNA has great importance in translation efficiency. If the 5'-end folds into stable secondary structures, it can reduce translational initiation (Kozak, 1986), therefore it is always advisable to keep the local secondary structure as "open" as possible within the first 50 nt of the open reading frame (ORF) to aid translational initiation (Jackson *et al.*, 2014). Also, motifs generating double stranded-RNA stems of 12 bp or more in any part of the ORF should be avoided to prevent translational stalling (Chou and Moyle, 2014).

Translation can be repressed by post-transcriptional gene silencing (PTGS) triggered as the consequence of excessive aberrant transcript levels (Que *et al.*, 1997). High expression of transgenes can be counter-productive if it leads to saturation of the RNA maturation machinery causing the production of improperly matured transcripts, thus the considerations regarding promoter and terminator use are of the uttermost importance for translation as well.

1.3.1.3. Computational tools for design

The use of CAD tools allows the synthetic biologist to optimize design parameters and test possible designs *in silico* before construction (Liu and Stewart, 2015). Gene synthetic design has gained popularity in the last years, due in part to the advances in DNA synthesis, giving birth to an extent of CAD tools that streamline the design of longer and more complex DNA sequences (Gould *et al.*, 2014; Webster *et al.*, 2017). Nevertheless, the predictive power from algorithms of the behaviours encoded in DNA sequences is still limited and iterations of the DBTL process are necessary to achieve the optimal sequence.

Codon Optimization OnLine (COOL) (Chin *et al.*, 2014) is one of the most complete CAD tools available for synthetic gene design. COOL offers a multi-objective optimization using metaheuristics, considering several user-defined parameters ranging from codon usage and GC content, to motifs and repeats removal and 5' RNA folding instability assessment (Figure 4). These parameters are the key elements of the design and need to be established specifically for each project from a thorough literature survey on the host organism of choice. The software provides various solutions, leaving the user the ultimate choice of the sequence that offers the best compromise between all the design criteria considered (Chin *et al.*, 2014).

Figure 4. Workflow followed in Codon Optimization OnLine.

Optimization settings as individual codon usage, which measures the frequency by which individual codons appear in a set of coding sequences, GC and GC3 content and 5' RNA folding instability were used alongside with the removal of motifs such as polyadenylation sequences, RNA instability motifs and repeats, providing a multi-objective optimization for synthetic gene design. Adapted from Yu *et al.* (2017).

1.3.2. Build

An important task within a SynBio project is the building of new or redesigned versions of DNA constructs. Traditionally, they were made using relatively time-consuming DNA manipulation protocols that limited the scope of research projects, e.g. DNA digestion using one or two restriction enzymes, dephosphorylation of vector ends or use of multiple-cloning sites for the insertion of a single DNA fragment into a vector. Although still used today, more efficient methods enable parallel directional assembly of multiple

parts of DNA in a single reaction, enabling the fast and easy generation of complex devices.

1.3.2.1. Modular cloning tools

Two critical factors that have changed the way new constructs are built are standardization of basic parts, allowing their reuse and exchange between users, and the development of modular assembly strategies, allowing the efficient association of these standard parts into more complex arrangements (Werner *et al.*, 2012).

The BioBrick standard was the first widely-adopted modular assembly method (Knight, 2003). Its key feature is idempotency, as BioBricks parts can be assembled resulting in new BioBricks modules that retain the same structure as the original parts regarding the flanking restriction sites (Knight, 2003; Shetty *et al.*, 2008). However, BioBrick assembly relies on the use of type II restriction enzymes, meaning that the two assembled parts are separated by an eight bp scar sequence and that the process is limited to the association of only two parts in a single step.

To overcome these constraints, new modular assembly systems have been developed in the last decade, such as Golden Gate (Engler *et al.*, 2009) which resulted in GoldenBraid (GB) (Sarrion-Perdigones *et al.*, 2011) and Modular Cloning (MoClo) (Werner *et al.*, 2012) assembly methods for plant SynBio. Golden Gate uses Type IIS restriction enzymes, capable of recognizing nonpalindromic sequence motifs and cleaving outside of their recognition site, resulting in custom-designed DNA overhangs. These overhangs enable parallel directional assembly of multiple parts using only one restriction enzyme and DNA ligase in a one-pot, one-step reaction (Engler *et al.*, 2009). Both GB and MoClo assembly methods have gained considerable popularity and several kits have been developed containing tools and parts for plant engineering (Emami *et al.*, 2013; Engler *et al.*, 2014; Lin *et al.*, 2014; Sarrion-Perdigones *et al.*, 2013; Vazquez-Vilar *et al.*, 2016, 2017).

In GB and MoClo assembly systems, the notion of genetic elements, e.g. promoter or coding sequence, has been converted into standard parts with positions defined by four bp codes flanking them, known as Level 0 parts or Phytobricks, and a common syntax for these codes has been agreed globally to enable their exchange between laboratories (Patron *et al.*, 2015).

Figure 5. Overview of Golden Gate MoClo Assembly Standard.

Standard (Level 0) parts are assembled through a *BpiI*-mediated restriction-ligation reaction from single or multiple PCR-amplified sequences with primers that contain the corresponding four bp adapters and *BpiI* restriction sites. The common syntax for plants defines the four base-pair overhangs that join standard parts which will determine the position of each piece within the future transcriptional unit (TU), enabling parts to span multiple fusion sites. Level 0 parts are assembled into Level 1 acceptor backbones to make complete TUs via a *BsaI*-mediated restriction-ligation reaction. The Level 1 acceptor chosen in the assembly will determine the position of that TU in the multigene assembly. Subsequently, up to six Level 1 TUs can be assembled in an orderly manner in a Level 2 or M acceptor backbones through a *BpiI*-mediated restriction-ligation reaction. Any Level 1 transcriptional unit can be substituted for a dummy piece and the correct end-linker must be used to join the last Level 1 unit to the acceptor. CarbR: carbenicillin resistance; KanR: kanamycin resistance; SpecR: spectinomycin resistance. Adapted from Engler *et al.* (2014).

The MoClo system contains three levels of hierarchy (Level 0, 1, or 2) which can be used in successive assembly steps (Figure 5) (Werner *et al.*, 2012). Basic genetics parts are PCR amplified from a DNA source (genomic DNA, cDNA or plasmids) using primers containing a four nt code and *BpiI* site, and cloned into a Level 0 universal vector. Level 0 standard parts are directionally assembled in a transcriptional unit (TU) via *BsaI* mediated restriction-ligation into Level 1 acceptor vectors. There are six different Level 1 positions available, which define the location of each TU in a multigene construct. Subsequently, MoClo allows the concurrent assembly up to six TUs via *BpiI* mediated restriction-ligation into acceptor vectors as Level 2, M or P, comprising the highest hierarchy level. Furthermore, the use of openable “end-linkers” and a combination of *BsaI*-*BpiI* enzymes allows an iterative process to generate Level 2 constructs larger than six TUs, enabling the creation of more complex circuits.

The implementation of these assembly methods has enabled plant SynBio, leading to the rapid generation of constructs efficiently and with high accuracy, facilitating their prototyping, testing, and iteration.

1.3.2.2. Computational tools

Due to the number of standard parts generated and the increasing complexity of the SynBio systems developed, it is necessary to deposit these parts along with their associated information in bioregistries, so they can be accessed and shared. Some well-known non-commercial bioregistries for Golden Gate standard parts are GB's (<https://gbcloning.upv.es>) or AddGene (<https://www.addgene.com>), which also serve as biorepositories. Tools for the management and building of parts and devices have also been developed by research groups, e.g. MoClo planner (Shaer *et al.*, 2013) or ePARTS (Pardo and Caro, 2017), and private companies e.g. Geneious (Kearse *et al.*, 2012) or Benchling (<https://www.benchling.com/>), allowing users to streamline their experiments.

1.3.3. Test

With the 'design' and 'build' of synthetic DNA modules becoming efficient and cheap, the bottleneck of the DBTL cycle for bioengineering is the limited number of screening tools available to rapidly test a large number of constructs (Pouvreau *et al.*, 2018). However, plants have a prominent advantage as they can be transiently or stably transformed, and transient expression platforms have the potential to break this bottleneck.

1.3.3.1. Transient expression

Transient expression assays, in which, after DNA introduction into plant cells, the transgene undergoes rapid and high transcription and translation without integrating into the genome, are particularly useful to rapidly perform tests on constructs and to obtain high amounts of recombinant protein in a short period of time while avoiding possible harmful long-term effects on the plant cell.

This rapid and high-expression can be achieved by delivering DNA using *Agrobacterium* or physical methods, as well as by a hybrid strategy based on integrating transgenes into complete or deconstructed genomes of viruses, which are then delivered to plant cells (Barampuram and Zhang, 2011; Naseri *et al.*, 2019).

Several techniques have been developed over the years, being agroinfiltration, magnification and protoplast transformation the most commonly used. Agroinfiltration is a method based on the introduction of a suspension of *Agrobacterium* bacteria carrying the recombinant DNA into a plant leaf, typically *N. benthamiana*, by direct injection or by vacuum infiltration, generating an infection of plant cells that express the transgene. It is a common procedure to co-infiltrate with the silencing suppressor P19 from the plant

pathogenic tomato bushy stunt virus to avoid PTGS and boost the expression of the transgenes of interest (Csorba *et al.*, 2015; Li, 2011; Muthamilselvan *et al.*, 2019). Conversely, magniffection uses the agroinfiltration procedure to deliver viral replicons together with the transgene of interest to plant cells, achieving an even higher expression yield (Gleba *et al.*, 2005).

Protoplasts consist of plant cells in which the cell wall has been previously digested. They are a proven versatile cell-based experimental system widely used in functional characterization of genes and protein analysis in several species, e.g. Arabidopsis, maize, rice or tobacco (Zhang *et al.*, 2011). The lack of the cell wall allows for molecules like DNA and proteins to be delivered using physical methods as PEG–calcium transfection or electroporation. After entering the cell, transgenes are then rapidly and transiently expressed. Protoplast regeneration into stably transformed plants is also possible when cultured in specialized plant growth conditions and sterile culture media (Damm *et al.*, 1989).

1.3.3.2. Stable expression

Stable transformation involves the integration of the introduced DNA into the plant genome, which is necessary to produce transgenic plants with heritable traits. In this approach, a marker gene is commonly integrated along with the genes of interest in order to detect positive transformants. However, both *Agrobacterium*-mediated transformation and biolistic methods lack control over the site, frequency and structure of exogenous DNA integration, which leads to time-consuming screenings in search of lines that express transgenes stably and in high levels (Butaye *et al.*, 2005; Pouvreau *et al.*, 2018).

Some stable transformation methods, such as floral dipping, directly transform female gametes (Clough and Bent, 1998), however, many species are not amenable to this procedure and rely on somatic cell transformation, which requires extensive tissue culture. The bottleneck of stable engineering lays in the step of plant regeneration from transformed tissues, as it involves long tissue culture periods with conditions that have to be set experimentally for each species (Altpeter *et al.*, 2016). This weakness limits the possibility to test large numbers of constructions in stably transformed plants of most crops (Pouvreau *et al.*, 2018).

1.3.4. Learn

The DBTL cycle ends at the ‘learn’ stage, in which all data produced in previous steps are analysed and combined to refine the original hypothesis, prompting a new one that

will be tested in a new cycle iteration (Pouvreau *et al.*, 2018). The process is then repeated until the target function is achieved, enabling a systematic design of biological systems.

2. Application of SynBio tools to the engineering of Biological Nitrogen Fixation in plants

2.1. The worldwide need for nitrogen

Cereal crops, mainly rice, wheat and maize, provide half of the world's daily calories consumed directly by humans (Awika, 2011). One of the crucial factors for crop productivity is nitrogen availability to plants (Erisman *et al.*, 2008). Since major staple cereal crops are unable to form symbioses with nitrogen-fixing bacteria, as occurs in legumes, the nitrogen needed for higher yields is supplied via synthetic nitrogen fertilizers. Ammonia fertilizer is industrially obtained by the Haber–Bosch process, in which molecular nitrogen gas is mixed with hydrogen at very high pressure (300 atm) and temperature (500°C) over a metal catalyst (Gilchrist and Benjamin, 2017).

The use of synthetic nitrogen fertilizers has increased steadily since the 1960s (Stewart and Roberts, 2012). Despite the food-security benefits offered by synthetic fertilizers since then, their industrial production and extensive use present environmental and socio-political issues (Erisman and Sutton, 2008). The Haber–Bosch process consumes vast amounts of non-renewable fossil fuels, increasing the atmospheric carbon dioxide pollution. Besides, inefficient assimilation into crops and excess application of synthetic fertilizers lead to leaks into the environment, resulting in water eutrophication (e.g. cyanobacterial blooms) and air pollution (e.g. nitrous oxide, a greenhouse gas) (Erisman *et al.*, 2015). Furthermore, the high prices these fertilizers reach makes them inaccessible to many smallholder farmers in developing regions (Mueller *et al.*, 2012).

2.2. Biological nitrogen fixation as an alternative

Biological nitrogen fixation (BNF) is the conversion of dinitrogen (N_2) into ammonia (NH_3) catalysed by a nitrogenase enzyme. Although this is an essential process that supports life on Earth, only a small group of bacteria and archaea known as diazotrophs (dinitrogen eaters) are able to fix nitrogen on their own (Zehr, 2013), accounting for about two-thirds of the world's total fixed nitrogen (Canfield *et al.*, 2010).

BNF has been envisioned as an alternative for providing biologically active nitrogen to cereal crops since the 1970s, as Normal Borlaug stated in his Nobel Prize lecture (Borlaug, 1972). Great advances in understanding nitrogenase biosynthesis and function followed, such as the functional transfer of nitrogen fixation genes from *Klebsiella oxytoca* to *E. coli* (Dixon and Postgate, 1972). Since then, technical developments and the birth of SynBio have spiked the interest in engineering BNF in cereals to sustain cereal

crop productivity. Three different strategies have been proposed to achieve this goal (Figure 6).

The first strategy mainly relies on enhancing nitrogen-fixing capabilities of bacteria that are naturally associated with cereals, either residing on or near the root surface (epiphytes) or within plant cells (endophytes) (Mus *et al.*, 2016). It is considered the approach with lower technical barriers (Bloch *et al.*, 2020). However, although inoculation of crops can improve growth and yield, the positive results obtained in greenhouse studies do not always correlate with the performance of these bioinoculants in the field (Compant *et al.*, 2019). Nevertheless, Ryu and colleagues recently engineered inducible nitrogenase expression, under the control of signals such as root exudates or phytohormones, in microorganisms associated with cereals. They demonstrated nitrogenase activity equivalent to the native diazotrophs in engineered variants of the endophyte *Azorhizobium caulinodans* and the epiphyte *Pseudomonas protegens* (Ryu *et al.*, 2020).

The second strategy focuses on engineering new associations between cereals and nitrogen-fixing bacteria to recreate nodule-forming symbiosis (Rogers and Oldroyd, 2014). Even though similarities have been found between the Nod factors, responsible for legume-rhizobia symbiosis, and the Myc factors, involved in arbuscular mycorrhizal symbiosis in cereals, strategies leading to the infection and nodule formation in cereals are to this day elusive (Rogers and Oldroyd, 2014).

The third strategy aims to generate varieties of cereals that require little or no nitrogen input by making plants fix their own nitrogen, via the direct transfer of prokaryotic *nitrogen fixation (nif)* genes needed for the biogenesis of nitrogenase into the plant genome (Curatti and Rubio, 2014). This approach has the advantage of providing technology that can be transmitted to the progeny in the seeds. Significant proofs of concept have been achieved in this field, some of them derived from the work developed in this thesis (Allen *et al.*, 2017; Burén *et al.*, 2019; Burén, Young, *et al.*, 2017; Eseverri *et al.*, 2020; Ivleva *et al.*, 2016; López-Torrejón *et al.*, 2016).

Figure 6. Biological nitrogen fixation in cereal crops as an alternative to industrial fertilizers.

Legumes form specialized symbioses with diazotrophic bacteria of the order Rhizobiales resulting in root nodule structures housing the rhizobia and providing them with sugars (photosynthate) in exchange for fixed nitrogen. Harnessing biological nitrogen fixation for cereal crops has been pursued via three different strategies: Enhancing nitrogen-fixing capabilities via genetic engineering of epiphytic or endophytic bacteria naturally associated with cereals and applying them to crops as inoculants; engineering new associations between cereals and nitrogen-fixing bacteria to recreate nodule-forming symbiosis; generating varieties of cereals that fix their own nitrogen via the direct transfer of prokaryotic *nif* genes into the plant genome and expression of nitrogenase in mitochondria and/or plastids. Adapted from Bloch *et al.* (2020).

2.3. Nitrogenase biochemistry

Nitrogenases can be classified into three groups according to their active site metal contents, i.e. vanadium (V) nitrogenase, iron only (Fe-only) nitrogenase and molybdenum (Mo) nitrogenase (Bishop and Joerger, 1990), being the last one the most abundant and ecologically relevant, therefore the best studied (Burén and Rubio, 2018).

Bacterial Mo-nitrogenase catalyses the reduction of dinitrogen into two molecules of ammonia in a reaction that requires eight electrons and the hydrolysis of at least 16 adenosine triphosphate molecules (ATP) (Seefeldt *et al.*, 2009). The Mo-nitrogenase catalytic components consist of two metalloproteins: the *nifH*-encoded Fe protein

homodimer and the *nifDK*-encoded MoFe protein heterotetramer (Figure 7) (Georgiadis *et al.*, 1992). The Fe protein, also named dinitrogenase reductase, is a reductase that accepts electrons from ferredoxins or flavodoxins and functions as obligate electron donor to the MoFe protein. Besides, the Fe protein is required for the maturation of P-clusters onto the MoFe protein and for the iron–molybdenum cofactor (FeMo-co) biosynthesis (Figure 8) (Burén *et al.*, 2020). The Fe protein is a homodimer of the *nifH* gene product that contains one Mg·ATP-binding site in each subunit and carries a single [4Fe-4S] cluster bridging the two subunits (Figure 8A) (Georgiadis *et al.*, 1992). The MoFe protein, the catalytic component, is a dinitrogenase that binds and reduces molecular nitrogen. It is formed by a heterotetramer of the *nifD* and *nifK* gene products that carries one pair of complex metallocusters in each NifDK half, the P-cluster and the FeMo-co (Figure 8B) (Kim and Rees, 1992).

Figure 7. Structure and reaction of Mo nitrogenase.

The reduction of dinitrogen gas (N_2) to ammonia (NH_3) is catalysed by the nitrogenase enzyme complex, composed of two homodimers of the Fe protein, NifH, and one heterotetramer of the MoFe protein, NifDK. Structure of *A. vinelandii* MoFe protein and Fe protein complex showing (i) FeMo-co, (ii) P-cluster and (iii) [4Fe-4S] cluster in one half of the enzyme. Mo nitrogenase reaction is shown at the bottom. Adapted from Burén *et al.* (2020).

Remarkably, NifH is the most abundant Nif protein required for nitrogen fixation in *A. vinelandii* (Poza-Carrion *et al.*, 2014). Its maturation consists of two steps: the correct folding and formation of the NifH homodimer, and the loading of the [4Fe-4S] cluster to generate a fully mature protein (Figure 8) (Burén *et al.*, 2020). The presence of [4Fe-4S] cluster confers NifH its oxygen-sensitivity, as exposure to air irreversibly damages them and inactivates the enzyme (Shah and Brill, 1973).

Since the Fe protein component of nitrogenase is more oxygen-sensitive and its maturation requirements are simpler and better characterized, it has been chosen to test the possibility of engineering an active nitrogenase in yeast and plants (Burén *et al.*, 2020; Shah and Brill, 1973).

Figure 8. Simplified model of Fe protein maturation and its roles in MoFe protein maturation.

(A) Fe protein maturation: NifM assists in the correct folding of NifH, NifU and NifS assemble and transfer the [4Fe-4S] cluster to NifH. **(B)** Requirement of Fe protein for MoFe protein maturation: Fe protein is required for the maturation of P-clusters onto MoFe protein and for FeMo-co biosynthesis outside of the MoFe protein. Fe protein is shown as NifH homodimer lacking [4Fe-4S] cluster to indicate that apo-Fe protein is competent in P-cluster formation and FeMo-co biosynthesis. **(C)** Formation of mature Mo nitrogenase enzyme complex. Adapted from Burén *et al.* (2020).

The prokaryote model organism used as source of nitrogenase genes and proteins in this thesis is *A. vinelandii*, a free-living soil and strict aerobe bacterium that performs nitrogen fixation, in which nitrogenase is protected from oxygen damage mainly by maintaining high respiratory rates. There are four genes in *A. vinelandii* that are necessary to produce active Fe protein, namely *nifH*, *nifM*, *nifS* and *nifU*. The *nifM* gene encodes a peptidyl-prolyl cis-trans isomerase that assists in the correct folding of NifH (Howard *et al.*, 1986). The *nifS* gene product is a cysteine desulfurase that mobilizes S for [Fe-S] cluster assembly that takes place on NifU (Zheng *et al.*, 1994). Then, NifU, an assembly scaffold, sequesters Fe and S for [Fe-S] groups and delivers them to nitrogenase target proteins for activity and cofactor synthesis (Fu *et al.*, 1994).

The activity of the Fe protein can be easily determined by its capacity to support acetylene to ethylene formation by the MoFe protein in standard nitrogenase activity assays. In this biochemical assays, the heterologous Fe protein is incubated *in vitro* with a mixture of the remaining essential components, namely the MoFe protein purified from *A. vinelandii*, an electron donor (dithionite) and ATP (Shah and Brill, 1973). This assay relies on the ability of the triple-bonded molecule acetylene to serve as substrate for nitrogenase, in addition to dinitrogen, and its reduction to ethylene that can be detected by gas chromatography.

2.4. Engineering eukaryotic nitrogen fixation

We face two main obstacles in the engineering of biological nitrogen fixation in plants. The first one is the complexity of the nitrogenase biosynthetic pathway, which requires the co-expression of eight to ten bacterial genes in a precise stoichiometry to achieve an enzyme with optimal function (Smanski *et al.*, 2014; Temme *et al.*, 2012; Vicente and Dean, 2017; Wang *et al.*, 2013). The second hurdle is the sensitivity of the nitrogenase enzyme and the accessory proteins involved in the synthesis of its metalloclusters towards oxygen, a by-product of photosynthesis in plants (Curatti and Rubio, 2014). In addition, for nitrogenase to function properly it requires abundant available reducing power and ATP.

The DBTL cycle has been used to overcome the complexity barrier, testing each one of the nitrogenase components and validating their functionality. For this purpose, the model eukaryote *Saccharomyces cerevisiae* and plant *N. benthamiana* have been used as chassis to gain relevant information with the idea of transferring it to cereal engineering.

To solve the oxygen sensitivity problem, the subcellular organelles mitochondria and chloroplast (plastid) have been proposed as possible compartments in which to engineer nitrogenase in plants (Beatty and Good, 2011; Curatti and Rubio, 2014). The mitochondrial matrix provides several advantages. It presents a low oxygen environment due to respiration, it can provide the ATP and reducing power required for nitrogenase catalytic activity, and carries iron-sulfur ([Fe-S]) cluster assembly machineries (Lill and Mühlenhoff, 2008). However, a recent study showed that mitochondrial ferredoxins cannot directly replace the function of their counterparts in bacteria by serving as electron donors to nitrogenase, increasing the complexity of engineering an active mitochondrial nitrogenase (Yang *et al.*, 2017). Alternatively, chloroplasts are considered as a potential subcellular location because of their availability of the immediate products of photosynthesis, NADPH and ATP, that could satisfy the demands of reducing power and energy, as well as, the presence of [Fe-S] cluster assembly machineries needed for nitrogenase biosynthesis (Balk and Pilon, 2011; Curatti and Rubio, 2014). Moreover, the same study from Yang and colleagues demonstrated that a number of ferredoxins found in plastids are able to serve as electron donors to nitrogenase (Yang *et al.*, 2017). However, an obvious disadvantage is the oxygen production as a by-product of photosynthesis that takes place in chloroplasts, which may be incompatible with the oxygen-labile nitrogenase (Curatti and Rubio, 2014).

All considered, chloroplasts may have the necessary properties to accommodate the assembly of a functional nitrogenase enzyme, but to overcome the oxygen sensitivity

problem, its biosynthesis might need to be temporally separated from photosynthesis, as it is the case in some photosynthetic nitrogen-fixing cyanobacteria known to accommodate photosynthesis and nitrogen fixation separating them temporally under the control of the circadian clocks (Grobelaar *et al.*, 1986; Mitsui *et al.*, 1986).

2.5. Coupling nitrogen fixation to plant metabolism

The subcellular locations proposed for the engineering of nitrogenase in plants need to consider the appropriate physiological environment for the enzyme, as well as the metabolic coupling to the plant host. Poor metabolic coupling of recombinant pathways has shown to directly affect performance in hosts (Ortiz-Marquez *et al.*, 2013) and it could lead to side effects influencing the host system itself (Wilson and Roberts, 2014). Even if the expression of a nitrogenase enzyme at sufficient levels in plant mitochondria or plastids is achieved, several factors may limit efficient nitrogen fixation.

The first constraint relates to the provision of sufficient reductant through the Fe protein as an obligated donor of electrons to the MoFe protein. The fact that the *in vivo* electron donor for the Fe protein is less strictly conserved, opens the possibility to find compatible plant counterparts which would allow reducing the number of genes required for nitrogenase engineering (Halbleib and Ludden, 2000; Yang *et al.*, 2017).

The second factor faces the biosynthesis of metalloclusters in plant organelles. In plants, [Fe-S] cluster assembly machineries capable of synthesizing [4Fe-4S] clusters are found in mitochondria (ISC pathway) and chloroplasts (SUF pathway) (Balk and Pilon, 2011). These machineries from eukaryotic organelles can substitute to an extent the activity of NifU and NifS in harbouring and incorporating the [4Fe-4S] clusters into NifH (Ivleva *et al.*, 2016; López-Torrejón *et al.*, 2016). However, the complex cofactors carried by the MoFe protein, FeMo-co and P-cluster, are only found in nitrogen fixers.

A third constraint is how to couple the ammonia production as a result of nitrogen fixation into the host pathways, as it can be toxic to plants. In plants, ammonia assimilation takes place primarily in chloroplasts and other plastids by the glutamine synthetase/glutamate synthase pathway, where it is incorporated into the formation of amino acids (Foyer *et al.*, 2011; Merrick and Dixon, 1984). Thus, it seems possible to easily couple the ammonia produced by nitrogenase into host metabolism in plastids. In the case of mitochondria, ammonia produced could be exported to the cytosol and assimilated by a cytosolic glutamine synthetase using glutamate from the ornithine catabolism or loaded into the xylem (Good, 2018; Witte, 2011).

The fourth factor is the energetic requirement, as nitrogenase uses large amounts of ATP. Both mitochondria and chloroplast can theoretically provide enough ATP for nitrogenase to function but the metabolic burden that it may carry is still unknown. Nevertheless, the theoretical energy requirement for nitrogen fixation is almost identical to that required for nitrate assimilation, the other main source of nitrogen for most field crops (Kennedy and Tchan, 1992).

2.6. State of the art in the engineering of nitrogen-fixing organisms

We encounter ourselves far from engineering a plant that is able to fix nitrogen. Nevertheless, significant advances have been achieved in the last years in photosynthetic prokaryotes and in eukaryotic organisms following different approaches.

2.6.1. Engineering nitrogen fixation in a non-diazotrophic cyanobacterium

Engineering nitrogen fixation in a non-diazotrophic cyanobacterium may prove insightful, as cyanobacteria are considered the ancestors of chloroplasts (Falcón *et al.*, 2010; Jarvis and López-Juez, 2013). Fifteen years ago, Cheng *et al.* (2005) proved that the *nifH* gene from *Klebsiella pneumoniae* M5a1 (now renamed as *K. oxytoca*) could partially restore the capacity for chlorophyll biosynthesis in the dark in *Chlamydomonas reinhardtii*. Recently, Tsujimoto *et al.* (2018) demonstrated the low, but functional, expression of an oxygen labile nitrogenase from the diazotrophic cyanobacterium *Leptolyngbya boryana* in an oxygenic photosynthetic organism, the non-diazotrophic cyanobacterium *Synechocystis* sp. PCC 6803. Furthermore, Liu *et al.* (2018) successfully engineered the same organism to express a *nif* gene cluster from *Cyanothece* sp. ATCC 51142, achieving nitrogenase activity up to 30% of the wild-type enzyme. Interestingly, the introduction of uptake hydrogenase genes showed to improve nitrogenase activity under micro-oxic conditions.

2.6.2. Engineering nitrogen fixation in yeast

S. cerevisiae is a widely used eukaryotic chassis for synthetic biology, serving as a starting point for complex engineering projects in higher eukaryotes. The formation of a functional nitrogenase enzyme has not yet been achieved in yeast mitochondria, although all the main Nif components have been individually expressed in it (Burén, Jiang, *et al.*, 2017; Burén, Young, *et al.*, 2017; López-Torrejón *et al.*, 2016; Pérez-González *et al.*, 2017). The first breakthrough came from the study by López-Torrejón *et al.* (2016), where active Fe protein was produced from *A. vinelandii* *nifH* and *nifM* genes co-expression in the mitochondrial matrix of yeast cultures growing under highly aerobic conditions. This study confirmed the protective function against oxygen offered by respiration in the

mitochondria and that the endogenous mitochondrial [Fe-S] cluster assembly machinery can substitute, at least to an extent, some of the functions of NifS and NifU. Burén, Young *et al.* (2017) demonstrated that expression and mitochondrial targeting of a nine-gene *nif* cluster of *A. vinelandii* in yeast resulted in NifDK tetramer formation, although the resulting MoFe protein was inactive. The second milestone was achieved by Burén *et al.* (2019) with the production of active NifB and its product NifB-co in yeast mitochondria, which are key to the biosynthesis of the Mo-nitrogenase active-site cofactor, i.e. FeMo-co, though in this case co-expression with NifU and NifS was required.

2.6.3. Engineering nitrogen fixation in plants

Engineering a staple crop capable of fixing its own nitrogen is the ultimate goal of the ‘direct gene transfer’ strategy. To date, the expression of Mo-nitrogenase components in both mitochondria and chloroplast of model plants, namely *N. benthamiana* or *Nicotiana tabacum*, has been achieved.

2.6.3.1. Mitochondria

Studies targeting mitochondria as a subcellular location for the expression of nitrogenase components have appeared in recent years. Allen *et al.* (2017) showed individual transient expression of 16 Nif proteins from *K. oxytoca* targeted to *N. benthamiana* mitochondria, but none of the structural components was tested for activity. In the case of NifD, a mitochondrial endoprotease was degrading the protein once imported into the mitochondria due to a specific motif found in its amino acid sequence. Its mutation generated NifD variants resistant to degradation in both plant and yeast (Allen *et al.*, 2020; Xiang *et al.*, 2020).

An exploration to identify superior Mo-nitrogenase components from other organisms, that could outperform those of *K. oxytoca* and *A. vinelandii* has been underway (Burén *et al.*, 2019). In this context, Jiang *et al.* (2020) just reported that the Fe protein from *Hydrogenobacter thermophilus* was found active when targeted to *N. benthamiana* mitochondria.

2.6.3.2. Chloroplast

More than twenty years ago, Dixon *et al.* (1997) showed that *nifH* and *nifM* genes from *K. pneumoniae* N-terminally fused to a transit peptide were correctly expressed and imported into chloroplast of *N. tabacum* plants. Unfortunately, the NifH protein levels achieved were very low and no activity could be determined. In an alternative approach, Ivleva *et al.* (2016) generated transplastomic tobacco plants expressing the *nifH* and *nifM*

genes of *A. vinelandii* and showed low Fe protein activity from plant enriched extracts, although only when plants were prior cultivated in a low-oxygen atmosphere. Given these scattered results after 25 years of work, it seemed plausible that the lack of positive reports could be due to the presence of oxygen-derived from the photosynthetic activity of plant chloroplasts, which could be avoiding the accumulation of functional nitrogenase proteins in this subcellular location.

2.7. Engineering of nitrogenase biosynthesis in plastids

SynBio projects in chloroplasts of higher plants have proven a useful strategy for the last two decades (Boehm and Bock, 2019). To definitely test the suitability of plastids as locations for the engineering of nitrogenase biosynthesis, we envisioned two technical possibilities.

2.7.1. Plastid transformation

The first one consists of the direct transformation of the chloroplast genome by stacking prokaryotic nitrogenase transgenes in synthetic operons, generating transplastomic plants. This approach presents important advantages, such as avoidance of gene silencing and epigenetic or positional effects, elevated protein expression levels due to high plastid DNA copy number and transgene containment due to plastid maternal inheritance in most crops (Adem *et al.*, 2017). However, chloroplast transformation methods are not yet in place for staple crop plants or are in the best case problematic, due to a naturally present point mutation that confers spectinomycin resistance in the plastid 16S rRNA genes of all cereals (Bock, 2015). Although chloroplast genomes of several major crops including cotton, soybean or sugar beet have been transformed, cereals such as maize and rice remain elusive to this technique (Clarke and Daniell, 2011). As our research aims to generate nitrogen-fixing cereals, this technical impossibility pushed our efforts towards the second possible strategy.

2.7.2. Nuclear transformation

The second strategy is based on nuclear genome transformation, following a monocistronic gene structure, and targeting of the recombinant cytosol-translated proteins into the chloroplast by fusing them to chloroplasts transit peptides.

The major advantage of nuclear transformation lies in the existence of techniques to transform most of the known plant species amenable to engineering. Some bottlenecks, like the assembly of large DNA constructs, enough to encode for all proteins required for nitrogenase biosynthesis, has been facilitated by the development of modular assembly

techniques in the last decade and there is a growing collection of characterized genetic modules, e.g. promoters, terminators, gene switches or insulators, that enables a tightly controlled regulation of transgene expression when integrated within the host genome (Bernabé-Orts *et al.*, 2020; Cai *et al.*, 2020; Engler *et al.*, 2014; Patron, 2020; Pérez-González *et al.*, 2018; Vazquez-Vilar *et al.*, 2016, 2017). Moreover, a relevant asset of nuclear transformation is the quick estimate of the performance of constructions that can be made for gene expression and protein targeting with transient expression assays.

For this approach to work, it is critical that protein import from transit peptides is highly efficient. The use of chloroplast transit peptides is a strategy that has already been used in many biotechnological applications to direct recombinant proteins to the chloroplast stroma (Carvalho *et al.*, 2011; Feng *et al.*, 2010; Kebeish *et al.*, 2007; Kiani *et al.*, 2013; Maier *et al.*, 2012; Shen *et al.*, 2019, 2017). However, targeting foreign proteins to chloroplasts can still be a troublesome task in some cases (Shen *et al.*, 2017) and there is a need for the characterization of specific signals that perform correctly in the species of choice.

The collection of transit peptides available at the moment in which we started this project, although functional, was not optimal. Most of the TPs described in the literature included a sequence longer than the one cleaved after import, leaving undesired amino acid residues in the N-terminus of the recombinant proteins, which can affect folding and function (Muthamilselvan *et al.*, 2019). Furthermore, although targeting of proteins to mitochondria and chloroplasts is generally highly specific, an increasing number of proteins imported dually into both organelles have been described (Peeters and Small, 2001).

The use of SynBio tools for the engineering of complex pathways in higher plant chloroplasts has proved its potential during the last years. In this thesis, we targeted chloroplasts from *N. benthamiana* and *O. sativa* and generated the proof of concept that nitrogenase biosynthesis can be engineered in plant plastids.

Material and Methods

3. DNA Techniques

3.1. Modular Cloning Techniques

All constructs used in this work were generated using MoClo (Werner *et al.*, 2012) and its adaptation for the direct DNA transfer to rice (Baysal *et al.*, 2020).

3.1.1. Standard MoClo conditions

MoClo restriction-ligations were set up in a final volume of 20 µl with a 2:1 molar ratio of insert:acceptor vector, 5U of the required restriction enzyme — *BpiI* (Thermo Fisher Scientific) for the generation of Level 0 and Level 2 plasmids, and *BsaI*-HFv2 (NEB) for the generation of Level 1 plasmids — 4.5U of T4 Ligase (Promega), 1.5 µl of Ligase 10x Buffer and 1.5 µl of 10x BSA (GE Healthcare). The reactions were incubated in a thermocycler with the following program: 20 s at 37°C, 26 cycles of 3 min at 37°C plus 4 min at 16°C, and 5 min at 50°C, 5 min at 80°C and hold at 16°C.

The reaction mixes were then added to chemically competent cells of *E. coli* DH5α or TOP10 (Thermo Fisher Scientific), which were then incubated in LB solid medium containing 20 µg/ml X-Gal (Duchefa Biochemie), 1 mM IPTG (Sigma-Aldrich), and the corresponding antibiotic, 50 µg/ml spectinomycin (Sigma-Aldrich) for Level 0 or Level M, 100 µg/ml carbenicillin (Formedium) for Level 1, or 50 µg/ml kanamycin (Formedium) for Level 2 constructs. Colonies were selected for plasmid DNA (pDNA) extraction using the QIAprep Spin Miniprep Kit (Qiagen). After pDNA extraction, concentrations were determined using NanoDrop® ND-1000 (Thermo Fisher Scientific). The fidelity of all plasmids was verified by Sanger sequencing (Eurofins Genomics) and results were analysed using Geneious version 10.2.2 software (Kearse *et al.*, 2012).

E. coli colonies corresponding to positive clones were stored in 60% glycerol stocks in cryovials (Nalgene) at -80°C.

3.1.2. Generation of modular pieces

The design of primers for amplification of DNA fragments to generate Level 0 plasmids was performed manually following the rules described in Weber *et al.* (2011). When DNA fragments contained internal restriction sites for either *BsaI* or *BpiI*, the fragment was domesticated using internal primers to eliminate the site. The design of domestication primers was performed using the Domesticator Tool

(<https://gbcloning.upv.es/do/domestication/>), subsequently modifying the tails of the primers to add a *BpiI* restriction site.

Modular DNA fragments were amplified by PCR using Phusion Hot Start II DNA Polymerase (Thermo Fisher Scientific), following manufacturer's protocol, and the primers and templates described in Supplemental Table 1. PCR products were analysed in 1% agarose gels to check for correct amplicon size, exclude possible non-specific amplicons and primer dimers, and gel purified using QIAquick Gel Extraction Kit (Qiagen).

All modular pieces generated in this thesis used the Universal Level 0 vector (pAGM9101) from the MoClo Toolkit (Werner *et al.*, 2012) as an entry vector. Modular pieces cloned for this work can be found in Supplemental Table 2.

3.1.3. Generation of Transcriptional Units

In the MoClo system, pieces are assembled into TUs in restriction/ligation reactions with destination vectors that define the position of each TU in a future multigene assembly.

Level 1 in all six different positions (L1P_x) were used as destination vectors, either binary vectors for *Agrobacterium* T-DNA delivery to plant cells (pICH47732-L1P1, pICH47742-L1P2, pICH47751-L1P3, pICH47761-L1P4, pICH47772-L1P5, and pICH47781-L1P6) (Werner *et al.*, 2012) or vectors adapted for particle bombardment transformation of plants (pUC57-L1P1, pUC57-L1P2, pUC57-L1P3, pUC57-L1P4, pUC57-L1P5, and pUC57-L1P6) (Baysal *et al.*, 2020 and Section 3.1.5).

A list of all Level 1 constructs generated and used in this work can be found in Supplemental Table 3.

3.1.4. Generation of multigene constructs

Multigene constructs were assembled from Level 1 constructs in acceptor backbones named Level 2 or M (Werner *et al.*, 2012). For this work, Level 2 binary vectors for *Agrobacterium* T-DNA delivery to plant cells (pAGM4673-L2 and pAGM8055-LM) (Werner *et al.*, 2012) or a Level 2 vector adapted for particle bombardment transformation of plants (pUC57-L2) (Baysal *et al.*, 2020) were used as destination vectors.

A list of all Level 2 and M constructs generated and used in this work can be found in Supplemental Table 4.

3.1.5. Adaptation of MoClo vectors for particle bombardment

The backbone of the pUC57-AmpR (GenScript) vector was PCR amplified using Phusion Hot Start II DNA Polymerase (Thermo Fisher Scientific) and primers 591/592, following the manufacturer's protocol. The PCR product was analysed in a 1% agarose gel and a band corresponding to over 2200 bp was purified using QIAquick Gel Extraction Kit (Qiagen) and digested with *AdeI* (Thermo Fisher Scientific) following the manufacturer's protocol.

The vectors pICH47732-L1P1, pICH47751-L1P3, pICH47761-L1P4, pICH47772-L1P5, and pICH47781-L1P6 (Werner *et al.*, 2012) were digested with *AdeI*, analysed in 1% agarose gels, and fragments of 650 bp corresponding to the MoClo cloning cassettes were purified.

The *AdeI*-digested cloning cassettes were ligated to the amplified pUC57-AmpR backbone using T4 Ligase (Promega), according to the manufacturer's indications. Ligation mixtures were transformed in *E. coli* DH5 α chemically competent cells, which were grown on LB solid medium containing 20 μ g/mL X-Gal (Duchefa) and 1mM IPTG (Sigma-Aldrich), supplemented with 100 μ g/mL carbenicillin (Formedium).

Blue carbenicillin resistant colonies were selected for plasmid DNA extraction using the GenElute Plasmid Miniprep Kit (Sigma-Aldrich). The ligation between backbone and cassette was verified by Sanger sequencing (Eurofins Genomics) and results were analysed using Geneious version 10.2.2 software (Kearse *et al.*, 2012).

Vector can be found in Addgene under the ID 109220 (pUC57-L1P1), 109222 (pUC57-L1P3), 109223 (pUC57-L1P4), 109224 (pUC57-L1P5), and 109225 (pUC57-L1P6).

3.2. Multiple Overlap Extension PCR

Point mutations in rice codon-optimized genes *nifH*, *nifM*, *nifU*, and *nifS* were performed via multiple overlap extension PCR (MOE-PCR) as described in (Kadkhodaei *et al.*, 2016) with slight modifications.

Firstly, genes were PCR amplified in several fragments with primers 887 to 910, introducing the silent mutations described in Supplemental Tables 1 and 2, using Phusion Hot Start II DNA Polymerase (Thermo Fisher Scientific), setting up reactions according to the manufacturer's protocol. Each fragment had an overlapping section of about 20 bp with the contiguous section, in order to set a correct order in the MOE-PCR step.

PCR products were analysed in 1% agarose gels to check for correct amplicon size and exclude possible non-specific amplicons and primer dimers, and gel purified using QIAquick Gel Extraction Kit (Qiagen).

In order to build the full *nif* genes, 100 ng of each linear fragment was mixed in a 50 µL MOE-PCR reaction containing all reagents and primers (613 to 618, 623, and 624) for the amplification of the modular piece. Thermal cycling conditions were as follows: 30 s at 95°C, [10 s at 98°C, 80 – 70°C (gradient as 0.4°C/s), 30 s at 72°C] for 30 cycles and a final extension of 5 min at 72°C.

After running the MOE-PCR reaction, the products were analysed in 1% agarose gels and gel purified using the QIAquick Gel Extraction Kit (Qiagen). Linear DNA was used in MoClo restriction/ligations reactions to generate modular pieces as described in Section 3.1.2.

3.3. Genomic DNA isolation

Genomic DNA isolation of *A. thaliana* Col-0, *N. tabacum* L. cv. Samsun, *O. sativa* subsp. *japonica* and *Pisum sativum* were necessary as a template for PCR amplification to clone modular pieces (see Supplemental Table 2). Isolation was performed using the DNeasy Plant Mini Kit (Qiagen) except for *P. sativum*, which was performed using Extract-N-Amp™ Plant Tissue PCR Kits (Sigma-Aldrich) following the manufacturer's protocol.

4. Bacterial Culture Techniques

4.1. *Escherichia coli*

DH5α strain (F⁻ Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *recA1 endA1 hsdR17*(r_k⁻, m_k⁺) *phoA supE44 thi-1 gyrA96 relA1 λ⁻*) (Thermo Fisher Scientific) and TOP10 strain (F-*mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ*ΔM15 Δ*lacX74 recA1 araD139 Δ(araIeu)*7697 *galU galK rpsL* (Str^R) *endA1 nupG*) (Thermo Fisher Scientific) were used for cloning purposes.

E. coli cells were grown at 37°C in LB solid or liquid medium (Bertani, 1951), supplemented with antibiotics, X-Gal (Duchefa) or IPTG (Sigma-Aldrich) as needed.

For plasmid transformation, chemically competent *E. coli* cells were prepared as described in Hanahan *et al.* (1991).

4.2. *Agrobacterium tumefaciens*

A. tumefaciens GV3101 (pMP90) strain (Koncz and Schell, 1986) cells were grown at 28-30°C in LB medium containing rifampicin (25µg/mL) (Sigma-Aldrich), and gentamicin (10µg/mL) (Sigma-Aldrich), supplemented with other selective antibiotics as needed. For liquid cultures, cells were grown in baffled flasks. For plasmid transformation, chemically competent *A. tumefaciens* cells were prepared following the same protocol as for *E. coli* described in Hanahan *et al.* (1991).

5. Plant Culture Techniques

5.1. *Nicotiana benthamiana*

5.1.1. Growth conditions

N. benthamiana plants were cultivated on 8 x 8 x 8 cm pots in a greenhouse in long-day conditions (16 h light/8 h dark) ensuring enough light with LED lamps at 26/22°C day/night temperature.

5.1.2. Transient expression assays

Overnight cultures of *A. tumefaciens* GV3101 strain cells carrying the plasmid of interest were diluted to an OD₆₀₀ between 0.3-0.6 in an infiltration solution containing 10 mM MES pH 5.5, 10 mM MgSO₄, and 150 µM acetosyringone (Sigma-Aldrich) and incubated at 150 rpm at room temperature (RT) for 3 h. When using single transcriptional units, an *A. tumefaciens* GV3101 strain carrying the silencing suppressor p19 (Li, 2011; Naim *et al.*, 2012) was co-diluted to an OD₆₀₀ of 0.3-0.6 (multigenic constructs already contain p19 transcriptional unit). Leaves of 3-4 weeks-old *N. benthamiana* plants were agroinfiltrated using a syringe without a needle and 3-4 days after infiltration, tissue was collected at the end of the dark period and used for protein extraction, chloroplast isolation, or confocal microscopy (protocols performed under ambient light).

5.2. *Arabidopsis thaliana*

5.2.1. Seed sterilization

Seeds were sterilized in 1.5mL tubes using a solution of 20% (v/v) commercial bleach and 0.1% (v/v) Tween 20 (VWR Chemicals). Seeds were incubated in that solution for 10 min under constant agitation, followed by five washes with sterile MilliQ water.

After sterilization, tubes were covered with tinfoil and seeds remained in the dark for 2-3 days at 4°C for stratification.

5.2.2. Culture media

A. thaliana Col-0 seeds were plated in MS medium pH 5.7 (Murashige and Skoog, 1962) (Mod.No.1B, Duchefa) with 1% (w/v) sucrose (Duchefa) and 1% (w/v) plant agar (Duchefa).

5.2.3. Growth conditions

Seedlings were grown in an environmentally controlled chamber under 16/8 h light/dark conditions at 22°C and 65% relative humidity (RH).

5.2.4. Protoplast isolation

Isolation of protoplast from Arabidopsis seedlings was performed as described in Yoo *et al.* (2007) and González-García *et al.* (2020) with modifications. Aerial parts and roots of ca. 100 seedlings were separated and cut into 0.5 – 1.0 mm strips using a razor blade. The strips were transferred to different Erlenmeyer flasks containing 10 ml of enzyme solution (1.25% (w/v) cellulase R10 (Duchefa), 0.3% (w/v) macerozyme R10 (Duchefa), 0.4 M D-mannitol, 20 mM MES at pH 5.7, 20 mM KCl, 10 mM CaCl₂ and 0.1% (w/v) BSA) and incubated in the dark for 3 h (aerial part) or 1 h and 45 min (roots) at RT with 80 rpm shaking to allow digestion of cell wall material. An equal amount of W5 solution (154 mM NaCl, 125 mM CaCl₂, 5 mM KCl and 2 mM MES at pH 5.7) was added to stop the digestion. Protoplasts were released by filtering through 40 µm nylon cell strainers and pelleted by centrifugation at 200 × *g* for 2 min (aerial part) or 500 × *g* for 10 min (roots) at RT. Protoplasts were resuspended in 2 ml MMG solution (0.4 M D-mannitol, 15 mM MgCl₂, and 4 mM MES at pH 5.7) and kept on ice.

5.2.5. Protoplast transfection

Transformation of protoplasts was carried out by DNA-PEG-calcium transfection as described in Yoo *et al.* (2007) and Page *et al.* (2019) with modifications. For each transformation, 10 µl of plasmid (5 to 10 µg DNA) and 100 µl of protoplasts were combined with 110 µl of a freshly-prepared PEG solution containing 40% (w/v) PEG 4000 (Merk), 0.2 M D-mannitol and 0.1 M CaCl₂, and incubated in the dark at RT for 15 min. Negative control samples, replacing plasmid with 10 µl Milli-Q H₂O, were carried out. W5 solution (440 µl) was slowly added to complete transformations. Protoplasts were pelleted by centrifugation at 100 × *g* for 2 min in the case of leaf protoplasts or 500 × *g* for 10 min for root protoplast, resuspended in 375 µl of WI solution (0.5 M D-mannitol, 20 mM KCl and 4 mM MES at pH 5.7), transferred to 96-well microplates (125 µl per well), and incubated at RT on the laboratory bench for 16 h.

5.3. *Oryza sativa*

5.3.1. Transient expression assays

5.3.1.1. Growth of plant material

Rice seeds (*Oryza sativa* L. cv. EYI 105) were sown into a 3:1 mixture of soil (Floragard) and vermiculite in $\varnothing$ 13 cm pots and grown for nine days after germination in an environmentally controlled chamber (Conviron) under 16/8 h light/dark conditions at 24°C and 70% RH.

5.3.1.2. Protoplast isolation

Isolation of protoplast from rice seedlings was performed as described in Zhang *et al.* (2011) and Page *et al.* (2019) with slight modifications. About 100 seedlings were harvested and approximately 10 cm of stem and sheath green tissue was retained and subsequently cut into 0.5 – 1 mm strips using a razor blade. The strips were immediately submerged into 0.6 M D-mannitol (Acros Organics) for 15 min in the dark at RT to initiate plasmolysis.

After discarding the mannitol, the tissue was transferred to an enzyme solution (1.5% (w/v) cellulase RS (Duchefa), 0.75% (w/v) macerozyme R10 (Duchefa), 0.6 M D-mannitol (Acros Organics), 20 mM MES at pH 5.7 (Duchefa), 10 mM KCl (Acros Organics), 10 mM CaCl₂ (Merk) and 0.1% (w/v) BSA (GE Healthcare Life Sciences)) and incubated in the dark for 4 hr at RT with 60 rpm shaking to allow digestion of cell wall material. An equal amount of W5 solution (154 mM NaCl, 125 mM CaCl₂, 5 mM KCl and 2 mM MES at pH 5.7) was added to terminate digestion.

Protoplasts were released by filtering through 40 μ m nylon cell strainers (Corning) with 3 washes of the strips using W5 solution. The pellets were collected by centrifugation at $300 \times g$ for 3 min at RT. The supernatant was decanted, and the pellets were washed by gentle resuspension in 10 ml W5 solution. After second centrifugation at $300 \times g$ for 3 min at RT, the pellets were resuspended in 1 to 2 ml MMG solution (0.4 M D-mannitol, 15 mM MgCl₂, and 4 mM MES at pH 5.7).

5.3.1.1. Protoplast transfection

Transformation of protoplasts was carried out by DNA-PEG-calcium transfection as described in (Page *et al.*, 2019). For each transformation, 10 μ l of plasmid (5 to 10 μ g DNA) and 120 μ l of protoplasts were combined with 140 μ l of a freshly-prepared PEG solution containing 40% (w/v) PEG 4000 (Merk), 0.2 M D-mannitol and 0.1 M CaCl₂, and incubated in the dark at RT for 25 min. Negative control samples, replacing plasmid

with 10 µl Milli-Q H₂O, were carried out. W5 solution (560 µl) was slowly added to complete transformations. Protoplasts were pelleted by centrifugation, resuspended in 375 µl of WI solution (0.5 M D-mannitol, 20 mM KCl and 4 mM MES at pH 5.7), transferred to 96-well microplates (125 µl per well), and incubated at RT on the laboratory bench for 12 to 16 h.

5.3.2. Stable transformation

5.3.2.1. Seed sterilization

Rice seeds (*Oryza sativa* L. cv. EYI 105) were dehusked, surface-sterilized in 70% (v/v) ethanol for 3 min, then in a 3.7% (v/v) sodium hypochlorite and 0.1% Tween-20 solution for 35 min, and lastly rinsed five times in sterile distilled water. Washed seeds were then transferred to sterile Petri dishes with autoclaved filter paper for drying.

5.3.2.2. Culture media

Fifteen rice seeds were plated in Petri dishes of callus induction media (MSP) (MS medium (Murashige and Skoog, 1962) (with Gamborg's B5 Vitamins, Duchefa) supplemented with 3% (w/v) sucrose (Duchefa), 2.5 mg/l 2,4-D (Duchefa), 0.05% (w/v) L-Proline (Duchefa), 0.03% (w/v) Casein hydrolysate (Duchefa) and solidified with 0.6% (w/v) Phytigel (Sigma-Aldrich)).

In subsequent steps the following media was used:

- Osmoticum media (MSO): MS medium (Murashige and Skoog, 1962) (with Gamborg's B5 Vitamins, Duchefa) supplemented with 3% (w/v) sucrose (Duchefa), 7.82% (w/v) D-Mannitol (Duchefa) 2.5 mg/l 2,4-D (Duchefa), 0.05% (w/v) L-Proline (Duchefa), 0.03% (w/v) Casein hydrolysate (Duchefa) and solidified with 0.3% (w/v) Phytigel (Sigma-Aldrich).
- Selection media (MSS): Callus induction media supplemented with 30 mg/ml hygromycin B (Duchefa).
- Shoot induction media (MSR): MS medium (Murashige and Skoog, 1962) (with Gamborg's B5 Vitamins, Duchefa) supplemented with 3% (w/v) maltose (Duchefa), 0.01% myo-inositol (Duchefa), 2 mg/l BAP (Sigma-Aldrich), 0.5 mg/l NAA (Sigma-Aldrich), 0.05% (w/v) L-Proline (Duchefa), 0.03% (w/v) Casein hydrolysate (Duchefa) and solidified with 0.25% (w/v) Phytigel (Sigma-Aldrich).
- Root induction media (MSR-R): Half strength MS medium (Murashige and Skoog, 1962) (with Gamborg's B5 Vitamins, Duchefa) supplemented with 3% (w/v) sucrose (Duchefa), 0.01% myo-inositol (Duchefa), 0.05% (w/v) L-Proline

(Duchefa), 0.03% (w/v) Casein hydrolysate (Duchefa) and solidified with 0.25% (w/v) Phytigel (Sigma-Aldrich).

The pH of the media was adjusted to 5.8 with KOH before autoclaving at 120°C for 18 min, except MRS, which was autoclaved for 8 min to avoid caramelization of maltose.

5.3.2.3. Growth conditions

Rice seeds and callus were grown in an *in vitro* growth chamber (Heratherm, Thermo Fisher Scientific) under dark conditions at 25°C.

Rice callus in shoot or root induction media were grown in an environmentally controlled chamber (Sanyo) under 16/8 h light/dark conditions at 25°C.

Regenerated plantlets that were transferred to soil grew in an environmentally controlled chamber (Convion) under 16/8 h light/dark conditions at 24°C and 70% RH.

5.3.2.4. Biolistic transformation

Mature rice seeds were handled as described in Christou *et al.* (1991), Sudhakar *et al.* (1998) and Valdez (1998). Gold particles were coated with 20 µg of pDNA. Level 1 pUC57 based transcriptional units containing eGFP fused to the individual chloroplast transit peptides, namely *AtmCAB6_{TP}*, *AtmTOCC_{TP}*, *AtmRCA_{TP}*, and *AtmBCCP1_{TP}*, were introduced separately into rice nature-embryo derived callus rice embryos, together with the *hpt* gene for selection in a 3:1 ratio. Level 2 pUC57 based plasmids carried the *nif* genes of interest plus the *hpt* selectable marker.

Per each reaction, 10 mg of gold particles of 0.76 µm size were prepared in an Eppendorf tube. The pDNA was then added and mixed with 100 µl XHO buffer, 100 µl 0.1M spermidine, 100 µl 25% PEG 1300-1600, and 100 µl 2.5M CaCl₂. After incubation for 10 min on vortex, the preparation was centrifuged and the pellet washed twice with 100% ethanol while sonicating briefly. The pDNA-coated gold particles were then resuspended in 9 ml of 100% ethanol and stored at -20°C until further use.

Six days-old rice embryos were excised, removing the expanded embryo from the seeds and incubating them on MSO for 4 h prior to bombardment with the scutellum facing upwards. Transformation of rice embryos with pDNA-coated gold particles was performed following Christou *et al.* (1991), in two bombardment steps at 15 kV in a Helium atmosphere with 4 h interval. Subsequently, embryos were incubated overnight on MSO plates and transferred to fresh MSP for two days. Then, embryos were transferred to MSS plates and incubated for 14-15 days until embryogenic callus

appeared, when they were transferred to fresh MSS plates (secondary selection) for verification of positive transformants. A minimum of two representative independent callus lines and the corresponding regenerated plants for each construct were selected for in-depth analyses.

5.3.2.5. Plant regeneration

After approximately 6 – 8 weeks, positive embryogenic callus expressing the protein of interest were sequentially transferred to MSR plates and incubated in a 16/8 h photoperiod as described in Section 5.3.2.3.

Once the emerged shoots had sufficiently developed, tissue was carefully transferred to MSR-R and incubated as described in Section 5.3.2.3.

Regenerated plantlets with a sufficiently developed root system were transferred to 8 x 8 x 8 cm pots containing a 3:1 mixture of soil (Floragard) and vermiculite and were grown under flooded conditions in a growth chamber in a 12/12 h photoperiod at 28/25°C light/dark with constant 80% RH. Plants were irrigated with a soluble Fe solution (Sequestrene 138 Fe G-100, Syngenta) until seed maturity.

6. Protein Techniques

6.1. Protein extraction

6.1.1. Preparation of Arabidopsis protoplast extracts

A. thaliana transformed protoplasts were recovered from 96-well plates by gentle pipetting, transferred to 1.5 ml Eppendorf tubes, and pelleted by centrifugation at $100 \times g$ for 2 min (mesophyll cells) or $500 \times g$ for 10 min (root cells) at RT. Supernatants were discarded and pellets were shock frozen in liquid nitrogen. Protoplasts were resuspended in 2x Laemmli buffer (125 mM Tris-HCl pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β -mercaptoethanol, 0.005% (w/v) bromophenol blue) and subsequently boiled for 10 min, cooled down and centrifuged at $14,000 \times g$ for 3 min at 4°C to obtain total protein extracts.

6.1.2. Preparation of tobacco extracts

Leaf discs from infiltrated *N. benthamiana* plants were harvested into 2-ml screw-cap tubes containing five 3-mm diameter glass beads and their weight was measured. Tubes were immediately frozen in liquid nitrogen and stored at -80°C for further use. Tissue was ground to a fine powder using a BeadBug homogenizer (Benchmark Scientific Inc.) at maximum speed for 45 s avoiding thawing. Samples were mixed with 4 volumes of 2x

Laemmli buffer, boiled for 10 min, cooled down and centrifuged at $14,000 \times g$ for 3 min at 4°C to obtain total protein extracts.

6.1.2.1. Chloroplast isolation assays

Isolation of chloroplasts from agroinfiltrated leaves was performed as described in Klinkenberg (2014), with slight modifications. All steps were performed on ice. From 500 to 700 mg of plant material was harvested and homogenized in a ratio 1:6 with ice-cold Chloroplast Isolation Buffer (CIB) (0.33 M Sorbitol, 50 mM HEPES pH7.5, 1 mM MgCl_2 , 0.1% (w/v) BSA) using a precooled Potter homogenizer (Sigma-Aldrich).

After filtration through a $47 \mu\text{m}$ nylon mesh (Merck) the intact chloroplasts were separated by centrifugation through a double Percoll (GE Healthcare Life Sciences) gradient (40/80%) at $3,200 \times g$ at 4°C for 15 min. The band that formed at the interface was collected and washed twice with CIB without BSA. Chloroplasts were shock frozen in liquid nitrogen, resuspended in ice-cold protein extraction buffer (100 mM HEPES pH 7.2, 1 mM MgCl_2 , 10 mM DTT, 10% (w/v) glycerol, 0.5% (v/v) Protease Inhibitor Cocktail) and centrifuged at $15,000 \times g$ at 4°C for 10 min. The supernatant “stroma fraction (S)” was recovered and the “pellet fraction (P)”, was re-suspended in the same volume of protein extraction buffer.

6.1.3. Preparation of rice extracts

6.1.3.1. Rice protoplasts

Rice transformed protoplasts were recovered from 96-well plates by gentle pipetting, transferred to 1.5 ml Eppendorf tubes, and pelleted by centrifugation at $300 \times g$ for 6 min at RT. Supernatants were discarded and pellets were shock frozen in liquid nitrogen. Protoplasts were resuspended in 2x Laemmli buffer and subsequently boiled for 10 min, cooled down, and centrifuged at $14,000 \times g$ for 3 min at 4°C to obtain total protein extracts.

6.1.3.2. Rice callus

Rice callus was harvested and placed into 1.5 ml Eppendorf tubes, weighted, immediately frozen in liquid nitrogen, and then stored at -80°C until further use. Callus tissue was homogenized in the presence of ice-cold protein extraction buffer composed of 100 mM HEPES pH 7.2, 1 mM MgCl_2 , 10 mM DTT, 10% (w/v) glycerol, and 0.5% (v/v) Protease Inhibitor Cocktail (Sigma-Aldrich) with the help of a stainless-steel pestle, and subsequently centrifuged at $14,000 \times g$ for 3 min at 4°C to obtain soluble protein extracts.

6.1.3.3. Rice leaves and roots

Rice leaf and root tissue samples were excised from plantlets when transferring them from Magenta GA-7 vessels (Magenta Corp.) to soil and harvested into 2-ml screw-cap tubes containing five 3-mm diameter glass beads, weighted, immediately frozen in liquid nitrogen, and then stored at -80°C until further use. Samples were ground to a fine powder with the help of a plastic pestle and a BeadBug homogenizer (Benchmark Scientific Inc.), mixed with 4 volumes of 2x Laemmli buffer, boiled for 10 min, cooled down, and centrifuged at $14,000 \times g$ for 3 min at 4°C to obtain total protein extracts.

6.2. Immunoblot assays

SDS-PAGE (Laemmli, 1970) and immunoblot (Towbin *et al.*, 1979) analysis were performed by standard methods. Samples processed with protein extraction buffer were resuspended in Laemmli sample buffer 2x, where total protein extracts were used directly. Samples were loaded in 12% electrophoresis polyacrylamide gels and run on a Mini-Protean Tetra cell (Bio-Rad). SDS gels were either stained with Coomassie (0.1% (w/v) Coomassie R-250 (Thermo Fisher Scientific), 40% ethanol, 10% glacial acetic acid) or electroblotted to 0.45 µm pore-size nitrocellulose membranes (Bio-Rad) using a Trans-Blot Semi-Dry Transfer cell (Bio-Rad).

Blotted membranes were stained with Ponceau S solution (0.2% (w/v) Ponceau S (Sigma-Aldrich) (w/v) in 5% glacial acetic acid) for quick checking of correct protein transfer and subsequently destained with TBS-T (pH 7.5, 200 mM Tris-base, 1.5 M NaCl, 0.2% (v/v) Tween-20).

For the immunodetection of proteins, primary antibodies prepared in 5% (w/v) BSA and 0.1% (v/v) NaN₃ were used as follows: in-house generated rabbit polyclonal antibodies detecting NifH (1:3500 dilution) (López-Torrejón *et al.*, 2016), NifU (1:2000 dilution) (López-Torrejón *et al.*, 2016), NifS (1:1200 dilution) (López-Torrejón *et al.*, 2016) and NifM (1:2500 dilution) (Burén, Young, *et al.*, 2017) were used. Commercially available antibodies against GFP (1:3000 dilution) (11814460001; Roche), Strep-tag II (1:10000 dilution) (Strep-Tactin-HRP, 2-1502-001; IBA Lifesciences), RbcL (1:10000 dilution) (AS03 037; Agrisera AB), Actin-11 (1:2000 dilution) (AS10 702; Agrisera AB) and IDH (1:5000 dilution) (AS06 203A; Agrisera AB) were used. Secondary horseradish peroxidase-conjugated antibodies were prepared in 2% (w/v) skimmed milk as follows: anti-rabbit IgG (1:25000 dilution) (A0545-1ML; Sigma-Aldrich) and anti-mouse IgG (1:10000 dilution) (AS11 1772; Agrisera AB).

Immunoblots were developed in an iBright FL1000 Imaging System (Thermo Fisher Scientific) using chemiluminescence mode. *Smart exposure* setting was used to develop blots and iBright Analysis Cloud Service (Thermo Fisher Scientific) was used for image processing, which was equally applied across the entire image including control lines.

6.2.1. Quantification of Immunoblot data

For protein accumulation measurements, short exposure blots were analysed in iBright Analysis Cloud Service (Thermo Fisher Scientific). The signal intensity for each band was measured using automatic band recognition and background subtraction.

6.2.1.1. Statistical analysis

The accumulation values for each Nif protein normalized using GFP signal shown in Figures 9, 10 and 19 were graphically represented using Prism (GraphPad). Significant differences in Nif protein expression were determined by applying a Student's t-test or Tukey's multiple comparisons test, where a p-value < 0.05 was considered statistically significant and a p-value < 0.01 highly significant.

The import efficiency values for each TP shown in Figures 23, 25 and 27 were graphically represented using Prism (GraphPad) and significant differences in eGFP protein import efficiency between tissues were determined by applying a Student's t-test, where a p-value < 0.05 was considered statistically significant.

6.3. Anaerobic purification of TwinStrep-tagged *NbNifH*

Agroinfiltrated *N. benthamiana* fresh leaves were ground in liquid nitrogen in the presence of quartz sand and resuspended in anaerobic lysis buffer (100 mM Tris-HCl pH 8.0, 250 mM NaCl, 2 mM sodium dithionite (DTH)) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM leupeptin, and 5 µg/ml DNaseI. After blender homogenization, the extract was filtered through a cloth, then centrifuged at 73,000 x g for 1 h at 4°C under anaerobic conditions, and the supernatant filtered through a Nalgene filter unit (Thermo Fisher Scientific) with 0.2 µm pore size, resulting in the cell-free extract (CFE).

Twin-Strep-tag *NbNifH* was purified by Strep-Tactin XT affinity chromatography under anaerobic conditions (<0.1 ppm oxygen) using an AKTA Prime FPLC system (GE Healthcare Life Sciences) inside an MBraun glovebox (M. Braun Inertgas-Systeme GmbH). All buffers were previously made anaerobic by sparging with nitrogen gas. The CFE was loaded at 2 ml/min into a 5 ml Streptactin-XT high capacity cartridge (IBA Lifesciences) previously equilibrated with lysis buffer, then washed with 20 column

volumes of lysis buffer and eluted with 6 column volumes of elution buffer (50 mM Tris-HCl pH 8.0, 250 mM NaCl, 5% glycerol, 2 mM DTH, 50 mM biotin). The elution fraction was concentrated using an Amicon Ultra Centrifugal Filter unit (Amicon Ultra-15, Merck) with a cut-off pore size of 30 kDa, and then desalted using a PD-10 column (GE Healthcare Life Sciences) equilibrated in buffer (50 mM Tris-HCl pH 8.0, 250 mM NaCl, 5% glycerol, 2 mM DTH) in order to remove the biotin. Purified *NbNifH* was frozen and stored in cryogenic tubes (Nalgene) in liquid nitrogen until used for acetylene reduction assays.

6.4. Edman Degradation

Three μ g of pure *NbNifH* were resolved by SDS-PAGE and blotted to an Immun-Blot PVDF membrane (pore size 0.45 μ m, Bio-Rad) using blot buffer (50 mM sodium borate pH 9.0, 20% methanol, and 0.1% SDS) at 100 mA for 12 h, and 400 mA for 3 additional h. The membrane was then stained with Coomassie Brilliant Blue R250 (Sigma-Aldrich) and dried. Afterwards, a single band corresponding to *NbNifH* was excised and submitted to Proteome Factory AG for sequence analysis. An ABI Procise 491 Protein Sequencer (Applied Biosystems) was used for automated N-terminal sequencing according to the manufacturer's protocol.

6.5. Peptide mass fingerprinting

SDS-PAGE of 10 μ g total protein from the concentrated *NbNifH* fraction was run and stained with Coomassie Brilliant Blue R250 (Sigma-Aldrich). As shown in Supplemental Figure 1, bands corresponding to supposed *NbNifH*, contaminants, and degradation products were manually excised from gels and stored in Milli-Q H₂O until analysis. The proteomic analysis was performed by the Proteomics Unit of CAI Técnicas Biológicas (Universidad Complutense de Madrid), as described below.

Proteins selected for analysis were in-gel reduced, alkylated, and digested with trypsin according to Sechi and Chait, 1998. Briefly, the samples were reduced with 10 mM dithioerythritol in 25 mM ammonium bicarbonate for 30 min at 56°C and subsequently alkylated with 25 mM iodoacetamide in 25 mM ammonium bicarbonate for 15 min in the dark. Finally, samples were digested with 12.5 ng/ μ l sequencing grade trypsin (Roche) in 25 mM ammonium bicarbonate (pH 8.5) overnight at 37°C.

After digestion, the supernatant was collected and 1 μ l was spotted onto a MALDI target plate and allowed to air-dry at RT. Then, 0.6 μ l of a 3 mg/ml of α -cyano-4-hydroxycinnamic acid matrix (Sigma-Aldrich) in 50% acetonitrile was added to the dried peptide digest spots and allowed again to air-dry at RT.

MALDI-TOF MS analyses were performed in a 4800 Plus Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems). The MALDI-TOF/TOF operated in positive reflector mode with an accelerating voltage of 20,000 V. All mass spectra were calibrated internally using peptides from the autodigestion of trypsin.

For protein identification SwissProt DB 2016_10 (552884 sequences; 197760918 residues) without taxonomy restriction and Home DB were searched using MASCOT 2.3 (www.matrixscience.com) through the software Global Protein Server v 3.6 (ABSciex). Search parameters were:

- Carbamidomethyl Cysteine as fixed modification and oxidized methionine as variable modification
- Peptide mass tolerance, 50 ppm (PMF)
- 1 missed trypsin cleavage site

In all protein identification, the probability scores were greater than the score fixed by mascot as significant with a p-value < 0.05.

6.6. Fe protein activity determination

Fe protein activity of *NbNifH* preparations was analysed by the acetylene to ethylene reduction assay. Fe protein was added to a 600 µl ml reaction mixture containing 13 µM MoFe protein (Fe protein to MoFe protein molar ratio 25:1) and an ATP-regenerating mixture (1.23 mM ATP, 18 mM phosphocreatine, 2.2 mM MgCl₂, 3 mM DTH, and 40 µg of creatine phosphokinase, 22 mM Tris-HCl pH 7.5) in a 9 ml serum vial sealed with a rubber stopper and flushed with 100% argon. After injection of 500 µl acetylene (Contse S.A.) the vial was incubated at 30°C for 15 min with shaking (Shah and Brill, 1973). The reaction was stopped by adding 100 µl of 8 M NaOH.

Ethylene formation was measured in a Shimadzu GC-2014 gas chromatograph (Shimadzu Corporation) equipped with a PoraPak N 80/100 column (Agilent Technologies). Positive control reactions were carried out with Fe protein and MoFe proteins purified from *A. vinelandii* under anaerobic conditions as described by Curatti *et al.* (2007).

6.7. Microscopy Techniques

6.7.1. Light-Microscopy

Protoplast from Arabidopsis and rice cells were placed on a Neubauer counting chamber (Marienfeld-Superior) and imaged using a Zeiss AxioPhot Microscope (Zeiss) equipped

with a Plan-Neofluar 40X/0.75 objective, coupled to a colour CCD Leica DFC 300FX camera (Leica) and LAS software. Bright Field images were processed with LAS software and assembled using Illustrator software (Adobe).

6.7.2. Stereomicroscopy

Rice callus was imaged using a Fluorescent Stereo Microscope Leica MZ10 F (Leica) equipped with a Plan-Apochromat 1.0x/0.125 objective and Leica Application Suite (LAS) v4.3 software (Leica). A GFP Plus filter was set with the following parameters to detect eGFP: excitation wavelength 460 – 500 nm/emission 510 nm. Images were processed with LAS software and assembled using Illustrator software (Adobe).

6.7.3. Confocal Microscopy

Samples were imaged using a Zeiss LSM880 confocal laser scanning microscope (Zeiss) equipped with a Plan-Apochromat 40X/1.2 water-immersion or Plan-Apochromat 63X/1.2 oil-immersion objective and ZEN 2.6 Black software (Zeiss). The excitation laser lines wavelength/emission bands used with PMT detectors were as follows: GFP (488 nm/493 to 556 nm), BFP (405 nm/410 to 529 nm), mCherry (561 nm/595 to 640 nm), and chlorophyll autofluorescence (633 nm/647 to 721 nm). To avoid overlap between the fluorescence channels, sequential scanning was used when necessary. Super-resolution (AS-SR) imaging was performed with an Airyscan detection unit, sequentially, with excitation as described above and with emission band-pass filters as follows: GFP (488 nm/BP 420-480 + BP 495-550), mCherry (BP 420-480 + BP 495-620), and chlorophyll autofluorescence (633 nm/ BP 570-620 + LP 645).

ZEN 2.6 Black software (Zeiss) was used to process the AS-SR acquired datasets with automatically-determined Airyscan Filtering. The software processes each of the 32 Airy detector channels by performing filtering, deconvolution, and pixel reassignment to obtain images with enhanced spatial resolution and improved signal-to-noise ratio (SNR) (Huff, 2015). Images were post-processed with ZEN 2.6 Blue (Zeiss) and assembled using Illustrator software (Adobe).

6.7.3.1. Tobacco leaves

Leaf discs from 4 to 5-week-old plants were excised and mounted onto microscope slides (Thermo Fisher Scientific) in a PBS solution using High-Performance cover glasses (Zeiss).

6.7.3.2. Arabidopsis and rice protoplasts

Protoplasts were resuspended with gentle pipetting and a small drop mounted onto a microscope slide inside an observation gel. A High-Performance cover glass (Zeiss) was placed on top of the sample. For protoplast image post-processing, Z-axis maximum intensity projections were performed in ZEN 2.6 (Zeiss).

6.7.3.3. Rice leaves

Leaf tissue samples (1 x 10 mm) were fixed with 2% (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) and cut into semithin sections (30 – 40 μ m) using a CM3050S Research Cryostat (Leica Microsystems). The sections were collected on standard glass microscope slides pre-coated with poly-L-lysine and images were captured using an FV1000 laser scanning confocal microscope (Olympus) with illumination at 488 nm (excitation wavelength of eGFP, multiline argon laser).

6.7.4. Immuno-electron microscopy

For analysis of rice tissues, samples were sent to the Servei de Microscòpia (Universitat de Lleida) and Servicio Central de Soporte a la Investigación Experimental (Universidad de Valencia) for preparation and analysis respectively. A detailed protocol is detailed below.

For electron microscopy immune detection, pieces of rice callus (1 mm³) or leaf tissue (1 x 10 mm) or root (10 mm) were fixed with 1% (w/v) glutaraldehyde and 1% (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for 16 – 24 h at 4°C. To enhance the penetration of fixative, samples were initially subjected to a light vacuum until the tissue pieces sank. Once fixed, pieces were washed three times (10 min) with the same buffer and dehydrated in an ethanol series (30 – 100%) before embedding in Lowicryl K4 M resin (Polysciences) in a cold chamber at –20°C to –35°C and inducing polymerization by exposure to ultraviolet light.

Semithin (2 μ m) and ultrathin (70 – 90 nm) sections were prepared using a Reichert–Jung ultra-cut E cryotome (Leica). The semithin sections were stained with Richardson's blue, covered with a drop of DPX slide mounting medium and a coverslip, and observed under a DM4000B microscope (Leica). Images were captured using a DFC300 FX 1.4-MP digital colour camera equipped with LAS v3.8 (Leica). The ultrathin sections were mounted on Formvar carbon-coated gold grids (200 mesh) and incubated for 30 min in blocking buffer for polyclonal antibodies (200 mM Tris–HCl at pH 7.4, 1% Tween-20, 0.1% gelatin (w/v), 1% BSA (w/v)) or monoclonal antibodies (10 mM Tris–HCl at pH

7.4, 0.9% NaCl, 0.05% PEG 20,000, 3% BSA). The grids were then washed in distilled water and incubated overnight at 4°C with primary polyclonal anti-eGFP antibody PA5-22688 (Thermo Fisher Scientific) diluted 1:200 in blocking buffer, or primary monoclonal anti-eGFP antibody 11814460001 (Sigma-Aldrich) diluted 1:250 in blocking buffer. After washing in distilled water, followed by a further 30-min incubation in the appropriate blocking buffer and another wash, the grids were incubated at RT for 1 h with the 15-nm gold-conjugated secondary antibody diluted 1:20 in the appropriate blocking buffer:goat-anti-rabbit IgG for the polyclonal antibody, or EM-grade goat-anti-mouse IgG for the monoclonal antibody (Electron Microscopy Sciences). Finally, the grids were contrasted with 1% (w/v) uranyl acetate in water (20 min) and Reynold's lead citrate (2 min) before observation in Jeol Jem-1010 Transmission Electron Microscope (Jeol Ltd.). A minimum of two grids per treatment and sample were analysed.

Results

7. Use of synthetic biology tools to optimize the production of active nitrogenase Fe protein in chloroplasts of tobacco leaf cells[†]

7.1. Introduction

Nitrogen is a critically important element for all organisms, the element that is required in amounts greater than all others except for carbon, oxygen, and hydrogen. It is very abundant in the atmosphere, which is composed of 78% molecular nitrogen gas; however, neither plants nor animals can obtain nitrogen directly from dinitrogen. Instead, they ultimately depend on a process performed by some archaea and bacteria known as biological nitrogen fixation, where dinitrogen is converted into usable ammonia by the nitrogenase enzyme.

Nitrogen availability often limits the productivity of natural and managed ecosystems, so much so that among all farmer-controlled input factors nitrogen is considered to have the second-largest impact on plant growth, only after water (Martin and Marschner, 1988). To increase crop yield, the use of nitrogen fertilizers has become widespread in developed countries, with enormous undesired environmental consequences that cannot be overlooked. On the other hand, the cost of chemical fertilizers is prohibitive for farmers in the developing countries, which is why there are regions in Sub-Saharan Africa and Southeast Asia where crop yields are extremely low and poverty and hunger prevail (Mueller *et al.*, 2012).

The use of biotechnology to boost biological nitrogen fixation has been considered, for at least the last 45 years, as one of the best strategies to increase crop yield to face population growth and general agronomic crop demand while preserving the environment (Beatty and Good, 2011; Burén and Rubio, 2018; Curatti and Rubio, 2014; Hardy and Havelka, 1975; Ladha and Reddy, 1995; Oldroyd and Dixon, 2014; Rogers and Oldroyd, 2014; Rosenblueth *et al.*, 2018). In this context, one of the proposed biotechnological strategies involves the direct transfer of the prokaryotic genes needed to assemble a functional molybdenum nitrogenase (*nif* genes) into the plant genome, a huge undertaking given the relatively large number of genes in the nitrogenase biosynthetic pathway and

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the extreme oxygen-sensitivity of nitrogenase structural components and the proteins involved in the synthesis of their metal cofactors (Curatti and Rubio, 2014). The molybdenum nitrogenase is composed of an Fe protein (encoded by *nifH*) and a MoFe protein (encoded by *nifD* and *nifK*), which require [Fe-S] clusters for activity. Since the nitrogenase Fe protein is the most sensitive component to oxygen, and its maturation requirements are simpler and better characterized than those of the MoFe protein, it has been chosen as a proof of concept by most previous efforts to express *nif* genes in eukaryotes. There are four genes in *A. vinelandii* required to render active Fe protein (*nifH*, *nifM*, *nifU*, and *nifS*) (Jacobson *et al.*, 1989). NifM is a putative peptidyl-prolyl cis-trans isomerase that assists in the proper folding of NifH (Gavini *et al.*, 2006). NifS is a cysteine desulfurase responsible for the mobilization and transfer of sulfur to NifU (Yuvaniyama *et al.*, 2000; Zheng *et al.*, 1993), where [Fe-S] clusters are synthesized and later donated to the cofactor-less apo-Fe protein for activation (Dos Santos *et al.*, 2004). These are oxygen-sensitive processes in which intermediates are generated and carried directly from one protein to another to protect them from reactive species (Zheng and Dos Santos, 2018).

The most successful efforts in transferring active nitrogenase components to eukaryotes to date have been the production of active NifU and NifH within the mitochondria of yeast cultures growing aerobically, overcoming the oxygen-sensitivity of Nif proteins (López-Torrejón *et al.*, 2016), and the production of active NifB and its product NifB-co which are key to the biosynthesis of the Mo-nitrogenase active-site cofactor, called FeMo-co (Burén *et al.*, 2019). In addition, Allen *et al.* (2017) showed that indeed, 16 *nif*- or *nif*-related genes from *K. oxytoca* could be individually expressed fused to mitochondrial targeting peptides to accumulate in the mitochondrial matrix of tobacco cells. However, no protein activity was demonstrated.

A different location where to engineer nitrogen fixation in plants is chloroplasts, suggested already in 1984 by Merrick and Dixon because of their availability of the immediate products of photosynthesis, NADPH, and ATP, that could satisfy nitrogen fixation demands of energy and reducing power. Moreover, the ammonia produced as a consequence of nitrogen fixation could be easily assimilated into the formation of amino acids by the glutamine synthetase/glutamate synthase pathway, found in plastids. In 1997, Dixon *et al.* expressed *nifH* and *nifM* in tobacco leaf chloroplasts. Even though it was shown that the proteins were stable when targeted to this organelle by transit peptides, they accumulated at levels too low for functionality tests. Using a different strategy, Cheng *et al.* (2005) transformed directly the chloroplast genome and showed that NifH was expressed and accumulated in the chloroplasts of the unicellular alga

Chlamydomonas reinhardtii. Although Fe protein activity was not proven, it could partially substitute for the function of the putative ‘chlorophyll iron protein’ encoded by *chlL*, a protein similar to NifH in both structure and function and also sensitive to oxygen. More recently, Ivleva *et al.* (2016) reported the generation of transplastomic tobacco plants expressing *nifH* and *nifM*. Their approach rendered a high amount of protein accumulating in the leaves of the plants, but NifH activity could only be detected when plants were incubated under low oxygen conditions (10% oxygen), and even then, the activities obtained were very low.

Thus, although it has been possible to express some nitrogenase proteins in chloroplasts, there are still problems to overcome. While direct transformation of the chloroplast would be ideal (Adem *et al.*, 2017), since chloroplast transformation methods are not yet in place for maize and very few successful applications have been developed for rice, we have focused our work on the expression of nuclear-encoded *nif* genes. In this context, increasing *nif* gene expression and optimizing the import of the recombinant proteins into chloroplasts were our main priorities in the pursuit of active Fe protein in chloroplasts using a strategy that can be directly transferred to cereals.

It is well known that the DNA sequence used to encode a protein can have dramatic effects on its expression and accumulation (Gustafsson *et al.*, 2012). mRNA structures near the ribosome binding site were long ago reported to reduce translational initiation (Kozak, 1986), as well as mRNA destabilization by the basal mRNA decay machinery and sequence-specific decay components (Gutiérrez *et al.*, 1999). The exclusion of these elements from synthetic genes, together with premature polyadenylation sites and cryptic intron splice sites, has been described to increase transcription and enhance translation in plants (Jackson *et al.*, 2014). In an attempt to design synthetic *nif* genes with a higher expression and stability, we eliminated these undesired elements from their sequence and had them match codon usage and GC content from the host as much as possible, which is well known to affect recombinant protein expression (Webster *et al.*, 2017).

Another step that could be limiting the availability of Nif proteins in the chloroplast is their import from the cytosol. It requires chloroplast transit peptides (TPs), N-terminal targeting sequences that direct proteins to their correct subcellular compartment and, after import through an ATP hydrolysis-dependent process, are cleaved by the stromal processing peptidase. The transit peptide of the small subunit of RuBisCo, the most commonly used TP, is very efficient in driving most recombinant proteins to the chloroplast stroma, although the N-terminal region of cargo proteins is also critical for TP activity (Shen *et al.*, 2017).

In the work presented here, the generation of synthetically designed genes to maximize Nif protein accumulation and the screening for the optimal chloroplast transit peptide for each Nif protein led to tobacco plants where NifH, M, U, and S accumulated as soluble proteins within the stroma of mesophyll cells and Fe protein activity could be shown for NifH.

7.2. Results

7.2.1. *nif* gene synthetic design

Previous studies had shown that nuclear-encoded *nifH* could not be expressed to high levels in tobacco chloroplasts using its original sequence from *K. oxytoca* (Dixon *et al.*, 1997). The codon adaptation index (CAI) is a simple and effective way of measuring synonymous codon usage bias and the likeliness of success of heterologous gene expression (Sharp and Li, 1987). The sequences of *A. vinelandii nifH*, *nifM*, *nifU*, and *nifS* codon-optimized for their expression in *S. cerevisiae* (López-Torrejón *et al.*, 2016) showed theoretical good optimization also for expression in tobacco, with a CAI of 0.82, 0.84, 0.81, and 0.82, respectively, and no presence of low frequency (<40%) codons (Biologics International Corp codon adaptation index calculator and rare codon analyser). These values strongly indicated that heterologous gene expression in tobacco using the yeast codon-optimized sequences could be successful, especially when compared with the data for original non-optimized *A. vinelandii* genes (CAIs of 0.60, 0.54, 0.55, and 0.53 and presence of 5, 16, 10, and 12 low-frequency codons for *nifH*, *nifM*, *nifU*, and *nifS*, respectively).

We decided to design *nif* synthetic genes specifically optimized for maximum expression in tobacco taking into account, not only codon usage patterns, but also additional parameters such as mRNA stability motifs, hidden stop codons and poly(A) signals that could impact protein production. The web server application Codon Optimization OnLine (COOL) (Chin *et al.*, 2014) was used for this purpose.

The specific parameters of the synthetic design used in this work involved using an individual codon usage (ICU) and a GC content matching that of tobacco coding sequences, both overall and specifically in the third base of codons (GC3) (Nakamura *et al.*, 2000). Moreover, some motifs were actively avoided, like any form of dicotyledonous plant polyadenylation signal, reported RNA instability motifs and repetitions of more than seven bp or A and T strings (Jackson *et al.*, 2014; Ji *et al.*, 2007; Shen *et al.*, 2008) (Supplemental Table 5). Synthetic designed *nif* sequences closely matched the ICU and GC3 content of tobacco compared to the yeast codon-optimized sequences. Furthermore, exclusion motifs and repetitions were effectively removed to a large degree. In contrast,

CAIs values pointed to slightly better performance of yeast codon-optimized sequences (Supplemental Table 6).

Figure 9. Effect of using synthetically designed *nif* genes optimized for tobacco.

(A) Schematic representation of the constructs used in *N. benthamiana*-transient expression assays leading to *nif* genes and eGFP simultaneous expression. **(B)** Western blot analysis of NifH, NifM, NifU, and NifS accumulation when expressed from yeast codon-optimized genes or synthetically designed genes optimized for expression in tobacco. Negative control refers to *N. benthamiana* leaves agroinfiltrated with *Agrobacterium* transformed with an empty vector. Expected sizes are Twin-Strep NifH: 35.3 kDa, NifM: 32.3 kDa, NifU: 34.4 kDa, and NifS: 44.3 kDa. **(C)** Western blot analysis of eGFP accumulation. Expected size: 27 kDa. **(D)** Quantification of Nif/eGFP accumulated protein. Mean $\pm$ SD (n=3 biological replicates). The given value for each Nif protein band was normalized using the value corresponding to the eGFP signal in the same lane. 'Ratio' refers to the ratio between Nif protein levels achieved using the tobacco synthetic design and the yeast codon-optimized sequences. * Represents Student's test significant differences ($P < 0.05$); ** represents Student's test highly significant differences ($P < 0.01$).

We generated multigenic constructs comprised of a transcriptional unit where the p35S promoter directs the expression of each yeast codon-optimized or synthetically designed *nif* gene and a second transcriptional unit where the p35S directs the expression of eGFP. Transient expression assays were performed to gauge the effect of the synthetic gene

design (Figure 9A). The accumulation of Nif proteins (Figure 9B) was compared, using the level of eGFP to normalize for differences in overall recombinant protein expression (Figure 9C). The results showed increased accumulation of all four Nif proteins when using the constructs with the synthetically optimized genes, validating the parameters established for their re-design (Figure 9D). The increase was specifically significant in the case of NifH, which accumulated 170 times more when the synthetic gene was used for expression (Figure 9D).

Figure 10. Time course accumulation of synthetically designed *nif* genes optimized for tobacco.

(A) Schematic representation of the construct used in *N. benthamiana*-transient expression assays leading to *nif* genes and eGFP simultaneous expression. **(B)** Time course of NifH (tobacco synthetic design) and eGFP accumulation after *N. benthamiana* agroinfiltration. N.C. refers to 'negative control', *N. benthamiana* leaves agroinfiltrated with *Agrobacterium* transformed with an empty vector. Expected sizes are Twin-Strep NifH: 35.3 kDa, NifM: 32.3 kDa, NifU: 34.4 kDa, NifS: 44.3 kDa, and eGFP: 27 kDa. **(C)** Quantification of Nif/eGFP accumulated protein. 'n.d.' refers to 'non-detected'.

We followed the accumulation of NifH, NifM, NifU, and NifS through a time-course from the moment of agroinfiltration to optimize the time of tissue collection, and found that protein levels steadily increased until the end of the dark period of the third day (Figure 10). This is in accordance with the standard method for transient expression in *N. benthamiana* (Li, 2011; Naim *et al.*, 2012), and shows that the oxygen derived from photosynthesis taking place during the light period of plant growth did not lead to a decrease in NifH polypeptide levels or any of the other Nif proteins accumulation.

7.2.2. Optimization of chloroplast targeting

In an effort to find the TP that best suited each specific Nif protein in study, 12 different TPs previously used in the literature to drive protein import into the chloroplast stroma were fused to each Nif protein, namely: *AtAROA*_{TP} (Klee *et al.*, 1987), *AtBCCP1*_{TP}, *AtCAB6*_{TP}, *AtDNAJ8*_{TP}, *AtGLTB2*_{TP}, *AtTOCC*_{TP} (Lee *et al.*, 2008), *NtRBS*_{TP} (Mazur and Chui, 1985), *AtRBS1A*_{TP} (Lee and Hwang, 2011), *PsRBS2*_{TP} (Coruzzi *et al.*, 1984), *AtRCA*_{TP} (Kim *et al.*, 2010), *NtSIR*_{TP} (Yonekura-Sakakibara *et al.*, 1998) and *synRBS*_{TP} (Engler *et al.*, 2014) (details in Supplemental Table 7) and analysed for protein accumulation upon agroinfiltration of *N. benthamiana* leaves.

The use of TP sequences that extend beyond the peptidase cleavage site (complete TP, c) would produce recombinant proteins with additional amino acids left at the N-terminus after TP cleavage. Since this can affect protein functionality, shorter TP versions were generated by cloning only the TP coding sequence up until the cleavage recognition site predicted by ChloroP 1.1 server (Emanuelsson *et al.*, 1999) (minimal TP, m). Constructs for expression of cytosolic Nif proteins (protein size control) and Nif fusions to complete and minimal versions for all 12 candidate TPs were generated (Figure 11A).

Figure 11. Screening of chloroplast transit peptides fused to NifH, NifM, NifU, and NifS proteins. (A) Schematic representation of the constructs used in *N. benthamiana*-transient expression assays leading to TP-*nif* expression. (B-E) Western blot analysis of the product of expressing 12 TPs in full version (c, complete) and up until their predicted cleavage site by stromal peptidase (m, minimal) fused to the N-terminus of NifH (B), NifM (C), NifU (D), and NifS (E). Negative control refers to *N. benthamiana* leaves agroinfiltrated with *Agrobacterium* transformed with an empty vector. Cytosolic versions were expressed for Nif correct size control, expected sizes are Twin-Strep NifH: 35.3 kDa, NifM: 32.3 kDa, NifU: 34.4 kDa, and NifS: 44.3 kDa. For TP sizes, see Table S2. Green dots mark Nif proteins with the same electrophoretic mobility as their cytosolic controls, consistent with complete cleavage of the TP. The triangles in red mark the expected mature form of the protein.

Obtaining Nif proteins with the same electrophoretic mobility as the cytosolic versions (Figure 11B-E, green dots, and Supplemental Table 8) suggested correct import and cleavage of the TP, generating a chloroplast-recombinant Nif protein with none, or very few, residual amino acids in the N-terminus. Nif proteins with an increased size coinciding with that of the Nif protein plus the TP suggested neither import nor cleavage had taken place. The appearance of two bands could be indicative of partial import and cleavage (Figure 11B-E and Supplemental Table 8).

Overall, the use of a minimal TP resulted in a higher motility band against the complete TP (Figure 11B-E). However, in cases such as *AtmAROATP*, *PsmRBS2TP*, *NtmRBS_{TP}*, and *SymRBS_{TP}* fusions, the minimal TP seemed to have lost its ability to import the Nif protein into the chloroplast as the band size coincided with that of the Nif protein plus the TP. It is noteworthy that only two minimal TPs, *AtmGLTB2_{TP}* and *AtmBCCP1_{TP}*, performed properly for all Nif proteins.

Figure 12. Confocal microscopy localization of stroma-targeted Nif proteins.

(A) Schematic representation of the constructs used in *N. benthamiana*-transient expression assays leading to Nif localization. (B) Confocal microscopy images of Nif proteins fused to tGFP. N.C. corresponds to ‘negative control’, a non-agroinfiltrated leaf. The three columns show the individual signals for tGFP (green, on the left) and chlorophyll autofluorescence (red, in the centre). On the right, the overlap of both signals (merge).

One of the best-performing TPs for each specific Nif protein was selected (*AtmRCA_{TP}* for NifH, *AtmBCCP1_{TP}* for NifM, *AtmTOCC_{TP}* for NifU, and *AtmCAB6_{TP}* for NifS), and Nif proteins were fused in their C-terminus to turbo GFP (tGFP) in order to visualize them within tobacco leaf mesophyll cells (Figures 12A and 13A). NifM, NifU, and NifS all showed a diffuse pattern of expression co-localized with chlorophyll, supporting correct targeting to the chloroplast stroma (Figure 12B). However, for NifH, we could observe tGFP concentrated in foci located within or around the chloroplasts, and that excluded chlorophyll. Chloroplast purification assays were performed in leaf tissue expressing Nif or Nif-tGFP fusion proteins. NifM, NifU, and NifS, as well as their corresponding tGFP fusions, were confirmed to accumulate mostly in the stroma soluble fraction, while the majority of NifH and NifH-tGFP appeared associated with membranes (Figure 13B). The similar behaviour of Nif and Nif-tGFP fusions validated the results obtained by microscopy, confirming that the fusion of the reporter protein did not lead to an artifactual localization and a clear indication of a NifH solubility problem. Anti-isocitrate dehydrogenase (IDH) antibody was used to check for the presence of mitochondrial contamination in the isolation of chloroplasts (Figure 13B), and the negative results obtained suggest that the NifH-tGFP foci observed do not correspond to mitochondria, but rather, to chloroplast-associated bodies.

(A)

(B)

Figure 13. Subcellular fractionation confirming the localization of stroma-targeted Nif proteins.

(A) Schematic representation of the constructs used in *N. benthamiana*-transient expression assays leading to Nif localization. (B) Chloroplast purification assays. Total proteins extract before chloroplast purification (CE). Intact chloroplasts were isolated and then broken to separate the soluble (S) and membrane-associated (P) fractions. Control refers to *N. benthamiana* plants agroinfiltrated with *Agrobacterium* transformed with an empty vector. Proteins were resolved by SDS-PAGE and detected by Western blot. Expected sizes are Twin-Strep NifH: 35.3 kDa, NifM: 32.3 kDa, NifU: 34.4 kDa, and NifS: 44.3 kDa (27 kDa more in the case of tGFP fusions), RbcL: 52.7 kDa, Actin-11: 41.6 kDa, and IDH: 39 kDa. Note that the IDH blot was re-probed after stripping anti-RuBisCO and some signal remains (upper band).

To elucidate the nature and origin of these NifH-accumulating foci we co-expressed the *AtmRCA_{TP}*-NifH-tGFP fusion with a *syncRbs_{TP}*-BFP, that accumulates in the stroma (Figure 14A). The co-localization of tGFP and BFP in a punctuate pattern (Figure 14B, arrows) supports that after NifH is imported into the chloroplast, it is included in vesicles together with other stroma soluble proteins, like BFP.

Figure 14. Expression of *nifH* alone leads to protein accumulation in foci originating from chloroplasts.

(A) Schematic representation of the constructs used in *N. benthamiana*-transient expression assays leading to NifH and stromal BFP co-localization. (B) Confocal microscopy images of NifH fused to tGFP and stromata targeted BFP. N.C. corresponds to ‘negative control’, a non-agroinfiltrated leaf. The four columns show the individual signals for tGFP (green), BFP (blue), and chlorophyll autofluorescence (red). On the right, the overlap of the three signals (merge). Note arrows pointing to the co-localization of green and blue signals in a punctuate pattern.

However, when both *nifH* and *nifM* genes were co-expressed, fused to *eGFP* and *mCherry*, respectively (Figure 15A), NifH punctuated accumulation disappeared and could only be found diffuse within the chloroplast stroma (Figure 15B). This observation was corroborated by chloroplast purifications from cells co-expressing *nifH*, *nifM*, *nifU*, and *nifS*, where NifH could now be found mostly in the soluble protein fraction coming from the stroma (Figure 16A, C). All these data suggest that when expressed alone, NifH

is accumulating in aggregates of immature protein that become included in vesicles, and that NifM is required for NifH to fold correctly and become soluble in the stroma.

Figure 15. NifM is necessary for the accumulation of soluble NifH in the stroma.

(A) Schematic representation of the construct used in *N. benthamiana*-transient expression assays leading to NifH and NifM co-localization. (B) Confocal microscopy images of co-expressed NifH and NifM proteins fused to tGFP and mCherry respectively. N.C. corresponds to ‘negative control’, a non-agroinfiltrated leaf. The four columns show the individual signals for tGFP (green), mCherry (blue), and chlorophyll autofluorescence (red). On the right, the overlap of the three signals (merge). Note the co-localization of green and blue signals in a diffuse pattern within the chloroplast.

7.2.3. Tobacco plants co-expressing *nifH*, *nifM*, *nifU*, and *nifS* exhibit Fe protein activity

Once optimal conditions had been established for Nif protein accumulation and import to the chloroplast stroma, a multigenic construct was assembled with the tobacco synthetic *nif* genes and their selected TPs (Figure 16A). For protein purification, a single vector containing *nifH*, *nifM*, *nifU*, and *nifS* in addition to the silencing suppressor P19 was used. Following this approach in transient expression assays assures that all cells infected by *Agrobacterium* will get the complete gene set, and the purified Nif protein will have been expressed and matured in cells containing simultaneously all of them.

Tobacco leaves were collected at the end of the dark period of the third day after agroinfiltration when all four *nif* genes were expressed (Figure 16B) and Nif proteins accumulated in a soluble form within the chloroplast stroma (Figure 16C).

Figure 16. Co-expression of *nifH*, *nifM*, *nifU*, and *nifS* in chloroplasts of *N. benthamiana*.

(A) Schematic representation of the multigenic construct used in *N. benthamiana*-transient expression assays leading to NifH, NifM, NifU, and NifS co-expression. **(B)** Western blot analysis of the agroinfiltrated tissue using antibodies targeting Strep-tagged NifH, NifM, NifU, and NifS as indicated. Control refers to *N. benthamiana* leaves agroinfiltrated with *Agrobacterium* transformed with an empty vector. Expected sizes are Twin-Strep NifH: 35.3 kDa, NifM: 32.3 kDa, NifU: 34.4 kDa, and NifS: 44.3 kDa. **(C)** Chloroplast purification assays. Total proteins extract before chloroplast purification (CE). Intact chloroplasts were isolated and then broken to separate the soluble (S) and membrane-associated (P) fractions. Control refers to *N. benthamiana* plants agroinfiltrated with *Agrobacterium* transformed with an empty vector. Proteins were resolved by SDS-PAGE and detected by Western blot. Expected sizes are RbcL: 52.7 kDa; Actin-11: 41.6 kDa and IDH: 39 kDa. The triangles in red mark the mature form of the protein.

NifH from *N. benthamiana* chloroplasts was purified using Twin-Strep tag for affinity chromatography (Figure 17A-D). Various bands could be detected in the elution fraction after NifH purification. Peptide mass fingerprinting identified them as a RuBisCo subunit

(probably a contaminant), the maturase NifM, and NifH degradation products or multimers (Supplemental Figure 1). NifH migrated as a close double band in SDS-PAGE (Figure 17C-D); however, it was not due to N-terminal degradation or unspecific cleavage of the TP by the stromal peptidase, since Edman degradation of the purified protein confirmed a unique N-terminal sequence starting at the predicted location for TP cleavage (Supplemental Figure 2). NifH doublets not related to post-translational modifications have been previously observed both in diazotrophic bacteria and in heterologous hosts (Howard *et al.*, 1986; Roberts *et al.*, 1978). Detection after Western blots using anti-NifM showed a band that corroborated some degree of co-elution due to interaction with NifH. The same was observed using an anti-NifU antibody (Supplemental Figure 3), suggesting the on-going maturation of a subpopulation of the Fe protein in the samples.

Figure 17. Purification of NifH from chloroplasts of *N. benthamiana* co-expressing *nifH*, *nifM*, *nifU*, and *nifS*.

(A) Schematic representation of the multigenic construct used in *N. benthamiana*-transient expression assays leading to NifH purification. (B) Coomassie staining illustrating the Strep-tactin purification procedure of *A. vinelandii* NifH expressed in leaves of *N. benthamiana*. CFE, cell-free extract (soluble fraction following centrifugation and filtering of disrupted leaf tissue); FT, Strep-Tactin flow-through fraction; W, wash fraction; E, biotin-eluted fraction; E_c, concentrated eluate. (C-D) Western blot analysis of the Strep-Tactin purification procedure. Antibodies targeting Strep-tag (C) or NifH (D) were used for visualization of the purified NifH protein.

Pure NifH isolated from chloroplasts exhibited a light yellowish/brownish colour, characteristic of Fe-S proteins. Its activity was determined by using *in vitro* acetylene reduction assays in which the *N. benthamiana* NifH was incubated with MoFe protein purified from *A. vinelandii*. *N. benthamiana* Fe protein activity varied among preparations, supporting a maximum specific activity of 189 nmol of ethylene formed per

minute and milligram of MoFe protein at a *N. benthamiana* Fe protein/*A. vinelandii* MoFe protein molar ratio of 25 (Table 1).

Table 1. Maximum nitrogenase MoFe protein activity supported by *N. benthamiana* NifH and *A. vinelandii* NifH. Highest result obtained in three positive independent biological experiments. *Defined as nmol of C₂H₄ formed per minute and milligram of MoFe protein (mean $\pm$ s.d., n=2 technical replicates).

Source of Fe protein	Acetylene reduction assay *	Fe protein to MoFe protein molar ratio
<i>N. benthamiana</i> NifH	189 $\pm$ 5	25
<i>A. vinelandii</i> NifH	1194 $\pm$ 65	25

Although the activities detected are low compared to those of *A. vinelandii* NifH (a maximum of 16% activity was obtained in chloroplast NifH compared to *A. vinelandii* NifH), these results indisputably show that tobacco chloroplasts are capable of accumulating functional Fe protein, at least when its maturation and [Fe-S] cluster assembly is separated in time from the process of photosynthesis (activity is shown for samples collected at the end of the dark period). Future efforts will be directed at designing strategies to protect NifH [Fe-S] clusters from oxygen as a means to increase its activity, and to test the effect of photosynthesis during the light period on the protein metal content and activity. The influence of the Twin-Strep-tag cannot be considered as a factor accounting for the lower chloroplast NifH activity, since purified *A. vinelandii* Strep-tagged NifH showed almost the same activity as the non-tagged version (Eseverri *et al.*, 2020).

In the chloroplast, other pathways exist for Fe-S protein assembly, like the sulfur mobilization pathway (SUF), raising the question of the requirement for NifU and NifS. To test their role in the assembly and delivery of [Fe-S] clusters to NifH within the chloroplast, we performed the same experiments in *N. benthamiana* leaves expressing only *nifH* and *nifM* (Figure 18A). NifH from *N. benthamiana* chloroplasts was successfully purified using Twin-Strep tag for affinity chromatography (Figure 18B-D). However, in the absence of NifU and NifS, no Fe protein activity could be detected, suggesting that the chloroplast assembly and transfer factors are not able to provide enough [Fe-S] clusters to result in functional Fe protein. These results open a feasible avenue of work on plastid engineering for the generation of nitrogen-fixing plants.

Figure 18. Purification of NifH from chloroplasts of *N. benthamiana* co-expressing *nifH* and *nifM*. (A) Schematic representation of the multigenic construct used in *N. benthamiana* transient expression assays leading to NifH purification. (B) Coomassie staining illustrating the Strep-tactin purification procedure of *A. vinelandii* NifH expressed in leaves of *N. benthamiana*. CFE, cell-free extract (soluble fraction following centrifugation and filtering of disrupted leaf tissue); FT, Strep-Tactin flow-through fraction; W, wash fraction; E, biotin-eluted fraction; Ec, concentrated eluate. (C) Western blot analysis of the Strep-tactin purification procedure using antibodies targeting Strep-tag. The expected sizes are Twin-Strep NifH: 35.3 kDa. (D) Western blot analysis of the Strep-tactin purification procedure using antibodies targeting NifH.

7.3. Discussion

7.3.1. Optimization of *nif* gene synthetic design and chloroplast targeting

The use of *nif* genes synthetically designed to maximize expression in tobacco led to much higher protein accumulation than the use of the yeast-optimized versions (Figure 9), although the CAIs for these synthetically designed genes (0.76, 0.77, 0.79 and 0.72 for *nifH*, *nifM*, *nifU*, and *nifS*, respectively) were in all cases slightly lower than those of the ones optimized for yeast (Supplemental Table 6). Adapting the codon usage frequencies of a foreign sequence to that of the host organism was not enough to guarantee high gene expression and protein accumulation, and the advantage obtained by the inclusion of more parameters in the design of the synthetic genes clearly outweighed the penalty of a small decrease in the CAI. More complex approaches, like the one used here, should be considered when the goal is to achieve high levels of recombinant protein. Note that using a complete synthetic design allowed us to obtain NifH protein levels 170 times higher than those achieved by using simply a codon-optimized sequence (Figure 9D), amounts that were enough for Fe protein purification and activity tests. Although no significant Nif degradation products could be detected linked to the light period (Figure

10B), oxygen damage could still affect Nif proteins in the form of cluster loss, which remains to be studied.

The use of the nuclear transformation approach requires that the protein import into the plastid must be optimized as well. The use of TPs is a strategy that has already been used in many biotechnological applications to direct recombinant proteins to the chloroplast stroma (Van den Broeck *et al.*, 1985; Hoppmann *et al.*, 2002; Kavanagh *et al.*, 1988; Kim *et al.*, 2009, 2010; Lee *et al.*, 2008; Zhong *et al.*, 2003). However, we found that most of the TPs described in the literature included a sequence longer than the transit peptide, leaving scar amino acid residues in the N-terminus of the imported proteins after TP cleavage. We used bioinformatics tools to predict the minimal sequence required to direct recombinant proteins to the subcellular compartment and that would leave the minimal scar after cleavage (ChloroP 1.1 server (Emanuelsson *et al.*, 1999)). In most cases, the extended version of the TP did not work better than the minimal version, thus having a longer sequence does not seem beneficial, and only the minimal versions should be used in the future, as they comprise cleaner and as efficient tools for recombinant protein targeting (Figure 11).

As expected, TPs showed different behaviour when fused to different Nif proteins, highlighting the influence of cargo proteins in import efficiency (Shen *et al.*, 2017). Although some TPs, like the one from the biotin carboxyl carrier protein (*AtmBCCP1_{TP}*) and ferredoxin dependent glutamate synthase (*AtmGLTB2_{TP}*), seemed to perform well in all four cases (Figure 11), diversifying the TPs used is desirable for synthetic biology efforts in which multiple *nif* genes are to be co-expressed. In this case, avoiding the repetition of TPs would be beneficial since homologous recombination between repeated sequences is a major cause of genomic rearrangements, producing insertion/deletions and translocations.

Targeting of proteins to mitochondria and chloroplasts is generally highly specific, but an increasing number of proteins imported dually into both organelles have been described (Peeters and Small, 2001). In this work, chloroplast purification assays clearly show that the selected TPs drive Nif proteins primarily to the chloroplast, and microscopy of the tGFP fusions confirms specific organelle targeting.

The observation of cleavage of the transit peptide in a Western blot is often indicative of correct localization of the recombinant protein but should be confirmed. Our results indicate that when expressed alone, NifH is imported into the organelle and the TP is correctly processed. However, NifM co-expression was necessary to lead to soluble stroma NifH accumulation. With a few exceptions, the production of an active NifH is

known to depend on NifM (Howard *et al.*, 1986), and our data suggest that this requirement extends to plant cells.

The observed compartmentalization of NifH in the absence of NifM is reminiscent of the bodies formed in plant cells during piecemeal chlorophagy, a process that involves the formation of small vesicle-like structures that bud from plastids and are delivered to the vacuole for degradation (Otegui, 2018). All the gathered information suggests improper folding of the NifH protein when expressed alone, which triggers this or another similar degradation pathway within the plant cell.

7.3.2. Purification of active NifH from tobacco chloroplasts

The recovery of relatively high Fe protein activity expressed in a plant is a major accomplishment. The activity obtained is still lower than that of endogenous *A. vinelandii* NifH or the one obtained from yeast mitochondria (López-Torrejón *et al.*, 2016), so future efforts should be directed at fine-tuning its maturation and/or preventing damage of its [4Fe-4S] cluster by oxygen. As NifH is extremely sensitive and irreversibly damaged by oxygen (Shah *et al.*, 1973), finding its functional form within the chloroplast indicates this organelle is able to provide an anoxygenic (enough) environment for the enzyme, at least during the night period, after which tissue collection took place. This is in accordance with the observation that *C. reinhardtii* mutants in *chlL*, an essential chloroplast gene required for chlorophyll biosynthesis in the dark, could be partially complemented with the *K. pneumoniae nifH* (Cheng *et al.*, 2005).

The co-elution of NifM with NifH could be interpreted in the light of NifM-mediated maturation of NifH (Supplemental Figure 3A). It is possible that in our transient expression system the time in which the two proteins cohabitate within the chloroplast is too short for complete maturation of NifH and in the future, it will be important to test whether, in stable transformants, this interaction diminishes leading to a higher activity of (properly matured) NifH. The co-elution of NifU with NifH points to an interaction between these two proteins, suggestive of an actual cluster transfer occurring between the two (Supplemental Figure 3B). In fact, *A. vinelandii* NifU isolated from *N. benthamiana* chloroplasts was found to be functional in transferring [4Fe-4S] clusters to apo-NifH in *in vitro* assays (Eseverri *et al.*, 2020).

Similarly, recent experiments co-expressing NifU and NifS targeted to chloroplast showed a fraction of NifS co-purifying with NifU, which carried a permanent [2Fe-2S] cluster per dimer (Eseverri *et al.*, 2020). However, when NifU was expressed alone, its

Fe content was much lower, suggesting that NifS was also active in transferring the sulfur necessary for the assembly of [Fe-S] clusters in the chloroplast.

The lack of NifH activity when it was only co-expressed with *nifM*, but not *nifU* or *nifS*, suggests that the chloroplast machinery cannot efficiently transfer [4Fe-4S] clusters to NifH. We cannot completely exclude, however, the possibility that the effect of NifU and NifS is indirect and being asserted by other Fe-S protein that in turn has a positive effect on NifH, or by reducing the possible stress caused by excessive free iron. Previously, only a very low and difficult to quantify NifH activity had been detected in transplastomic plants expressing NifH and NifM after incubation of whole plants under a low oxygen atmosphere (Ivleva *et al.*, 2016). By using a different technical approach and incorporating NifU and NifS, we have obtained significant levels of activity for NifH in plants grown with no manipulation of the atmospheric oxygen content, demonstrating that the nuclear-encoding of plastid-targeted *nif* genes constitutes a worthy strategy for engineering nitrogenase assembly in the plastids of higher plants.

8. Optimization of nitrogenase Fe protein biosynthesis in rice: Arabidopsis transit peptides as tools for trait engineering in rice plastids[‡]

8.1. Introduction

Plastids are organelles found within plants and algae cells. They are enclosed by two membranes, the outer and inner envelope membranes, that separate the cytosol from the stroma. Phylogenetic analyses show that plastids originated from the endosymbiosis of a cyanobacterial ancestor (Yoon *et al.*, 2004). In higher plants, all plastids derive from undifferentiated pro-plastids that develop into different morphological and functional types in a developmental and tissue-specific manner and which have distinct functions mediated by different proteomes (Daher *et al.*, 2010; Kleffmann *et al.*, 2004; Siddique *et al.*, 2006). Among them are chloroplasts, housing green chlorophyll pigments that mediate photosynthesis; leucoplasts, colourless plastids found in non-photosynthetic tissues which include starch-storing amyloplasts and lipid-storing elaioplast; and chromoplasts, responsible for the carotenoid pigments found in flowers and fruits (Wise, 2007). Plastids carry out a diverse set of metabolic functions, both in assimilation carbon, sulfur, and nitrogen, and in the production of lipids, amino acids, plant hormones, and many secondary metabolites (Waters and Pyke, 2005).

Plastid genomes of land plants have suffered a huge reduction by transferring genes to the nuclear genome (Kleine *et al.*, 2009). More than 95% of chloroplast proteins are nucleus-encoded and synthesized on cytosolic ribosomes (Shi and Theg, 2010), which account for around 2,100 in *A. thaliana* and up to 4,800 proteins in *O. sativa* (Richly and Leister, 2004). Such transfer underscores the importance of differentially expressing nuclear genes encoding plastid proteins according to tissue and developmental stage (Kleffmann *et al.*, 2004) and the regulation of the import of proteins from the cytosol (Chu *et al.*, 2020; Chu and Li, 2018; Jarvis and López-Juez, 2013), as these processes ultimately determine plastid biogenesis and plant development.

Nuclear-encoded plastid-targeted proteins are synthesized as precursors containing an N-terminal targeting sequence called the transit peptide (Chua and Schmidt, 1978; Dobberstein *et al.*, 1977; Kleffmann *et al.*, 2004). TPs bind the targeting receptors

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associated with the translocons at the outer/inner envelope membrane of plastids (TOC and TIC, respectively) and direct the import of precursor proteins across the organellar double membrane (reviewed in Chotewutmontri *et al.*, 2017). After precursor translocation into the stroma, the TP is cleaved off by the Stromal Processing Peptidase (SPP), and the mature protein folds into its native conformation and stays within the stroma or continues its journey to the thylakoids (Richter and Lamppa, 2002).

TPs are necessary and sufficient for protein import into plastids; the removal of the gene part coding for it renders a protein that remains in the cytosol, while the addition of the TP to a non-plastid protein can direct it to the organelle (Bruce, 2001). The length of TPs is very heterogeneous, ranging from 20 to 150 amino acids, depending on the position of the processing site by the SPP (Balsera *et al.*, 2009). TP primary sequence alignments have been used to identify conserved motifs responsible for specific import, but their conservation, amino acid composition, and organization are very reduced (Bruce, 2000).

Contrarily, the general import pathway seems to be very conserved, as evidenced by the fact that most TOC/TIC components are maintained in all land plant species (Tello-Ruiz *et al.*, 2016). Their expression is highly regulated. In *A. thaliana*, the receptors for the initial pre-protein binding, atToc59 and atToc33 are highly expressed in green tissues, while atToc132/120 and atToc34 are uniformly expressed across all tissues (reviewed in Chu and Li, 2018). Arabidopsis and rice TIC/TOC systems are very conserved in terms of members of the complex and their specific expression patterns (Supplemental Figure 4).

Recent data suggest that the availability of TOC isoforms that bind with different specificity to each transit peptide can determine the import of precursor proteins (Chu *et al.*, 2020; Chu and Li, 2018; Dutta *et al.*, 2014; Jarvis and López-Juez, 2013). *In vitro* binding assays showed that atToc159 binds preferentially to transit peptides of photosynthetic proteins and atToc132 binds transit peptides of non-photosynthetic proteins (Inoue *et al.*, 2010; Ivanova *et al.*, 2004; Smith *et al.*, 2004). Swapping of TPs among proteins confirmed their role in the determination of plastid-type import selectivity (Wan *et al.*, 1996; Yan *et al.*, 2006). The precursor-specificity of the pathway seems to be restricted to the TOC, since TIC components were found to associate equally with all kinds of plastid imported proteins (Chen *et al.*, 2002; Jarvis, 2008; Kovacheva *et al.*, 2005).

Detailed characterization of different TPs, and their specific plastid-type import preference, is of the utmost importance to engineer useful traits determined by nuclear-encoded recombinant plastid proteins, as it is the case in the engineering of NifH

biosynthetic pathway in rice plastids. Here we show that the TPs of four *Arabidopsis* proteins related to photosynthetic, photo-protection processes, and fatty acid biosynthesis direct highly efficient import of a recombinant reporter protein to chloroplasts and leucoplasts. These TPs also show 100% import efficiency in rice callus and the shoot and root tissues of rice plants, suggesting that translocon components mediating import of the reporter protein are distributed equally in these tissues or that the TPs bind promiscuously to predominant TOC/TIC isoforms.

Further work on the optimization of NifH biosynthetic pathway in rice plastids consisted in the improvement of Nif protein production and accumulation. Protoplast transient expression assays showed the importance of codon usage and GC content criteria in gene optimisation, and the requirement of NifM for NifH soluble protein accumulation in the stroma of rice cells. Although our efforts to generate *nif*-expressing stable rice lines had a negative outcome, further efforts should consider these findings in the design phase of further iterations of the DBTL cycle.

8.2. Results

8.2.1. *nif* gene synthetic design for rice

In an attempt to translate the knowledge gained from the experiments in tobacco to the engineering of nitrogenase Fe protein biosynthesis in the staple crop rice, we decided to start by focusing our efforts on maximizing *nif* gene expression within the context of rice cells. For that, we tried two different approaches. On the one hand, we ordered the synthesis of rice codon-optimized sequences for *A. vinelandii nifH*, *nifM*, *nifU*, and *nifS* to GenScript (Piscataway), which, using their proprietary algorithm, designed versions with CAI values of 0.90, 0.89, 0.87 and 0.87, respectively (Biologics International Corp codon adaptation index calculator and rare codon analyser).

On the other hand, in an effort to enhance *nif* gene optimization specifically for rice, we analyzed the *nifH*, *nifM*, *nifU*, and *nifS* sequences provided by GenScript in search of mRNA instability motifs, monocotyledonous plant polyadenylation signals, and AT strings (Jackson *et al.*, 2014; Ji *et al.*, 2007; Shen *et al.*, 2008) (Supplemental Table 9), and partially removed them by PCR (Supplemental Table 10). This approach allowed us to test the influence of these undesired motifs in the accumulation of Nif proteins. As part of our previous work in nitrogenase engineering, we also had available the endogenous *A. vinelandii nifH*, *nifM*, *nifU*, and *nifS* gene sequences, yeast codon-optimized sequences (as used in López-Torrejón *et al.*, 2016) and tobacco synthetic designs (as used in Section 7).

Therefore, we compared protein accumulation in rice derived from those five different *nif* gene variants. We generated multigenic constructs comprised of a transcriptional unit where the pZmUbi1 promoter directed the expression of each *nif* variant and a second transcriptional unit where the same promoter directed the expression of eGFP. Transient expression assays in *O. sativa* protoplasts isolated from sheath and stem tissues were performed to assess the fitness of each of the designs (Figures 19A and 20A). Nif protein accumulation (Figure 19B) was compared using the level of eGFP to normalize for differences in overall recombinant protein expression and relativized to the value of the *A. vinelandii* endogenous sequences (Figure 19C).

The results showed a consistent pattern in both NifH and NifM accumulation, with the yeast codon-optimized sequences ranking as the worst-performing ones, followed by the synthetic designs for tobacco (Figure 19D). The rice codon-optimizations had a similar performance to the original *A. vinelandii* sequences, only overcome by the synthetic design for rice, in the case of NifM. This result partially supported our theory that the removal of deleterious motifs can be beneficial for the expression and accumulation of Nif proteins (Supplemental Table 11).

The sequences of *nifH* and *nifM*, optimized for their expression in yeast (López-Torrejón *et al.*, 2016) and tobacco (Section 7.2.1) showed a theoretical detrimental optimization for expression in rice due to codon usage, with CAIs of 0.57 and 0.60 in the case of yeast optimization, and 0.53 and 0.56 in the case of the tobacco synthetic design, respectively (Supplemental Table 11). Conversely, the CAIs of *nifH* and *nifM* originally from *A. vinelandii* (0.83 and 0.85), the rice codon-optimization (0.90 and 0.89), and the rice synthetic design (0.89 and 0.89), already pointed to a successful heterologous gene expression in rice and matched with the NifH and NifM protein accumulation actual patterns observed experimentally. Interestingly, for latter three versions of *nifH* and *nifM* direct correlation between GC and GC3 content values and higher protein accumulation was observed (Supplemental Table 11).

Figure 19. Effect of using diverse *nifH* and *nifM* gene optimizations in rice.

(A) Schematic representation of the constructs used in *O. sativa*-transient expression assays leading to *nifH* or *nifM* gene and eGFP simultaneous expression. (B) Western blot analysis of protoplasts isolated from rice accumulating NifH (left) and NifM (right) when expressed from its native *A. vinelandii* gene, a yeast codon-optimized sequence, a synthetically designed gene optimized for expression in tobacco, a rice codon-optimized sequence, or a synthetically designed gene optimized for expression in rice. N.C. refers to non-transformed protoplasts. The triangles in red mark the expected mature form of the protein. Intense 30 and 35 kDa non-specific bands can be observed when using α -NifM antibodies. Expected sizes are Twin-Strep NifH: 35.3 kDa and NifM: 32.3 kDa. (C) Western blot analysis of eGFP accumulation. Expected size: 27 kDa. (D) Quantification of Nif/eGFP accumulated protein normalized to native *A. vinelandii* sequence protein accumulation. Mean $\pm$ SD (n=3 for NifH, n=2 for NifM). The given value for each Nif protein band was normalized using the value corresponding to the eGFP signal in the same lane. Means not sharing any letter are significantly different by Tukey's multiple comparisons test at the 95% level of significance.

In the case of NifU, antibodies available in our laboratory presented a pattern of unspecific bands that made its detection by immunoblot impossible (Figure 20B). Regarding NifS, no signal could be detected at the expected size for any of the optimizations, although the eGFP internal control present in each plasmid was detected in all cases, indicating that transformation worked properly and that NifS accumulation levels were too low for detection (Figure 20B, C).

Figure 20. *nifU* and *nifS* gene optimizations led to no detectable protein in rice.

(A) Schematic representation of the constructs used in *O. sativa*-transient expression assays leading to *nifU* or *nifS* gene and eGFP simultaneous expression. (B) Rice protoplasts western blot analysis of NifU (left) and NifS (right) accumulation when expressed from its native *A. vinelandii* gene, a yeast codon-optimized sequence, a synthetically designed gene optimized for expression in tobacco, a rice codon-optimized sequence, or a synthetically designed gene optimized for expression in rice. N.C. refers to non-transformed protoplasts. The triangles in red mark the expected mature form of the protein. An intense 35 kDa non-specific band can be observed when using α -NifU and α -NifS antibodies. Expected sizes are NifU: 34.4 kDa and NifS: 44.3 kDa. (C) Western blot analysis of eGFP accumulation. Expected size: 27 kDa.

8.2.2. Import plastid-type specificity

We had previously validated the minimal versions of four Arabidopsis TPs that were able to direct Nif recombinant proteins from bacterial origin to tobacco chloroplasts (Section 7.2.2). In an effort to gauge the utility of these TPs as tools for cereal engineering, we decided to study their species and plastid specificity.

With that purpose, we tested the efficiency of the previously selected four minimal Arabidopsis TPs, which come from proteins directly involved in photosynthesis (CAB6, chlorophyll a/b binding protein; RCA, RuBisCO activase), photo-protection (TOCC, tocopherol cyclase), and fatty acid biosynthesis (BCCP1, Biotin carboxyl carrier protein)

and of one complete TP involved in the assimilation of ammonium into amino-acids (GLTB2, ferredoxin dependent glutamate synthase 2) (Figure 21A and Supplemental Figure 5). CAB6, RCA, TOCC, and BCCP1 are highly expressed in green tissue (Figure 21B, left panel), and accumulate preferentially in leaves (Figure 21B, right panel), while GLTB2 expression and protein accumulation are most abundant in roots, as evidenced by data from the Plant eFP browser (Waese *et al.*, 2017) and AtProteome (Baerenfaller *et al.*, 2008) databases. It is interesting to note that TOCC was also expressed at low levels in roots, consistent with recent studies that suggest a role of tocopherols in the adaptation to drought (Cela *et al.*, 2011) and heavy metals (Collin *et al.*, 2008).

Figure 21. Transit Peptides from *A. thaliana* and *O. sativa*.

(A) Amino acid sequence of Transit Peptides (TPs) from *A. thaliana* and *O. sativa*. Colouring according to amino acid polarity as follows: Yellow: Non-polar; Green: Polar, uncharged; Red: Polar, acidic; Blue: Polar, basic. Red arrows indicate the predicted Stromal Processing Peptidase (SPP) cleavage site. (B) Gene expression and protein levels of corresponding TP genes and mature proteins in *A. thaliana* and *O. sativa*. Gene expression data were retrieved from the Plant eFP browser, showing log₂ of fold change from microarray transcriptomics (Waese *et al.*, 2017). Protein data were retrieved from AtProteome (Baerenfaller *et al.*, 2008). Heatmap shows the log₂ ratio from shotgun proteomics.

We generated constructs in which these five Arabidopsis TPs together with a rice TP coming from the Rubisco small chain 1 protein (*OsRBS1*_{TP}), which is highly expressed in leaves (Figures 21A and B), were fused to the reporter protein eGFP and cytosolic eGFP was used as control of mature protein size (Figures 22A, 23A, and 25A). In order to analyse import mediated by the different TPs, protoplasts obtained from Arabidopsis mesophyll (Figures 22B, 24A, and Supplemental Figure 6) and root cells (Figure 23B, 25B, and Supplemental Figure 6) were transformed.

Figure 22. *In vivo* chloroplast targeting of TP-eGFP fusion proteins in *Arabidopsis* leaf protoplasts. (A) Schematic representation of the constructs used in *A. thaliana* transient expression assays leading to eGFP expression and localization. (B) Confocal laser scanning microscopy images of *A. thaliana* leaf protoplast expressing indicated TP-eGFP fusions. N.C. corresponds to “Negative Control”, non-transformed protoplasts. The columns show individual signals for eGFP (green, on the left) and chlorophyll autofluorescence (red, in the centre). On the right, the overlap of both signals (merge). Scale bars = 5 μ m.

Chloroplasts were easily identified in protoplasts of mesophyll cells by their red autofluorescence (Figure 22B). The plastid-targeted eGFP coincided with chlorophyll fluorescence for all *Arabidopsis* TPs, whereas for the rice *OsRBS1_{TP}* TP and the cytosolic control, eGFP fluorescence was localized outside the chloroplasts (Figure 22B). These results are consistent with our previous observations in tobacco (Section 7.2.2) and demonstrate that the selected *Arabidopsis* TP sequences contain all necessary motifs to correctly direct import of nuclear-encoded recombinant proteins into chloroplasts.

Figure 23. *In vivo* leucoplast targeting of TP-eGFP fusion proteins in *Arabidopsis* root protoplasts. (A) Schematic representation of the constructs used in *A. thaliana* transient expression assays leading to eGFP expression and localization. (B) Confocal laser scanning microscopy images of *A. thaliana* root protoplast expressing indicated TP-eGFP fusions. N.C. corresponds to “Negative Control”, non-transformed protoplasts. The four columns show individual signals for eGFP (green, on the far left), mCherry (red, on the left), and transmitted light (BF, on the far right). On the right, the overlap of eGFP and mCherry signals (merge). Scale bars = 5 μ m.

Expression of the Arabidopsis TP-eGFP fusions in protoplasts obtained from Arabidopsis root cells rendered a fluorescence overlapping with that produced by the expression of *AtRecA*_{TP}-mCherry, an established root plastid marker (Nakata *et al.*, 2018) and different to the one observed for the rice *OsRBS1*_{TP} TP and the cytosolic control (Figure 23). This result suggests that the selected Arabidopsis TPs also mediate successful import of eGFP into root leucoplasts.

Figure 24. Import efficiency of *A. thaliana* and *O. sativa* Transit Peptides in Arabidopsis leaf protoplasts.

(A) Western-blot analysis of import experiments. Protoplast isolated from *A. thaliana* leaves were transformed with indicated TP-eGFP fusions and total protein extracts were analysed by Western blotting using an anti-GFP antibody. The experiments were performed in three biological replicates and representative data are shown. White arrows (pr-eGFP) indicate precursor form; black arrows (eGFP) indicate, processed form; blue arrow indicates partially processed form. **(B)** Quantification of import efficiency of TPs into Arabidopsis chloroplast. Import efficiency was defined as the percentage of the processed faster-migrating protein form relative to the total amount of expressed eGFP protein in each line. Data represent means (n=3) with SD.

SDS-PAGE and immunoblot analysis of protein extracts from Arabidopsis mesophyll (Figure 24A) and root protoplasts (Figure 25A, B) taken at two different time points (12 and 16 h after transformation) made the evaluation of the dynamics of protein import possible. The results show that although some increase in total protein abundance could be observed after 16 h, import efficiency is mostly stable and not dependent on time. The immunoblots showed the presence of lower mobility bands, in some cases, with a size

corresponding to the pre-protein (pr-eGFP), in others, smaller, which could correspond to processing within the TP; and a higher mobility band with a size consistent with the processed eGFP protein after SPP cleavage during import. The ratio of processed to total protein in each lane provides an estimate of import efficiency for each TP in leaf and root plastids (Figure 24B and 25C, respectively).

Figure 25. Import efficiency of *A. thaliana* and *O. sativa* Transit Peptides in Arabidopsis root protoplasts.

(A) Schematic representation of the constructs used in *A. thaliana* transient expression assays leading to eGFP expression. **(B)** Western-blot analysis of import experiments. Protoplast isolated from *A. thaliana* roots were transformed with indicated TP-eGFP fusions and total protein extracts were analysed by Western blotting using an anti-GFP antibody. The experiments were performed in three biological replicates and representative data are shown. White arrows (pr-eGFP) indicate precursor form; black arrows (eGFP) indicate, processed form; blue arrow indicates partially processed form. **(C)** Quantification of import efficiency of TPs into Arabidopsis root plastids. Import efficiency was defined as the percentage of the processed faster-migrating protein form relative to the total amount of expressed eGFP protein in each line. Data represent means (n=3) with SD, except GLTB2 and RBS1 TPs, where n=2.

Although some preference was noted for chloroplast import in the case of *AtmCAB6*_{TP} (96% and 95%, at 12 h and 16 h respectively) and *AtmRCA*_{TP} TPs (92% and 94%), both still showed relatively high efficiency in the import to root leucoplasts (61% – 70% and 56% – 54%, respectively). However, *AtmBCCP1*_{TP} exhibited the highest efficiency in the import to both chloroplast and root leucoplasts (99% – 99% and 97% – 98%, respectively)

of the TPs set studied here. Conversely, *AtmTOCC*_{TP} and *AtcGLTB2*_{TP} TPs showed medium import efficiency into chloroplasts (71% – 63% and 57% – 79%), and also a bit lower in leucoplasts (51% – 52% and 51% – 49%, respectively). This is a surprising result for *AtcGLTB2*_{TP}, given that its actual function is performed in the root tissue where it is preferentially expressed. Nevertheless, possible processing within the *AtcGLTB2*_{TP} TP resulted in an additional band, decreasing its import efficiency. The rice *OsRBS1*_{TP} TP was unable to correctly target eGFP into chloroplasts (2% – 2%, at 12 h and 16 h respectively) or leucoplasts (3% – 2%) of Arabidopsis, accumulating as a pre-protein (Figure 24A and 25B), and suggesting a species-specific import mechanism. For full data and statistics, please see Supplemental Table 12.

8.2.3. Conservation of the import mechanism between species

The identification of Arabidopsis TPs that mediate effective plastid import of recombinant proteins in both, Arabidopsis (*AtmCAB6*_{TP}, *AtmRCA*_{TP}, *AtmTOCC*_{TP}, *AtmBCCP1*_{TP} and *AtcGLTB2*_{TP}) and tobacco (*AtmCAB6*_{TP}, *AtmRCA*_{TP}, *AtmTOCC*_{TP} and *AtmBCCP1*_{TP}, Section 7.2.2), and of a rice TP that is not able to mediate import in Arabidopsis (*OsRBS1*_{TP}) provided a basis for further investigation. Therefore, we deepened our study on the extent of conservation of plastid translocation mechanisms between Arabidopsis and rice. To this end, eGFP constructs analogous to those described above were designed for expression in monocot cells (Figure 26A) and used to transform protoplasts of cells from rice sheath and stem (Supplemental Figure 6). The plastid-targeted eGFP signal coincided with chlorophyll fluorescence in all cases, in contrast to that of the cytosolic eGFP (Figure 26B), confirming that all the selected TPs were functional in rice cells, as opposed to previous results in Arabidopsis.

Figure 26. Arabidopsis TPs are able to direct eGFP into rice chloroplast.

(A) Schematic representation of the constructs used in *O. sativa* transient expression assays leading to eGFP expression and localization. (B) Confocal laser scanning microscopy images of rice protoplast expressing indicated TP-eGFP fusions. N.C. corresponds to “Negative Control”, non-transformed protoplasts. The three columns show individual signals for eGFP (green, on the left), chlorophyll autofluorescence (red, in the centre), and overlap of both signals (right). Scale bars = 5 μ m.

SDS-PAGE and immunoblot analysis of transformed rice protoplast extracts showed a lower mobility band corresponding to pre-eGFP and a higher mobility band corresponding to processed eGFP (Figure 27A). Interestingly, import efficiencies observed were, in the case of *AtmCAB6_{TP}* (80% – 81%, at 12 h and 16 h respectively) and *AtmRCA_{TP}* (85% – 88%), slightly lower than those observed for Arabidopsis chloroplasts; higher in the case of *AtmTOCC_{TP}* (89% – 92%) and *AtcGLTB2_{TP}* (97% – 99%); and equal for *AtmBCCP1_{TP}* (94% – 98%) (Figure 27B). The rice endogenous *OsRBS1_{TP}* was now able to direct eGFP into chloroplasts, but, surprisingly, less efficiently (68% – 68%, at 12 h and 16 h respectively) than its counterparts from Arabidopsis. For full data and statistics, please see Supplemental Table 12.

Figure 27. Import efficiency of *A. thaliana* and *O. sativa* Transit Peptides in rice protoplast.

(A) Western-blot analysis of import experiments. Protoplasts isolated from rice were transformed with indicated TP-eGFP fusions and total protein extracts were analysed by western blotting using an anti-GFP antibody. The experiments were performed in three biological replicates and representative data are shown. White arrows (pr-eGFP) indicate precursor form; black arrows (eGFP) indicate, processed form. **(B)** Quantification of import efficiency of TPs into rice chloroplast. Import efficiency was defined as the percentage of the processed faster-migrating protein form relative to the total amount of expressed eGFP protein in each line. Data represent means (n=3) with SD.

8.2.4. Arabidopsis TPs as tools for plastid import in whole rice plants

In light of the previous results supporting the efficiency of Arabidopsis TPs in rice green tissue, we generated transgenic rice lines in which rice embryos were co-transformed with a plasmid carrying a hygromycin selection cassette and the *AtmCAB6*_{TP}, *AtmRCA*_{TP}, *AtmTOCC*_{TP}, and *AtmBCCP1*_{TP} TPs fused to the N-terminus of eGFP (Figure 28A). Several independent embryogenic callus lines were recovered from each combination of plasmids in which eGFP was expressed to such levels as to be visible under the stereomicroscope (Figure 28B). Lines recovered after transformation with the construct containing the *AtmTOCC*_{TP} TP had lower expression levels compared to *AtmCAB6*_{TP}, *AtmRCA*_{TP}, or *AtmBCCP1*_{TP} TPs (as evidenced by fluorescence emission and protein accumulation). Immunoblot analyses confirmed in all four cases, that only the fully cleaved version of eGFP was detected in callus proplastids, indicating almost 100% import efficiency (Figure 28C, Supplemental Table 12).

Figure 28. Stably transformed rice lines accumulate eGFP in callus stage.

(A) Schematic representation of the constructs used for stable transformation of *O. sativa* leading to eGFP expression and localization. (B) Stereomicroscope fluorescence images from two independent transformed callus lines expressing *AtmCAB6*_{TP}-eGFP, *AtmRCA*_{TP}-eGFP, *AtmTOCC*_{TP}-eGFP or *AtmBCCP1*_{TP}-eGFP. N.C. corresponds to “Negative Control”, a transformed callus line expressing only the HPT resistance cassette. Scale bars = 1 mm. (C) Western blot analysis of callus soluble protein extracts from two independent transformed rice lines with the indicated constructs incubated with an anti-GFP antibody. eGFP produced in *N. benthamiana* was used as a positive size control. Import efficiency was defined as the percentage of the processed faster-migrating protein form relative to the total amount of expressed eGFP protein. Data shown are means ± SD of three independent extractions.

Localization of eGFP within callus proplastids was confirmed using immunogold labelling and electron microscopy. When using a monoclonal antibody against eGFP, callus lines showed very low levels of labelling, but a very specific association of particles with the interior of plastids (Figure 29). Labelling using polyclonal antibodies against eGFP produced significant non-specific labelling, also visible in the wild-type callus, especially in nuclei and cytoplasm, but also higher signal density could still be observed within plastids (Supplemental Figure 7), consistent with our earlier results (Figure 29). An estimated quantification of the immunogold labelling results can be found in Supplemental Table 13.

Whole transgenic rice plants could be regenerated from the eGFP expressing callus lines for *AtmCAB6*_{TP}, *AtmRCA*_{TP}, and *AtmTOCC*_{TP} TPs to study the Arabidopsis TPs import efficiency in different rice tissues. All attempts to regenerate *AtmBCCP1*_{TP} TP lines resulted in embryogenic callus death. In leaves, immunoblot analysis revealed an almost 100% import efficiency for all three TPs (Figure 30A, Supplemental Table 12), much higher than the one observed in the protoplast transient expression system (Figure 27). Electron microscopy immunogold detection showed labelling of chloroplasts, easily identifiable by their thylakoid structures, both by monoclonal (Figures 30B, C, D, and E) and polyclonal antibodies (Supplemental Figure 7). An estimated quantification of the immunogold labelling results can be found in Supplemental Table 13. Furthermore,

confocal microscopy analysis showed co-localization of the eGFP signal with chlorophyll autofluorescence (Supplemental Figure 8), supporting correct plastid targeting mediated by the TPs in study.

Figure 29. Stably transformed rice callus lines exhibit eGFP targeting into rice pro-plastids by Arabidopsis TPs.

(A-E) Immunogold labelling of eGFP in proplastids of rice callus cells using a GFP-specific monoclonal antibody (diluted 1:250). **A** Wild-type cells. **B** *AtmCAB6_{TP}-eGFP*. **C** *AtmRCA_{TP}-eGFP*. **D** *AtmTOCC_{TP}-eGFP*. **E** *AtmBCCP1_{TP}-eGFP*. i: Light microscopy (LM) image of studied section, except in E; ii-v: Transmission electron microscope (TEM) images. (CW: cell wall, m: mitochondria, N: nucleus, p: plastid like, St: starch; bars i = 50 μ m, ii-v = 500 nm; gold particle size = 15 nm).

Figure 30. Regenerated rice plants show eGFP targeting into chloroplast driven by Arabidopsis TPs.

(A) Western blot analysis of leaf total protein extracts from two plants regenerated from the same transformed callus line expressing the indicated constructs above, incubated with an anti-GFP antibody. Negative Control represents a wild-type plant. Import efficiency was defined as the percentage of the processed faster-migrating protein form relative to the total amount of expressed eGFP protein. Data shown are means $\pm$ SD of three independent extractions. **(B-E)** Immunogold labelling of eGFP in chloroplasts of rice leaf cells using a GFP-specific monoclonal antibody (diluted 1:250). **B** Wild-type cells. **C** *AtmCAB6*_{TP}-eGFP. **D** *AtmRCA*_{TP}-eGFP. **E** *AtmTOCC*_{TP}-eGFP. i: Light microscopy (LM) image of studied section; ii-v: Transmission electron microscope (TEM) images. (Ch: chloroplast, CW: cell wall, m: mitochondria, N: nucleus, p: plastid like organelle, St: starch; bars i = 50 μ m, ii-v = 500 nm; gold particle size = 15 nm).

Similar import efficiency was observed in roots of transgenic rice plants. A single band corresponding to mature eGFP was observed for the three TPs in their respective immunoblots, indicating again almost 100% import efficiency (Figure 31A, Supplemental Table 12). Immunogold labelling using monoclonal antibodies showed weaker signals than in leaves or callus, but the gold particles were localized within

leucoplasts (Figures 31B, C, D, and E). Polyclonal antibodies rendered non-specific labelling (also visible in the wild-type roots, especially in the cytoplasm) but the highest signal density was observed within leucoplasts (Supplemental Figure 7). For an estimated quantification of the signal, see Supplemental Table 13.

Taken together these results support the use of any of these TPs, *AtmCAB6*_{TP}, *AtmRCA*_{TP} and *AtmTOCC*_{TP} as tools for the engineering of rice chloroplasts or leucoplasts.

Figure 31. Roots from regenerated rice plants show eGFP targeting into leucoplasts driven by Arabidopsis TPs.

(A) Western blot analysis of root total protein extracts from two plants regenerated from the same transformed callus line expressing the indicated constructs above, incubated with an anti-GFP antibody. Negative Control represents a wild-type plant. Import efficiency was defined as the percentage of the processed faster-migrating protein form relative to the total amount of expressed eGFP protein. Data shown are means $\pm$ SD of three independent extractions. (B-E) Immunogold labelling of eGFP in plastids of rice root cells using a GFP-specific monoclonal antibody (diluted 1:250). B Wild-type cells. C *AtmCAB6*_{TP}-eGFP. D *AtmRCA*_{TP}-eGFP. E *AtmTOCC*_{TP}-eGFP. i: Light microscopy (LM) image of

studied section; ii: detail of i (central cylinder); iii-vii: Transmission electron microscope (TEM) images. (CW: cell wall, m: mitochondria, N: nucleus, Nu: nucleolus, p: plastid-like, p-c: plastid showing an isolated cytoplasmic region; bars i = 0.2 mm, ii = 50 μ m, iii-vii = 500 nm; gold particle size = 15 nm).

8.2.5. NifM remains a requirement for NifH solubility in rice

Following the optimization pipeline implemented in our work on tobacco, we next focused on evaluating the role and functionality of NifM in rice cells. For that purpose, we generated constructs consisting of *AtmRCA*_{TP}-NifH-tGFP and *AtmBCCP1*_{TP}-NifM-mCherry fusions, in which the expression of the rice codon-optimized *nifH* and *nifM* genes were driven by the pZmUbi1 promoter. We used them to transiently transform rice sheath and stem protoplast (Supplemental Figure 6) and visualize tGFP and mCherry signals to assess their subcellular localization after individual and co-expression of NifH with NifM proteins (Figure 32A).

The individual expression of NifM showed a diffuse pattern colocalizing with chlorophyll (Figure 32B), suggestive of a stromatal localisation. NifH-tGFP visualization, however, showed punctuate signal accumulation around the chloroplasts, similar to what was observed in tobacco and consistent with the accumulation of an improperly folded protein (Figures 14B and 32B). Co-expression of NifH and NifM resulted in diffuse eGFP and mCherry signals, both colocalizing with chlorophyll, confirming previous observations in tobacco (Section 7.2.2) and yeast (López-Torrejón *et al.*, 2016) that NifM is required for the accumulation of soluble NifH. Furthermore, the co-localization of NifH and NifM proteins in chloroplasts indicated that their corresponding TPs, *AtmRCA*_{TP} and *AtmBCCP1*_{TP} respectively, were functional in driving their import into the organelle, an important result, since their efficiency had only been previously tested using eGFP as cargo protein.

Figure 32. NifM remains necessary for the accumulation of soluble NifH in the stroma of rice cells.

(A) Schematic representation of the construct used in *O. sativa*-transient expression assays leading to NifH and NifM co-localization. (B) Confocal microscopy images of co-expressed NifH and NifM proteins fused to tGFP and mCherry respectively. N.C. refers to non-transformed protoplasts. The four columns show the individual signals for tGFP (green), mCherry (yellow), and chlorophyll autofluorescence (red). On the right, the overlap of the three signals (merge). Note the co-localization of green and yellow signals in a diffuse pattern within the chloroplast.

8.2.6. Stable transformation of rice embryos co-expressing *nifH*, *nifM*, *nifU*, and *nifS*

Once optimal conditions had been established for Nif protein accumulation and import into the chloroplast stroma, multigenic constructs were assembled with the rice codon-optimized *nif* genes and fused to the selected TPs for stable transformation. Three different versions of vectors were generated, all containing chloroplast-targeted Strep-tagged *nifH* and non-tagged versions of *nifM*, *nifU*, and *nifS* together with the *hpt* gene for selection (Figure 33A). In the first version (v1) all *nif* genes expressions were driven by the pZmUbi1+1i promoter. In the second version (v2), the design tried to achieve the optimal *nif* gene expression ratio of 100:25:50:25 for *nifH*:*nifM*:*nifS*:*nifU*, mimicking their ratios in nitrogen-fixing cells of *A. vinelandii* (Poza-Carrion *et al.*, 2014), by using

different promoters to drive the expression of the different *nif* genes, i.e. pZmUbi1+1i for *nifH*, 2xp35s for *nifM* and *nifU* and pOsActin1 for *nifS*. In the third version (cyto), gene expression was driven by pZmUbi1+1i for all genes, and no TP was used so that all Nif proteins were targeted to the cytosol and used as size controls (Figure 33A).

Prior to embryo transformation, constructs described above were checked in transformation experiments of protoplasts obtained from cells of rice sheath and stem (Supplemental Figure 6). SDS-PAGE and immunoblot analysis of rice protoplasts extracts enabled the detection of a band corresponding to NifH, both with antibodies specific to the protein and against Strep-tag, after transformation with all three plasmids (v1, v2, and cyto) (Figure 33B). NifM was likewise detected with specific antibodies in all cases. However, in the case of NifU and NifS, no clear signal could be identified due to the same detection problems previously described.

Figure 33. Multigenic constructs carrying *nifH*, *nifM*, *nifU*, and *nifS* for rice protoplast transformation.

(A) Schematic representation of the three multigenic constructs (v1, v2, and cyto) used in *O. sativa*-protoplast transient expression assays. **(B)** Western blot analysis of transformed protoplasts isolated from rice using antibodies targeting Strep-Tag, NifH, NifM, NifU, and NifS as indicated. N.C. refers to non-transformed protoplasts. The triangles in red mark the expected mature form of the protein. An intense 35 kDa non-specific band can be observed when using α -NifM, α -NifU, and α -NifS antibodies. Expected sizes are Twin-Strep NifH: 35.3 kDa, NifM: 32.3 kDa, NifU: 34.4 kDa, and NifS: 44.3 kDa.

Transformation of rice embryos was carried out with v1, v2, and cyto plasmids (Figures 34A, C, and E, respectively). For each of the two first versions, 26 independent hygromycin resistant callus primordia were recovered out of 75 bombarded embryos. Regarding the cytosolic control, eight resistant lines were recovered. All 60 independent callus lines were screened for NifH expression by SDS-PAGE and immunoblot analysis with antibodies against NifH and Strep-tag, but no positive results were obtained in any case (Figures 34B, D, and F). Further studies will be conducted to determine the reasons preventing Nif proteins accumulation.

Figure 34. Stable transformation of rice embryos with multigenic plasmids carrying *nifH*, *nifM*, *nifU* and *nifS*.

(A, B, C) Western blot screening of recovered callus lines stably transformed with the three multigenic constructs (A) v1, (B) v2, and (C) cyto using antibodies targeting Strep-Tag and NifH. N.C. refers to a transformed callus line expressing only the HPT resistance cassette. Strep tagged-NifH produced in *N. benthamiana* was used as a positive size control. The triangles in red mark the expected mature form of the protein. Expected size is Twin-Strep NifH: 35.3 kDa.

8.3. Discussion

Transient expression experiments provided proof of concept of tobacco chloroplasts being able to host active Fe protein at the end of the dark period (Section 7.2.3). However, the goal of ubiquitous expression of Nif proteins throughout plant tissues raises the question of whether chloroplast TPs could drive the import of fused cargo proteins into other plastid types, such as proplastids or leucoplasts. The latter ones are a promising location to host active nitrogenase in crops, as the cultivation procedure of rice plants involves waterlogged environments devoid of oxygen. Therefore, we embarked on the optimization of *nif* genes for their expression in rice and the characterization of previously validated minimal TPs in order to study their tissue and species specificity.

8.3.1. Optimization of NifH biosynthetic pathway in rice plastids

The use of *nifH* and *nifM* genes synthetically designed for maximum expression in rice led to higher protein accumulation than other optimized versions (Figure 19). The rice codon-optimized and original *A. vinelandii* sequences scored comparable though slightly lower result. Noteworthy, the *nifM* rice synthetic design, which lacked two exclusion motifs, allowed us to obtain protein levels significantly higher than those achieved by using simply a codon-optimized sequence (Figure 19D, Supplemental Table 11). This constitutes a proof of concept that the removal of deleterious motifs in the DNA sequence can aid the expression and accumulation of a heterologous protein.

Regarding codon optimization, Nif accumulation patterns followed the same trend as the CAI (Figure 19, Supplemental Table 11). Remarkably, higher GC and GC3 content correlated with better gene expression and protein accumulation (Supplemental Table 11). GC content in rice codon-optimized and synthetic-designed sequences was between 60 – 64% and GC3 above 80%, much higher than those of the CDS of rice (GC 55.26% and GC3 61.61%). A similar correlation has been previously observed in maize, where using codon biasing strategies that increased GC and GC3 content above the average codon usage resulted in higher protein accumulation (Sidorenko *et al.*, 2017).

Detection of NifU and NifS was not possible by immunoblot due to the presence of a pattern of unspecific bands or a lack of signal, respectively, while eGFP controls were detected (Figure 20). Plasmid construction mistakes or frameshift mutations were ruled out, as the standard pieces leading to NifU and NifS accumulation were successfully tested in other experiments and Sanger sequencing was performed in all constructions (Figures 9 and 19). Future efforts should be aimed at solving this technical problem. Possible alternatives include the cleaning polyclonal antibodies against a WT rice extract to eliminate non-specific binding, the use of specific antibodies against a fused protein

tag, or the analysis of soluble extracts by targeted proteomics, a high-sensitivity, quantitative technique.

The observed compartmentalization of NifH in the absence of NifM is similar to what was observed in mesophyll cells from agroinfiltrated leaves of *N. benthamiana* (Figure 15), suggesting an incorrect maturation of the NifH protein when expressed in the absence of NifM and confirming its requirement for the correct maturation of NifH also in rice cells (Figure 32B).

8.3.2. Arabidopsis TPs as tools for plastid import

Earlier studies have identified preprotein specificities of import (Chu *et al.*, 2020; Chu and Li, 2018; Fitzpatrick and Keegstra, 2001; Jarvis and López-Juez, 2013). However, our limited knowledge on this subject still makes a difficult task trying to infer pre-protein recognition and translocation efficiencies from the simple analysis of TPs' primary sequence and TP behaviour needs to be confirmed experimentally.

Apart from a general enrichment in alanine, serine, and uncharged amino acid residues, no consensus sequence motifs have been identified among TPs (Ivey *et al.*, 2000; Zybaylov *et al.*, 2008). The TP sequences used here to direct the nuclear-encoded eGFP marker protein into plastids exhibit these characteristics, i.e. a prevalence in serine, alanine, and uncharged residues (cysteine, threonine, tyrosine, asparagine, and glutamine) compared to that of the mature protein (Figure 21A and Supplemental Figure 9).

Two motifs were identified in the TP of Arabidopsis RuBisCO small subunit that are responsible for its Toc159-dependent import pathway into chloroplasts (Lee *et al.*, 2009). The first one, (DITSITSNGG), could not be found among the TPs in study here (Figure 21A). The second motif, consisting of a group of serine residues (SS), was present in *AtmCAB6*_{TP}, *AtmTOCC*_{TP}, *AtmBCCP1*_{TP}, *AtcGLTB2*_{TP}, and rice *OsRBS1*_{TP} TPs. On the other hand, Vojta *et al.* (2004) proposed that positively charged amino acid residues at the C-terminus of TPs (positions -8 and -1 from the cleavage site) were involved in atToc34 recognition and import. This seems to be the case for the five Arabidopsis TPs studied here since they present positively charged residues around the fore-mentioned positions (Figure 21A). Contrary, *OsRBS1*_{TP} TP only presents two positively charged residues at -2 and -4.

Our experimental results support that *AtmCAB6*_{TP}, *AtmRCA*_{TP}, and *AtmBCCP1*_{TP} TPs follow the atToc159-dependent import mechanism specific for photosynthetic proteins, which would explain their higher import efficiency into chloroplasts (Figure 24B). However, they must also be able to interact with the translocon machinery that is

expressed constitutively across all tissues (mainly atToc132, atToc120, and atToc34) to be efficiently imported into leucoplasts (Figure 25C). The case of *AtmBCCP1*_{TP} TP is interesting as it presents a high import efficiency into both chloroplasts and leucoplasts. This behaviour could be explained as BCCP1 is involved in fatty acid biosynthesis, which primarily takes place in chloroplasts of green tissues and plastids of non-photosynthetic tissues, from acetyl CoA, an abundant direct product of photosynthesis (Supplemental Figure 5). Moreover, expression and protein accumulation of BCCP1 between leaf and root tissues exhibit a lesser difference when compared with other TP related proteins (Figure 21B). For *AtmTOCC*_{TP} and *AtcGLTB2*_{TP} TPs, which show a similar degree of import rate in both plastid types (Figure 24B and 25C), our results support a preferential interaction with the TOC family members uniformly expressed across shoot and root.

The case of rice *OsRBS1*_{TP} TP is surprising, showing only the ability to mediate import to plastids in rice (Figure 24B, 25C, and 27B). This would suggest some differences between the TIC/TOC systems of the two species analysed that avoid its recognition by Arabidopsis members. This idea is supported by some previous observations that point to a differential behaviour of TPs depending on the species they were tested. Pea CAB TP was shown to mediate preferential import of proteins into chloroplasts (Wan *et al.*, 1996), while RCA TP conferred no such specificity, exhibiting comparable efficiencies in the import of nuclear-encoded proteins in chloroplasts and leucoplasts (Wan *et al.*, 1995, 1996). As we have shown in this work, in Arabidopsis, the set of TPs are more effective in their capacity to import eGFP into chloroplasts, although eGFP import into leucoplasts was still quite efficient (Figure 24B). On the other hand, all Arabidopsis TPs performed very efficiently in rice chloroplasts (Figure 27B), and *AtmCAB6*_{TP}, *AtmRCA*_{TP}, *AtmTOCC*_{TP}, and *AtmBCCP1*_{TP} abolished completely plastid type specificity in terms of their capacity of protein import (Figures 28C, 30A, and 31A). It seems that all the Arabidopsis TPs in study here lose their plastid type import specificity, which is prevalent in their native species-environment when expressed in rice cells. Moreover, *OsRBS1*_{TP} TP was completely unable to mediate plastid import in Arabidopsis, suggesting that the mechanisms regulating the import of nuclear-encoded proteins into plastids may not be highly conserved and appear to vary between species.

In general, protoplast transient expression experiments resulted in lower import efficiencies than those observed in plant stable expression. This could be explained by high protein expression being able to saturate the translocon machinery and leading to partial import. Thus, caution should be taken when TPs are characterized using only protoplasts, onion, or BY-2 cells experiments where these artificial effects are possible.

Our results show low protein accumulation in transgenic plant roots (Figure 31) although the promoter used has been reported to drive higher expression in rice roots relative to leaves (Green *et al.*, 2002). Whether this is due to lower protein production or degradation before plastid import remains to be studied. In addition, we note that all lines recovered after transformation with the *AtmTOCC_{TP}*-eGFP construct showed lower eGFP expression and protein accumulation. Future studies should be aimed at understanding if this can be avoided by any strategy such as specific codon usage optimization for rice.

Furthermore, the correct import of specific Nif proteins carried out by *AtmRCA_{TP}* and *AtmBCCP1_{TP}* validate their use to drive NifH and NifM into the chloroplasts of rice (Figures 32 and 33).

8.3.3. Efforts in the generation of *nif* expressing rice lines

Use of the v1 construct aimed to achieve high levels of protein accumulation by using a strong constitutive promoter, i.e. pZmUbi1, to drive the expression of all *nif* genes (Figures 33, 34A, and 34B). Contrary to dicotyledonous species, the repeated use of the same promoter in multi-gene combinatorial experiments has not been related to any issues of homology-dependent gene silencing in rice or maize (Kohli *et al.*, 2006). However, no signal for any Nif protein could be observed and the involvement of gene silencing remains to be determined in further studies.

The design of the v2 construct was an attempt to tailor expression using the few well-established constitutive promoters in rice (Figures 33, 34C, and 34D) (McElroy and Brettell, 1994). Recent work has characterized a set of standardized promoters and terminators for engineering legumes and cereals (Feike *et al.*, 2019), which could help refine the engineering of nitrogenase biosynthesis even further. The fact that this strategy where the promoter driving *nifH* is unique supports the hypothesis that the problem inhibiting *nif* gene expression is not uniquely due to homology-driven silencing.

Whereas the multigenic constructs rendered NifH and NifM accumulation in rice protoplast transient expression experiments (Figure 33B), the attempt at generating stably transformed rice lines identified further barriers associated with the transformation procedure, as no lines accumulated detectable NifH in immunoblot assays (Figures 34B, D, and F). Further studies will be aimed at deciphering where the main bottleneck for Nif protein accumulation in rice resides. The analysis of the inserted DNA integrity and copy number determination of *nif* transgenes within the host genome will determine if the transformation method was appropriate. Additionally, mRNA Northern blots or Real-Time quantitative PCR analysis will be used to analyse whether the lines positive for

correct and complete insertions do express the integrated transgenes or not, in order to determine if the main problem resides at transgene expression (Bai *et al.*, 2016; Sudhakar *et al.*, 1998). Preliminary experiments were already done in some of the above-mentioned lines to assess if the problem could be derived from protein degradation due to the presence of oxygen in rice chloroplasts. This does not seem the case, although this line of investigation should also be continued before discarding this possibility. The data obtained from all these experiments will shed some light on the constraints we are facing and hopefully point a way to resume engineering of NifH biosynthesis in rice.

Discussion

Plant synthetic biology seeks to engineer novel traits into plants in a predictable and understandable manner and plays a fundamental role in facing current challenges of climate change and world population rise. One major aspect of these challenges is the nitrogen availability to crops, which is a limiting factor of crop yield so much so that the use of synthetic nitrogen fertilizers is prevalent in developed countries to increase crop yield. The generation of nitrogen-fixing crops is one of the SynBio projects that could lead to a new Green Revolution and have a global impact on agriculture and environment preservation. The idea of generating plants that do not need nitrogen fertilizers is far from being new (Hardy and Havelka, 1975). Many groups have worked in the transfer of the genes encoding for the nitrogenase enzyme to eukaryotic hosts, however, most attempts failed in getting active protein (Burén *et al.*, 2020; Burén and Rubio, 2018).

The nitrogenase Fe protein has been the protein of choice to assay the suitability of subcellular compartments to acquire and maintain its activity, given that it is highly oxygen-sensitive and its maturation steps are simpler than those of the MoFe protein. Within this context, this thesis has focused on testing the ability of plant plastids to host active nitrogenase Fe protein, for that we engaged in the optimization of prokaryotic *nif* genes for their expression in higher plants and the import of Nif proteins into their plastids.

***Azotobacter nif* gene optimization for expression in higher plants**

We researched and established a set of parameters intended to increase *nif* gene expression and translation in plants, specifically, tobacco and rice, and with the aid of CAD tools, generated *nif* synthetic gene variants that resulted in enhanced accumulation of Nif proteins.

Transient expression assays in *N. benthamiana* proved that *nifH*, *M*, *U*, and *S* genes synthetically designed to maximize expression in tobacco led to a much higher protein accumulation than the yeast-optimized ones, although those were theoretically also optimum for recombinant protein production in the tobacco system regarding codon optimization parameters. These results show that the extra-factors considered in our synthetic design beyond codon optimization had a determinant role in stimulating Nif protein production and stabilizing it.

Our work has been essential to enable the study of NifH functionality within plant cells. For a long time, plant engineering strategies seeking to achieve high levels of expression

of transgenes were only based on the use of strong constitutive promoters. Dixon *et al.* (1997) proved that *K. pneumoniae nifH* and *nifM* original sequences could be expressed in tobacco using the cauliflower mosaic virus 35S promoter. They showed that genes were transcribed at relatively high levels in the plants. However, Western blotting experiments showed very little Nif protein accumulation, below the limits of detection for an activity assay. Thus, the question regarding the functionality of NifH in plant cells remained unanswered for more than 20 years.

A different strategy was used by Ivleva *et al.* (2016), using a transplastomic approach and benefiting from the prokaryotic nature of the organelle that assured efficient transcription and translation of original prokaryotic *nif* sequences. In this case, transplastomic plants carrying the original *A. vinelandii nifH* and *nifM* sequences rendered sufficient levels of protein to detect very low Fe protein activity in enriched extracts, but it was required to grow plants in an oxygen-depleted atmosphere. Since direct plastid genome transformation remains a challenge for most crops, this approach is not readily transferrable to cereals, and for those, our optimization pipeline showed great promise.

We followed a similar strategy as with *N. benthamiana* and generated *nif* synthetic gene variants optimized for *O. sativa* using monocot-specific parameters. Detection of NifU and NifS was not possible due to unspecific binding of their antibodies to several proteins within the rice extracts. However, *nifH* and *nifM* could be detected, and it was observed that the synthetic design led to a comparable protein accumulation to the one obtained using the rice codon-optimized and original *A. vinelandii* sequences, but much higher than the one obtained when using the yeast-optimized ones, as predicted by CAI values. Remarkably, a tight correlation between sequences with high GC and GC3 content values and higher protein accumulation was observed for both *nifH* and *nifM* genes consistent with data previously reported (Sidorenko *et al.*, 2017). The exclusion of deleterious motifs in the sequence of *nif* genes showed a tendency to improve protein accumulation, confirming our previous observations in tobacco.

Overall, our results show that adapting the codon usage frequencies of a foreign sequence to that of your host organism is not always enough to guarantee high gene expression and protein accumulation, and more complex strategies can be included in synthetic biology approaches in which obtaining high protein levels is a challenge.

Optimization of protein import into plastids

Import of nuclear-encoded proteins into plastids is an important bottleneck in fundamental plant research and many applications of plant biotechnology. The characterization of a set of TPs that facilitate recombinant protein import into different

plastid types and that work with high efficiency in both model plants and crops increases the portfolio of standard parts available for plant SynBio and can be very useful for plant engineering projects. Even more in many staple crops including food security crops such as rice or maize, which are not amenable to direct plastid transformation.

The limited knowledge on the motifs responsible for TPs functionality impedes the prediction of their behaviour when carrying recombinant cargo proteins and their functionality needs to be confirmed experimentally. We have characterized a set of minimal transit peptides and analysed their behaviour in the import of eGFP and specific Nif proteins into the plastids of tobacco and rice, as a means to studying their modularity and orthogonality.

In *N. benthamiana*, we tested a library of 12 TPs fused to each of our four Nif proteins of interest. Most of the TPs previously described in the literature span past the SPP cleavage site and thus, leave scar amino acid residues in the N-terminus of the imported protein after their cleavage, which could affect cargo protein activity and folding. Thus, we also tested minimal versions of all 12 TPs, which included the TP coding sequence up until the predicted cleavage site plus the following alanine. Overall, the use of a minimal TP resulted in a higher mobility band compared to the complete version. However, in some cases, the minimal TP seemed to lose its ability to import the Nif protein into the chloroplast, possibly due to the removal of a motif that was needed for its recognition by the import machinery (Li and Teng, 2013).

It is noteworthy, but not surprising, that only two minimal TPs *AtmGLTB2_{TP}* and *AtmBCCP1_{TP}* performed properly for all four Nif proteins. It has been previously reported that tertiary structures of the cargo protein next to the TP sequence affect its function (Shen *et al.*, 2017). Also, several reports have noted that a portion of the mature protein, usually an unfolded region following the TP, is in some cases required for proper import (Bionda *et al.*, 2010). Therefore, experiments performed with carrier proteins as GFP or mCherry are useful to characterize a TP but do not accurately predict its behaviour when fused to the specific cargo protein of interest.

In an attempt to characterize useful TPs, we selected four of the best-performing minimal TPs in tobacco (*AtmRCA_{TP}*, *AtmBCCP1_{TP}*, *AtmTOCC_{TP}*, and *AtmCAB6_{TP}*) together with one commonly used from the target host (*OsRBS1_{TP}*), and, to test how promiscuous TPs are in different tissues, we incorporated a TP coming from a gene highly expressed in roots (*AtcGLTB2_{TP}*). This library was tested for eGFP import efficiency in leaves and roots cells of Arabidopsis and rice. In Arabidopsis, we observed that the set of TPs selected is generally more effective in their capacity to import eGFP into chloroplasts

compared to leucoplasts, with the noteworthy exception of *OsRBS1*_{TP}, which was unable to mediate plastid import, and *AtmBCCP1*_{TP}, which presented a high import efficiency into both plastid types. All Arabidopsis TPs performed very efficiently in eGFP import into rice chloroplasts from protoplast, losing their species import specificity, whereas *OsRBS1*_{TP}, although functional, was significantly less efficient. Interestingly, *OsRBS1*_{TP} was the only TP presenting two positively charged residues, involved in atToc34 recognition and import, at different positions than the ones described by Vojta *et al.* (2004), possibly interfering in its function in Arabidopsis; whereas its low efficiency in rice may be due to the import competition with the endogenous RuBisCO protein. We further confirmed that all four minimal TPs were functional and highly efficient in driving eGFP into proplastids, chloroplast, and leucoplast of stably transformed rice plants, confirming the results obtained in the transient system. Additionally, we proved that *AtmRCA*_{TP} and *AtmBCCP1*_{TP} minimal TPs were effective in targeting NifH and NifM to chloroplasts of rice cells, validating their use as engineering tools for future projects.

NifM, a protein with similarity to prolyl isomerases, is essential for NifH activity in its original host (Howard *et al.*, 1986) and when expressed in both mitochondria and cytoplasm of yeast (López-Torrejón *et al.*, 2016). Our results in tobacco and rice cells clearly show that, when expressed alone, NifH was correctly imported into plastids, but accumulated in a punctuated pattern. Simultaneously expressing a soluble marker of the stroma traced the compartmentalisation of NifH to a point after their import, possibly following a pathway for degradation of improperly folded proteins. Yet, when co-expressed with NifM, soluble stroma NifH accumulation was obtained, showing that the maturase is also a requirement for achieving a functional Fe protein in plant cells.

Engineering nitrogenase Fe protein biosynthesis in plastids

The production of Nif proteins that accumulate to high levels in *N. benthamiana* leaf cells and that are correctly imported into chloroplasts facilitated the recovery of active nitrogenase Fe protein, with no doubt, a major accomplishment. Although oxygenic photosynthesis might be providing an unfavourable environment for nitrogenase Fe protein maturation during the day, our results show that at least at night, leaf cell chloroplasts constitute an environment with low enough oxygen for nitrogenase Fe protein accumulation in active form. The activity obtained is less than a half of the one obtained from yeast mitochondria (López-Torrejón *et al.*, 2016) and about a sixth of the one from *A. vinelandii*, so there is still room for improvement in the engineering but constitutes a considerable achievement. Previously, only very low NifH activity had been detected in transplastomic plants expressing NifH and NifM, and only after incubation of the plants in an oxygen-depleted atmosphere (Ivleva *et al.*, 2016). Our results constitute

the first definitive proof of concept that engineering Fe protein biosynthesis in plastids of higher plants is an achievable strategy. However, future efforts should be directed at fine-tuning NifH maturation and protection of its Fe-S clusters from oxidative damage, perhaps by the engineering of a temporal separation of nitrogen fixation and photosynthesis (Curatti and Rubio, 2014) or by co-expressing the FeSII protein that could bind NifH for cluster protection (Moshiri *et al.*, 1995; Schlesier *et al.*, 2016).

Nitrogenase activity relies on its metal clusters, which require significant amounts of iron and molybdenum. The [Fe-S] clusters need to be properly assembled and delivered into nitrogenase components, a task carried out in diazotrophs by NifS, which mobilizes the S required, and NifU, where cluster assembly takes place for its subsequently delivery.

Previously, NifU and NifS were found not to be necessary for Fe protein activity in the mitochondria of yeast, suggesting that mitochondrial [Fe-S] cluster assembly machinery can substitute at least some of the functions from these Nif proteins (López-Torrejón *et al.*, 2016), though NifU and NifS were in fact required for NifB activity in yeast mitochondria (Burén *et al.*, 2019). However, our results show that, in plant plastids, NifH lacks any activity when only co-expressed with NifM, suggesting that the chloroplast [Fe-S] cluster machinery cannot, at least in transient assays, efficiently replace the function of NifU and NifS. Nevertheless, plants are rich in [Fe-S] clusters due to their requirement in the photosynthesis machinery, and its assembly pathways, primarily located in mitochondria and chloroplasts, are similar to those found in bacteria (Balk and Pilon, 2011). Given the importance of [Fe-S] clusters in plants, it is conceivable that [Fe-S] carrier proteins in plastids play redundant roles and under certain conditions transfer clusters promiscuously, a possibility that may be exploited in the future to eliminate the need for *nifU* and *nifS* in the engineering of an active nitrogenase (Couturier *et al.*, 2013).

Perspectives of nitrogen-fixing plants

SynBio tools can be of great help in the optimization of the expression of foreign genes, protein accumulation, and other biological processes like organelle transport. The use of transient expression assays together with modular cloning techniques and fast-growing model plants have proved extremely useful in our optimization pipeline. We were able to perform several iterations of the DBTL cycle in a short time, enabling the optimization of each of the different elements that constituted an engineering challenge, in order to prove that chloroplasts are indeed a suitable location to host active Fe protein, a goal not achieved for more than 20 years.

SynBio allowed us to decouple the complex problem of Fe protein biosynthesis in plants into smaller and simpler steps, that were addressed individually. After the optimization

of each specific transcriptional unit for each *nif* gene, it was fairly straightforward to pile them all up to test their co-expression and NifH functionality in transient expression assays within tobacco. But our objective is to transfer these results to stably transformed crops.

As a further step in this direction, we used rice protoplast transient expression assays. Although this seems useful to fine-tune host species-specific preferences, there are still significant differences between transiently and stably transformed plants. Protoplast assays showed lower chloroplast import efficiencies than those observed in stably transformed rice plants and, while NifH presence could be detected in rice protoplasts, no stably transformed lines accumulated detectable NifH when checked on immunoblot assays. Though it is technically possible to engineer rice plants expressing four or even more transgenes (Zhu *et al.*, 2008), failure to express transgenic proteins in plants has frequently been observed. A number of different causes have been linked to those failures, including positional effects of the chromatin environment due to the random nature of the integration (Butaye *et al.*, 2005) or gene silencing (Vaucheret and Fagard, 2001). When using biolistic transformation, severe genome rearrangements have been described in the genome of host plants (Liu *et al.*, 2019), and transgene aberrant structures are commonly generated (Fu *et al.*, 2000). Therefore, the use of other transformation methods for rice, such as *Agrobacterium*-mediated T-DNA transfer, could be an approach worth considering (Hiei and Komari, 2008).

Despite the success in obtaining NifH activity in tobacco, the engineering of the different *nif* modules necessary to achieve an active nitrogenase enzyme in plants is lagging behind, but SynBio approaches can help tackle these problems. NifB from *A. vinelandii*, a key enzyme in the nitrogenase pathway, was found insoluble in mitochondria of yeast and tobacco, but bioinformatic mining in databases found a relative from *Methanothermobacter thermautotrophicus* that accumulated as soluble protein and was functional (Burén *et al.*, 2019). Moreover, NifD from *A. vinelandii* and *K. oxytoca* suffered from N-terminal degradation in mitochondria of yeast and tobacco due to a recognition motif for a mitochondrial endoprotease. The identification of the motif and engineering of amino acid mutations resulted in variants that were resistant to degradation, as well as retained function solving a bottleneck to the assembly of active MoFe protein (Allen *et al.*, 2020; Xiang *et al.*, 2020).

The development of tools that facilitate crop engineering is of the uttermost importance for plant SynBio. Many efforts in the characterization of genetic modules, gene optimization and protein targeting in crops are currently underway (Baysal *et al.*, 2020;

Feike *et al.*, 2019). In the future, it will be interesting to study whether the tools developed in this thesis can work with other Nif proteins required for nitrogenase biosynthesis. In any case, the pipeline described for the optimization of each element used in their engineering provides an example of how complex projects can be achieved. In the future, we will focus on the transition from model to staple crops like rice and maize, where our efforts can lead to the final goal of generating nitrogen-fixing cereals.

Conclusions

1. We established a set of species-specific parameters to increase gene expression and protein accumulation and generated *nif* synthetic gene variants that resulted in enhanced accumulation of Nif proteins.
2. The experimental validation of transit peptides is critical. In *Nicotiana benthamiana*, in some cases, the minimal transit peptides seemed to lose their ability to import the cargo protein into chloroplasts and their behaviour was influenced by different Nif cargo proteins.
3. Four minimal transit peptides, namely *AtmRCA*_{TP}, *AtmBCCP1*_{TP}, *AtmTOCC*_{TP}, and *AtmCAB6*_{TP}, proved that they correctly and accurately drive the import of NifH, NifM, NifU and NifS respectively, into the chloroplast stroma of *Nicotiana benthamiana* cells.
4. Soluble stroma NifH accumulation required of NifM co-expression, confirming that the maturase is a requirement for achieving soluble Fe protein in both plant cells assayed, tobacco and rice.
5. We recovered active nitrogenase Fe protein from chloroplasts of tobacco mesophyll cells, providing the proof of concept that, at least at night, this location constitutes a suitable environment for engineering Fe protein biosynthesis, which is dependent on NifU and NifS for cluster delivery.
6. We observed that *AtmRCA*_{TP}, *AtmTOCC*_{TP}, and *AtmCAB6*_{TP} transit peptides were more effective in their capacity to import eGFP into chloroplasts compared to leucoplasts of *Arabidopsis thaliana*, while *AtmBCCP1*_{TP} presented a high import efficiency into both plastid types.
7. *AtmRCA*_{TP}, *AtmBCCP1*_{TP}, *AtmTOCC*_{TP}, and *AtmCAB6*_{TP} transit peptides were functional and highly efficient in driving eGFP into proplastids and chloroplast from *Oryza sativa*, losing their species import specificity. *AtmRCA*_{TP}, *AtmTOCC*_{TP}, and *AtmCAB6*_{TP} further mediated eGFP import efficiently into rice leucoplast.
8. We demonstrated that *AtmRCA*_{TP} and *AtmBCCP1*_{TP} were also effective in targeting NifH and NifM cargo proteins to the stroma of chloroplasts from *Oryza sativa* cells.

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Supplemental Material

Supplemental Table 1. List of primers used in this thesis.

Primer number	Primer sequence (5' – 3')	Comment
406	ATGAAGACATCTCGGGCTGCTCGTGGATGCTGCTGCGTT	
407	ATGAAGACATCTCAAATGGCGCAAGTTAGCAGAATC	
408	GCGCGAAGACTGAAAACGCAGCAGCATCCACG	Mutation G 105 > A
409	ATGAAGACATCTCGGGCTTCCGCCGTGGAACAGACGACATGACC	Mutation T 210 > G
410	GCGCGAAGACCGTTTTCAGAGAAACCGATAAGGG	Mutation G 105 > A
411	ATGAAGACATCTCGGGCTGCTTTCACCACAGGGTAGC	
412	ATGAAGACATCTCAAATGGCGTCGTCGTCGTTCTC	Mutation T 9 > G
413	GCGCGAAGACGTCTTCTGCTAAGCCCAAGCT	Mutation C 120 > T
414	ATGAAGACATCTCGGGCTCCATCAACTTTGGCAGCAT	
415	GCGCGAAGACAGAAAGACGGAAAGAAACTCTGC	Mutation C 120 > T
416	ATGAAGACATCTCGGGCTGCTGGTCGTGGCTCGCCAG	
417	ATGAAGACATCTCAAATGGCGTCGAACTCGCTTATG	
418	ATGAAGACATCTCGGGCTCCAAAGTCACCAGGAGCAG	
419	ATGAAGACATCTCGGGCTGAACAAACGACTTTTGATCTGT	
420	ATGAAGACATCTCAAATGACAATTGCTTTAACGATCGG	
421	ATGAAGACATCTCGGGCTTTAGCGAGTTGTCTGAAAGCT	
422	ATGAAGACATCTCGGGCTGCTTTCACCGAAAACGGCGAC	
423	ATGAAGACATCTCAAATGGCTCTACAGTCTCCCGGAG	
424	ATGAAGACATCTCGGGCTGCTCGGTCAGAATTAAGGATCG	
425	ATTAAGGATCGCTTTCACCGAAAACGGCGACCTCAGCGAG	Mutation T 201 > G
426	ATGAAGACATCTCGGGCTGCAGCACTAGCACGAGTAG	
427	ATGAAGACATCTCAAATGGCTTCTTCTATGCTTTCTT	
428	ATGAAGACATCTCGGGCTGCTCGAACTCTTCCTCCGTTA	
432	ATGAAGACATCTCGGGCTGCTTGAGCAACATTGCTGC	
433	ATGAAGACATCTCAAATGGCTTCCTCAGTTCTTTCCT	

434	ATGAAGACATCTCGGGCTGAGCATTGCACTCTTCCGCCGT	
435	ATGAAGACATCTCGGGCTGAGCAGTTAACTCTTCCGCCGT	
436	ATGAAGACATCTCAAATGGCTTCCTCTATGCTCTCT	
437	ATGAAGACATCTCGGGCTGAATCGGTAAGGTCAGGAAGG	
438	ATGAAGACATCTCGGGCTGCGGATTGCCCCCTAGAGG	
439	ATGAAGACATCTCAAATGGCTTCTATGATATCCTCTTCC	
440	ATGAAGACATCTCGGGCTGAGCACTTTACTCTTCCACCAT	
441	ATGAAGACATCTCGGGCTGCCAACACCTTGAATGATCC	
442	ATGAAGACATCTCAAATGGCCGCCGCAGTTTCCAC	
443	GCGCGAAGACAAGATAAAACAAACCGATGGAGAC	Mutation C 189 > T
444	ATGAAGACATCTCGGGCTGAATCAGAAGTGTCGTAGGCAA	
445	GCGCGAAGACTTTATCTTCTTTACAGCCAACAC	Mutation C 189 > T
446	ATGAAGACATCTCGGGCTGCGCGGACAATAGAACTAGG	
447	ATGAAGACATCTCAAATGACGACGTCGTTTGGAGC	
448	GCGCGAAGACGTCTGCACGTTTTTCAGTCCTTT	Mutation T 126 > G
449	ATGAAGACATCTCGGGCTGACGTAGATACGGCGCGGACAA	
450	GCGCGAAGACGTGCAGACGCCTGCTAAGCAAC	Mutation T 126 > G
451	ATGAAGACATCTCGGGCTGCCGAAACCCTAGAAATGG	
452	ATGAAGACATCTCAAATGGAGATACGGAGCTTGATTG	
453	ATGAAGACATCTCGGGCTGAGTGAGGAGTCCGGAGTT	
454	ATGAAGACATCTCATTCGATGGCCATGAGACAATGTGC	
455	ATGAAGACATCTCGAAGCTTATACTTCTTCAGCAGTTTTACCG	
456	ATGAAGACATCTCAAGCCATGGCCTCAGAAAGATTAG	
457	ATGAAGACATCTCGAAGCTTAACCATGTGCTAAGTTTTC	
458	ATGAAGACATCTCAAGCCATGGCCGACGTTTACTTGGA	
459	ATGAAGACATCTCGAAGCTCAACCATAGACAGGAGCAAAG	
460	AGACAGGAGCAAAGGCTTTACCAGGATCTTCAACAG	Mutation C 1176 > T
461	ATGAAGACATCTCAAGCCATGTGGGACTACTCTGAAAAGG	
462	ATGAAGACATCTCGAAGCTCAGACTTCCATTTGGGCGTG	
463	ATGAAGACATCTCATTCGATGGCAATGAGACAATGTGCTA	

465	ATGAAGACATCTCAAGCCATGGCATCAGAAAGATTAGCTG	
467	ATGAAGACATCTCAAGCCATGGCAGACGTTTATTTGGACA	
469	ATGAAGACATCTCAAGCCATGTGGGATTACTCAGAGAAAG	
471	ATGAAGACATCTCAAGCCTCTGCATGGAGTCATCCTCAG	
479	ATGAAGACATCTCAGGAGGAATTCCAATCCCACA	
480	ATGAAGACATCTCGCATTGCGTGTCTCTCCAAATGA	
495	ATGAAGACATCTCGCGAAGATTTTCAAATTGTGGATGTG	
502	ATGAAGACATCTCGAAGCTTACACTTCCTCAGCGGTTT	
503	ATGAAGACATCTCGAAGCTTATCCATGCGCCAAATTCTCT	
504	ATGAAGACATCTCGAAGCTTATCCGTAAACGGGCGCGAA	
505	ATGAAGACATCTCGAAGCTTATACTTCCATCTGAGCATG	
535	ATGAAGACATCTCGGGCTGCGTGTCTCTCCAAATGA	
576	ATGAAGACATCTCAGCAGATGAGAGGATCTGGATCTG	
577	ATGAAGACATCTCGAAGCCTATTCCTCACCAGCATCAGC	
578	ATGAAGACATCTCGCTGCCCACCGCTTCCACCACCT	Partial linker
579	CTTCCACCACCTGAACCTCCCATTCTCTCAGCGGTTTGC	No stop codon. Partial linker
580	CTTCCACCACCTGAACCTCCTCCATGCGCCAAATTCTCTA	No stop codon. Partial linker
581	CTTCCACCACCTGAACCTCCTCCGTAAACGGGCGCG	No stop codon. Partial linker
582	CTTCCACCACCTGAACCTCCTACTTCCATCTGAGCATGTG	No stop codon. Partial linker
595	ATGAAGACATCTCAGGAGTGCAGTGCAGCGTGACCCGGTC	
596	ATGAAGACATCTCGCATTCTGCAGAAGTAACACCAAACAA	
597	ATGAAGACATCTCAGGAGTAGCTAGCATACTCGAGGTCAT	
598	GCGCCGTCTCGCAAGACCGAGACGAAAAAAAAAAC	
599	GCGCCGTCTCGCTTGATCTTTGGCCTTGGTAG	
600	ATGAAGACATCTCGCATTCTTCTACCTACAAAAAAGCTCC	
613	ATGAAGACATCTCAAGCCTGGGACTACAGCGAGAAGGTGA	
614	ATGAAGACATCTCGAAGCTCAGACCTCCATCTGGGCATGG	
615	ATGAAGACATCTCAAGCCGCGGACGTGTACCTCGATAACA	
616	ATGAAGACATCTCGAAGCTCATCCGTAGACAGGGGCGAAT	
617	ATGAAGACATCTCAAGCCGCCTCAGAGAGGCTCGCGGACG	

618	ATGAAGACATCTCGAAGCTCAGCCGTGCGCCAGATTCTCG	
621	ATGAAGACATCTCAAGCCGTGAGCAAGGGCGAGGAGCTGT	
622	ATGAAGACATCTCGAAGCTTACTTGTACAGCTCGTCCATG	
623	ATGAAGACATCTCATTCGGCCATGAGGCAGTGCGCGATCT	
624	ATGAAGACATCTCGAAGCTCACACTTCCTCGGCCGTCTTG	
629	ATGAAGACATCTCAGCTTATGGGGCATCGGCGACGAGCAC	
630	ATGAAGACATCTCGAGCGTCGATCTGCCCAGTTAAGATCA	
712	ATGAAGACATCTCAGCAGATGAGCGAGCTGATTAAGGAGA	
713	ATGAAGACATCTCGAAGCTCAATTAAGCTTGTGCCCCAG	
714	ATGAAGACATCTCAGCAGATGGTGAGCAAGGGC	
715	ATGAAGACATCTCGAAGCTCACTTGTACAGCTCGTCCA	
758	ATGAAGACATCTCGGGCTGACATTCTGCAGAAGTAACACC	
759	ATGAAGACATCTCAAATGGCCCCCTCCGTGATG	
760	ATGAAGACATCTCGGGCTGCGCACCTGATCCTGCCGCCAT	
765	ATGAAGACATCTCAAATGAAAAAGCCTGAACTCACC	
766	ATGAAGACATCTCGAAGCTATTCCTTTGCCCTCGGAC	
771	ATGAAGACATCTCAAGCCTCTGCCTGGTCACACCCT	
772	ATGAAGACATCTCGCGAAGATTTCTCGAACTGCGGATGG	
812	ATGAAGACATCTCAGGAGGTCAACATGGTGAGC	
813	ATGAAGACATCTCATTCGATGGCTATGCGTCAATGCG	
814	ATGAAGACATCTCGAAGCTCAGACTTCTTCGGCGGTTTTGCCGAC- GATGGATTCATCTTCG	
815	ATGAAGACATCTCAAGCCATGGCATCTGAGCGTCTCG	
816	ATGAAGACATCTCGAAGCTCATCCATGGGCGAGGTTC	
817	ATGAAGACATCTCAAGCCATGTGGGATTATTCGGAAAAAG	
818	ATGAAGACATCTCGAAGCTCAGACCTCCATCTGCGC	
819	ATGAAGACATCTCAAGCCATGGCTGACGTCTATCTCG	
820	ATGAAGACATCTCGAAGCTCAGCCGTAGACCG	
823	GCGCGAAGACTTCATCTTCCAGCTCCAGATCT	Mutation C 225 > T
824	GCGCGAAGACAAGATGTGCTGAAGGCTGGCTA	Mutation C 225 > T
825	GCGCGAAGACTTTCATCTTCGTAGGCGCCTTC	Mutation C 354 > T

826	GCGCGAAGACAAATGATCTGGACTTCGTATTCTAC	Mutation C 354 > T
827	GCGCGAAGACTTCTCATCTTCGCGGTTCGGTGT	Mutation C 582 > T
828	GCGCGAAGACAATGAGCTGATCATCGCTCTGG	Mutation C 582 > T
829	GCGCGAAGACTTTTGTCTCGATGCGCGTCCGG	Mutation C 516 > A
830	GCGCGAAGACAAACAATCCTCAAGCGGCTGCG	Mutation C 516 > A
831	GCGCGAAGACTTCAAAGACTTCACCGCGAGCG	Mutation C 204 > T
832	GCGCGAAGACAATTTGTCTCGCGCCCCGATAAA	Mutation C 204 > T
833	GCGCGAAGACTTCTGTCTCGATGCGACGGATG	Mutation C 714 > A
834	GCGCGAAGACAAACAGTGCTGGCGGCGATCCG	Mutation C 714 > A
835	GCGCGAAGACTTTAAAGACGATTTCCGAATCGTG	Mutation C 204 > T
836	GCGCGAAGACAATTTACCTCCTGCGGCACCGA	Mutation C 204 > T
837	GCGCGAAGACTTCTGTCTCGTTGTTGGCCAC	Mutation C 459 > A
838	GCGCGAAGACAAACAGGTACCCTGTTCCCGAT	Mutation C 459 > A
883	CTTCCACCACCTGAACCTCCCACTTCCTCGGCCGTCTTG	No stop codon. Partial linker
884	CTTCCACCACCTGAACCTCCGCCGTGCGCCAGATTCTC	No stop codon. Partial linker
887	GCACTTCACGCCACCGTATC	Overlapping PCR Mutation C 243 > T
888	GATACGGTGGCGTGAAGTGC	Overlapping PCR Mutation C 243 > T
889	CTGTGATGACTCCGCGGC	Overlapping PCR Mutation C 303 > A
890	GCCGCGGAGTCATCACAG	Overlapping PCR Mutation C 303 > A
891	GAATCCGCCACAGACCACG	Overlapping PCR Mutation C 396 > A
892	CGTGGTCTGTGGCGGATTC	Overlapping PCR Mutation C 396 > A
893	CACTCACTGTGCTTCATGGC	Overlapping PCR Mutation TTCA 570 > CAGT
894	CTCAGCTCACCGCGGGCCATC	Overlapping PCR Mutation TTCA 570 > CAGT
895	GCCATGAAGCACAGTGAGTG	Overlapping PCR Mutation C 672 > T
896	GATGGCCCGCGGTGAGCTGAG	Overlapping PCR Mutation C 243 > T
897	ACCCGATCTATTTCTTCCTCCG	Overlapping PCR Mutation G 1083 > A & C 1086 > A
898	CGGAGGAAGAAATAGATCGGGT	Overlapping PCR Mutation G 1083 > A & C 1086 > A

899	TCCCCACAGGACAGTGAGC	Overlapping PCR Mutation C 102 > T & C 105 > G
900	GCTCACTGTCCTGTGGGGA	Overlapping PCR Mutation C 102 > T & C 105 > G
901	GTCCTCGATTGTCTCTCCGC	Overlapping PCR Mutation C 366 > A
902	GCGGAGAGACAATCGAGGAC	Overlapping PCR Mutation C 366 > A
903	CATGCAGAGCAGCCACCG	Overlapping PCR Mutation C 507 > T
904	CGGTGGCTGCTCTGCATG	Overlapping PCR Mutation C 507 > T
905	GAGCACTGTCTCAATACTGCG	Overlapping PCR Mutation G 702 > T & C 705 > T
906	CGCAGTATTGAGACAGTGCTC	Overlapping PCR Mutation G 702 > T & C 705 > T
907	TCGACGTCGATTAGCTCCAC	Overlapping PCR Mutation G 765 > A
908	GTGGAGCTAATCGACGTCG	Overlapping PCR Mutation G 765 > A
909	CTCCTCAATGAGCCGCTGC	Overlapping PCR Mutation G 864 > C & C 867 > T
910	GCAGCGGCTCATTGAGGA	Overlapping PCR Mutation G 864 > C & C 867 > T
947	ATGAAGACATCTCAAATGGATTACAGCTAGTCTTG	
948	ATGAAGACATCTCGGGCTGCGGCGTAGACGGTAACCGGAG	

Supplemental Table 1 (continued). List of primers used in this thesis.

Primer number	Primer sequence (5' – 3') Internal primers used for Sanger sequencing.	Comment
261	AGCGAGGAAGCGGAAGAGCG	L1Px_pUC57 Fwd
262	GCCACCTGACGTCTAAGAAACC	L1Px_pUC57 Rev
263	GGATAAACCTTTTCACGCCC	MoClo RB
266	GTGGTGTAACAAATTGACGC	MoClo LB
393	ACTTCAAGATCCGCCACAACA	<i>GFP</i> Fwd
394	ATGCCGTTCTTCTGCTTGTC	<i>GFP</i> Rev
405	GACACCGCGCGCGATAATTT	tNOS Rev
475	GGACAGGTATCCGGTAAGCG	pAGM9121 (L0) Fwd
476	AACAAGCCATGAAAACCGCC	pAGM9121 (L0) Rev
526	TCATACCGTGACTCCCTTAATTCTC	pNOS Rev
527	AGCGCGCAAAGTAGGATAAATTATC	tNOS Fwd
528	TCCTAAAACCAAAATCCAGTACT	t35S Fwd
583	AACAGGCTGATGAGTACCGC	<i>nifH</i> tsd Fwd
584	CCTTCGGATCACAGCCAACT	<i>nifH</i> tsd Rev
585	GGCTAGGGGCGAATTATCCC	<i>nifM</i> tsd Fwd
586	CTAACGCCTGCTGAAGAGCT	<i>nifM</i> tsd Rev
587	ACATGCCTTCGTACAGGAG	<i>nifS</i> tsd Fwd
588	AAGATTGCGGTGCTGTCAGA	<i>nifS</i> tsd Rev
589	CGCAAGGGGTGAAGTGTTTG	<i>nifU</i> tsd Fwd
590	GCTTCGTCCAATGTCAAGCC	<i>nifU</i> tsd Rev
698	GCTAGCGTTCGTACACGGAT	pMaize Ubi1+1i Fwd
739	CCGTCAGGGCGCGTC	L2_pUC57 Fwd
746	CACGCGACAATGTGGTCC	<i>nifH</i> rco Fwd
747	TCGGCCGCCATCTCCA	<i>nifH</i> rco Rev
748	CGAGTCTCCTATCGGCTTCC	<i>nifM</i> rco Fwd
749	CCTCTGGTGACTCGTAGCG	<i>nifM</i> rco Rev
750	CCTTCACACGTGATGAGGGC	<i>nifS</i> rco Fwd

751	GCTGCGCCTTCAGGG	<i>nifS</i> rco Rev
752	CCTCCAGCGGGATAAGGG	<i>nifU</i> rco Fwd
753	GAAGGTCTGGAAGCCGGC	<i>nifU</i> rco Rev
870	ACTGTCGGGCGTACACAAAT	<i>HPT</i> Fwd
905	CGATGTCGACGGCAAGAACGTTTATGTCAA	<i>nifS</i> nco Fwd
908	CATCCTGCTGCTGCTGAACAAGGTC	<i>nifU</i> nco Fwd
911	CATACAAAAATGACGTATCAAAG	pRice Actin1 Rev
928	TGAGTCCGGTACTGGAATCG	<i>nifM</i> nco Fwd
936	AGAGCATTGGCCAGAAAGGT	<i>nifH</i> yco Fwd
936	CCGCCAACACATCTCCAA	<i>nifH</i> nco Fwd
937	CCAGCAAGACAATTGACCTTGG	<i>nifM</i> yco Fwd
940	TACAGCTGCACACGGTACTG	<i>nifS</i> yco Fwd
941	ACGTCAAATTGACCGGTGCT	<i>nifU</i> yco Fwd
942	CGCGAACTCTTCAGCATCTC	<i>P19</i> Fwd
1429	GACGTAAGGGATGACGCACA	p35S Fwd
2288	CTACGAGGGCACCCAGACC	<i>BFP</i> Fwd
2289	CTGCCACCTCGTGCTGCT	<i>BFP</i> Rev

Supplemental Table 2. List of modular pieces generated in this thesis.

Modular piece	Backbone	Position	Type	Domesticated for	Amplification Primers (Mutagenesis primers)	Template
<i>Atm</i> AROATP	pAGM9121	B3	TP	<i>BsaI</i> , <i>BpiI</i>	406/407 (408/410)	Arabidopsis genomic DNA
<i>Atc</i> AROATP	pAGM9121	B3	TP	<i>BsaI</i> , <i>BpiI</i>	406/409 (409)	Arabidopsis genomic DNA
<i>Atm</i> BCCP1TP	pAGM9121	B3	TP	<i>BsaI</i> , <i>BpiI</i>	411/412 (413/415/412)	Arabidopsis cDNA
<i>Atc</i> BCCP1TP	pAGM9121	B3	TP	<i>BsaI</i> , <i>BpiI</i>	414/412 (413/415/412)	Arabidopsis cDNA
<i>Atm</i> DNAJ8TP	pAGM9121	B3	TP	<i>BsaI</i> , <i>BpiI</i>	419/420	Arabidopsis genomic DNA
<i>Atc</i> DNAJ8TP	pAGM9121	B3	TP	<i>BsaI</i> , <i>BpiI</i>	421/420	Arabidopsis genomic DNA
<i>Atm</i> CAB6TP	pAGM9121	B3	TP	<i>BsaI</i> , <i>BpiI</i>	416/417	Arabidopsis genomic DNA
<i>Atc</i> CAB6TP	pAGM9121	B3	TP	<i>BsaI</i> , <i>BpiI</i>	418/417	Arabidopsis genomic DNA
<i>Atm</i> GLTB2TP	pAGM9121	B3	TP	<i>BsaI</i> , <i>BpiI</i>	422/423 (425)	Arabidopsis genomic DNA
<i>Atc</i> GLTB2TP	pAGM9121	B3	TP	<i>BsaI</i> , <i>BpiI</i>	423/424 (425)	Arabidopsis genomic DNA
<i>Ntm</i> RBS _{TP}	pAGM9121	B3	TP	<i>BsaI</i> , <i>BpiI</i>	432/433	<i>Nicotiana tabacum</i> genomic DNA
<i>Ntc</i> RBS _{TP}	pAGM9121	B3	TP	<i>BsaI</i> , <i>BpiI</i>	434/433	<i>Nicotiana tabacum</i> genomic DNA
<i>Atm</i> RBS1ATP	pAGM9121	B3	TP	<i>BsaI</i> , <i>BpiI</i>	435/436	Arabidopsis cDNA
<i>Atc</i> RBS1ATP	pAGM9121	B3	TP	<i>BsaI</i> , <i>BpiI</i>	437/436	Arabidopsis cDNA
<i>Psm</i> RBS2TP	pAGM9121	B3	TP	<i>BsaI</i> , <i>BpiI</i>	438/439	<i>Pisum sativum</i> genomic DNA
<i>Psc</i> RBS2TP	pAGM9121	B3	TP	<i>BsaI</i> , <i>BpiI</i>	440/439	<i>Pisum sativum</i> genomic DNA
<i>Atm</i> RCA _{TP}	pAGM9121	B3	TP	<i>BsaI</i> , <i>BpiI</i>	441/442 (443/445)	Arabidopsis cDNA
<i>Atc</i> RCA _{TP}	pAGM9121	B3	TP	<i>BsaI</i> , <i>BpiI</i>	444/442 (443/445)	Arabidopsis cDNA
<i>Ntm</i> SIR _{TP}	pAGM9121	B3	TP	<i>BsaI</i> , <i>BpiI</i>	446/447 (448/450)	<i>Nicotiana tabacum</i> genomic DNA

<i>NtcSIR</i> _{TP}	pAGM9121	B3	TP	<i>BsaI, BpiI</i>	449/447 (448/450)	<i>Nicotiana tabacum</i> genomic DNA
<i>AtmTOCC</i> _{TP}	pAGM9121	B3	TP	<i>BsaI, BpiI</i>	451/452	Arabidopsis genomic DNA
<i>AtcTOCC</i> _{TP}	pAGM9121	B3	TP	<i>BsaI, BpiI</i>	453/452	Arabidopsis genomic DNA
<i>synmRBS</i> _{TP}	pAGM9121	B3	TP	<i>BsaI, BpiI</i>	426/427	pICH78133 (MoClo ¹)
<i>syncRBS</i> _{TP}	pAGM9121	B3	TP	<i>BsaI, BpiI</i>	428/427	pICH78133 (MoClo ¹)
<i>OsRBS1</i> _{TP}	pAGM9121	B3	TP	<i>BsaI, BpiI</i>	759/760	Oryza sativa genomic DNA
<i>AtRecA</i> _{TP}	pAGM9121	B3	TP	<i>BsaI, BpiI</i>	947/948	Arabidopsis genomic DNA
<i>nifH</i> yco	pAGM9121	B5	CDS	<i>BsaI, BpiI</i>	454/455	Synthesis by Proteogenix
<i>nifM</i> yco	pAGM9121	B4-B5	CDS	<i>BsaI, BpiI</i>	456/457	Synthesis by GenScript
<i>nifS</i> yco	pAGM9121	B4-B5	CDS	<i>BsaI, BpiI</i>	458/459 (460)	Synthesis by GenScript
<i>nifU</i> yco	pAGM9121	B4-B5	CDS	<i>BsaI, BpiI</i>	461/462	Synthesis by GenScript
<i>nifH</i> tsd	pAGM9121	B5	CDS	<i>BsaI, BpiI</i>	463/502	Synthesis by NZYTech
<i>nifM</i> tsd	pAGM9121	B4-B5	CDS	<i>BsaI, BpiI</i>	465/503	Synthesis by NZYTech
<i>nifS</i> tsd	pAGM9121	B4-B5	CDS	<i>BsaI, BpiI</i>	467/504	Synthesis by NZYTech
<i>nifU</i> tsd	pAGM9121	B4-B5	CDS	<i>BsaI, BpiI</i>	469/505	Synthesis by NZYTech
Twin-Strep	pAGM9121	B4	Tag	<i>BsaI, BpiI</i>	471/495	Synthesis by NZYTech
CaMV p35S	pAGM9121	A1-B2	Promoter	<i>BsaI, BpiI</i>	479/480	pICH51266 (MoClo ¹)
CaMV p35S	pAGM9121	A1-B3	Promoter	<i>BsaI, BpiI</i>	479/535	pICH51266 (MoClo ¹)
CaMV p35S (short)	pAGM9121	A1-B2	Promoter	<i>BsaI, BpiI</i>	812/480	pICH41388 (MoClo ¹)
2x CaMV p35s + 5'UTR TMV	pICH41295	A1-B2	Promoter	<i>BsaI, BpiI</i>	-	pICH51288 (MoClo ¹)
<i>nifH</i> tsd	pAGM9121	B5a	CDS	<i>BsaI, BpiI</i>	463/578/579	Synthesis by NZYTech
<i>nifM</i> tsd	pAGM9121	B4-B5a	CDS	<i>BsaI, BpiI</i>	465/578/580	Synthesis by NZYTech

<i>nifS</i> tsd	pAGM9121	B4-B5a	CDS	<i>BsaI, BpiI</i>	467/578/581	Synthesis by NZYTech
<i>nifU</i> tsd	pAGM9121	B4-B5a	CDS	<i>BsaI, BpiI</i>	469/578/582	Synthesis by NZYTech
<i>tGFP</i>	pAGM9121	B5b	CDS	<i>BsaI, BpiI</i>	576/577	pICSL80005 (MoClo ¹)
<i>mCherry</i>	pAGM9121	B5b	CDS	<i>BsaI, BpiI</i>	714/715	pICSL80007 (MoClo ¹)
<i>mCherry</i>	pAGM1301	B5	CDS	<i>BsaI, BpiI</i>	-	pICSL50004 (MoClo ¹)
<i>BFP</i>	pAGM9121	B4-B5	CDS	<i>BsaI, BpiI</i>	712/713	GB0025 ²
<i>eGFP</i>	pICH41308	B3-B5	CDS	<i>BsaI, BpiI</i>	-	pICH41531 (MoClo ¹)
<i>eGFP</i>	pAGM9121	B4-B5	CDS	<i>BsaI, BpiI</i>	621/622	pICH41531 (MoClo ¹)
T35s	pICH41276	B6-C1	Terminator	<i>BsaI, BpiI</i>	-	pICH41414 (MoClo ¹)
pNOS+Ω	pICH41295	A1-B2	Promoter	<i>BsaI, BpiI</i>	-	pICH87633 (MoClo ¹)
<i>p19</i>	pICH41308	B3-B5	Silencing inhibitor	<i>BsaI, BpiI</i>	-	pICH44022 (MoClo ¹)
tNOS	pICH41276	B6-C1	Terminator	<i>BsaI, BpiI</i>	-	pICH41421 (MoClo ¹)
pMaize Ubi1+1i	pAGM9121	A1-B2	Promoter	<i>BsaI, BpiI</i>	595/596	Synthesis by GenScript
pMaize Ubi1+1i	pAGM9121	A1-B3	Promoter	<i>BsaI, BpiI</i>	595/758	Synthesis by GenScript
pRice Actin1	pAGM9121	A1-B2	Promoter	<i>BsaI, BpiI</i>	597/600 (598/599)	Synthesis by GenScript
tADPGG	pAGM9121	B6-C1	Terminator	<i>BsaI, BpiI</i>	629/630	Synthesis by GenScript
HPT	pAGM9121	B3-B5	CDS	<i>BsaI, BpiI</i>	765/766	Paul Christou's lab ³
Twin-Strep rco	pAGM9121	B4	Tag	<i>BsaI, BpiI</i>	771/772	Synthesis by GenScript
<i>nifH</i> rco	pAGM9121	B5	CDS	<i>BsaI, BpiI</i>	623/624	Synthesis by GenScript
<i>nifM</i> rco	pAGM9121	B4-B5	CDS	<i>BsaI, BpiI</i>	617/618	Synthesis by GenScript
<i>nifS</i> rco	pAGM9121	B4-B5	CDS	<i>BsaI, BpiI</i>	615/616	Synthesis by GenScript
<i>nifU</i> rco	pAGM9121	B4-B5	CDS	<i>BsaI, BpiI</i>	613/614	Synthesis by GenScript

<i>nifH</i> rco	pAGM9121	B5a	CDS	<i>BsaI, BpiI</i>	578/623/883	Synthesis by GenScript
<i>nifM</i> rco	pAGM9121	B4-B5a	CDS	<i>BsaI, BpiI</i>	578/617/884	Synthesis by GenScript
<i>nifS</i> rco	pAGM9121	B4-B5a	CDS	<i>BsaI, BpiI</i>	578/615/886	Synthesis by GenScript
<i>nifU</i> rco	pAGM9121	B4-B5a	CDS	<i>BsaI, BpiI</i>	578/613/885	Synthesis by GenScript
<i>nifH</i>	pAGM9121	B5	CDS	<i>BsaI, BpiI</i>	813/814 (823/824/825/ 826/827/828)	<i>Azotobacter vinelandii</i> gDNA
<i>nifM</i>	pAGM9121	B4-B5	CDS	<i>BsaI, BpiI</i>	815/816 (829/830)	<i>Azotobacter vinelandii</i> gDNA
<i>nifS</i>	pAGM9121	B4-B5	CDS	<i>BsaI, BpiI</i>	819/820 (835/836/837/ 838)	<i>Azotobacter vinelandii</i> gDNA
<i>nifU</i>	pAGM9121	B4-B5	CDS	<i>BsaI, BpiI</i>	817/818 (831/832/833/ 834)	<i>Azotobacter vinelandii</i> gDNA
<i>nifH</i> rsd	pAGM9121	B5	CDS	<i>BsaI, BpiI</i>	623/624 (887/888/889/ 890/891/892)	Synthesis by GenScript
<i>nifM</i> rsd	pAGM9121	B4-B5	CDS	<i>BsaI, BpiI</i>	617/618 (893/894/895/ 896)	Synthesis by GenScript
<i>nifS</i> rsd	pAGM9121	B4-B5	CDS	<i>BsaI, BpiI</i>	615/616 (897/898)	Synthesis by GenScript
<i>nifU</i> rsd	pAGM9121	B4-B5	CDS	<i>BsaI, BpiI</i>	613/614 (899/900/901/ 902/903/904/ 905/906/907/90 8/909/910)	Synthesis by GenScript

Abbreviation: m: minimal; c: complete; yco: Yeast codon optimization; tsd: Tobacco synthetic design; rco: Rice codon optimization; rsd: Rice synthetic design.

Supplemental Table 3. List of assembled Transcriptional Units.

Transcriptional Units (Level 1)	Entry vector	Modular pieces used
<i>Atm</i> AROATP- <i>nifH</i> _{tsd}	pICH47732	p35S:: <i>Atm</i> AROATP-Twin-Strep- <i>nifH</i> _{tsd} -T35s
<i>Atc</i> AROATP- <i>nifH</i> _{tsd}	pICH47732	p35S:: <i>Atc</i> AROATP-Twin-Strep- <i>nifH</i> _{tsd} -T35s
<i>Atm</i> BCCP1 _{TP} - <i>nifH</i> _{tsd}	pICH47732	p35S:: <i>Atm</i> BCCP1 _{TP} -Twin-Strep- <i>nifH</i> _{tsd} -T35s
<i>Atc</i> BCCP1 _{TP} - <i>nifH</i> _{tsd}	pICH47732	p35S:: <i>Atc</i> BCCP1 _{TP} -Twin-Strep- <i>nifH</i> _{tsd} -T35s
<i>Atm</i> DNAJ8 _{TP} - <i>nifH</i> _{tsd}	pICH47732	p35S:: <i>Atm</i> DNAJ8 _{TP} -Twin-Strep- <i>nifH</i> _{tsd} -T35s
<i>Atc</i> DNAJ8 _{TP} - <i>nifH</i> _{tsd}	pICH47732	p35S:: <i>Atc</i> DNAJ8 _{TP} -Twin-Strep- <i>nifH</i> _{tsd} -T35s
<i>Atm</i> CAB6 _{TP} - <i>nifH</i> _{tsd}	pICH47732	p35S:: <i>Atm</i> CAB6 _{TP} -Twin-Strep- <i>nifH</i> _{tsd} -T35s
<i>Atc</i> CAB6 _{TP} - <i>nifH</i> _{tsd}	pICH47732	p35S:: <i>Atc</i> CAB6 _{TP} -Twin-Strep- <i>nifH</i> _{tsd} -T35s
<i>Atm</i> GLTB2 _{TP} - <i>nifH</i> _{tsd}	pICH47732	p35S:: <i>Atm</i> GLTB2 _{TP} -Twin-Strep- <i>nifH</i> _{tsd} -T35s
<i>Atc</i> GLTB2 _{TP} - <i>nifH</i> _{tsd}	pICH47732	p35S:: <i>Atc</i> GLTB2 _{TP} -Twin-Strep- <i>nifH</i> _{tsd} -T35s
<i>Ntm</i> RBS _{TP} - <i>nifH</i> _{tsd}	pICH47732	p35S:: <i>Ntm</i> RBS _{TP} -Twin-Strep- <i>nifH</i> _{tsd} -T35s
<i>Ntc</i> RBS _{TP} - <i>nifH</i> _{tsd}	pICH47732	p35S:: <i>Ntc</i> RBS _{TP} -Twin-Strep- <i>nifH</i> _{tsd} -T35s
<i>Atm</i> RBS1A _{TP} - <i>nifH</i> _{tsd}	pICH47732	p35S:: <i>Atm</i> RBS1A _{TP} -Twin-Strep- <i>nifH</i> _{tsd} -T35s
<i>Atc</i> RBS1A _{TP} - <i>nifH</i> _{tsd}	pICH47732	p35S:: <i>Atc</i> RBS1A _{TP} -Twin-Strep- <i>nifH</i> _{tsd} -T35s
<i>Psm</i> RBS2 _{TP} - <i>nifH</i> _{tsd}	pICH47732	p35S:: <i>Psm</i> RBS2 _{TP} -Twin-Strep- <i>nifH</i> _{tsd} -T35s
<i>Psc</i> RBS2 _{TP} - <i>nifH</i> _{tsd}	pICH47732	p35S:: <i>Psc</i> RBS2 _{TP} -Twin-Strep- <i>nifH</i> _{tsd} -T35s
<i>Atm</i> RCA _{TP} - <i>nifH</i> _{tsd}	pICH47732	p35S:: <i>Atm</i> RCA _{TP} -Twin-Strep- <i>nifH</i> _{tsd} -T35s
<i>Atc</i> RCA _{TP} - <i>nifH</i> _{tsd}	pICH47732	p35S:: <i>Atc</i> RCA _{TP} -Twin-Strep- <i>nifH</i> _{tsd} -T35s
<i>Ntm</i> SIR _{TP} - <i>nifH</i> _{tsd}	pICH47732	p35S:: <i>Ntm</i> SIR _{TP} -Twin-Strep- <i>nifH</i> _{tsd} -T35s
<i>Ntc</i> SIR _{TP} - <i>nifH</i> _{tsd}	pICH47732	p35S:: <i>Ntc</i> SIR _{TP} -Twin-Strep- <i>nifH</i> _{tsd} -T35s

<i>AtmTOCC_{TP}-nifH_{tsd}</i>	pICH47732	p35S:: <i>AtmTOCC_{TP}-Twin-Strep-nifH_{tsd}-T35s</i>
<i>AtcTOCC_{TP}-nifH_{tsd}</i>	pICH47732	p35S:: <i>AtcTOCC_{TP}-Twin-Strep-nifH_{tsd}-T35s</i>
<i>synmRBS_{TP}-nifH_{tsd}</i>	pICH47732	p35S:: <i>synmRBS_{TP}-Twin-Strep-nifH_{tsd}-T35s</i>
<i>syncRBS_{TP}-nifH_{tsd}</i>	pICH47732	p35S:: <i>syncRBS_{TP}-Twin-Strep-nifH_{tsd}-T35s</i>
<i>AtmAROATP-nifM_{tsd}</i>	pICH47742	p35S:: <i>AtmAROATP-nifM_{tsd}-T35s</i>
<i>AtcAROATP-nifM_{tsd}</i>	pICH47742	p35S:: <i>AtcAROATP-nifM_{tsd}-T35s</i>
<i>AtmBCCP1_{TP}-nifM_{tsd}</i>	pICH47742	p35S:: <i>AtmBCCP1_{TP}-nifM_{tsd}-T35s</i>
<i>AtcBCCP1_{TP}-nifM_{tsd}</i>	pICH47742	p35S:: <i>AtcBCCP1_{TP}-nifM_{tsd}-T35s</i>
<i>AtmDNAJ8_{TP}-nifM_{tsd}</i>	pICH47742	p35S:: <i>AtmDNAJ8_{TP}-nifM_{tsd}-T35s</i>
<i>AtcDNAJ8_{TP}-nifM_{tsd}</i>	pICH47742	p35S:: <i>AtcDNAJ8_{TP}-nifM_{tsd}-T35s</i>
<i>AtmCAB6_{TP}-nifM_{tsd}</i>	pICH47742	p35S:: <i>AtmCAB6_{TP}-nifM_{tsd}-T35s</i>
<i>AtcCAB6_{TP}-nifM_{tsd}</i>	pICH47742	p35S:: <i>AtcCAB6_{TP}-nifM_{tsd}-T35s</i>
<i>AtmGLTB2_{TP}-nifM_{tsd}</i>	pICH47742	p35S:: <i>AtmGLTB2_{TP}-nifM_{tsd}-T35s</i>
<i>AtcGLTB2_{TP}-nifM_{tsd}</i>	pICH47742	p35S:: <i>AtcGLTB2_{TP}-nifM_{tsd}-T35s</i>
<i>NtmRBS_{TP}-nifM_{tsd}</i>	pICH47742	p35S:: <i>NtmRBS_{TP}-nifM_{tsd}-T35s</i>
<i>NtcRBS_{TP}-nifM_{tsd}</i>	pICH47742	p35S:: <i>NtcRBS_{TP}-nifM_{tsd}-T35s</i>
<i>AtmRBS1ATP-nifM_{tsd}</i>	pICH47742	p35S:: <i>AtmRBS1ATP-nifM_{tsd}-T35s</i>
<i>AtcRBS1ATP-nifM_{tsd}</i>	pICH47742	p35S:: <i>AtcRBS1ATP-nifM_{tsd}-T35s</i>
<i>PsmRBS2_{TP}-nifM_{tsd}</i>	pICH47742	p35S:: <i>PsmRBS2_{TP}-nifM_{tsd}-T35s</i>
<i>PscRBS2_{TP}-nifM_{tsd}</i>	pICH47742	p35S:: <i>PscRBS2_{TP}-nifM_{tsd}-T35s</i>
<i>AtmRCA_{TP}-nifM_{tsd}</i>	pICH47742	p35S:: <i>AtmRCA_{TP}-nifM_{tsd}-T35s</i>
<i>AtcRCA_{TP}-nifM_{tsd}</i>	pICH47742	p35S:: <i>AtcRCA_{TP}-nifM_{tsd}-T35s</i>

<i>NtmSIR_{TP}-nifM_{tsd}</i>	pICH47742	p35S:: <i>NtmSIR_{TP}-nifM_{tsd}</i> -T35s
<i>NtcSIR_{TP}-nifM_{tsd}</i>	pICH47742	p35S:: <i>NtcSIR_{TP}-nifM_{tsd}</i> -T35s
<i>AtmTOCC_{TP}-nifM_{tsd}</i>	pICH47742	p35S:: <i>AtmTOCC_{TP}-nifM_{tsd}</i> -T35s
<i>AtcTOCC_{TP}-nifM_{tsd}</i>	pICH47742	p35S:: <i>AtcTOCC_{TP}-nifM_{tsd}</i> -T35s
<i>synmRBS_{TP}-nifM_{tsd}</i>	pICH47742	p35S:: <i>synmRBS_{TP}-nifM_{tsd}</i> -T35s
<i>syncRBS_{TP}-nifM_{tsd}</i>	pICH47742	p35S:: <i>syncRBS_{TP}-nifM_{tsd}</i> -T35s
<i>AtmAROA_{TP}-nifU_{tsd}</i>	pICH47761	p35S:: <i>AtmAROA_{TP}-nifU_{tsd}</i> -T35s
<i>AtcAROA_{TP}-nifU_{tsd}</i>	pICH47761	p35S:: <i>AtcAROA_{TP}-nifU_{tsd}</i> -T35s
<i>AtmBCCP1_{TP}-nifU_{tsd}</i>	pICH47761	p35S:: <i>AtmBCCP1_{TP}-nifU_{tsd}</i> -T35s
<i>AtcBCCP1_{TP}-nifU_{tsd}</i>	pICH47761	p35S:: <i>AtcBCCP1_{TP}-nifU_{tsd}</i> -T35s
<i>AtmDNAJ8_{TP}-nifU_{tsd}</i>	pICH47761	p35S:: <i>AtmDNAJ8_{TP}-nifU_{tsd}</i> -T35s
<i>AtcDNAJ8_{TP}-nifU_{tsd}</i>	pICH47761	p35S:: <i>AtcDNAJ8_{TP}-nifU_{tsd}</i> -T35s
<i>AtmCAB6_{TP}-nifU_{tsd}</i>	pICH47761	p35S:: <i>AtmCAB6_{TP}-nifU_{tsd}</i> -T35s
<i>AtcCAB6_{TP}-nifU_{tsd}</i>	pICH47761	p35S:: <i>AtcCAB6_{TP}-nifU_{tsd}</i> -T35s
<i>AtmGLTB2_{TP}-nifU_{tsd}</i>	pICH47761	p35S:: <i>AtmGLTB2_{TP}-nifU_{tsd}</i> -T35s
<i>AtcGLTB2_{TP}-nifU_{tsd}</i>	pICH47761	p35S:: <i>AtcGLTB2_{TP}-nifU_{tsd}</i> -T35s
<i>NtmRBS_{TP}-nifU_{tsd}</i>	pICH47761	p35S:: <i>NtmRBS_{TP}-nifU_{tsd}</i> -T35s
<i>NtcRBS_{TP}-nifU_{tsd}</i>	pICH47761	p35S:: <i>NtcRBS_{TP}-nifU_{tsd}</i> -T35s
<i>AtmRBS1A_{TP}-nifU_{tsd}</i>	pICH47761	p35S:: <i>AtmRBS1A_{TP}-nifU_{tsd}</i> -T35s
<i>AtcRBS1A_{TP}-nifU_{tsd}</i>	pICH47761	p35S:: <i>AtcRBS1A_{TP}-nifU_{tsd}</i> -T35s
<i>PsmRBS2_{TP}-nifU_{tsd}</i>	pICH47761	p35S:: <i>PsmRBS2_{TP}-nifU_{tsd}</i> -T35s
<i>PscRBS2_{TP}-nifU_{tsd}</i>	pICH47761	p35S:: <i>PscRBS2_{TP}-nifU_{tsd}</i> -T35s

<i>AtmRCA_{TP-nifU_{tsd}}</i>	pICH47761	p35S:: <i>AtmRCA_{TP-nifU_{tsd}}</i> -T35s
<i>AtcRCA_{TP-nifU_{tsd}}</i>	pICH47761	p35S:: <i>AtcRCA_{TP-nifU_{tsd}}</i> -T35s
<i>NtmSIR_{TP-nifU_{tsd}}</i>	pICH47761	p35S:: <i>NtmSIR_{TP-nifU_{tsd}}</i> -T35s
<i>NtcSIR_{TP-nifU_{tsd}}</i>	pICH47761	p35S:: <i>NtcSIR_{TP-nifU_{tsd}}</i> -T35s
<i>AtmTOCC_{TP-nifU_{tsd}}</i>	pICH47761	p35S:: <i>AtmTOCC_{TP-nifU_{tsd}}</i> -T35s
<i>AtcTOCC_{TP-nifU_{tsd}}</i>	pICH47761	p35S:: <i>AtcTOCC_{TP-nifU_{tsd}}</i> -T35s
<i>synmRBS_{TP-nifU_{tsd}}</i>	pICH47761	p35S:: <i>synmRBS_{TP-nifU_{tsd}}</i> -T35s
<i>syncRBS_{TP-nifU_{tsd}}</i>	pICH47761	p35S:: <i>syncRBS_{TP-nifU_{tsd}}</i> -T35s
<i>AtmAROA_{TP-nifS_{tsd}}</i>	pICH47772	p35S:: <i>AtmAROA_{TP-nifS_{tsd}}</i> -T35s
<i>AtcAROA_{TP-nifS_{tsd}}</i>	pICH47772	p35S:: <i>AtcAROA_{TP-nifS_{tsd}}</i> -T35s
<i>AtmBCCP1_{TP-nifS_{tsd}}</i>	pICH47772	p35S:: <i>AtmBCCP1_{TP-nifS_{tsd}}</i> -T35s
<i>AtcBCCP1_{TP-nifS_{tsd}}</i>	pICH47772	p35S:: <i>AtcBCCP1_{TP-nifS_{tsd}}</i> -T35s
<i>AtmDNAJ8_{TP-nifS_{tsd}}</i>	pICH47772	p35S:: <i>AtmDNAJ8_{TP-nifS_{tsd}}</i> -T35s
<i>AtcDNAJ8_{TP-nifS_{tsd}}</i>	pICH47772	p35S:: <i>AtcDNAJ8_{TP-nifS_{tsd}}</i> -T35s
<i>AtmCAB6_{TP-nifS_{tsd}}</i>	pICH47772	p35S:: <i>AtmCAB6_{TP-nifS_{tsd}}</i> -T35s
<i>AtcCAB6_{TP-nifS_{tsd}}</i>	pICH47772	p35S:: <i>AtcCAB6_{TP-nifS_{tsd}}</i> -T35s
<i>AtmGLTB2_{TP-nifS_{tsd}}</i>	pICH47772	p35S:: <i>AtmGLTB2_{TP-nifS_{tsd}}</i> -T35s
<i>AtcGLTB2_{TP-nifS_{tsd}}</i>	pICH47772	p35S:: <i>AtcGLTB2_{TP-nifS_{tsd}}</i> -T35s
<i>NtmRBS_{TP-nifS_{tsd}}</i>	pICH47772	p35S:: <i>NtmRBS_{TP-nifS_{tsd}}</i> -T35s
<i>NtcRBS_{TP-nifS_{tsd}}</i>	pICH47772	p35S:: <i>NtcRBS_{TP-nifS_{tsd}}</i> -T35s
<i>AtmRBS1A_{TP-nifS_{tsd}}</i>	pICH47772	p35S:: <i>AtmRBS1A_{TP-nifS_{tsd}}</i> -T35s
<i>AtcRBS1A_{TP-nifS_{tsd}}</i>	pICH47772	p35S:: <i>AtcRBS1A_{TP-nifS_{tsd}}</i> -T35s

<i>PsmRBS2_{TP-nifS_{tsd}}</i>	pICH47772	p35S:: <i>PsmRBS2_{TP-nifS_{tsd}}</i> -T35s
<i>PscRBS2_{TP-nifS_{tsd}}</i>	pICH47772	p35S:: <i>PscRBS2_{TP-nifS_{tsd}}</i> -T35s
<i>AtmRCA_{TP-nifS_{tsd}}</i>	pICH47772	p35S:: <i>AtmRCA_{TP-nifS_{tsd}}</i> -T35s
<i>AtcRCA_{TP-nifS_{tsd}}</i>	pICH47772	p35S:: <i>AtcRCA_{TP-nifS_{tsd}}</i> -T35s
<i>NtmSIR_{TP-nifS_{tsd}}</i>	pICH47772	p35S:: <i>NtmSIR_{TP-nifS_{tsd}}</i> -T35s
<i>NtcSIR_{TP-nifS_{tsd}}</i>	pICH47772	p35S:: <i>NtcSIR_{TP-nifS_{tsd}}</i> -T35s
<i>AtmTOCC_{TP-nifS_{tsd}}</i>	pICH47772	p35S:: <i>AtmTOCC_{TP-nifS_{tsd}}</i> -T35s
<i>AtcTOCC_{TP-nifS_{tsd}}</i>	pICH47772	p35S:: <i>AtcTOCC_{TP-nifS_{tsd}}</i> -T35s
<i>synmRBS_{TP-nifS_{tsd}}</i>	pICH47772	p35S:: <i>synmRBS_{TP-nifS_{tsd}}</i> -T35s
<i>syncRBS_{TP-nifS_{tsd}}</i>	pICH47772	p35S:: <i>syncRBS_{TP-nifS_{tsd}}</i> -T35s
<i>cytonifH_{tsd}</i>	pICH47742	p35S::Twin-Strep- <i>nifH_{tsd}</i> -T35s
<i>cytonifM_{tsd}</i>	pICH47761	p35S:: <i>nifM_{tsd}</i> -T35s
<i>cytonifS_{tsd}</i>	pICH47772	p35S:: <i>nifS_{tsd}</i> -T35s
<i>cytonifU_{tsd}</i>	pICH47761	p35S:: <i>nifU_{tsd}</i> -T35s
<i>AtmRCA_{TP-nifH_{tsd}}- tGFP</i>	pICH47732	p35S:: <i>AtmRCA_{TP}</i> -Twin-Strep- <i>nifH_{tsd}</i> - <i>tGFP</i> -T35s
<i>AtmBCCP1_{TP-nifM_{tsd}}- tGFP</i>	pICH47742	p35S:: <i>AtmBCCP1_{TP-nifM_{tsd}}</i> - <i>tGFP</i> -T35s
<i>AtmBCCP1_{TP-nifM_{tsd}}- mCherry</i>	pICH47742	p35S:: <i>AtmBCCP1_{TP-nifM_{tsd}}</i> - <i>mCherry</i> -T35s
<i>AtmTOCC_{TP-nifU_{tsd}}- tGFP</i>	pICH47761	p35S:: <i>AtmTOCC_{TP-nifU_{tsd}}</i> - <i>tGFP</i> -T35s
<i>AtmCAB6_{TP-nifS_{tsd}}- tGFP</i>	pICH47772	p35S:: <i>AtmCAB6_{TP-nifS_{tsd}}</i> - <i>tGFP</i> -T35s
<i>AtmRCA_{TP-nifH_{yco}}</i>	pICH47732	p35S:: <i>AtmRCA_{TP}</i> -Twin-Strep- <i>nifH_{yco}</i> -T35s
<i>AtmBCCP1_{TP-nifM_{yco}}</i>	pICH47742	p35S:: <i>AtmBCCP1_{TP-nifM_{yco}}</i> -T35s
<i>AtmTOCC_{TP-nifU_{yco}}</i>	pICH47761	p35S:: <i>AtmTOCC_{TP-nifU_{yco}}</i> -T35s

<i>AtmCAB6_{TP}-nifS_{yc}</i>	pICH47772	p35S:: <i>AtmCAB6_{TP}-nifS_{yc}</i> -T35s
<i>syncRBS_{TP}-BFP</i>	pICH47781	p35S:: <i>syncRBS_{TP}-BFP</i> -T35s
<i>AtRecA_{TP}-mCherry</i>	pICH47742	2x p35S:: <i>AtRecA_{TP}-mCherry</i> -T35s
P19	pICH47751	pNOS+Ω:: <i>p19</i> -tNOS
<i>cytoeGFP</i>	pICH47781	p35S:: <i>eGFP</i> -T35s
<i>cytoeGFP</i>	pICH47732	p35S:: <i>eGFP</i> -T35s
<i>AtmRCA_{TP}-eGFP</i>	pICH47732	2x p35S:: <i>AtmRCA_{TP}-eGFP</i> -tNOS
<i>AtmBCCP1_{TP}-eGFP</i>	pICH47732	2x p35S:: <i>AtmBCCP1_{TP}-eGFP</i> -tNOS
<i>AtmTOCC_{TP}-eGFP</i>	pICH47732	2x p35S:: <i>AtmTOCC_{TP}-eGFP</i> -tNOS
<i>AtmCAB6_{TP}-eGFP</i>	pICH47732	2x p35S:: <i>AtmCAB6_{TP}-eGFP</i> -tNOS
<i>AtcGLTB2_{TP}-eGFP</i>	pICH47732	2x p35S:: <i>AtcGLTB2_{TP}-eGFP</i> -tNOS
<i>OsRBS1_{TP}-eGFP</i>	pICH47732	2x p35S:: <i>OsRBS1_{TP}-eGFP</i> -tNOS
pUC57-L1P1	pUC57-AmpR	Cloning cassette pICH47732
pUC57-L1P2	pUC57-AmpR	Cloning cassette pICH47742
pUC57-L1P3	pUC57-AmpR	Cloning cassette pICH47751
pUC57-L1P4	pUC57-AmpR	Cloning cassette pICH47761
pUC57-L1P5	pUC57-AmpR	Cloning cassette pICH47772
pUC57-L1P6	pUC57-AmpR	Cloning cassette pICH47781
HPT	pUC57-L1P3	P35s (short):: <i>HPT</i> -tNOS
<i>cytoeGFP</i>	pUC57-L1P1	pMaize Ubi1 +1:: <i>eGFP</i> -tNOS
<i>cytoeGFP</i>	pUC57-L1P3	pMaize Ubi1 +1:: <i>eGFP</i> -tNOS
<i>AtmRCA_{TP}-eGFP</i>	pUC57-L1P1	pMaize Ubi1 + 1:: <i>AtmRCA_{TP}-eGFP</i> -tNOS

<i>AtmBCCP1_{TP}-eGFP</i>	pUC57-L1P1	pMaize Ubi1 +1:: <i>AtmBCCP1_{TP}-eGFP</i> -tNOS
<i>AtmTOCC_{TP}-eGFP</i>	pUC57-L1P1	pMaize Ubi1 +1:: <i>AtmTOCC_{TP}-eGFP</i> -tNOS
<i>AtmCAB6_{TP}-eGFP</i>	pUC57-L1P1	pMaize Ubi1 +1:: <i>AtmCAB6_{TP}-eGFP</i> -tNOS
<i>AtcGLTB2_{TP}-eGFP</i>	pUC57-L1P1	pMaize Ubi1 +1:: <i>AtcGLTB2_{TP}-eGFP</i> -tNOS
<i>OsRBS1_{TP}-eGFP</i>	pUC57-L1P1	pMaize Ubi1 +1:: <i>OsRBS1_{TP}-eGFP</i> -tNOS
<i>cytonifH_{rco}</i>	pUC57-L1P1	pMaize Ubi1 +1::Twin-Strep rco- <i>nifH_{rco}</i> -tNOS
<i>cytonifM_{rco}</i>	pUC57-L1P2	pMaize Ubi1 +1:: <i>nifM_{rco}</i> -tNOS
<i>cytonifS_{rco}</i>	pUC57-L1P4	pMaize Ubi1 +1:: <i>nifS_{rco}</i> -tNOS
<i>cytonifU_{rco}</i>	pUC57-L1P5	pMaize Ubi1 +1:: <i>nifU_{rco}</i> -tNOS
<i>AtmRCA_{TP}-nifH_{rco}</i>	pUC57-L1P1	pMaize Ubi1 +1:: <i>AtmRCA_{TP}</i> -Twin-Strep rco- <i>nifH_{rco}</i> -tNOS
<i>AtmBCCP1_{TP}-nifM_{rco}</i>	pUC57-L1P2	pMaize Ubi1 +1:: <i>AtmBCCP1_{TP}-nifM_{rco}</i> -tNOS
<i>AtmTOCC_{TP}-nifU_{rco}</i>	pUC57-L1P4	pMaize Ubi1 +1:: <i>AtmTOCC_{TP}-nifU_{rco}</i> -tNOS
<i>AtmCAB6_{TP}-nifS_{rco}</i>	pUC57-L1P5	pMaize Ubi1 +1:: <i>AtmCAB6_{TP}-nifS_{rco}</i> -tNOS
<i>AtmBCCP1_{TP}-nifM_{rco}</i> (b)	pUC57-L1P2	2x p35S:: <i>AtmBCCP1_{TP}-nifM_{rco}</i> -tNOS
<i>AtmTOCC_{TP}-nifU_{rco}</i> (b)	pUC57-L1P4	2x p35S:: <i>AtmTOCC_{TP}-nifU_{rco}</i> -tADPGG
<i>AtmCAB6_{TP}-nifS_{rco}</i> (b)	pUC57-L1P5	pRice Actin 1:: <i>AtmCAB6_{TP}-nifS_{rco}</i> -tADPGG
<i>AtmRCA_{TP}-nifH_{rco}-tGFP</i>	pICH47732	pMaize Ubi1 +1:: <i>AtmRCA_{TP}</i> -Twin-Strep rco- <i>nifH_{rco}-tGFP</i> -tNOS
<i>AtmBCCP1_{TP}-nifM_{rco}-mCherry</i>	pICH47742	pMaize Ubi1 +1:: <i>AtmBCCP1_{TP}-nifM_{rco}-mCherry</i> -tNOS
<i>AtmRCA_{TP}-nifH_{nco}</i>	pUC57-L1P1	pMaize Ubi1 +1:: <i>AtmRCA_{TP}</i> -Twin-Strep nco- <i>nifH_{nco}</i> -tNOS
<i>AtmBCCP1_{TP}-nifM_{nco}</i>	pUC57-L1P2	pMaize Ubi1 +1:: <i>AtmBCCP1_{TP}-nifM_{nco}</i> -tNOS
<i>AtmTOCC_{TP}-nifU_{nco}</i>	pUC57-L1P4	pMaize Ubi1 +1:: <i>AtmTOCC_{TP}-nifU_{nco}</i> -tNOS
<i>AtmCAB6_{TP}-nifS_{nco}</i>	pUC57-L1P5	pMaize Ubi1 +1:: <i>AtmCAB6_{TP}-nifS_{nco}</i> -tNOS

<i>AtmRCA_{TP}-nifH_{yco}</i>	pUC57-L1P1	pMaize Ubi1 +1:: <i>AtmRCA_{TP}</i> -Twin-Strep _{yco} - <i>nifH_{yco}</i> -tNOS
<i>AtmBCCP1_{TP}-nifM_{yco}</i>	pUC57-L1P2	pMaize Ubi1 +1:: <i>AtmBCCP1_{TP}</i> - <i>nifM_{yco}</i> -tNOS
<i>AtmTOCC_{TP}-nifU_{yco}</i>	pUC57-L1P4	pMaize Ubi1 +1:: <i>AtmTOCC_{TP}</i> - <i>nifU_{yco}</i> -tNOS
<i>AtmCAB6_{TP}-nifS_{yco}</i>	pUC57-L1P5	pMaize Ubi1 +1:: <i>AtmCAB6_{TP}</i> - <i>nifS_{yco}</i> -tNOS
<i>AtmRCA_{TP}-nifH_{tsd}</i>	pUC57-L1P1	pMaize Ubi1 +1:: <i>AtmRCA_{TP}</i> -Twin-Strep _{tsd} - <i>nifH_{tsd}</i> -tNOS
<i>AtmBCCP1_{TP}-nifM_{tsd}</i>	pUC57-L1P2	pMaize Ubi1 +1:: <i>AtmBCCP1_{TP}</i> - <i>nifM_{tsd}</i> -tNOS
<i>AtmTOCC_{TP}-nifU_{tsd}</i>	pUC57-L1P4	pMaize Ubi1 +1:: <i>AtmTOCC_{TP}</i> - <i>nifU_{tsd}</i> -tNOS
<i>AtmCAB6_{TP}-nifS_{tsd}</i>	pUC57-L1P5	pMaize Ubi1 +1:: <i>AtmCAB6_{TP}</i> - <i>nifS_{tsd}</i> -tNOS
<i>AtmRCA_{TP}-nifH_{rsd}</i>	pUC57-L1P1	pMaize Ubi1 +1:: <i>AtmRCA_{TP}</i> -Twin-Strep _{rsd} - <i>nifH_{rsd}</i> -tNOS
<i>AtmBCCP1_{TP}-nifM_{rsd}</i>	pUC57-L1P2	pMaize Ubi1 +1:: <i>AtmBCCP1_{TP}</i> - <i>nifM_{rsd}</i> -tNOS
<i>AtmTOCC_{TP}-nifU_{rsd}</i>	pUC57-L1P4	pMaize Ubi1 +1:: <i>AtmTOCC_{TP}</i> - <i>nifU_{rsd}</i> -tNOS
<i>AtmCAB6_{TP}-nifS_{rsd}</i>	pUC57-L1P5	pMaize Ubi1 +1:: <i>AtmCAB6_{TP}</i> - <i>nifS_{rsd}</i> -tNOS

Abbreviation: m: minimal; c: complete; yco: Yeast codon optimization; tsd: Tobacco synthetic design; rco: Rice codon optimization; rsd: Rice synthetic design.

Supplemental Table 4. List of assembled multigenic constructions.

Multigenic constructions (Level 2)	Entry vector	Transcriptional Units used
mChloro- <i>nifH</i> - <i>nifM</i> - <i>nifU</i> - <i>nifS</i> -P19	pAGM4673	<i>AtmRCA</i> _{TP} - <i>nifH</i> _{tsd} + <i>AtmBCCP1</i> _{TP} - <i>nifM</i> _{tsd} + <i>AtmTOCC</i> _{TP} - <i>nifU</i> _{tsd} + <i>AtmCAB6</i> _{TP} - <i>nifS</i> _{tsd} + P19
mChloro- <i>nifH</i> - <i>nifM</i> -P19	pAGM4673	<i>AtmRCA</i> _{TP} - <i>nifH</i> _{tsd} + <i>AtmBCCP1</i> _{TP} - <i>nifM</i> _{tsd} + P19
mChloro- <i>nifH</i> (<i>tGFP</i>)- <i>nifM</i> (<i>mCherry</i>)-P19	pAGM4673	<i>AtmRCA</i> _{TP} - <i>nifH</i> _{tsd} - <i>tGFP</i> + <i>AtmBCCP1</i> _{TP} - <i>nifM</i> _{tsd} - <i>mCherry</i> + P19
mChloro- <i>nifH</i> (<i>tGFP</i>)-cChloro- <i>BFP</i> -P19	pAGM4673	<i>AtmRCA</i> _{TP} - <i>nifH</i> _{tsd} - <i>tGFP</i> + <i>syncRBS</i> _{TP} - <i>BFP</i> + P19
mChloro- <i>nifM</i> (<i>tGFP</i>)-cChloro- <i>BFP</i> -P19	pAGM4673	<i>AtmBCCP1</i> _{TP} - <i>nifM</i> _{tsd} - <i>tGFP</i> + <i>syncRBS</i> _{TP} - <i>BFP</i> + P19
mChloro- <i>nifU</i> (<i>tGFP</i>)-cChloro- <i>BFP</i> -P19	pAGM4673	<i>AtmTOCC</i> _{TP} - <i>nifU</i> _{tsd} - <i>tGFP</i> + <i>syncRBS</i> _{TP} - <i>BFP</i> + P19
mChloro- <i>nifS</i> (<i>tGFP</i>)-cChloro- <i>BFP</i> -P19	pAGM4673	<i>AtmCAB6</i> _{TP} - <i>nifS</i> _{tsd} - <i>tGFP</i> + <i>syncRBS</i> _{TP} - <i>BFP</i> + P19
mChloro- <i>nifH</i> _{tsd} -cytoe <i>GFP</i> -P19	pAGM4673	<i>AtmRCA</i> _{TP} -Twin-Strep- <i>nifH</i> _{tsd} + cytoe <i>GFP</i> + P19
mChloro- <i>nifM</i> _{tsd} -cytoe <i>GFP</i> -P19	pAGM4673	<i>AtmBCCP1</i> _{TP} - <i>nifM</i> _{tsd} + cytoe <i>GFP</i> + P19
mChloro- <i>nifU</i> _{tsd} -cytoe <i>GFP</i> -P19	pAGM4673	<i>AtmTOCC</i> _{TP} - <i>nifU</i> _{tsd} + cytoe <i>GFP</i> + P19
mChloro- <i>nifS</i> _{tsd} -cytoe <i>GFP</i> -P19	pAGM4673	<i>AtmCAB6</i> _{TP} - <i>nifS</i> _{tsd} + cytoe <i>GFP</i> + P19
mChloro- <i>nifH</i> _{yco} -cytoe <i>GFP</i> -P19	pAGM4673	<i>AtmRCA</i> _{TP} -Twin-Strep- <i>nifH</i> _{yco} + cytoe <i>GFP</i> + P19
mChloro- <i>nifM</i> _{yco} -cytoe <i>GFP</i> -P19	pAGM4673	<i>AtmBCCP1</i> _{TP} - <i>nifM</i> _{yco} + cytoe <i>GFP</i> + P19
mChloro- <i>nifU</i> _{yco} -cytoe <i>GFP</i> -P19	pAGM4673	<i>AtmTOCC</i> _{TP} - <i>nifU</i> _{yco} + cytoe <i>GFP</i> + P19
mChloro- <i>nifS</i> _{yco} -cytoe <i>GFP</i> -P19	pAGM4673	<i>AtmCAB6</i> _{TP} - <i>nifS</i> _{yco} + cytoe <i>GFP</i> + P19
mChloro- <i>nifH</i> - <i>nifM</i> - <i>nifU</i> - <i>nifS</i> -cytoe <i>GFP</i> -P19	pAGM4673	<i>AtmRCA</i> _{TP} - <i>nifH</i> _{tsd} + <i>AtmBCCP1</i> _{TP} - <i>nifM</i> _{tsd} + <i>AtmTOCC</i> _{TP} - <i>nifU</i> _{tsd} + <i>AtmCAB6</i> _{TP} - <i>nifS</i> _{tsd} + cytoe <i>GFP</i> + P19
cytoe <i>GFP</i> -P19	pAGM4673	cytoe <i>GFP</i> + P19
<i>AtmRCA</i> _{TP} - <i>eGFP</i> -P19	pAGM4673	<i>AtmRCA</i> _{TP} - <i>eGFP</i> + P19
<i>AtmBCCP1</i> _{TP} - <i>eGFP</i> -P19	pAGM4673	<i>AtmBCCP1</i> _{TP} - <i>eGFP</i> + P19
<i>AtmTOCC</i> _{TP} - <i>eGFP</i> -P19	pAGM4673	<i>AtmTOCC</i> _{TP} - <i>eGFP</i> + P19

<i>AtmCAB6_{TP}-eGFP-P19</i>	pAGM4673	<i>AtmCAB6_{TP}-eGFP + P19</i>
<i>AtcGLTB2_{TP}-eGFP-P19</i>	pAGM4673	<i>AtcGLTB2_{TP}-eGFP + P19</i>
<i>OsRBS1_{TP}-eGFP-P19</i>	pAGM4673	<i>OsRBS1_{TP}-eGFP + P19</i>
<i>cytoeGFP + AtRecA_{TP}-mCherry</i>	pAGM4673	<i>cytoeGFP + P19 + AtRecA_{TP}-mCherry</i>
<i>AtmRCA_{TP}-eGFP + AtRecA_{TP}-mCherry</i>	pAGM4673	<i>AtmRCA_{TP}-eGFP + P19 + AtRecA_{TP}-mCherry</i>
<i>AtmBCCP1_{TP}- eGFP + AtRecA_{TP}-mCherry</i>	pAGM4673	<i>AtmBCCP1_{TP}- eGFP + P19 + AtRecA_{TP}-mCherry</i>
<i>AtmTOCTP- eGFP + AtRecA_{TP}-mCherry</i>	pAGM4673	<i>AtmTOCTP- eGFP + P19 + AtRecA_{TP}-mCherry</i>
<i>AtmCAB6_{TP}- eGFP + AtRecA_{TP}-mCherry</i>	pAGM4673	<i>AtmCAB6_{TP}- eGFP + P19 + AtRecA_{TP}-mCherry</i>
<i>AtcGLTB2_{TP}- eGFP + AtRecA_{TP}-mCherry</i>	pAGM4673	<i>AtcGLTB2_{TP}- eGFP + P19 + AtRecA_{TP}-mCherry</i>
<i>OsRbs1_{TP}- eGFP + AtRecA_{TP}-mCherry</i>	pAGM4673	<i>OsRbs1_{TP}- eGFP + P19 + AtRecA_{TP}-mCherry</i>
<i>AtmRCA_{TP}-nifH_{nco}-cytoeGFP</i>	pAGM4673	<i>AtmRCA_{TP}-nifH_{nco} + cytoeGFP</i>
<i>AtmBCCP1_{TP}-nifM_{nco}-cytoeGFP</i>	pAGM4673	<i>AtmBCCP1_{TP}-nifM_{nco} + cytoeGFP</i>
<i>AtmTOCC_{TP}-nifU_{nco}-cytoeGFP</i>	pAGM8055	<i>AtmTOCC_{TP}-nifU_{nco} + cytoeGFP</i>
<i>AtmCAB6_{TP}-nifS_{nco}-cytoeGFP</i>	pAGM8055	<i>AtmCAB6_{TP}-nifS_{nco} + cytoeGFP</i>
<i>AtmRCA_{TP}-nifH_{yc}-cytoeGFP</i>	pAGM4673	<i>AtmRCA_{TP}-nifH_{yc} + cytoeGFP</i>
<i>AtmBCCP1_{TP}-nifM_{yc}-cytoeGFP</i>	pAGM4673	<i>AtmBCCP1_{TP}-nifM_{yc}-cytoeGFP</i>
<i>AtmTOCC_{TP}-nifU_{yc}-cytoeGFP</i>	pAGM8055	<i>AtmTOCC_{TP}-nifU_{yc} + cytoeGFP</i>
<i>AtmCAB6_{TP}-nifS_{yc}-cytoeGFP</i>	pAGM8055	<i>AtmCAB6_{TP}-nifS_{yc} + cytoeGFP</i>
<i>AtmRCA_{TP}-nifH_{isd}-cytoeGFP</i>	pAGM4673	<i>AtmRCA_{TP}-nifH_{isd} + cytoeGFP</i>
<i>AtmBCCP1_{TP}-nifM_{isd}-cytoeGFP</i>	pAGM4673	<i>AtmBCCP1_{TP}-nifM_{isd} + cytoeGFP</i>
<i>AtmTOCC_{TP}-nifU_{isd}-cytoeGFP</i>	pAGM8055	<i>AtmTOCC_{TP}-nifU_{isd}-cytoeGFP</i>
<i>AtmCAB6_{TP}-nifS_{isd}-cytoeGFP</i>	pAGM8055	<i>AtmCAB6_{TP}-nifS_{isd} + cytoeGFP</i>

<i>AtmRCA_{TP}-nifH_{rco}-cytoeGFP</i>	pAGM4673	<i>AtmRCA_{TP}-nifH_{rco} + cytoeGFP</i>
<i>AtmBCCP1_{TP}-nifM_{rco}-cytoeGFP</i>	pAGM4673	<i>AtmBCCP1_{TP}-nifM_{rco} + cytoeGFP</i>
<i>AtmTOCC_{TP}-nifU_{rco}-cytoeGFP</i>	pAGM8055	<i>AtmTOCC_{TP}-nifU_{rco} + cytoeGFP</i>
<i>AtmCAB6_{TP}-nifS_{rco}-cytoeGFP</i>	pAGM8055	<i>AtmCAB6_{TP}-nifS_{rco} + cytoeGFP</i>
<i>AtmRCA_{TP}-nifH_{rsd}-cytoeGFP</i>	pAGM4673	<i>AtmRCA_{TP}-nifH_{rsd} + cytoeGFP</i>
<i>AtmBCCP1_{TP}-nifM_{rsd}-cytoeGFP</i>	pAGM4673	<i>AtmBCCP1_{TP}-nifM_{rsd} + cytoeGFP</i>
<i>AtmTOCC_{TP}-nifU_{rsd}-cytoeGFP</i>	pAGM8055	<i>AtmTOCC_{TP}-nifU_{rsd} + cytoeGFP</i>
<i>AtmCAB6_{TP}-nifS_{rsd}-cytoeGFP</i>	pAGM8055	<i>AtmCAB6_{TP}-nifS_{rsd} + cytoeGFP</i>
mChloro- <i>nifH_{rco}</i> (tGFP)- <i>nifM_{rco}</i> (mCherry)	pAGM4673	<i>AtmRCA_{TP}-nifH_{rco}-tGFP + AtmBCCP1_{TP}- nifM_{rco}-mCherry</i>
mChloro _{rco} - <i>nifH-nifM-nifU-nifS</i> (v1)	pUC57-L2	<i>AtmRCA_{TP}-nifH_{rco} + AtmBCCP1_{TP}-nifM_{rco} + AtmTOCC_{TP}-nifU_{rco} + AtmCAB6_{TP}-nifS_{rco}</i>
mChloro _{rco} - <i>nifH-nifM-nifU-nifS</i> (v2)	pUC57-L2	<i>AtmRCA_{TP}-nifH_{rco} + AtmBCCP1_{TP}-nifM_{rco} (b)+ AtmTOCC_{TP}-nifU_{rco} (b)+ AtmCAB6_{TP}- nifS_{rco} (b)</i>
cyto _{rco} - <i>nifH-nifM-nifU-nifS</i>	pUC57-L2	<i>cytonifH_{rco} + cytonifM_{rco} + cytonifU_{rco} + cytonifS_{rco}</i>

Abbreviation: m: minimal; c: complete; yco: Yeast codon optimization; tsd: Tobacco synthetic design; rco: Rice codon optimization; rsd: Rice synthetic design.

Supplemental Table 5. Optimization and motif settings used in Codon Optimization On-Line for tobacco *nif* synthetic designed genes.

Optimization settings

Parameter	Setting
Individual Codon Usage	Maximize
Codon Context	Ignore
Codon Adaptation Index	Ignore
Number of Hidden Stop Codons	Ignore
5' RNA Folding Instability	Maximize in the first 50 bp
GC Total Content	43.38%
GC3 Content	39.53%
Custom Codon Usage Pattern Values	Imported from http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=4097

Motif settings

Parameter	Setting			
Exclusion sequences	Constraint	Description	Sequence	Reference
		PolyA.1	AATGAA	4
		PolyA.2	AATATT	
		PolyA.3	GATAAA	
		PolyA.4	AATTAA	
		PolyA.5	AATAAT	
		FUE1	AATGTA	5
		FUE2	TAGTAG	
		FUE3	TGATTC	
		FUE4	CAAGTT	
		FUE5	TAATAA	
		FUE6	TTCTTC	
		NUE1	AATAAA	
		Cryptic splice donor	MAGGTRAGT	6
		Cryptic splice acceptor	YYYYNTAGG	
		RNA destabilizing sequence	ATTTA	
		RNA instability determinant	ATAGAT	
		Shine Dalgarno	AGGAGGTNNNNNDTG	
		ATRICH1	ASAAAA	
		ATRICH2	AASAAA	
		ATRICH3	AAASAA	
		ATRICH4	AAAASA	
		ATRICH5	TSAAAA	
		ATRICH6	TTSTTT	
		ATRICH7	TTTSTT	
		ATRICH8	TTTTST	
		NUE2	AAAAAA	
		NUE2	TTTTTT	

		FUE-TRE-CE	TTTT
		FUE-ORY1	AAAAGT
		FUE-ORY1	TTTTGT
		FUE-ORY2	GTGTG
		FUE-ORY3	TGTAW
		FUE-ORY4	TGTGT
Parameter	Setting		
Restriction sites	Constraint	Description	Sequence
		<i>BsaI</i>	GGTCTC
			GAGACC
		<i>BpiI (BbsI)</i>	GAAGAC
			GTCTTC
		<i>BsmBI</i>	CGTCTC
			GCAGAG
		<i>BtgZI</i>	GCGATG
			CGCTAC
Parameter	Setting		
Motif Repeated Consecutively	Constraint	Description	Value
		Length of Nucleotide Motif	1
		Minimum Number of Instances before removal	12
Motif repeated Regardless of Location	Constraint	Description	Value
		Length of Nucleotide Motif	7
		Minimum Number of Instances before removal	2

Abbreviation: ICU: Individual Codon Usage; GC: Distribution of Guanine and Cytosine (GC) content in ORFs; GC3: Distribution of Guanine and Cytosine (GC) content in the third codon position; FUE: Far Upstream Element; NUE: Near Upstream Element.

Supplemental Table 6. Characteristics of yeast codon-optimized and tobacco synthetic designed sequences.

	<i>AvnifH</i>		<i>AvnifM</i>		<i>AvnifU</i>		<i>AvnifS</i>	
	yco	tsd	yco	tsd	yco	tsd	yco	tsd
CAI	0.82	0.76	0.84	0.77	0.81	0.79	0.82	0.72
ICU Fitness	-0.19641	-0.086724	-0.1717	-0.08324	-0.17922	-0.088254	-0.1632	-0.07813
Total GC Content	40.09%	43.87%	40.7%	43.76%	43.34%	43.77%	41.11%	45.24%
GC3 Content	33.68%	39.52%	28.57%	35.71%	34.19%	34.5%	30.77%	35.98%
Exclusion Motifs (bp)	81	5	115	0	88	0	149	6
Repetitions (bp)	463	14	461	0	454	0	644	0

Abbreviation: yco: Yeast codon optimization; tsd: Tobacco synthetic design; CAI: Codon Adaptation Index; ICU Fitness: how far the sequence deviates from the target Individual Codon Usage; GC: Distribution of Guanine and Cytosine (GC) content in ORFs; GC3: Distribution of Guanine and Cytosine (GC) content in the third codon position.

Supplemental Table 7. Summary of Chloroplast Transit Peptides studied in this thesis.

TP name		Length	Sequence	~Mw (kDa)	Related protein (UniProt ID)	Reference
<i>At</i> AROATP	m	1 – 42	MAQVSRICNGVQNPSLISNLSKSSQKRSPLSVSLKTQ QHPRA	4.57	P05466	7
	c	1 – 76	MAQVSRICNGVQNPSLISNLSKSSQKRSPLSVSLKTQ QHPRAYPISSSWGLKKSGMTLIGSELRLKVMSSVST AE	8.20		
<i>At</i> BCCP1TP	m	1 – 62	MASSSFVSVTSPAAAASVYAVTQTSSHFP IQNRSRRVS FRLSAKPKLRFLSKPSRSSYPVVKA	6.73	Q42533	8
	c	1 – 80	MASSSFVSVTSPAAAASVYAVTQTSSHFP IQNRSRRVS FRLSAKPKLRFLSKPSRSSYPVVKAQSNKVSTGASSN AAKVDG	8.43		
<i>At</i> CAB6TP	m	1 – 56	MASNSLMSCGIAAVYPSLLSSSKSKFVSAGVPLPNAG NVGRIRMAAHWMPGEPRPA	5.77	Q01667	8,9
	c	1 – 67	MASNSLMSCGIAAVYPSLLSSSKSKFVSAGVPLPNAG NVGRIRMAAHWMPGEPRPAYLDGSAPGDFG	6.85		
<i>At</i> DNAJ8TP	m	1 – 47	MTIALTIGNGFSGLPGSSSFSSSSSSSFR LKNSRRKNT KMLNRSKVVC	5.01	Q9SAG8	8
	c	1 – 80	MTIALTIGNGFSGLPGSSSFSSSSSSSFR LKNSRRKNT KMLNRSKVVCSSSSSVMDPYKTLKIRPDSSEYEVKKA FRQLAK	8.77		
<i>At</i> GLTB2TP	m	1 – 73	MALQSPGATGASSSVSRLLSAKLSSTKTIFSVDFVR SYCISKGTKRRELNSGFRGYSPLLKSSLRSPFSVKA	7.79	Q9T0P4	8
	c	1 – 80	MALQSPGATGASSSVSRLLSAKLSSTKTIFSVDFVR SYCISKGTKRRELNSGFRGYSPLLKSSLRSPFSVKAI LNSDRA	8.56		
<i>Nr</i> RBS _{TP}	m	1 – 20	MASSVLSSAAVATRSNVAQA	1.92	P69249	10
	c	1 – 57	MASSVLSSAAVATRSNVAQANMVAPFTGLKSAASFPV SRKQNLDTITSIASNGGRVQC	5.77		
<i>At</i> RBS1ATP	m	1 – 55	MASMLSSATMVASPAQATMVAPFNGLKSSAAFPATR KANNDITSITSNGGRVNC	5.53	P10795	9
	c	1 – 79	MASMLSSATMVASPAQATMVAPFNGLKSSAAFPATR KANNDITSITSNGGRVNCMQVWPPIGKKKFETLSYLP DLTDS	8.31		
<i>Ps</i> RBS2TP	m	1 – 22	MASMISSAVTTVSRASRGQSA	2.18	P00869	11
	c	1 – 57	MASMISSAVTTVSRASRGQSAAVAPFGGLKSMTGFP VKKVNTDITSITSNGGRVKC	5.75		

Supplemental Material

<i>AtRCA_{TP}</i>	m	1 – 59	MAAAVSTVGAINRAPLSLNGSGSGAVSAPASTFLGKK VVTVSRFAQSNKKSNGSFKVLA	5.81	P10896	12
	c	1 – 80	MAAAVSTVGAINRAPLSLNGSGSGAVSAPASTFLGKK VVTVSRFAQSNKKSNGSFKVLAVKEDKQTDGDRWRGL AYDTSD	8.25		
<i>NtSIR_{TP}</i>	m	1 – 59	MTTSFGAAINIAVADDPNPKLQIHNFSGLKSTSNLL LSRRLHVFQSFSPSNPSSIVRA	6.34	O82802	13
	c	1 – 62	MTTSFGAAINIAVADDPNPKLQIHNFSGLKSTSNLL LSRRLHVFQSFSPSNPSSIVRAVST	6.63		
<i>AtTOCC_{TP}</i>	m	1 – 48	MEIRSLIVSMNPNLSSFELSRPVSPLTRSLVPFRSTK LVPRSISRVS	5.36	Q94FY7	8
	c	1 – 80	MEIRSLIVSMNPNLSSFELSRPVSPLTRSLVPFRSTK LVPRSISRVSASISTPNSSETDKISVKPVYVPTSPNRE LRTPHS	8.88		
<i>synRBS_{TP}</i>	m	1 – 19	MASSMLSSAAVVATRASAA	1.78		1
	c	1 – 59	MASSMLSSAAVVATRASAAQASMVAPFTGLKSAASFP VTRKQNNLDITSIASNGGRVRA	5.93		
<i>AtRecA_{TP}</i>	c	1 – 53	MDSQLVLSLKLNPSTPLSPLFPFTPCSSFSPLRFS SCYSRRLYSPVTYAA	5.91	Q39199	14
<i>OsRBS1_{TP}</i>	c	1 – 47	MAPSVMASATTVPFQGLKSTAGMPVARRSGNSSFG NVSNGGRIRC	4.71	Q0INY7	15

Abbreviation: m: minimal; c: complete.

Supplemental Table 8. Summary of processing products from TP-Nif fusions.

Western blot signals of the product of expressing 12 TPs in full version (complete) and up until their predicted cleavage site by stromal peptidase (minimal) fused to the N-terminus of NifH, NifM, NifU and NifS, on a scale indicating: -: Nif proteins with the same electrophoretic mobility as their cytosolic controls; +: Nif proteins with lower electrophoretic mobility as their cytosolic controls; and =: Nif proteins with incomplete processing.

	NifH	NifM	NifU	NifS
<i>Atm</i> AROATP	-	=	+	=
<i>Atc</i> AROATP	+	-	-	-
<i>Atm</i> CAB6TP	+	=	-	-
<i>Atc</i> CAB6TP	+	+	+	=
<i>Atm</i> DNAJ8TP	+	=	-	-
<i>Atc</i> DNAJ8TP	+	=	+	=
<i>Atm</i> GLTB2TP	-	-	-	-
<i>Atc</i> GLTB2TP	+	+	+	+
<i>Atm</i> TOCCTP	-	=	-	+
<i>Atc</i> TOCCTP	+	+	+	+
<i>Ntm</i> RBS _{TP}	-	+	+	+
<i>Ntc</i> RBS _{TP}	-	=	-	-
<i>Psm</i> RBS2TP	=	+	+	+
<i>Psc</i> RBS2TP	+	=	-	-
<i>Atm</i> BCCP1TP	-	-	-	-
<i>Atc</i> BCCP1TP	+	+	+	+
<i>Ntm</i> SIRTP	-	=	-	-
<i>Ntc</i> SIRTP	-	=	-	+
<i>Atm</i> RBS1ATP	-	=	=	-
<i>Atc</i> RBS1ATP	+	=	+	+
<i>Atm</i> RCA _{TP}	-	+	-	-
<i>Atc</i> RCA _{TP}	+	+	+	+
<i>synm</i> RBS _{TP}	-	+	+	+
<i>sync</i> RBS _{TP}	-	=	-	-

Supplemental Table 9. Motif settings used for rice *nif* synthetic designed genes.

Motif settings				
Parameter	Setting			
Exclusion sequences	Constraint	Description	Sequence	Reference
		PolyA.1	AATGAA	4
		PolyA.2	AATATT	
		PolyA.3	GATAAA	
		PolyA.4	AATTAA	
		PolyA.5	AATAAT	
		FUE1	TTAATT	5
		FUE2	TGTAAA	
		FUE3	AATAAA	
		FUE4	TTCAGA	
		FUE5	TAGTAG	
		FUE6	TTCTTT	
		FUE7	GCGGCG	
		NUE1	AATAAA	
		NUE2	TTAATT	
		NUE3	TAGTAG	
		NUE4	GATCGA	
		CE1	TAATTA	
		Cryptic splice donor	MAGGTRAGT	6
		Cryptic splice acceptor	YYYYNTAGG	
		RNA destabilizing sequence	ATTTA	
		RNA instability determinant	ATAGAT	
		Shine Dalgarno	AGGAGGTNNNNNDTG	
		ATRICH1	ASAAAA	
		ATRICH2	AASAAA	
		ATRICH3	AAASAA	
		ATRICH4	AAAASA	
		ATRICH5	TSAAAA	
		ATRICH6	TTSTTT	
		ATRICH7	TTTSTT	
		ATRICH8	TTTTST	
		NUE2	AAAAAA	
		FUE-TRE-CE	TTTT	
		FUE-ORY1	TTTTGT	
		FUE-ORY2	GTGTG	
		FUE-ORY3	TGTAW	
		FUE-ORY4	TGTGT	
		FUE-ORY5	WTGTA	

Parameter	Setting		
Restriction sites	Constraint	Description	Sequence
		<i>BsaI</i>	GGTCTC
			GAGACC
		<i>BpiI (BbsI)</i>	GAAGAC
			GTCTTC
		<i>BsmBI</i>	CGTCTC
			GCAGAG
		<i>BtgZI</i>	GCGATG
			CGCTAC

Abbreviation: FUE: Far Upstream Element; NUE: Near Upstream Element.

Supplemental Table 10. Exclusion motifs present in codon-optimized *nif* genes.

Number of times the following exclusion motifs were found in rice codon-optimized *nif* genes provided by Genescript, which were subsequently removed by PCR.

Name	Motif	<i>nifH</i>	<i>nifM</i>	<i>nifU</i>	<i>nifS</i>
Removed by PCR					
FUE7	GCGGCG	3	1	3	
FUE4	TTCAGA		1		
NUE4	GATCGA			3	1
Maintained					
Shine D'algarno	GCAGAG	4	4	5	5
FUE ORY2	WTGTA	2	1		
FUE ORY5	CGCTAC	1			
<i>BsmBI</i>	GCAGAG	1	2		
<i>BtgZI</i>	CGCTAC		1		

Supplemental Table 11. Characteristics of *nifH* and *nifM* sequences regarding expression in rice.

The CAI index was obtained from the Biologics International Corp codon adaptation index calculator and rare codon analyser using *Zea mays* as target setting.

	<i>AvnifH</i>					<i>AvnifM</i>				
	<i>A. vinelandii</i> gene	Yeast codon optimization	Tobacco synthetic design	Rice codon optimized	Rice synthetic design	<i>A. vinelandii</i> gene	Yeast codon optimization	Tobacco synthetic design	Rice codon optimized	Rice synthetic design
CAI	0.83	0.57	0.60	0.90	0.89	0.85	0.53	0.56	0.89	0.89
Total GC Content	58.42%	39.86%	43.87%	61.03%	60.69%	65.42%	40.73%	43.76%	64.73%	64.73%
GC3 Content	76.98%	33.33%	39.52%	85.86%	84.83%	84.01%	28.67%	35.71%	83.96%	83.96%
Exclusion Motifs	7	23	4	11	8	11	21	1	10	8

Abbreviation: CAI: Codon Adaptation Index; GC: Distribution of Guanine and Cytosine (GC) content in ORFs; GC3: Distribution of Guanine and Cytosine (GC) content in the third codon position.

Supplemental Table 12. Statistics of import efficiency of TPs.

(A) Import efficiency of TPs into Arabidopsis chloroplast and root plastids, and rice chloroplast in transient expression protoplast experiments. **(B)** Import efficiency of TPs into rice proplastids, chloroplast and root plastids. Import efficiency was defined as the percentage of the processed faster-migrating protein form relative to the total amount of expressed eGFP protein. Data represent means (n=3) with SD, except GLTB2 and RBS1 TPs tested in root plastids, where n=2. **(C-E)** Multiple Student's test between tissues in protoplast transient experiments at (C) 12 hours and (E) 16h post-transfection and (D) stable transgenic rice lines. Student's test significant differences ($P < 0.05$).

(A)

Protoplast	Arabidopsis chloroplast			Arabidopsis leucoplast			Rice chloroplast		
	12 h	16 h	N	12 h	16 h	N	12 h	16 h	N
<i>AtmCAB6_{TP}</i>	95,82 ± 1,94	95,20 ± 0,96	3	60,57 ± 1,36	71,24 ± 9,51	3	80,95 ± 4,03	81,06 ± 2,98	3
<i>AtmRCA_{TP}</i>	92,32 ± 5,66	94,32 ± 4,88	3	56,16 ± 3,34	53,28 ± 0,75	3	85,60 ± 4,06	88,50 ± 2,16	3
<i>AtmTOCC_{TP}</i>	71,41 ± 15,94	63,09 ± 17,67	3	50,80 ± 3,53	52,01 ± 8,79	3	89,87 ± 0,44	92,10 ± 1,30	3
<i>AtmBCCP1_{TP}</i>	98,99 ± 0,90	99,57 ± 0,19	3	97,51 ± 0,31	96,70 ± 3,39	3	94,47 ± 1,95	98,42 ± 1,40	3
<i>AtcGLBT2_{TP}</i>	56,87 ± 2,66	79,46 ± 7,93	3	50,78 ± 1,99	49,18 ± 3,95	2	97,64 ± 1,92	99,07 ± 0,68	3
<i>OsRBS1_{TP}</i>	2,57 ± 1,74	2,39 ± 0,76	3	3,28 ± 3,36	2,29 ± 1,88	2	68,38 ± 0,53	68,24 ± 4,47	3

(B)

Stable rice lines	Callus proplastids		Leaf chloroplast		Root leucoplast	
	% ± SD	N	% ± SD	N	% ± SD	N
<i>AtmCAB6_{TP}</i>	99,41 ± 0,78	3	98,64 ± 0,98	3	99,93 ± 0,07	3
<i>AtmRCA_{TP}</i>	99,55 ± 0,16	3	98,03 ± 1,46	3	99,46 ± 0,09	3
<i>AtmTOCC_{TP}</i>	97,83 ± 1,39	3	99,66 ± 0,28	3	99,68 ± 0,19	3
<i>AtmBCCP1_{TP}</i>	99,75 ± 0,38	3	-	-	-	-

(C)

12 hours	Arabidopsis chloroplast	Arabidopsis leucoplast	p-value	Arabidopsis chloroplast	Rice chloroplast	p-value
<i>AtmCAB6</i> _{TP}	95,82	60,57	0,000014	95,82	80,95	0,004532
<i>AtmRCA</i> _{TP}	92,32	56,16	0,000676	92,32	85,60	0,169540
<i>AtmTOCC</i> _{TP}	71,41	50,80	0,094104	71,41	89,87	0,115386
<i>AtmBCCP1</i> _{TP}	98,99	97,51	0.055017	98,99	94,47	0.021856
<i>AtcGLBT2</i> _{TP}	56,87	50,78	0,072910	56,87	97,64	0,000027
<i>OsRBS1</i> _{TP}	2,568	3,280	0,766732	2,568	68,38	<0,00000 1

(D)

16 hours	Arabidopsis chloroplast	Arabidopsis leucoplast	p-value	Arabidopsis chloroplast	Rice chloroplast	p-value
<i>AtmCAB6</i> _{TP}	95,20	71,24	0,012263	95,20	81,06	0,001446
<i>AtmRCA</i> _{TP}	94,32	53,28	0,000135	94,32	88,50	0,131998
<i>AtmTOCC</i> _{TP}	63,09	52,01	0,386146	63,09	92,10	0,047101
<i>AtmBCCP1</i> _{TP}	99,57	96,70	0.217164	99,57	98,42	0.232171
<i>AtcGLBT2</i> _{TP}	79,46	49,18	0,016899	79,46	99,07	0,012967
<i>OsRBS1</i> _{TP}	2,390	2,288	0,934972	2,390	68,24	0,000015

(E)

Stable rice lines	Callus proplastids	Leaf chloroplast	p-value	Callus proplastids	Root leucoplast	p-value	Leaf chloroplast	Root leucoplast	p-value
<i>AtmCAB6</i> _{TP}	99,41	98,64	0,345617	99,41	99,93	0,313229	98,64	99,93	0,085177
<i>AtmRCA</i> _{TP}	99,55	98,03	0,147788	99,55	99,46	0,443386	98,03	99,46	0,165593
<i>AtmTOCC</i> _{TP}	97,83	99,66	0,088105	97,83	99,68	0,084426	99,66	99,68	0,949813
<i>AtmBCCP1</i> _{TP}	-	-	-	-	-	-	-	-	-

Supplemental Table 13. Levels of immunogold labelling.

Number of gold particles observed on organelles and cytoplasm of cells of different samples and lines after using a monoclonal or polyclonal antiserum, on a scale indicating: - (not labelling), +/- (very light labelling), + (weak but specific labelling), ++ (specific labelling), +++ (more specific labelling) and ++++ (specific and strong labelling).

Antiserum: Monoclonal		WT	<i>AtmCAB6</i> ^{TP} - eGFP	<i>AtmRCA</i> ^{TP} - eGFP	<i>AtmTOCC</i> ^{TP} - eGFP	<i>AtmBCCP1</i> ^{TP} - eGFP
Callus	Plastids	-	+	++	+	+
	Mitochondria	-	-	-	-	-
	Nuclei/nucleoli	-	-	-	-	+/-
	Cytoplasm	-	-	-	-	-
Leaf	Chloroplasts	-	+	+++	+	n/a
	Mitochondria	-	-	-	-	n/a
	Nuclei/nucleoli	-	-	-	-	n/a
	Cytoplasm	-	-	-	-	n/a
Root	Plastids	-	+	++	+	n/a
	Mitochondria	-	-	-	-	n/a
	Nuclei/nucleoli	-	-	-	-	n/a
	Cytoplasm	-	-	-	-	n/a
Antiserum: Polyclonal		WT	<i>AtmCAB6</i> ^{TP} - eGFP	<i>AtmRCA</i> ^{TP} - eGFP	<i>AtmTOCC</i> ^{TP} - eGFP	<i>AtmBCCP1</i> ^{TP} - eGFP
Callus	Plastids	-	+++	++++	++	n/a
	Mitochondria	-	-	-	-	n/a
	Nuclei/nucleoli	+	+	+	+	n/a
	Cytoplasm	+/-	+/-	+/-	+/-	n/a
Leaf	Chloroplasts	+/-	+++	++++	++	n/a
	Mitochondria	-	-	-	-	n/a
	Nuclei/nucleoli	+	+	+	+	n/a
	Cytoplasm	+/-	+/-	+/-	+/-	n/a
Root	Plastids	-	++	++++	++	n/a
	Mitochondria	-	-	-	-	n/a
	Nuclei/nucleoli	+	+	+	+	n/a
	Cytoplasm	+/-	+/-	+/-	+/-	n/a

Supplemental Figure 1. Mass spectrometry results of bands present in NifH purification.

(A) Estimation of the purity of NifH preparation expressed in *N. benthamiana*. The band corresponding to the full-size NifH protein amounts for 59,05% of the total protein present in the preparation, quantified using ImageJ ¹⁶. **(B)** Peptide mass fingerprint. Mascot search results against SwissProt database

(A)

Start - End	Observed	Mr (expt)	Mr (calc)	ppm	Miss	Sequence
19 - 32	1826.8606	1825.8533	1825.8774	-13.2	1	K.EYKLTYYTPEYQTK.D
22 - 32	1406.6692	1405.6619	1405.6765	-10.4	0	K.LTYTTPPEYQTK.D
22 - 41	2409.2219	2408.2146	2408.1900	10.2	1	K.LTYTTPPEYQTKDILAAFR.V
33 - 41	1021.5566	1020.5493	1020.5240	24.8	0	K.DTDILAAFR.V
84 - 89	896.4885	895.4813	895.4334	53.4	1	R.CYRIER.V
132 - 139	985.6098	984.6025	984.5716	31.4	1	R.ALRLEDLR.I
135 - 146	1413.7994	1412.7921	1412.8027	-7.50	1	R.LEDLRIPPAYVK.T
147 - 159	1465.7362	1464.7289	1464.7474	-12.6	0	K.TFQGPPIHQIQUVER.D
147 - 161	1708.8497	1707.8424	1707.8693	-15.7	1	K.TFQGPPIHQIQUVERDK.L
165 - 177	1502.8287	1501.8214	1501.8439	-15.0	0	K.YGRPLLGTIKPK.L
188 - 194	910.4882	909.4809	909.4378	47.4	0	R.AVYECLR.G
195 - 213	2169.9834	2168.9761	2168.9797	-1.64	1	R.GGLDFTKDDENVNSQPFMR.W
195 - 213	2185.9893	2184.9820	2184.9746	3.39	1	R.GGLDFTKDDENVNSQPFMR.W + Oxidation (M)
202 - 213	1451.6112	1450.6039	1450.6147	-7.42	0	K.DDENVNSQPFMR.W
216 - 227	1516.7455	1515.7382	1515.7544	-10.7	1	R.DRFLFCAEALFK.A
218 - 227	1245.6310	1244.6237	1244.6264	-2.12	0	R.FLFCAEALFK.A
237 - 253	1950.9048	1949.8975	1949.9087	-5.75	1	K.GHYLNATAGTCEEMIKR.A
237 - 253	1966.9031	1965.8958	1965.9037	-3.98	1	K.GHYLNATAGTCEEMIKR.A + Oxidation (M)
259 - 285	3022.6309	3021.6236	3021.4477	58.2	0	R.ELGVPIVMHDYLTGGFTANTSLAHYCR.D
259 - 285	3038.6343	3037.6270	3037.4426	60.7	0	R.ELGVPIVMHDYLTGGFTANTSLAHYCR.D +
Oxidation (M)						
286 - 295	1187.6713	1186.6640	1186.6571	5.86	0	R.DNGLLLHIHR.A
296 - 303	912.5040	911.4968	911.4647	35.2	0	R.AMHAVIDR.Q
296 - 305	1168.5988	1167.5915	1167.6182	-22.9	1	R.AMHAVIDRQK.N
304 - 312	1136.6182	1135.6109	1135.5999	9.71	1	R.QKNHGIHFR.V
306 - 312	880.4976	879.4903	879.4464	49.9	0	K.NHGIHFR.V
320 - 339	2082.0024	2080.9951	2080.9960	-0.43	1	R.MSGGDHHSHTVVGKLEGER.D + Oxidation (M)
335 - 350	1845.9656	1844.9583	1844.9996	-22.4	1	K.LEGERDITLGFVDLLR.D
340 - 350	1261.7120	1260.7047	1260.7078	-2.44	0	R.DITLGFVDLLR.D
340 - 358	2266.1362	2265.1289	2265.1277	0.52	1	R.DITLGFVDLLRDDFVEQDR.S
432 - 446	1712.8455	1711.8382	1711.8601	-12.8	1	R.NEGRDLAQEGNQIIR.E
436 - 446	1256.6583	1255.6510	1255.6520	-0.81	0	R.DLAQEGNQIIR.E
464 - 477	1579.8053	1578.7980	1578.8294	-19.8	0	K.EIVFNFAAVDVLK.-

Band 3

Match to: **NIFH1_AZOVI** Score: 152 Expect: 3.5e-010
Nitrogenase iron protein 1 OS=Azotobacter vinelandii GN=nifH1 PE=1 SV=2

Nominal mass (M_r): **31895**; Calculated pI value: **4.68**

Fixed modifications: Carbamidomethyl (C)

Variable modifications: Oxidation (M)

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Number of mass values searched: **65**

Number of mass values matched: **25**

Sequence Coverage: **50%**

Matched peptides shown in **Bold Red**

1	MAMRQCAIYG	KGIGKSTTT	QNLVAALAE	GKK	VMIVGCD	PKADSTRLL
51	HSK AQNTIME	MAAEAGTV	LELEDVLKAG	YGGVK	CVESG	GPEPGVGCAG
101	RGVITAINFL	EEEGAYEDDL	DFVFYDVLGD	VVCGGFAMPI	RENK AQEIYI	
151	VCSGEMMAMY	AANNISKGIV	KYANGSVRL	GGLICNSRNT	DREDELI	AL
201	ANKLGTQMIH	FVPRDNVQR	AEIRMTVIE	YDPKAKQADE	YRALAR	KVVD
251	NKLLVIPNPI	TMDELEELLM	EFGIMEVEDE	SIVGKTAEV		
Start - End	Observed	Mr (expt)	Mr (calc)	ppm	Miss	Sequence
17 - 32	1634.7944	1633.7871	1633.8345	-29.0	0	K.STTTQNLVAALAE MGK .K
17 - 33	1762.8884	1761.8811	1761.9295	-27.4	1	K.STTTQNLVAALAE MGK .V
17 - 33	1778.8793	1777.8720	1777.9244	-29.4	1	K.STTTQNLVAALAE MGK .V + Oxidation (M)
54 - 78	2720.2229	2719.2156	2719.2932	-28.5	0	K.AQNTIMEMAAEAGTV EDLELEDVLK .A
54 - 78	2736.2214	2735.2141	2735.2881	-27.0	0	K.AQNTIMEMAAEAGTV EDLELEDVLK .A + Oxidation (M)
54 - 78	2752.2166	2751.2093	2751.2830	-26.8	0	K.AQNTIMEMAAEAGTV EDLELEDVLK .A + 2
Oxidation (M)						
54 - 85	3352.5371	3351.5298	3351.6214	-27.3	1	K.AQNTIMEMAAEAGTV EDLELEDVLKAGYGGVK .C
54 - 85	3384.5283	3383.5210	3383.6112	-26.7	1	K.AQNTIMEMAAEAGTV EDLELEDVLKAGYGGVK .C + 2
Oxidation (M)						
145 - 167	2594.0999	2593.0926	2593.1684	-29.2	0	K.AQEIIYVCSGEMMAMY AANNISK .G
145 - 167	2610.0969	2609.0896	2609.1633	-28.3	0	K.AQEIIYVCSGEMMAMY AANNISK .G + Oxidation (M)
145 - 167	2626.0823	2625.0750	2625.1583	-31.7	0	K.AQEIIYVCSGEMMAMY AANNISK .G + 2 Oxidation (M)
168 - 179	1250.6654	1249.6581	1249.6779	-15.8	1	K.GIVKYANGSV R .L
172 - 179	853.3817	852.3744	852.4090	-40.5	0	K.YANGSV R .L
180 - 188	989.4786	988.4714	988.5124	-41.5	0	R.LGGLICNS R .N
189 - 203	1714.8528	1713.8455	1713.8897	-25.8	1	R.NTDREDELI ALANK .L
204 - 214	1298.6680	1297.6607	1297.6965	-27.6	0	K.LGTQMIHFV PR .D
204 - 214	1314.6600	1313.6527	1313.6914	-29.5	0	K.LGTQMIHFV PR .D + Oxidation (M)
204 - 220	2010.0171	2009.0098	2009.0629	-26.4	1	K.LGTQMIHFV PRDNVQR .A
204 - 220	2026.0066	2024.9993	2025.0578	-28.9	1	K.LGTQMIHFV PRDNVQR .A + Oxidation (M)
225 - 234	1251.6139	1250.6066	1250.6329	-21.0	1	R.RMTVIEYDPK A
225 - 234	1267.6006	1266.5933	1266.6278	-27.2	1	R.RMTVIEYDPK A + Oxidation (M)
226 - 234	1095.5027	1094.4954	1094.5318	-33.2	0	R.MTVIEYDPK A
226 - 236	1294.6339	1293.6266	1293.6638	-28.8	1	R.MTVIEYDPK AQ
235 - 242	980.4462	979.4390	979.4723	-34.0	1	K.AQ QADEYR .A
237 - 246	1192.5792	1191.5719	1191.5996	-23.2	1	K.QA DEYRALAR .K

Supplemental Material

Band 4

Match to: **NIFH1_AZOVI** Score: 173 Expect: 2.8e-012

Nitrogenase iron protein 1 OS=Azotobacter vinelandii GN=nifH1 PE=1 SV=2

Nominal mass (M_r): **31895**; Calculated pI value: **4.68**

Fixed modifications: Carbamidomethyl (C)

Variable modifications: Oxidation (M)

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Number of mass values searched: **65**

Number of mass values matched: **28**

Sequence Coverage: **59%**

Matched peptides shown in **Bold Red**

1	MAMRQCAIYG	KGGIGKSTTT	QNLVAALAEM	GKKVMIVGCD	PKADSTRLLIL		
51	HSKAQNTIME	MAAEAGTVED	LELEDVLKAG	YGGVKCVESG	GPEPGVGCAG		
101	RGVITAINFL	EEEGAYEDDL	DFVFYDVLGD	VVCGGFAMPI	RENKAQEIIYI		
151	VCSGEMMAMY	AANNISKGIV	KYANSGSVRL	GGLICNSRNT	DREDELIIAL		
201	ANKLGTQMIH	FVPRDNVVQR	AEIRMTVIE	YDPKAKQADE	YRALARKVVD		
251	NKLLVIPNPI	TMDELEELLM	EFGIMEVEDE	SIVGKTAEEV			
Start - End	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Sequence	
17 - 32	1634.8141	1633.8068	1633.8345	-16.9	0	K.STTTQNLVAALAEMGK.K	
17 - 32	1650.8110	1649.8037	1649.8294	-15.6	0	K.STTTQNLVAALAEMGK.K + Oxidation (M)	
17 - 33	1762.9119	1761.9046	1761.9295	-14.1	1	K.STTTQNLVAALAEMGK.V	
17 - 33	1778.9048	1777.8975	1777.9244	-15.1	1	K.STTTQNLVAALAEMGK.V + Oxidation (M)	
34 - 42	1018.5162	1017.5089	1017.4987	10.0	0	K.VMIVGCDPK.A	
34 - 47	1548.7299	1547.7226	1547.7436	-13.6	1	K.VMIVGCDPKADSTR.L	
34 - 47	1564.7418	1563.7345	1563.7385	-2.56	1	K.VMIVGCDPKADSTR.L + Oxidation (M)	
54 - 78	2720.3606	2719.3533	2719.2932	22.1	0	K.AQNTIMEMAAEAGTVEDLELEDVLK.A	
54 - 78	2736.3652	2735.3579	2735.2881	25.5	0	K.AQNTIMEMAAEAGTVEDLELEDVLK.A + Oxidation	
(M)							
54 - 78	2752.3560	2751.3487	2751.2830	23.9	0	K.AQNTIMEMAAEAGTVEDLELEDVLK.A + 2	
Oxidation (M)							
54 - 85	3352.8301	3351.8228	3351.6214	60.1	1	K.AQNTIMEMAAEAGTVEDLELEDVLKAGYGGVK.C	
54 - 85	3368.8384	3367.8311	3367.6163	63.8	1	K.AQNTIMEMAAEAGTVEDLELEDVLKAGYGGVK.C +	
Oxidation (M)							
86 - 101	1588.6731	1587.6658	1587.6770	-7.04	0	K.CVESGGPEPGVGCAGR.G	
145 - 167	2594.2185	2593.2112	2593.1684	16.5	0	K.AQEIIYIVCSGEMMAMYAANNISK.G	
145 - 167	2610.2239	2609.2166	2609.1633	20.4	0	K.AQEIIYIVCSGEMMAMYAANNISK.G + Oxidation	
(M)							
145 - 167	2626.2085	2625.2012	2625.1583	16.4	0	K.AQEIIYIVCSGEMMAMYAANNISK.G + 2 Oxidation	
(M)							
172 - 179	853.4465	852.4392	852.4090	35.5	0	K.YANSGSVR.L	
180 - 188	989.5368	988.5295	988.5124	17.3	0	R.LGGLICNSR.N	
189 - 203	1714.8777	1713.8704	1713.8897	-11.2	1	R.NTDREDELIIALANK.L	
204 - 214	1298.6947	1297.6874	1297.6965	-7.01	0	K.LGTQMIHFVPR.D	
204 - 214	1314.6890	1313.6817	1313.6914	-7.39	0	K.LGTQMIHFVPR.D + Oxidation (M)	
204 - 220	2010.0569	2009.0496	2009.0629	-6.61	1	K.LGTQMIHFVPRDNVVQR.A	
204 - 220	2026.0487	2025.0414	2025.0578	-8.10	1	K.LGTQMIHFVPRDNVVQR.A + Oxidation (M)	
225 - 234	1251.6360	1250.6287	1250.6329	-3.33	1	R.RMTVIEYDPK.A	
225 - 234	1267.6428	1266.6355	1266.6278	6.09	1	R.RMTVIEYDPK.A + Oxidation (M)	
226 - 234	1095.5449	1094.5376	1094.5318	5.34	0	R.MTVIEYDPK.A	
226 - 236	1294.6613	1293.6540	1293.6638	-7.59	1	R.MTVIEYDPKAK.Q	
237 - 246	1192.6123	1191.6050	1191.5996	4.56	1	K.QADEYRALAR.K	

Band 5

Match to: **NIFM_AZOVI** Score: 70 Expect: 0.055

Putative peptidyl-prolyl cis-trans isomerase Nifm OS=Azotobacter vinelandii GN=nifM PE=3 SV=1

Nominal mass (M_r): **33181**; Calculated pI value: **5.45**

Fixed modifications: Carbamidomethyl (C)

Variable modifications: Oxidation (M)

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Number of mass values searched: **65**

Number of mass values matched: **10**

Sequence Coverage: **39%**

Matched peptides shown in **Bold Red**

1	MASERLADGD	SRYYLLKVAH	EQFGCAPGEL	SEDQLQQADR	IIGRQRHIED		
51	AVLRSPDAIG	VVIPPSQLEE	AWAHIASRYE	SPEALQQALD	AQALDAAGMR		
101	AMLARELRVE	AVLDCVCAGL	PEISDTDVSL	YYFNHAEQFK	VPAQHKAHIL		
151	VTINEDFPEN	TREAAARTIE	TILKRLRGKP	ERFAEQAMKH	SECPAMQGG		
201	LLGEVVPCTL	YPELDACLFQ	MARGELSPVL	ESPIGFHVLY	CESVSPARQL		
251	TLEEILPRLR	DRLQRLQRKA	YQRKWLVCIL	QQNATLENLA	HG		
Start - End	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Sequence	
6 - 17	1413.7352	1412.7279	1412.7299	-1.43	1	R.LADGDSRYYLLK.V	
45 - 54	1236.6891	1235.6818	1235.6734	6.78	1	R.QRHIEDAVLR.S	
47 - 54	952.5436	951.5363	951.5138	23.7	0	R.HIEDAVLR.S	
55 - 78	2543.3379	2542.3306	2542.3180	4.98	0	R.SPDAIGVVIPPSQLEEAWAHIASR.Y	
79 - 100	2348.1182	2347.1109	2347.1114	-0.19	0	R.YESPEALQQALDAQALDAAGMR.A	
79 - 100	2364.1147	2363.1074	2363.1063	0.48	0	R.YESPEALQQALDAQALDAAGMR.A + Oxidation (M)	
79 - 105	2922.4561	2921.4488	2921.4011	16.3	1	R.YESPEALQQALDAQALDAAGMRAMLAR.E + 2	
Oxidation (M)							
167 - 174	973.6284	972.6211	972.5968	25.1	1	R.TRIETILK.R	
224 - 248	2743.4026	2742.3953	2742.3687	9.71	0	R.GELSPVLESPIGFHVLYCESVSPAR.Q	
249 - 258	1211.7137	1210.7064	1210.6921	11.8	0	R.QLTLEEILPR.L	

Supplemental Figure 2. Determination of N-terminal sequence of TwinStrep-NifH using the standard Edman degradation method.

Blank, standard and first six amino acids were determined (mV vs retention time in mins).

AA step	1	2	3	4	5	6
1. AA	A	S	A	?	Q/S	H
2. AA	S	V	P	Q	H	

Green: TP
 Red: TwinStrep
 Blue: NifH
 Black: cloning scars

MAAAVSTVGAINRAPLSLNGSGSGAVSAPASTFLGKKVTVSRFAQSNKKSNGSFKVLA**ASAWSH**PQFEK
 GGGSGGGSGGSAW**SH**PQFEKSSMAMRQCAIYGKGGIGKSTTTQNLVAALAEMGKKVMIVGCDPKADST
 RLILHSAQNTIMEMAAEAGTVEDLELDVLKAGYGGVKCVESGGPEPGVGCAGRGVITAINFLEEGAYED
 DLDFVFYDVLGDVVCGGFAMPIRENKAQEYIVCSGEMMAMYAANNISKGIVKYANSGSVRLGGLICNSRN
 TDREDELIALANKLGTQMIHFVPRDNVVRQAEIRRMVIEYDPKAKQADEYRALARKVVDNKLIVIPNPIT
 MDELELLMEFGIMEVEDESIVGKTAEV

Supplemental Figure 3. NifM and NifU co-elution of with Strep-tagged NifH.

Strep-tactin purification procedure of *A. vinelandii* NifH expressed in leaves of *N. benthamiana*. CFE, cell-free extract (soluble fraction following centrifugation and filtering of disrupted leaf tissue); FT, Strep-tactin flow-through fraction; W, wash fraction; E, biotin-eluted fraction; E_c, concentrated biotin-eluted fraction. **(A)** Western blot using antibodies against NifM. **(B)** Western blot using antibodies against NifU. **(C)** Western blot using antibodies against NifS. Arrows point at the correct size of the proteins, the higher mobility bands in NifS blot correspond to the signal from α -NifM which was developed before α -NifS incubation.

Supplemental Figure 4. Gene expression of TOC and TIC family members in *A. thaliana* and *O. sativa*.

Data was retrieved from the Plant eFP browser, showing $\log_2$ ratio from microarray transcriptomics of *Arabidopsis thaliana* (A) and *Oryza sativa* (B) tissues¹⁷. (C) Homologs of *A. thaliana* TOC and TIC family members in *O. sativa* ssp. japonica. In the case of Toc132-Toc120 and Toc33-Toc34, the same one homolog was retrieved respectively.

(A)

(B)

(C)

Protein	<i>Arabidopsis thaliana</i>	<i>Oryza sativa</i>
Toc159	AT4G02510	Os05g0151400
Toc90	AT5G20300	Os12g0197400
Toc132	AT2G16640	Os10g0548800
Toc120	AT3G16620	Os10g0548800
Toc33	AT1G02280	Os03g0240500
Toc34	AT5G05000	Os03g0240500
Tic20-I	AT1G04940	Os07g0568500
Tic20-IV	AT4G03320	-
Tic22-III	AT3G23710	Os07g0290800
Tic22-IV	AT4G33350	Os06g0213200

Supplemental Figure 5. Biological context of *A. thaliana* genes containing selected Transit Peptides.

(A) Schematic representation of the metabolic pathways in which genes *AT3G54890* (*AtCAB6*), *AT4G32770* (*AtTOCC*), *AT2G39730* (*AtRCA*), *AT5G16390* (*AtBCCP1*) and *AT2G41220* (*AtGLTB2*) are involved. Illustration obtained from MapMan¹⁸ overview of plant metabolism.
(B) Gene Ontology Annotations.

(A)

(B)

	<i>AtCAB6</i> (<i>AT3G54890</i>)	<i>AtRCA</i> (<i>AT2G39730</i>)	<i>AtTOCC</i> (<i>AT4G32770</i>)	<i>AtBCCP1m</i> (<i>AT5G16390</i>)	<i>AtGLTB2</i> (<i>AT2G41220</i>)
GO Biological Process	Photosynthesis, light harvesting in photosystem I	Response to light stimulus, jasmonic acid	Vitamin E biosynthetic process	Fatty acid biosynthetic process	Ammonia assimilation cycle, glutamate biosynthetic process
GO Cellular Component	Chloroplast (envelope, thylakoid)	Chloroplast (envelope, stroma, thylakoid)	Chloroplast (envelope, thylakoid)	Chloroplast (envelope, stroma)	Chloroplast (envelope, stroma)
GO Molecular Function	Chlorophyll binding	ADP and ATP binding, ribulose-1,5-bisphosphate carboxylase/oxygenase activator activity	Tocopherol cyclase activity	Biotin binding, acetyl-CoA carboxylase activity	Glutamate synthase (ferredoxin) activity

Supplemental Figure 6. Isolated protoplasts imaged using light microscopy.

Isolated protoplasts from **(A)** *Arabidopsis thaliana* aerial part, **(B)** roots and **(C)** *Oryza sativa* stem and sheath tissue were imaged with a Zeiss AxioPhot Microscope (Zeiss) equipped with a Plan-Neofluar 40X/0.75 objective. Scale bar represents (A) 40 μm or (B-C) 20 μm .

(A)

(B)

(C)

Supplemental Figure 7. Immunogold Transmission Electron Microscopy images treated with polyclonal antibody.

Immunogold labelling of eGFP in (A-D) proplastids of rice callus cells, (E-H) chloroplast of rice leaf cells and (I-L) plastids of rice root cells using a GFP polyclonal antibody (diluted 1:250). **A, E, I** Wild-type cells. **B, F, J** *AtmCAB6_{TP}-eGFP*. **C, G, K** *AtmRCA_{TP}-eGFP*. **D, H, L** *AtmTOCC_{TP}-eGFP*. i-vi: Transmission electron microscope (TEM) images. (CW: cell wall, m: mitochondria, N: nucleus, Nu: nucleolus, p: plastid like, St: starch, Ch: chloroplast, p-c: plastid showing an isolated cytoplasmic region; bars = 500 nm; gold particle size = 15 nm)

(A)

WT

(B)

AtCAB6_{TP}-eGFP

(C)

(D)

(E)

WT

(F)

*AtCAB6*_{TP}-eGFP

(I)

WT

(J)

*AtCAB6*_{TP}-eGFP

Supplemental Figure 8. Confocal laser scanning microscopy images of wild-type and transgenic lines rice leaves.

Confocal laser scanning microscopy images of wild-type (WT) rice leaves and leaves from transgenic lines transformed with *AtmCAB6_{TP}-eGFP*, *AtmRCA_{TP}-eGFP* and *AtmTOCC_{TP}-eGFP* fusions. The four panels show the individual signals for eGFP (a) chlorophyll autofluorescence (b), transmitted light (c) and the overlap of eGFP and chlorophyll autofluorescence (d). Scale bars, 20 μ m.

Supplemental Figure 9. Characteristics of *A. thaliana* and *O. sativa* Transit Peptides.

Relative abundance of Alanine, Serine and uncharged amino acids (Serine, Cysteine, Threonine, Tyrosine, Asparagine, Glutamine) in the minimal TPs and the mature protein.

Additional File 1. Sequences of genes used in this thesis.

The complete nucleotide sequences of the *nifH*, *nifM*, *nifU* and *nifS* yeast codon-optimized genes and tobacco synthetic designed genes have been deposited into GenBank under the accession numbers MN920692 to MN920699.

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>Yeast codon optimization *AvnifH*

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>Tobacco synthetic design *AvnifH*

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>Rice synthetic design *AvnifH*

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>*AvnifM*

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>Yeast codon optimization *AvnifM*

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>Tobacco synthetic design *AvniFM*

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>Rice codon optimization *AvniFM*

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>Rice synthetic design *AvniFM*

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>*AvniFU*

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>Yeast codon optimization *AvnifU*

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>Tobacco synthetic design *AvnifU*

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>Rice codon optimization *AvnifU*

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>Rice synthetic design *AvnifU*

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>*AvnifS*

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 CGCTTCTCCTGTGCGCTACACCACCGAGGAGGAGATCGACCGGGTGATCCGCGAGGTTCCGCCGATCG
 TGGCCCAGTTGCGCAAGCTGTGCCCCACTGGAGCGGCAACGGTCCGGTGGAGGACCCGGGCAAGGCCTT
 CGCTCCGGTCTACGGCTGA

>Yeast codon optimization *AvnifS*

ATGGCCGACGTTTACTTTGGATAATAACGCTACTACAAGAGTCGATGACGAAATAGTACAAGCTATGTTGC
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 TGCTCCTGTCTATGTTGA

>Tobacco synthetic design *Avni/S*

ATGGCAGACGTTTATTTGGACAACAATGCCACTACAAGAGTTGACGATGAGATTGTTTCAGGCAATGTTAC
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 GAGCCGGGACGGAAAAATGCAGCATCTATTATTGGCTTAGGCGTGGCTGCTGAAAGAGCTTTACAATTCAT
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 AGGTTCACTAGAGCCTTCACACGTTATGCGAGCTATGGATAATCCGTATACAGCAGCTCATGGTACTGTT
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 CGCGCCCGTTTACGGATAA

>Rice codon optimization *Avni/S*

ATGGCGGACGTGTACCTCGATAACAATGCCACCACACGGGTGGACGATGAGATCGTCCAGGCCATGCTCC
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 CGCCCCGTGTCTACGGATGA

>Rice synthetic design *Avni/S*

ATGGCGGACGTGTACCTCGATAACAATGCCACCACACGGGTGGACGATGAGATCGTCCAGGCCATGCTCC
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 TGGCACAGCTCCGCAAGCTGTGCGCTTACTGGTCCGGCAATGGCCCTGTGGAGGATCCTGGCAAGGCATT
 CGCCCCGTGTCTACGGATGA

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