Development of genetic tools for the symbiosis *Azolla*

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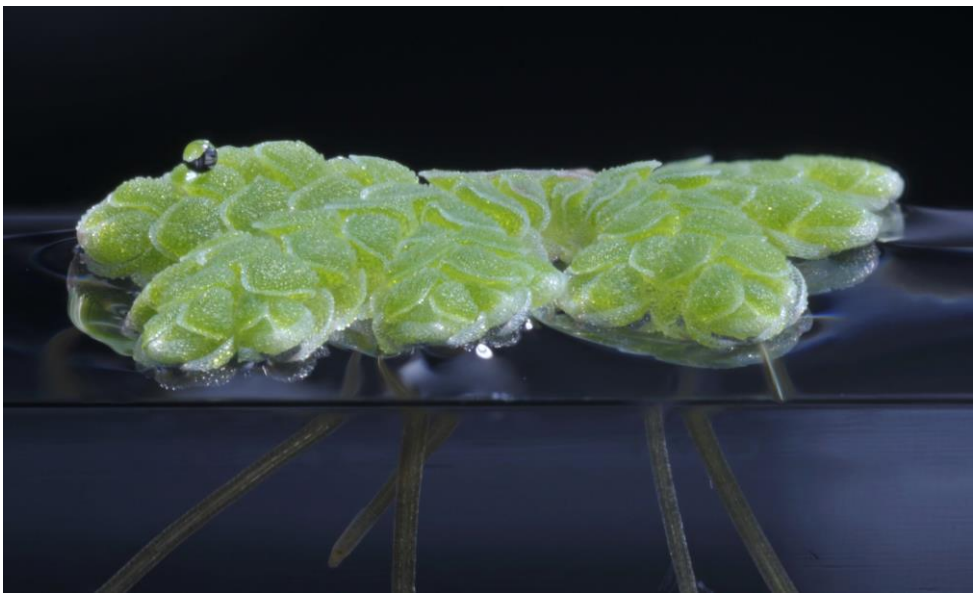

Azolla for the Circular Economy

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Abstract

Azolla is a symbiosis with a great potential to become a novel crop and study model. It consists of an aquatic fern and the filamentous cyanobacteria *N. azollae*. The genetic manipulation of neither of its components has been accomplished, but it is much needed for its domestication. Progress has been made, as both components have been transiently transformed. Combining such methods with novel genetic resources could finally unlock access to their genomes. A method to induce meristems without the need of tissue culture was tested in the fern *A. filiculoides* through *Agrobacterium*-mediated transformation. Expression of the reporter gene was detected after up to 10 days without the appearance of new meristems. Targeting pre-existing ones after shoot tip dissection appears as a promising alternative. For the cyanobacteria, a genetic toolbox for testing a novel CRISPR system relying on transposition rather than nuclease activity was developed. The efficiency of the system was demonstrated in *E. coli*. A library of plasmids was created to evaluate different aspects of the system in *Anabaena sp.*, a close relative of *N. azollae*. The plasmids were proofed effective transforming cyanobacteria through triparental conjugation.

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Introduction

Azolla

Current production systems are incapable of securing a healthy future for humanity and the planet. In recent years their repercussions have become evident. Climate change, ecosystems destruction, loss of biodiversity, water scarcity, socioeconomic gaps, emergence of diseases, among other major problems have been enlarged by this human activity. We urgently need more effective and sustainable alternatives that do not increase the pressure we put on the environment. Plant feedstocks for food, energy, chemicals and materials are a hopeful prospect, but they are limited by arable land and water availability. We must find novel crops that help transform our economy into a sustainable one.

Azolla is a genus of aquatic ferns species; the ferns (henceforth referred to as Azolla) have a unique symbiosis with the filamentous heterocyst-forming cyanobacteria *Nostoc azollae*, Fig 1. The symbiosis is a relation of high interdependency, where the fern provides a safe environment for the cyanobacteria throughout its life cycle and *N. azollae* fixes atmospheric dinitrogen for both. *N. azollae* can be found in the shoot tips of the fern and in special pockets located in its upper leaf lobes, Fig 1B. In each leaf pocket, Fig 1C, a pore establishes adequate gas exchange, Fig 1D, and some cyanobacteria cells differentiate into heterocysts at a high frequency, Fig 1E, ensuring enough nitrogen supply (Brouwer et al., 2017; De Vries & De Vries, 2018). This relationship makes *Azolla* relevant and challenging to study.

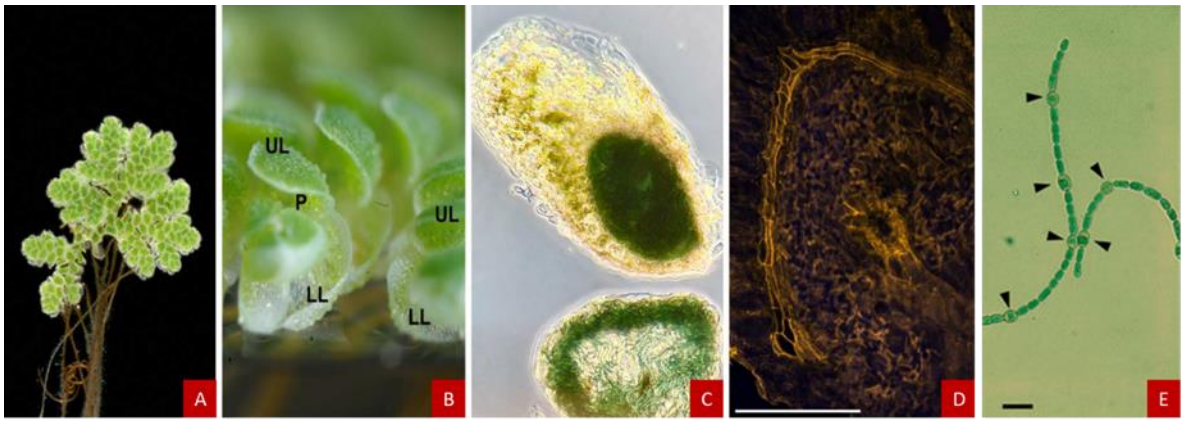


Fig. 1. Morphology of *Azolla*. A. *Azolla filiculoides* sporophyte from a top view (Brouwer, 2017). **B.** Azolla viewed from the shoot apex; upper leaf lobe (UL), leaf pocket (P), lower leaf lobe (LL) (Borel, 2019). **C.** Light microscope photo of an Azolla leaf showing its leaf pocket. Underneath a dissected leaf pocket (Okinawa Institute of Science and Technology, 2020). **D.** Confocal microscope photo of a leaf pocket with its pore in the center. Scale bar: 200 µm (Brouwer et al., 2017). **E.** Light microscopy photo of *N. azollae* filaments with arrows indicating differentiated nitrogen-fixing heterocysts. Scale bar: 5 µm (Vigil-Stenman et al., 2015).

The symbiosis has the potential to become a valuable resource in the transition into a sustainable society (Brouwer et al., 2014). *Azolla* thrives without any nitrogen fertilizers, a major benefit considering most are produced through the energy intensive and fossil fueled Haber-Bosch process (M. Wang et al., 2021). Without nitrogen limitations, it displays exceptional growth rates, doubling its biomass in two days under favorable conditions (Wagner, 1997). As a free-floating plant, it may be cultivated on non-arable lands, a space increasingly demanded for diverse applications. It is easily

harvested, for instance, in the case of *A. filiculoides* and *A. pinnata*, 60% of the standing canopy may be harvested weekly yielding 30-50 ton DW biomass annually experimentally. Moreover, the chemical composition of its biomass is favorable, with up to 35% w/w protein with balanced essential amino acids (Brouwer et al., 2018).

Part of the promise of *Azolla* resides in its numerous applications. The fern can be incorporated directly to the ground as fertilizer and for soil building (Akhtar et al., 2020; Setiawati et al., 2018). Perhaps its most relevant uses come when considered a sustainable crop. Its composition makes it a well suited source of protein and it can be used for food and feed purposes; although its large amounts of polyphenols limit its digestibility, interfere with its processing and limit the rate at which it can be included in diets (Brouwer et al., 2019). The symbiosis can also be used as feedstock for the production of chemicals and bioenergy (Brouwer et al., 2016; Golzary et al., 2019; Miranda et al., 2016), but its application as a bioremediation agent seems more adequate for its attributes. It may be deployed as a crop in re-wetted regions or in contained paludiculture to re-circularize nutrients on farms. It is its extremely effective capturing CO_2 (Baso et al., 2021; Speelman et al., 2009) and phosphorus (Akhtar et al., 2020; H. Wang et al., 2019), which both represent an environmental concern and relevant compounds to be recycled.

The characteristics and applications of *Azolla* makes it a promising resource, but its domestication is yet to be achieved (Brouwer et al., 2014). Although major milestones have been accomplished in recent years, for instance, the *A. filiculoides* genome was the first fern genome to be assembled and annotated (Li et al., 2018), there is still a long way to go. Well-developed methods for its dissemination, breeding, preservation and transformation are crucially required.

The challenges of working with *Azolla* derive from its nature as a fern and its particular symbiosis. Regarding the former, there is little knowledge on molecular mechanisms and no established methods of experimentation for this group of organisms. Most have to be translated with poor efficiency from methods used for soil angiosperms. Spores, particularly those from heterosporous ferns, differ greatly from seeds in the manner by which they are collected or preserved, complicating severely their handling. Besides, the hydrophobic character of the floating ferns in particular demands additional considerations.

Still, the major hardships come from maintaining its symbiosis. *N. azollae* cannot survive outside its host, since its genome has been degraded through millennia of evolution (Ran et al., 2010). Furthermore, the fern can survive without the cyanobacteria if a nitrogen source is provided, but there is no method to reintroduce the latter into its host.

N. azollae can only be transmitted vertically to new generations (Brouwer et al., 2014; Wagner, 1997). Colonies of dividing undifferentiated cells are maintained in the shoot apex. From here, they enter developing organs of the fern, including the leaf pockets and the sporocarps, specialized spore producing structures, Fig 2A. In the megasporocarp that eventually generate the female reproductive organs called archaegonia, the cyanobacteria are located under the indusium cap, where they differentiate into akinetes, their resting stage, Fig 2B. After germination and with the development of the sporeling, Fig 2C, the akinetes germinate and divide into vegetative cells that colonize the shoot apex of the new fern. Cyanobacteria can then turn into hormogonia, motile

filaments that colonize developing leaf pockets or sporocarps again concluding the life-cycle of the symbiosis, Fig 2D.

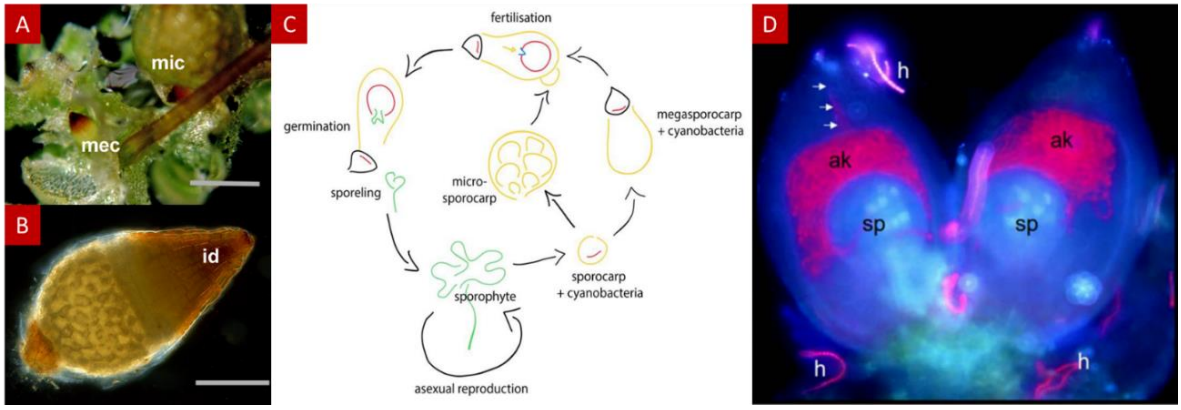


Fig 2. Life cycle of *Azolla*. A. Megasporocarps (mec) and microsporocarp (mic) at the underside of a sporulating plant. Scale bar: 1 mm. **B.** Detached megasporocarp with indusium cap (id). Scale bar: 0.2 mm (Brouwer et al., 2014). **C.** Sexual reproduction of *Azolla* (Güngör, 2018). **D.** Fluorescence microscopy photo of cyanobacteria colonizing a pair of megasporocarps; hormogonia (h), akinetes (ak), megaspores (sp), white arrows indicate the channels cyanobacteria use to enter the sporocarp (Ran et al., 2010).

Genetic engineering is a tool that can boost considerably the speed at which *Azolla* may be domesticated to achieve high-yields more reproducible or alter the biomass characteristics for specific uses. It is critically required for the understanding of gene functions, since only a minor proportion of the genes can be assigned a function using sequence homology. Nevertheless, genetic techniques are difficult to apply to *Azolla* due to its peculiar characteristics. Transformation of the fern is limited by the lack of knowledge and impossibility to re-introduce *N. azollae*, which translates to no use of sterile tissue culture. In addition, few selection markers are available that will permit selection of the fern but leave intact the cyanobacteria inside or vice versa. Most antibiotics will result in death of the non-transformed half of the symbiosis. Introducing erythromycin resistance into the cyanobacteria is one of the few possibilities, as the fern can survive the selection. Transformation of *N. azollae* is limited by its location. It is likely necessary to target the stem cells in the shoot apex, or germinating akinetes from under the indusium cap, for transformed bacterial cells to be disseminated to further parts of the plant or new generations. These are well protected by several layers of hydrophobic tissue.

Despite the challenges, some steps have been done towards establishing transformation in *Azolla*. Transient transformation of the fern has been achieved through the use of *Agrobacterium* for up to two days. Biobalistics, tissue culture and targeting key stages of its sexual life cycle have also been attempted (Güngör, 2018). Regarding the cyanobacterial genetic modification, *N. azollae* extracted from the leaves and *Anabaena* PCC 7120, cyanobacteria closely related to the former, have been transiently modified through triparental conjugation. In addition, fluorescent bacteria were shown to be able to access the shoot apical meristem colonies of *N. azollae* inside the fern through an adapted floral dip protocol. Moreover, both conjugation under the detergent and sugar concentrations used to infiltrate the fluorescent bacteria proved compatible, an encouraging keystone to develop the technology further (Borel, 2019).

Engineering cyanobacteria

Cyanobacteria have the potential to become sustainable food and chemical production platforms. Despite successful proof of principles, large scale applications are limited by low yields. To fulfill their promise, the genetic and metabolic engineering of these organisms must be developed to a level comparable to that of *E. coli* and *S. cerevisiae*, but synthetic biology strategies for cyanobacteria are still in their infancy (Wang et al., 2020).

Modification of cyanobacterial genomes is a simple but time-consuming process with considerable areas of improvement (Hitchcock et al., 2020). The most common method involves homologous recombination of constructs that are transferred by natural transformation, conjugation or electroporation. Several challenges hinder the development of the process. Cyanobacteria are relatively slow growing, when compared to *E. coli*. They are oligo or polyploid organisms, with some species having up to 53 chromosome copies per cell (Zerulla et al., 2016), requiring slow segregation procedures to generate homozygous strains. There is a limited number of antibiotic markers available for them, making marker-less editing a desirable trait for future technologies. Most replicative vectors are derived from a single plasmid due to stability issues, pRSF1010, which is able to replicate in a broad range of gram-negative bacteria, including *E. coli*.

The lack of options for engineering cyanobacteria must be addressed. Recent advances include specialized regulatory elements, e.g. riboswitches; genetic libraries, e.g. SyneBrick and CyanoGate; and CRISPR-Cas systems (F. Wang et al., 2020). Regarding the latter, CRISPR-Cas genome editing has been employed successfully in several cyanobacterial species (Behler et al., 2018). Although control and off target issues must be addressed to reduce cytotoxicity, it is a promising technology with a number of advantages, including marker-less selection, the characteristic of being easily adaptable and delivered, possibility for transcription regulation through CRISPR interference (CRISPRi) and simultaneous repression of multiple genes (multiplexing), (Pattharaprachayakul et al., 2020). CRISPR-Cas systems could hold the key to unlock the potential of cyanobacteria.

CyanoGate

Synthetic biology aims to simplify the process of designing, constructing and modifying complex biological systems; requiring robust and efficient procedures. Genetic engineering, as similar fields, requires parts and methods that follow engineering principles, such as standardization, modularity and simplicity (Andreou & Nakayama, 2018). They are essential for accelerating the “design, build, test, and learn” cycle, a central tenet of synthetic biology (McArthur IV et al., 2015). Moreover, the exponential increase of genomic information available demands a standardized system that can be characterized, exchanged and assembled cheaply, easily, and in an automated way (Patron et al., 2015).

The recent adoption of standards for assembly methods represents a major step in the right direction. Among them, the methods employing Golden Gate cloning, that is based on type IIS restriction enzymes, are one of the most promising and widely used. Golden Gate cloning permits simultaneous assembly of multiple DNA fragments in a defined linear order using a one-pot, one-step reaction. It is faster, cheaper, more flexible and efficient than traditional cloning techniques, especially when making multigene constructs (Weber et al., 2011). Its only limitation is the presence of internal recognition sites for the enzymes used, which have to be removed through a process

called domestication. Modular Cloning (MoClo) is the most popular assembly standard that employs Golden Gate cloning, having toolkits for various model systems, including heterotrophic bacteria (Moore et al., 2016), yeast (Lee et al., 2015), microalgae (Crozet et al., 2018), plants (Engler et al., 2014) and, most recently, cyanobacteria (Vasudevan et al., 2019). Vectors of >50 kb can be generated through MoClo assembly (Werner et al., 2012).

CyanoGate is a new MoClo system for cyanobacteria developed by Vasudevan et al. It represents a significant milestone for the synthetic biology of these microorganisms, which was lagging behind others. It is built on the syntax of the Plant Golden Gate MoClo kit, Fig 3A. The system includes components for making marked/unmarked knock-outs or integrations using an integrative vector, and transient multigene expression and repression systems using replicative vectors, having been tested in *Synechocystis sp.* PCC 6803 and *Synechococcus elongatus* UTEX 2973. An online tool was developed alongside.

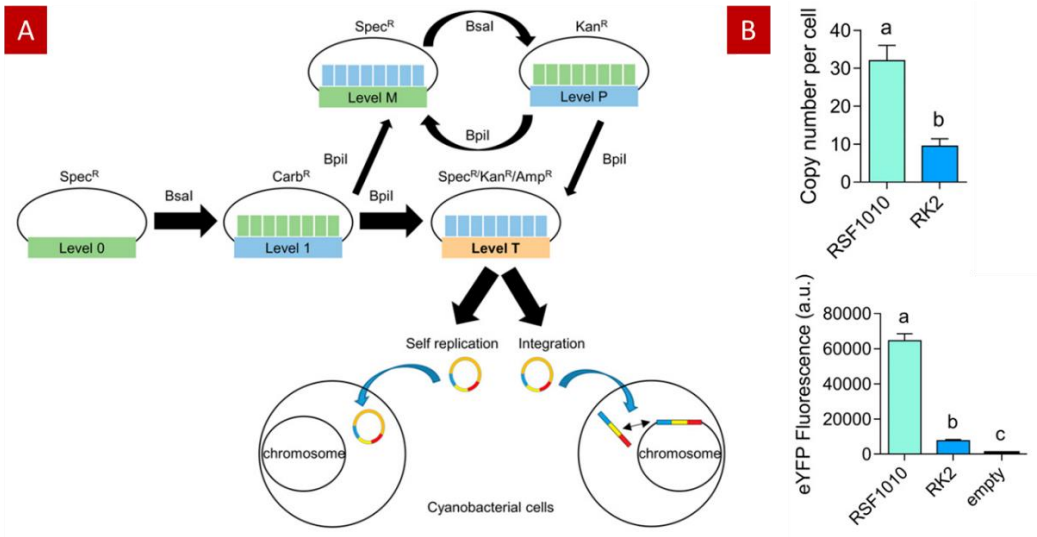


Fig. 3. CyanoGate system. A. Plant Golden Gate MoClo Assembly Standard for cyanobacterial transformation. **B.** Plasmid copy number and expression levels of eYFP in *Synechocystis* with RSF1010 and RK2 replicative origins (Vasudevan et al., 2019).

The toolkit contains genetic parts, both, previously characterized and specifically created. Most work was done to establish heterologous and synthetic promoters with diverse characteristics and expression levels, terminators, CRISPRi components and alternative replicative vectors to pRSF1010 derived ones. Regarding the latter, the RK2 replication origin derived from pSEVA421 was the only functional. However, RK2 has a reduced copy number relative to RSF1010, which impacts expression levels, Fig 3B. Therefore, the kit includes two replicative (pCAT.000 with an RSF1010-derived ori and pCAT.011 with an RK2 ori) and two integrative (pCAT.015 and .334) level T vectors. The modular nature of the CyanoGate kit allows for the necessity of testing different parts and their combinations. The kit was tested in filamentous cyanobacteria that mostly require conjugation for effective transformation, but CyanoGate lacks conjugative vectors that do not replicate inside cyanobacteria.

CRISPR-associated transposases (CAST)

The CRISPR-Cas system is one of the most relevant technologies developed in the 21st century. The general public cannot perceive its importance yet, but there is no doubt as to how impactful it will be for our society. It is already a resourceful tool in biological research and will be transcendental in numerous areas, including medicine, food, chemical production, environmental remediation, among others. Still, considerable work remains to be done, with major challenges to overcome.

The CRISPR-Cas systems characterized thus far are naturally efficient at knocking out genes, but achieving knock-ins is still an inconsistent process because of the dependence on the host cell repair machinery. After DNA cleavage, the Cas proteins used thus far introduce a double-strand break (DSB), which can be repaired by two general pathways: non-homologous end joining (NHEJ) or homology directed repair (HDR). The efficient but error-prone NHEJ pathway is the most active and predominant mechanism, resulting in a diverse array of mutations and knock-outs. Precise integration of new DNA can only be achieved through the less efficient but high fidelity HDR pathway. Numerous methods to inhibit NHEJ or enhance HDR have been developed (Liu et al., 2019), nevertheless, efficient genome editing of even model organisms remains challenging (Wolabu et al., 2020).

Alternative CRISPR-Cas systems to produce knock-ins or specific mutations without DSBs are desirable. Recent advances include deactivated Cas (dCas) proteins linked to nickases to generate single-strand breaks or deaminases to achieve base editing. Still, neither of them are capable of precisely inserting DNA. Transposases associated to CRISPR-Cas systems could accomplish it.

Recently, the association between Tn7-like transposons and several subtypes of CRISPR-Cas systems was reported in various prokaryotic organisms (Faure et al., 2019; Klompe et al., 2019; Peters et al., 2017). Similarly to other CRISPR-CAS systems, they could serve as an adaptive immunity mechanism against mobile genetic elements, such as viruses, plasmids and other transposons. Strecker et al. characterized the CRISPR-Cas12k associated transposase (CAST) from the cyanobacteria *Scytonema hofmanni* (ShCAST) and *Anabaena cylindrica* (AcCAST). It is a subtype V-K CRISPR-CAS system, which are the simplest since they function with a single-protein Cas effector, namely Cas12k. The CRISPR-Cas locus is conserved within the transposon ends, next to the Tn7-like transposon genes (*tnsB*, *tnsC* and *tniQ*) and the cargo genes, Fig 4A, making it a self-propagating system.

Fig 4B showcase a model of the CAST system in action. The Cas12k effector, with the guide of a specific RNA molecule, locates its target sequence and generates an R-loop to facilitate the insertion of the transposon, which is defined by the left and right ends. As any CRISPR-Cas system, it requires a PAM sequence upstream its target site to work, in this case being GTN. The transposon is inserted 60-66 bp (mostly 61 or 62) downstream in the case of ShCAST and 49-56 bp (mostly 51 or 52) in AcCAST, Fig 4C. Because staggered DNA breaks are caused and repaired during the process, 5 bp motifs are duplicated around the insertion site, which is consistent with Tn7 behaviour. In nature, the RNA molecule is composed of two parts: the crRNA (CRISPR RNA) with the spacer that specifies the target sequence and the tracrRNA (trans-activating CRISPR RNA) that helps mature the crRNA and links it with the Cas protein, Fig 4D. Strecker et al. designed a sgRNA molecule that combines both and can be modified to target any specific sequence.

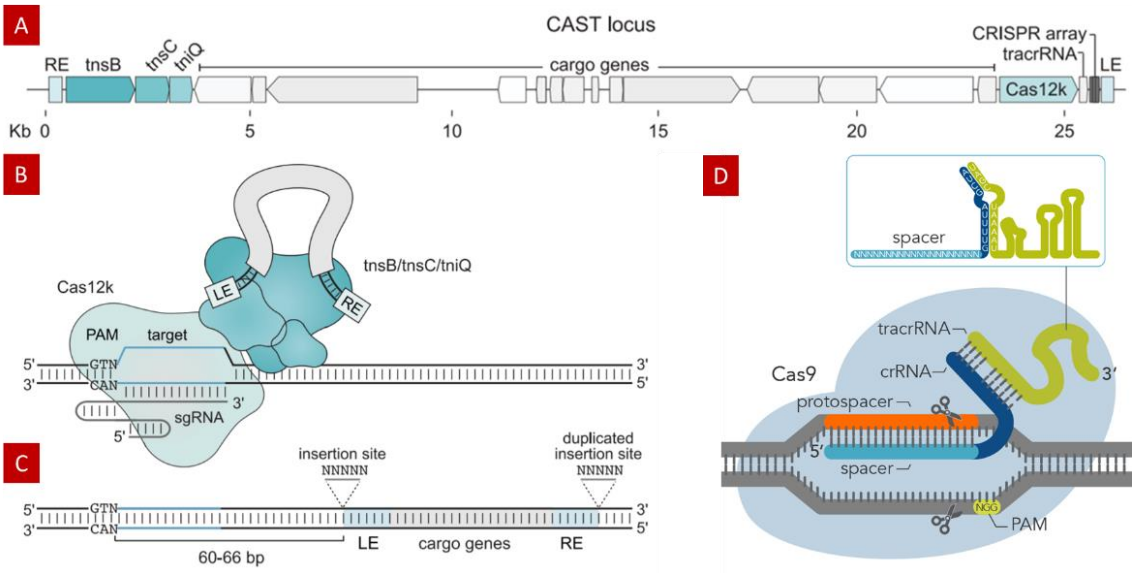


Fig 4. CRISPR-Cas12k-associated transposase (CAST) systems (Strecker et al., 2019). **A.** *S. hofmanni* CAST locus containing Tn7-like proteins, the CRISPR-Cas effector Cas12k, and a CRISPR array. **B.** Model of the CAST system in action. **C.** Transposon insertion structure by CAST. **D.** Parts of the original gRNA which was engineered by Strecker et al into a single guide RNA (sgRNA) for easier usage.

The CAST system effectively catalyzes RNA-guided DNA transposition without nuclease activity nor homologous recombination *in vitro* and in *E.coli*. When tested against plasmid and genomic material it proved to be highly effective, in the latter case 80% of the *E.coli* colonies recovered contained the transposon at the target location without applying selection. CAST can insert up to 10 kb DNA. The insertion occurs only in one site and in an approximate unidirectional known position. Off targets can occur, although they are rare. The system appears not suitable for targeting highly transcribed genes (González Linares, 2020). Still, CAST is a promising strategy to modify the genome of reluctant organisms.

Aim and Objectives

The aim of this study is to establish tools and techniques for the genetic modification of the symbiosis *Azolla*. Correspondingly, it is composed of two parts, one focused on the transformation of the fern and another on resources for editing the genome of the cyanobacteria.

The specific objectives of the fern section are:

a) Optimize the transient transformation of *A. filiculoides* through a previously tested floral dip protocol.

b) Test the induction of meristems in *A. filiculoides* through the developmental regulators WUS and ipt.

The specific objectives of the cyanobacteria section are:

a) Replicate the work done by Strecker et al. applying a CAST system from cyanobacteria in *E. coli*.

b) Construct a library of plasmids that employs CAST to target the genome from *Anabaena PCC* 7120, a close relative of *N. azollae*.

c) Transform *Anabaena* PCC 7120 with CAST through triparental conjugation.

d) Combine previously successful transient transformation methods for *N. azollae* with CAST system.

Transformation of *A. filiculoides*

Materials and Methods

Medium and culture conditions

A. filiculoides cultures were maintained in IRRI medium as described by Watanabe et al. with the modifications described by Brouwer, 2017 in 8 L polypropylene boxes. They were kept at a temperature of 22 °C with 100 umol m^{-2} s^{-1} light during 16 h in the diel cycle. Sporophytes were kept at high densities to cover the surface and inhibit the growth of algae. Dead or infected plant material was manually picked out.

Agrobacterium tumefaciens strains were maintained in solid Luria-Bertani (LB) medium supplemented with 50 mg/L of rifampicin and the respective antibiotic for each plasmid. The plates were grown at 28°C.

Transformation of *A. filiculoides*

A. filiculoides was transformed through an *Agrobacterium*-mediated method that employs an adapted floral dip protocol (Davis et al., 2009; Zhang et al., 2006). Plasmids constructed by Maher et al. were used, namely pMK057, 058, 059 and 060, (AddGene #133312-133315) and pMM131 (AddGene #127214) in the strain AGL1. These encode for luciferase as a reporter gene and developmental regulators with the capacity to induce meristems from somatic cells, Table 1. The latter can be delivered simultaneously with gene-editing reagents, such as CRISPR-Cas systems, enabling plant gene editing without the need of tissue culture or antibiotic selection.

Construct	Module A	Module B	Module C	Module D
pMM131	35S:LUC	AtU6:gRNA (NbPDS)	Empty	CmYLCV:AtSTM
pMKV060	Empty	AtUBQ10:LUC	Empty	Empty
pMKV059	Empty	AtUBQ10:LUC	Empty	35S:ipt
pMKV058	Empty	AtUBQ10:LUC	nos:ZmWus2	Empty
pMKV057	Empty	AtUBQ10:LUC	nos:ZmWus2	35S:ipt

Table 1. Plasmids from Maher et al. used to transform *A. filiculoides*.

The base protocol consist of the following steps. Cultures of *A. tumefaciens* with different plasmids were grown overnight in LB with appropriate antibiotics at 28°C, 180 rpm. On the day of the transformation, the bacteria was harvested by centrifuging 10 ml of liquid culture at 3,500 g for 10 min and resuspended in 1 ml of 0.5X MS supplemented with 5% sucrose (w/v) and 0.02% silwet L-77 (v/v). Sporophytes were cut into small pieces of 1-2 cm and maintained in IRRI medium. Afterwards, they were treated with 1.5% cellulase and 0.5% maceroenzyme for 2 hours at 28°C, as it has been shown cell wall digestion of fern gametophytes is critical to achieve transformation (Bui et al., 2015). Residual enzymes were removed by washing the fern pieces 3 times with 0.5X MS. The treated sporophytes were co-incubated with *Agrobacterium* solution at room temperature for 1 h. Then, they were washed three times with 0.5X MS and transferred to 1:1 IRRI:0.5X MS.

The following day, the samples were assayed for luciferase activity by capturing the low-light emitted with a Hamamatsu IEEE1394B camera equipped with the Nikon 60 mm lens and controlled by ImagEM-X2 program. To prepare 100X luciferin solution in its free acid form, luciferin was mixed in equimolar concentration of 10 mM with Tris base. For the working solution, luciferin was diluted

to 0.1 mM and Triton 0.01% v/v added. Samples were submerged in luciferin working solution for 1 minute before signal detection. After the assay, they were washed 3 times with IRRI medium.

In order to achieve longer transient or permanent transformation, several factors of the protocol were tested. These include pre-culture time of and co-incubation with *A. tumefaciens*, enzymatic digestion time, dissection and cutting specific sections of the sporophytes, addition of acetosyringone and different surfactants.

Results

Initially, signals came from pMKV057, 060 and pMM131, Fig 5, it is unclear why pMKV059 and 059 did not generate any. The strongest luciferase activity appeared with pMM131, reason why it was chosen as the plasmid to optimize the transformation protocol. After testing all of the factors mentioned, it was found that pure liquid cultures of bacteria containing pMM131 presented luciferase activity by themselves, Fig 6. Such founding made all of the previous results false positives. Luciferase signals, and therefore presence of bacteria, were still detected after up to 34 days. The testing had to be redone with pMKV057. It was found that pre-culture time of *Agrobacterium* was the most relevant factor of the protocol, Fig 7. Luciferase signals were detected after up to 7 days when pre-cultures were grown for 2 days.

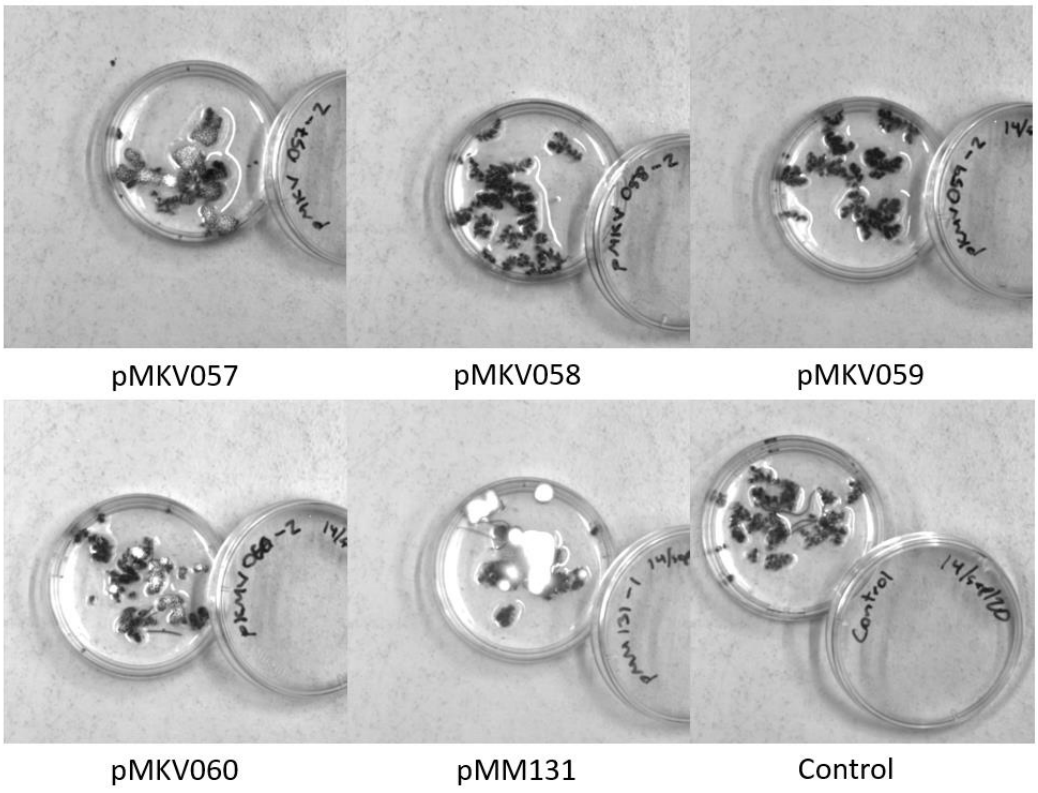


Fig 5. *A. filiculoides* after 1 day of *Agrobacterium* mediated transformation with pMK and pMM plasmids from Maher et al. 2019. Control is fern pieces without any treatment.

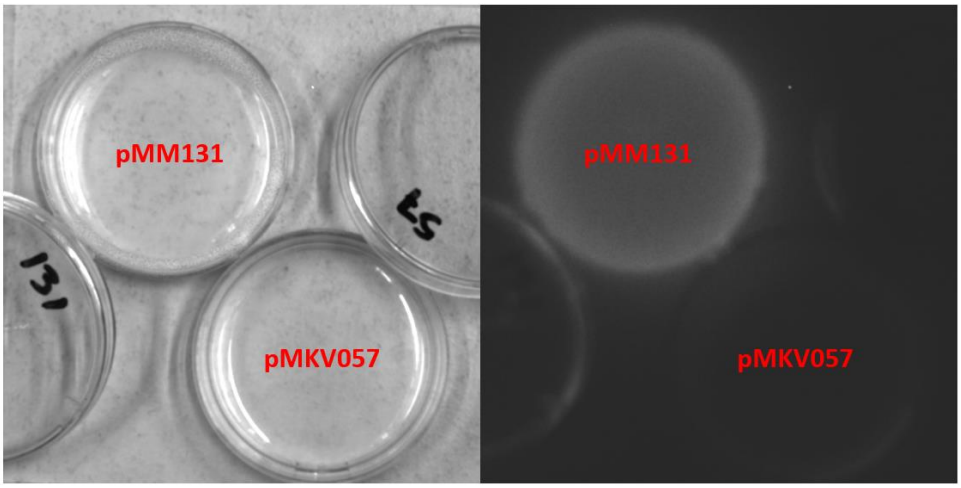


Fig 6. Liquid cultures of *A. tumefaciens* AGL1 with pMM131 and pMKV057 assayed for luciferase activity.

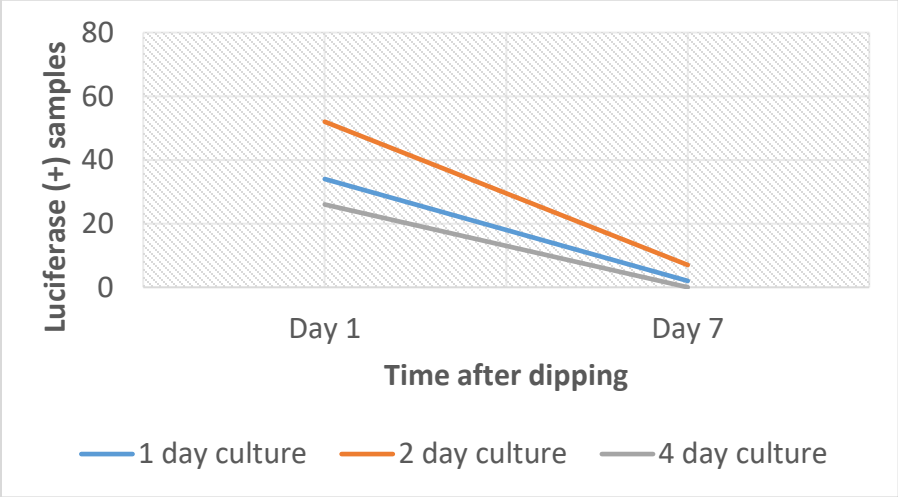


Fig 7. Luciferase positive pieces of *A. filiculoides* after co-incubation with *Agrobacterium* cultures of 1, 2 and 4 days old. N=80 pieces per treatment.

In addition to the base protocol, dissection of the shoot tips prior to transformation was also investigated. Its purpose was to expose their meristems, Fig 8, and allow the access of *Agrobacterium*. This method exhibited the sharpest and clearest signals, Fig 9A. In most cases, the dissected section died after a couple days, but a few samples stayed with white color instead of turning brown, and produced luciferase signal after up to 10 days, Fig 9B.

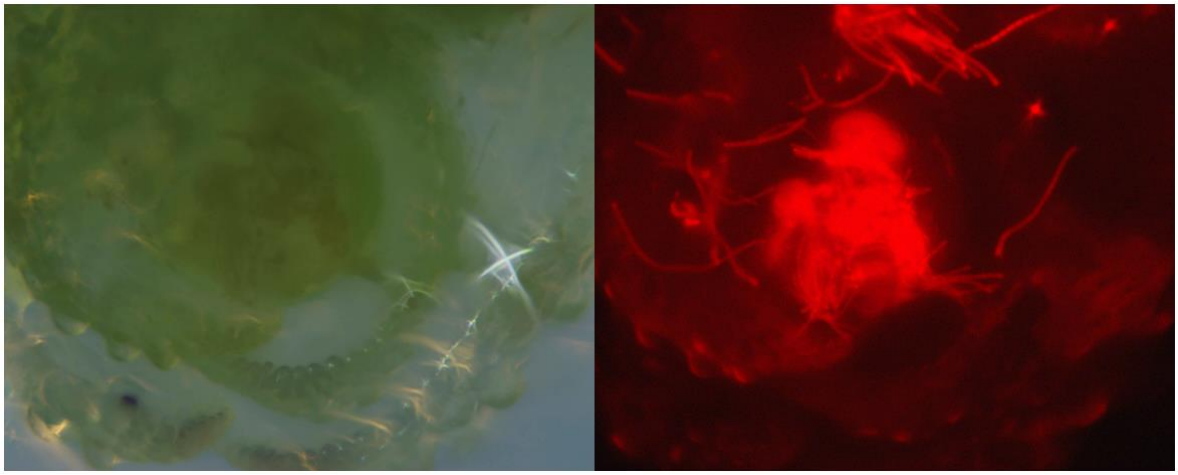


Fig 8. Dissected shoot tip of *A. filiculoides* under white light and red fluorescent protein filter after excitation with a wavelength of 590 nm. The apical cyanobacteria demonstrates the proximity to the meristems.

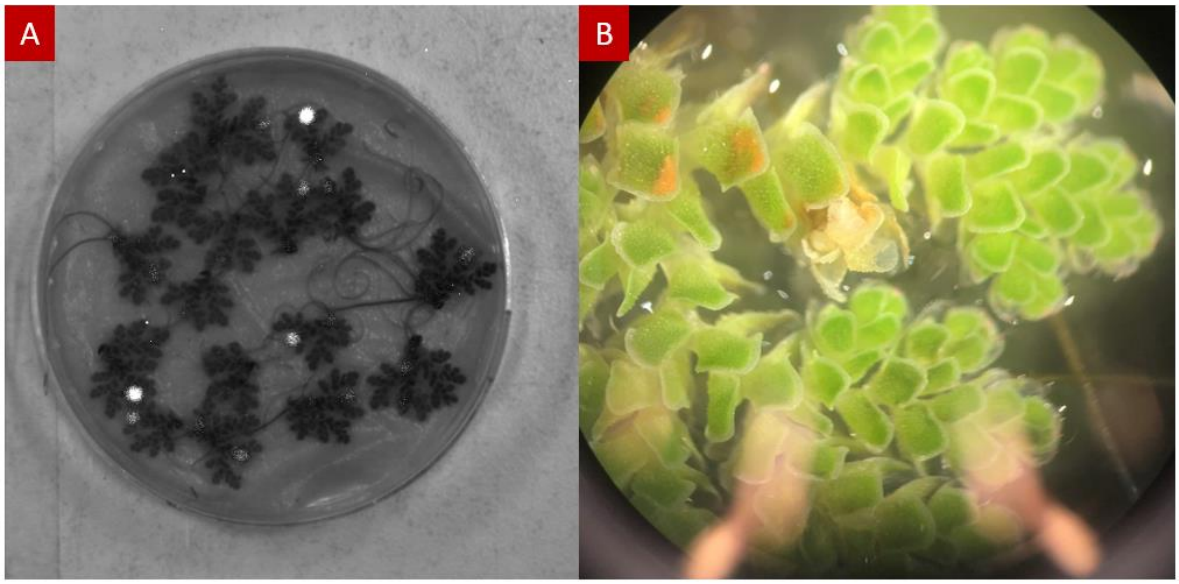


Fig 9. A. Dissected shoot tips showing luciferase activity. **B.** Single dissected tip remaining alive and producing signal after 10 days.

Discussion

A considerable number of tests done in this study were deceptive due to the lack of proper controls and an adequate detection system. It is especially relevant to design experiments correctly when working with a non-model organism. In this particular case, controls should include: pure liquid culture of bacteria, ferns untreated and treated with *Agrobacterium* wild type to avoid false positives, and plant material expressing luciferase as a positive control. Besides, a better detection system that is consistent is critical. Ideally, it should be quantitative, fast, with high resolution and personnel with expertise on the matter.

Nevertheless, several observations can be made. First, CaMV 35S promoter in the pMM131 plasmid drives the luciferase gene expression in *Agrobacterium*, demonstrated by the signals emitted by

pMM131. The bacteria containing the pMM131 can survive on the sporophytes after more than one month, which could be detrimental for *Azolla* health, therefore methods to ensure its removal after the co-incubation step should be developed. The state of the *Agrobacterium* culture is crucial for the success of the transformation, which is reflected by the pre-culture time results. Standardization through spectrophotometry must be applied, and addition of acetosyringone could also improve the transformation efficiency.

Luciferase signals with pMKV057 were detected after several days, but appearance of new meristems was not detected. This suggests that the expression of WUS and ipt does not induce new meristems in *Azolla*. Targeting pre-existing ones or the reproductive cycle of the fern seem to be the only options to transform without the use tissue culture. Dissecting sporophytes is a promising alternative, although it is time consuming, requires highly skilled ability and the survival rate has to be improved.

Conjugation of *Anabaena sp.*

Materials and Methods

Medium and culture conditions

The cyanobacteria *Anabaena sp.* PCC 7120 was cultured in solid and liquid BG-11 medium as described by Arévalo Díaz, 2020. Cultures were grown at 30°C under constant light of 20-30 umol m^{-2} s^{-1} and 100 rpm in the case of the liquid medium. To start new cultures, two elements should be taken into account; first, that they are inoculated with sufficient biomass to avoid light inhibition, and second, that the correct type of agar is used for making plates (Bacto Agar from Becton, Dickinson and Co. Ref 214010).

Genetic constructs

To generate the plasmids for the conjugation of *Anabaena*, several molecular techniques were employed. Unless specified differently, all the procedures were realized in *E. coli* DH5α with LB medium and the corresponding antibiotic for each plasmid. Heat shock was used to transform the *E. coli*, which was confirmed by colony PCR or restriction analysis. Commercial kits were employed for plasmid extraction (E.Z.N.A Plasmid DNA Mini Kit from Omega) and gel purification (MinElute PCR purification Kit from Qiagen) following the specifications of the manufacturer.

PCR were realized with Taq polymerase for verification of transformants, and Phusion for domestication and amplification of fragments used for cloning. The following mixture and conditions were employed:

		Working stock	Volume (µl)	Final concentration
Taq	Buffer Taq	10X	2	1X
	Taq pol	5 U/µl	0.2	0.05 U/µl
Phusion	Buffer Phu	5X	4	1X
	Phu pol	2 U/µl	0.2	0.02 U/µl
Both	dNTPs	2 mM	2	200 µM
	Primers	5 µM	1 each	0.25 µM
	MilliQ water	-	to 20	-
		Total	20	

Table 2. Components for PCR reaction with Taq pol and Phusion.

As template, 50 ng of plasmid DNA were used. In the case of colony PCR, a single colony was suspended in 15 µl of MilliQ water and 1 µl used as template.

	Taq	Phusion
Denaturation temperature	95°C	98°C
Extension time	1 min/kb	30 sec/kb
Cycles	30	30

Table 3. Conditions for PCR reaction with Taq pol and Phusion.

Besides confirmation through PCR, all samples were sequenced to verify that they were assembled properly.

Testing of the CAST system in *E. coli*

In order to verify the efficiency of the CAST system, the work done by Strecker et al., 2019, was replicated. It consists on the introduction of three different plasmids, pHelper (Addgene #127922), pDonor (Addgene #127924) and pTarget (Addgene #127926), into a single *E. coli*. The plasmids were received with the following characteristics:

Plasmid	Strain	Vector backbone	Origin of replication	Copy number	Resistance	Size (bp)
pHelper	NEB stable	pUC19	pMB1 (mutant)	High	Amp	8150
pTarget	DH5α	pACYC	p15A	Low	Chl	2899
pDonor	Pir1	pUC19	R6K	Low	Kan	3016

Table 4. Constructs to replicate plasmid targeting in *E. coli* and their characteristics (Strecker et al., 2019).

The constructions were verified through restriction analysis. Next, Pir1 cells containing pDonor were transformed with the other two plasmids independently and simultaneously to test the reliability of the system. The reason for using this specific strain is that the R6K ori requires the presence of the pir gene for replication, so standard *E. coli* cannot work with it. After selection with the corresponding three antibiotics, insertion of the cargo genes into pTarget was detected through PCR amplification in both ends of the insertion.

MoClo procedures

All GoldenGate MoClo reactions were realized with the following mixture and conditions:

Buffer G 10X	2 µl
ATP 100 mM	0.2 µl
Vector	100 ng
Insert	Molar ratio 2:1 insert:vector
BsaI/BpiI	1 µl
T4 ligase	0.4 µl
MilliQ	to 20 µl
Total	20

Table 5. Components of a MoClo reaction.

Temperature (°C)	Time (min)	
37	10	3 cycles
22	10	
37	10	
65	20	
12	∞	

Table 6. Program parameters for MoClo reaction.

For blue-white screening, plates of LB supplemented with the corresponding antibiotics, IPTG 0.2 mM and X-gal 40 µg/ml were used.

The components of the CAST system were introduced into MoClo vectors. All were extracted from pHelper_sgRNA (Addgene #127921). Primers were designed to amplify DNA fragments with overhangs corresponding to their vectors. Tns B, C and Q and Cas12k sequences had to be domesticated, as they contained BsaI and BpiI sites. The genes were split in fragments, initially cloned in a level -1 vector and assembled as CDS in a level 0 vector. The complete cassette to express the sgRNA was directly amplified and cloned in a level 1 vector. The additional element used for the constructions that is not part of the MoClo toolkit or the CyanoGate kit is the GlnA promoter, which is strong and inducible under nitrogen starvation, and was amplified from pRL3845 (Arévalo Díaz, 2020).

sgRNA design and cloning

Three gRNAs were designed to target the GFP gene of Anabaena CSVT15 and CSAM137 (Arévalo Díaz, 2020) to avoid toxic side effects and have an easily detectable visual marker. Their structure, sequence and characteristics are the following:

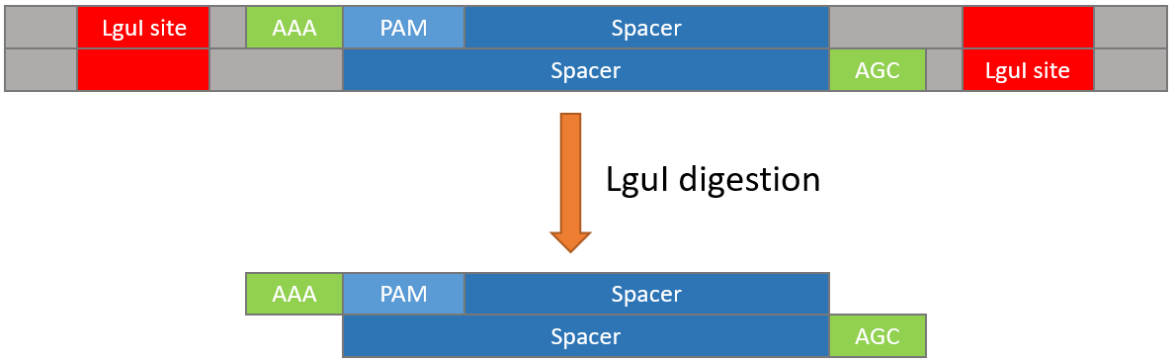


Fig 10. Structure of sgRNAs for cloning through LguI digestion.

Label	Orientation	Sequence	PAM	Target strand
A	Fwd	TTGCTCTTCCAAAGGTTATGTACAGGAAAG AACTATATTTTTCAAAGGCTGGAAGAGCAA	GGTT	Sense
	Rev	TTGCTCTTCCAGCCTTTGAAAAATATAGTT CTTTCCTGTACATAACCTTTGGAAGAGCAA		
B	Fwd	TTGCTCTTCCAAAGGTTGTCTGGTAAAAGG ACAGGGCCATCGCCAATGCTGGAAGAGCAA	GGTT	Antisense
	Rev	TTGCTCTTCCAGCATTGGCGATGGCCCTGT CCTTTTACCAGACAACCTTTGGAAGAGCAA		
C	Fwd	TTGCTCTTCCAAAGTTCCATGGCCAACACT TGTCACTACTTTCGCGGCTGGAAGAGCAA	GTT	Sense
	Rev	TTGCTCTTCCAGCCGCGAAAGTAGTGACAA GTGTTGGCCATGGAACTTTGGAAGAGCAA		

Table 7. Primers sequence to generate sgRNAs.

For cloning of the sgRNAs, LguI sites are included in the original cassette and in the primers that compose the insert. The primers are annealed using the following mixture and parameters:

	µl
Fwd primer (500 µM)	1
Rev primer (500 µM)	1
T4 ligase buffer (10X)	2
Milli-Q	19

Table 8. Components for primer annealing to generate sgRNA.

Place at 95°C for 5 min and cool down at a ramp of 5°C/min.

Afterwards, both the vector and insert can be digested with LguI and ligated in a one pot reaction using the same mixture and parameters as that of a MoClo reaction.

Anabaena conjugation

Anabaena sp. PCC 7120 was transformed through triparental conjugation as described by Arévalo Díaz, 2020. To achieve it, two strains of *E. coli* were employed:

- Strain ED8654 (Murray et al., 1977) carrying the conjugative plasmid pRL443 (Amp^R) (Elhai & Wolk, 1988)
- Strain HB101 (Boyer & Roulland-dussoix, 1969) carrying the helper plasmid pRL623 (Chl^R) (Elhai et al., 1997) and the cargo plasmid (variable antibiotic resistance)

Besides them, a liquid culture of the cyanobacteria of high density (grown at least 5 days under optimal conditions) is needed. One day before the conjugation, 3 ml cultures of each *E. coli* strain were started in LB medium with the corresponding antibiotics and incubated overnight at 37°C, 200 rpm.

On the conjugation day, two flasks containing 10 ml of LB with antibiotics were inoculated with 350 µl of HB101 and 250 µl of ED8654 each, and incubated for 2.5 hours in the same culture conditions. Pellets were washed 3 times with 10 ml of LB after centrifugation at 4000G for 5 min, and both strains were combined in a maximum volume of 400 µl. The mixed cultures were incubated at room temperature for a minimum of 2.5 hours.

The *Anabaena* culture was centrifuged at 4000G for 10 min and concentrated fivefold in its same medium. During decantation, care should be taken, as cyanobacteria resuspends easily. From the concentrated *Anabaena*, 100 µl are separated to realize a chlorophyll determination. This assay consists on mixing the 100 µl of cyanobacteria with 900 µl of methanol, vortex for 1 min, centrifuging at 13,000 rpm for 3 minutes and measuring the OD_{665} of the supernatant. To quantify the chlorophyll, the following formula is employed:

$$\frac{\mu g\ of\ chlorophyll}{ml} = (OD_{665})(10)(13.45)$$

For conjugation, the volume of concentrated *Anabaena* equivalent to 10 µg of chlorophyll were gently homogenized with the *E. coli*. The mixed culture was spread on a nitrocellulose filter (Immobilon NC from Merck Millipore Ref HATF08550) on a plate of BG11 medium + 5% LB (v/v) without antibiotics. The dish was incubated in darkness at 30°C for 2 hours and then transferred to light. After 24 hours, the filter was transferred to a plate of BG-11, and 24 hours later, to one

supplemented with the corresponding antibiotic. Every 48 hours afterwards the filter is transferred to a new plate until antibiotic-resistant colonies appear.

Results

CAST testing in *E. coli*

The plasmids with the components of the CAST system were firstly verified through restriction analysis, showing all the expected bands. Afterwards, they were combined in one host, and insertion of the cargo genes into pTarget was detected through PCR amplification on both ends of the sequence, Fig 11 and 12. The CAST system efficiently integrated the cargo gene in all of the samples tested and only when the three plasmids were present. The non-expected bands on the RB were confirmed as non-specific products. Abnormal growth appeared in the plates containing pHelper and pDonor after 2 weeks, Fig 13. The biomass could not be cultivated further.

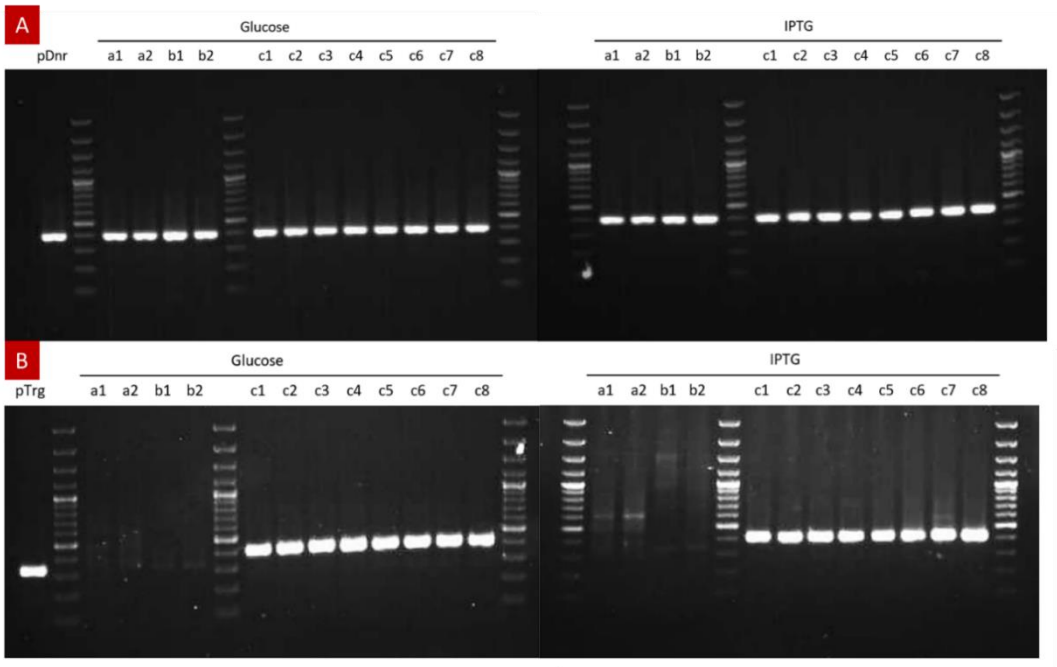


Fig 11. PCR detection of the left border (LB) in pTarget. A. Positive control amplifying sequence with the LB in pDonor. **B.** Amplification of sequence with the LB inserted in pTarget. a) pDonor + pHelper b) pDonor + pTarget c) pDonor + pHelper + pTarget.

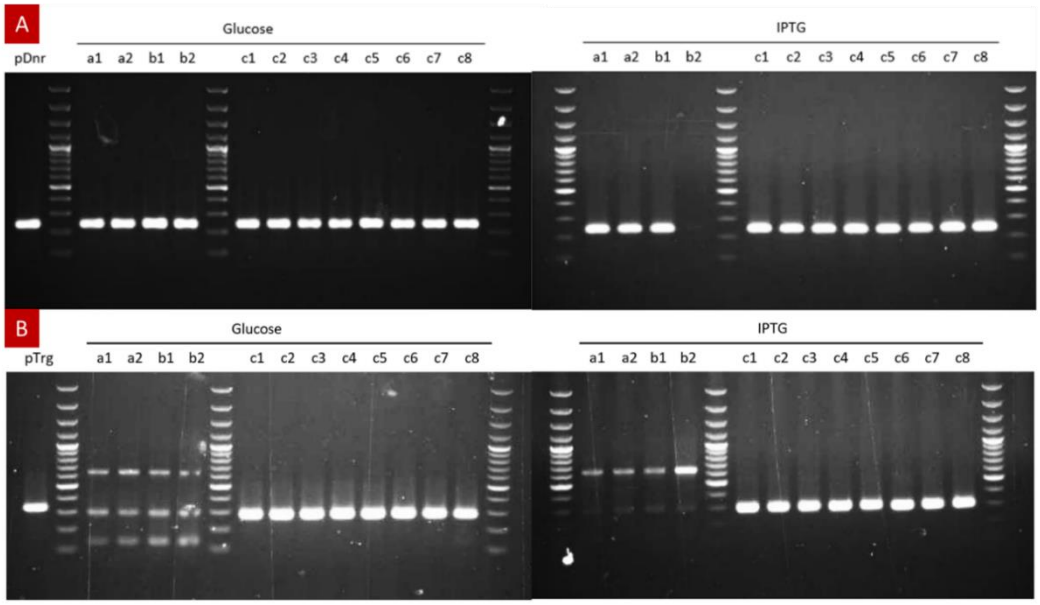


Fig 12. PCR detection of the right border (RB) in pTarget. A. Positive control amplifying sequence with the RB in pDonor. **B.** Amplification of sequence with the RB inserted in pTarget. a) pDonor + pHelper b) pDonor + pTarget c) pDonor + pHelper + pTarget.

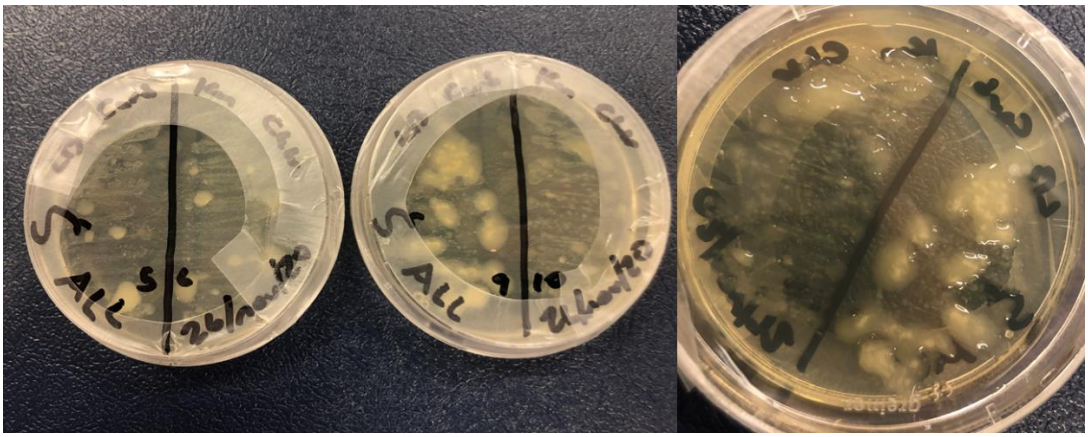


Fig 13. Plates with *E. coli* containing all the components of the CAST system showing abnormal growth after 2 weeks.

Plasmid library

A library of plasmids to test the CAST system in Anabaena was generated. To create the final plasmids for conjugation, constructs in all the levels of the MoClo syntax were required. Table 9 includes all the plasmids generated in this study and their characteristics. Verification of their proper construction was done through restriction analysis, PCR and sequencing.

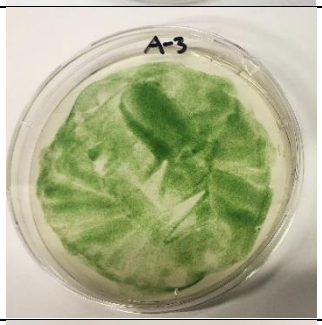
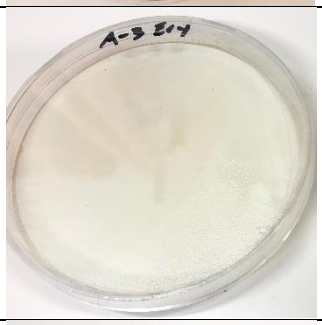
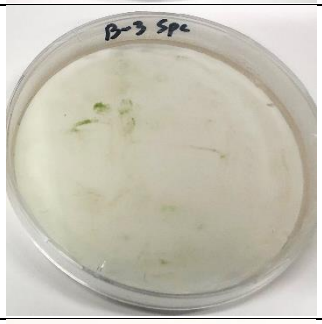
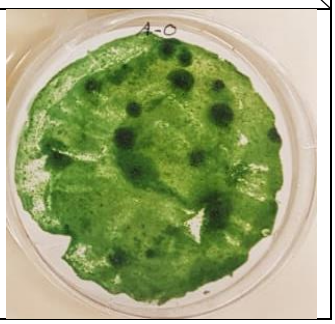
Name	Level	Antibiotic	Backbone	Content
pAzU0.1	0	Spec	pICH41295	GlnA promoter
pAzU0.2	0	Spec	pICH41308	Domesticated Tns B, C and Q
pAzU0.3	0	Spec	pICH41308	Domesticated Cas12k

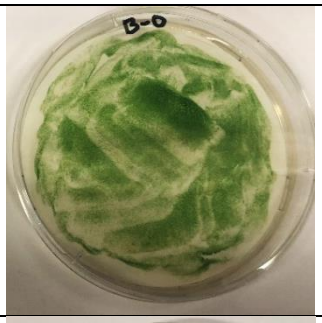
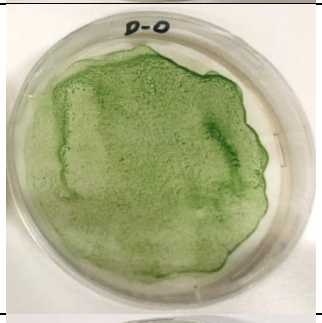
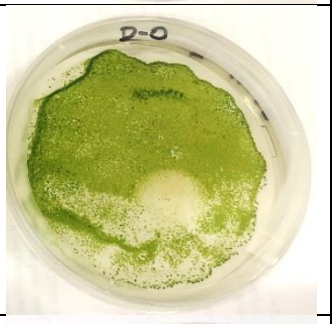
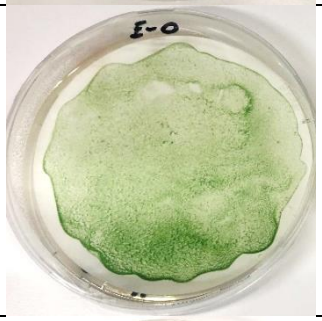
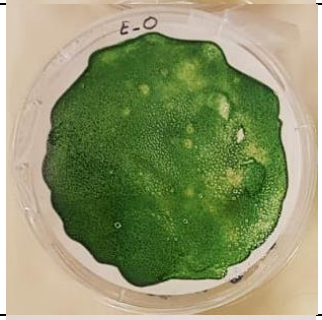
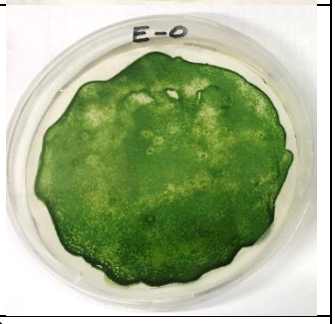
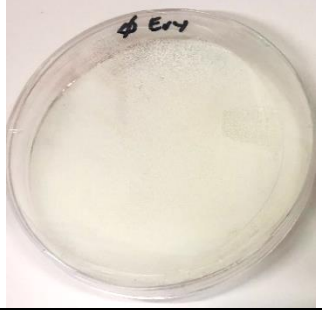
pAzU0.4	0	Spec	pICH41308	Domesticated erythromycin resistance				
pAzU0.5	0	Spec	pICH41308	Domesticated spectinomycin resistance				
Name	**Level**	**Antibiotic**	**Backbone**	**Promoter**	**CDS**		**Terminator**	
pAzU1.1	1-1	Carb/Amp	pICH47732	GlnA	Tns B, C and Q		PheA	
pAzU1.2	1-2	Carb/Amp	pICH47742	GlnA	Cas12k		PsaB	
pAzU1.3	1-3	Carb/Amp	pICH47751	J23119	sgRNA with LguI sites		T7Te	
pAzU1.3.1	1-3	Carb/Amp	pICH47751	J23119	sgRNA_A		T7Te	
pAzU1.3.2	1-3	Carb/Amp	pICH47751	J23119	sgRNA_B		T7Te	
pAzU1.3.3	1-3	Carb/Amp	pICH47751	J23119	sgRNA_C		T7Te	
pAzU1.4	1-4	Carb/Amp	pICH47761	LB + RnpB	eYFP		rrnB	
pAzU1.5	1-5	Carb/Amp	pICH47841	RB + Cpc560	Erythromycin resistance (antisense)		cpc_operon	
pAzU1.6	1-1	Carb/Amp	pICH47732	RnpB	eYFP		rrnB	
pAzU1.7	1-2	Carb/Amp	pICH47742	Cpc560	Erythromycin resistance		cpc_operon	
pAzU1.8	1-2	Carb/Amp	pICH47742	Cpc560	Spectinomycin resistance		cpc_operon	
pAzU1.9	1-4	Carb/Amp	pICH47761	RnpB	eYFP		rrnB	
pAzU1.10	1-5	Carb/Amp	pICH47841	Cpc560	Erythromycin resistance		cpc_operon	
pAzU1.11	1-5	Carb/Amp	pICH47841	Cpc560	Spectinomycin resistance		cpc_operon	
Name	**Level**	**Antibiotic**	**Backbone**	**Position 1**	**Position 2**	**Position 3**	**Position 4**	**Position 5**
pAzUT.0	T	Kan	pCAT.000	Dummy	pAzU1.7 (EryR)	-	-	-
pAzUT.1	T	Spec	pCAT.334	pAzU1.6 (eYFP)	pAzU1.7 (EryR)	-	-	-
pAzUT.2	T	Spec	pCAT.334	pAzU1.6 (eYFP)	pAzU1.8 (SpecR)	-	-	-
pAzUT.3	T	Kan	pCAT.000	pAzU1.6 (eYFP)	pAzU1.7 (EryR)	-	-	-
pAzUT.4	T	Kan	pCAT.000	pAzU1.6 (eYFP)	pAzU1.8 (SpecR)	-	-	-
pAzUT.5	T	Kan	pCAT.000	pAzU1.1 (Tns)	pAzU1.2 (Cas12k)	pAzU1.3 (sgRNA)	pAzU1.9 (eYFP)	pAzU1.10 (EryR)
pAzUT.6	T	Kan	pCAT.000	pAzU1.1 (Tns)	pAzU1.2 (Cas12k)	pAzU1.3 (sgRNA)	pAzU1.9 (eYFP)	pAzU1.11 (SpecR)
pAzUT.7	T	Kan	pCAT.000	Dummy	pAzU1.2 (Cas12k)	pAzU1.3 (sgRNA)	pAzU1.9 (eYFP)	pAzU1.10 (EryR)
pAzUT.8	T	Kan	pCAT.000	Dummy	pAzU1.2 (Cas12k)	pAzU1.3 (sgRNA)	pAzU1.9 (eYFP)	pAzU1.11 (SpecR)
pAzUT.9	T	Kan	pCAT.000	pAzU1.1 (Tns)	pAzU1.2 (Cas12k)	pAzU1.3 (sgRNA)	pAzU1.4 (LB+eYFP)	pAzU1.5 (EryR+RB)
pAzUT.10	T	Kan	pCAT.000	pAzU1.1 (Tns)	pAzU1.2 (Cas12k)	pAzU1.3.1 (sgRNA_A)	pAzU1.4 (LB+eYFP)	pAzU1.5 (EryR+RB)
pAzUT.11	T	Spec	pCAT.334	pAzU1.1 (Tns)	pAzU1.2 (Cas12k)	pAzU1.3.1 (sgRNA_A)	pAzU1.4 (LB+eYFP)	pAzU1.5 (EryR+RB)
pAzUT.12	T	Kan	pCAT.000	pAzU1.1 (Tns)	pAzU1.2 (Cas12k)	pAzU1.3.2 (sgRNA_B)	pAzU1.4 (LB+eYFP)	pAzU1.5 (EryR+RB)
pAzUT.13	T	Spec	pCAT.334	pAzU1.1 (Tns)	pAzU1.2 (Cas12k)	pAzU1.3.2 (sgRNA_B)	pAzU1.4 (LB+eYFP)	pAzU1.5 (EryR+RB)
pAzUT.14	T	Kan	pCAT.000	pAzU1.1 (Tns)	pAzU1.2 (Cas12k)	pAzU1.3.3 (sgRNA_C)	pAzU1.4 (LB+eYFP)	pAzU1.5 (EryR+RB)
pAzUT.15	T	Spec	pCAT.334	pAzU1.1 (Tns)	pAzU1.2 (Cas12k)	pAzU1.3.3 (sgRNA_C)	pAzU1.4 (LB+eYFP)	pAzU1.5 (EryR+RB)

Table 9. Plasmid library generated in this study.

Anabaena conjugation

Anabaena PCC 7120 (wild type strain) was conjugated with the plasmids pAzUT.0 to pAzUT.7 in order to evaluate the efficiency of the protocol. Antibiotic controls of cyanobacteria treated without *E. coli* were included in duplicates. Table 10 shows the selection of transformants through time.

	Days in antibiotic		
Plasmid	Day 0	Day 11	Day 20
pAzUT.0			
pAzUT.1			
pAzUT.2			
pAzUT.3			

pAzUT.4			
pAzUT.5			
pAzUT.6			
pAzUT.7			
Ery ctrl_1			

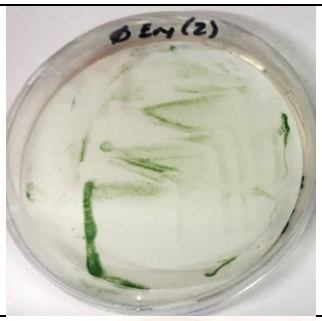
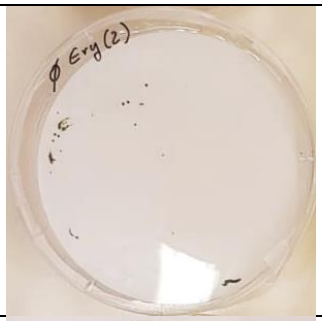
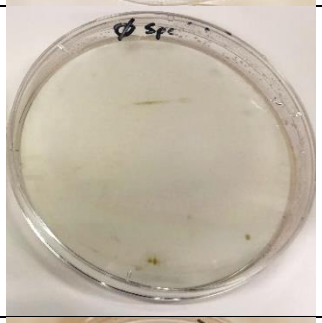
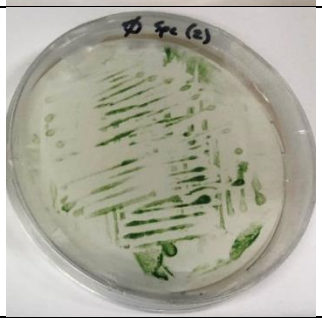
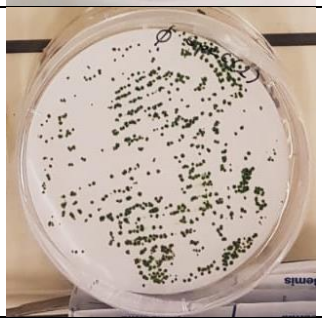
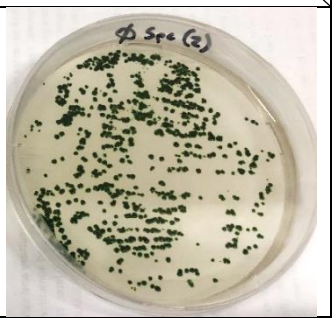
Ery ctrl_2			
Spec ctrl_1			
Spec ctrl_2			

Table 10. Selection of Anabaena transformants after conjugation.

Discussion

The CAST system proved greatly effective when targeting plasmid DNA in *E. coli*. All of the samples showed insertion of the cargo genes in pTarget. However, the system most probably generates toxic side effects after a period of time, which is reflected in the abnormal growth of the bacteria. It is unclear if such toxicity is caused by the Tns genes, Cas12k or the entire CAST system. Further work is required to determine it, but certain off targets and toxicity can be expected when there is an active transposase or CRISPR-Cas in an organism.

A wide range of plasmids were generated to test different aspects of the CAST system and *Anabaena* conjugation. These include controls for the marker genes (pAzUT.0 to pAzUT.4), the effects of the system when introduced in a host without (pAzUT.5 and pAzUT.6) and with defined cargo genes (pAzUT.9), identifying the toxic component of the system (pAzUT.7 to pAzUT.8) and its effectiveness targeting different PAMs and strands of the target sequence (pAzUT.10 to pAzUT.15). There was a focus on developing plasmids with erythromycin resistance, as it is one the few selection markers that can be used in *Azolla*.

Still, the design of the vectors could be improved very much. The vector pCAT.334 is non-conjugative and therefore is useful for work with species naturally transformable, such as *Synechocystis sp.,* but has no real use for the protocols applied in filamentous cyanobacteria. Combining that many

different promoters and terminators unnecessarily complicates the constructions and interferes with result analysis. Having an erythromycin resistance cassette in the opposite direction of the others is irrelevant if it cannot be compared properly. The cargo genes flanked by the borders for the CAST system have no flexibility, as they cannot be replaced. Besides, sgRNAs targeting the genome of *Anabaena* wild type, including different types of genes regarding significance and levels of expression, should be included. Coupling YFP with a GFP emitting strains could complicate fluorescence detection. These short-comings showcase the relevance of planning properly beforehand when trying to create a toolkit for genetic engineering.

The plasmids pAzUT.0 to pAzUT.7 were initially used to test the effectiveness of the conjugation protocol and get a first impression of the how the CAST system could affect the cyanobacteria. The conjugations were successful. The subjects transformed with pCAT.000 survived and grew in the presence of antibiotics after 20 days, while the negative controls and samples containing pCAT.334 were dead after less than 11 days. One of the antibiotic controls survived, which can be attributed to the fact that the antibiotic concentration might not be sufficient.

Considerable work remains to be done. Several aspects of the conjugation protocol should be improved. The antibiotic concentrations must be optimized, because the current values do not allow the isolation of transformed cells. Most probably, the cells that were transformed are not fully segregated, and a method to achieve it is necessary. It would be beneficial to include a level T vector that is conjugative, but not replicative, as it wouldn´t be ideal to maintain the system in the host for long.

Remarkably, the CAST system remains to be tested in the cyanobacteria. Since it is driven by an inducible promoter that requires nitrogen starvation to be active, the current study does not investigate its effectiveness or toxicity. The tightness of such control should be assessed. Successful plasmid insertion must be verified through fluorescence microscopy and chromosome recombination by molecular techniques. Besides, full segregation and stability of the transformants should be evaluated as well.

Several aspects could be improved to create a more efficient method and adaptable toolkit. For the latter, the design of the constructs could be modified as shown in Fig 14A. Taking advantage of the MoClo's flexibility, genes that are constant in the CAST system could be easily fused into one same module. Introducing them separately to characterize the system is no longer necessary, since plasmids for such task have been created in this study. By doing so, up to three spaces could be left empty to introduce any cargo genes through standard MoClo procedures. For instance, diverse selective and visual markers, regulatory elements or enzymes for metabolic engineering could be rapidly combined. Since level 1 and P components are compatible, the cargo genes could be ready to use after assembly in level 1. Moreover, the target sequence for the spacer could be easily introduced in a simple level P plasmid, as it would only contain the scaffold for the sgRNA and left border.

The plasmids and protocol of this study lack a counter selection method, which is required when using a replicative plasmid. Hereby it is proposed to include SacB as such, since it has been proven effective in *Anabaena sp.* PCC 7120 (Cai & Wolk, 1990). By fusing it to the exterior of the right border, the gene would not be integrated and prevent the use of an extra slot, Fig 14B. Alternately, a conjugative but non-replicative level T plasmid could avoid the need of a counter selection step.

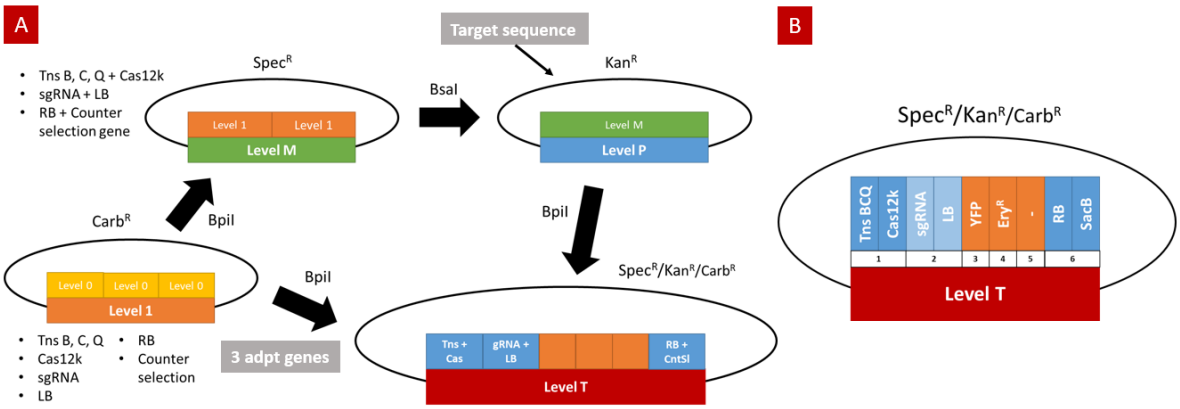


Fig 14. Proposed alternative to improve plasmid library. A. Cloning procedure to generate a vector adaptable for cargo genes. **B.** Example of a replicative level T construct.

It would be advantageous to modify the regulatory genes of the plasmids as well. Since each Cas12k effector recruits its own transposase, their expression could be controlled simultaneously. This could be done by having all the genes under one same promoter or eliminating the terminator between them. Furthermore, it would be insightful to use a tunable promoter to explore different expression levels of the CAST system. For instance, the rhamnose inducible promoter is tightly regulated and has been successfully implemented in cyanobacteria (Kelly et al., 2018). The expression levels of any cargo gene should be optimized as well through promoters of different strength. Such analysis can be time consuming, but it is compulsory to understand the effects in transformed cyanobacteria.

Conclusion

The two biological organisms of the *Azolla* symbiosis are reluctant to genetic manipulation. There has been progress in the right direction, achieving transient genetic modification on both. Fern samples showed luciferase activity after up to 10 days, although their viability should be improved. During such period, WUS and ipt did not induce new meristems. The state of the bacteria culture is the most critical element of the *Agrobacterium* mediated transformation, and dissection of the shoot tips is the most promising method to target key meristematic cells.

The CAST system remains to be tested in *Anabaena sp.* and *N. azollae*. It is an effective system that could overcome the challenges of their conjugation. A library of plasmids to attempt it was generated in this study. Their effectiveness in *Anabaena* conjugation was demonstrated. Once the CAST system is proved successful, it will be possible to combine it with transient transformation methods of *N. azollae* to achieve the genetic edition of this cyanobacteria. There is considerable work before the genetic manipulation of *Azolla* is unlocked, but its potential makes it worth the effort.

References

Akhtar, M., Sarwar, N., Ashraf, A., Ejaz, A., Ali, S., & Rizwan, M. (2020). Beneficial role of Azolla sp. in paddy soils and their use as bioremediators in polluted aqueous environments: implications and future perspectives. In *Archives of Agronomy and Soil Science*. https://doi.org/10.1080/03650340.2020.1786885

Andreou, A. I., & Nakayama, N. (2018). Mobius assembly: A versatile golden-gate framework towards universal DNA assembly. *PLoS ONE*. https://doi.org/10.1371/journal.pone.0189892

Arévalo Díaz, S. (2020). *Proteins influencing the septal junctions in heterocystous cyanobacteria*. Universidad de Sevilla.

Baso, N. C., Coetzee, J. A., Ripley, B. S., & Hill, M. P. (2021). The effects of elevated atmospheric CO2 concentration on the biological control of invasive aquatic weeds. *Aquatic Botany*. https://doi.org/10.1016/j.aquabot.2020.103348

Behler, J., Vijay, D., Hess, W. R., & Akhtar, M. K. (2018). CRISPR-Based Technologies for Metabolic Engineering in Cyanobacteria. In *Trends in Biotechnology*. https://doi.org/10.1016/j.tibtech.2018.05.011

Borel, F. (2019). *Towards genetic engineering of Nostoc azollae* (Issue December). Utrecht University.

Boyer, H. W., & Roulland-dussoix, D. (1969). A complementation analysis of the restriction and modification of DNA in Escherichia coli. *Journal of Molecular Biology*. https://doi.org/10.1016/0022-2836(69)90288-5

Brouwer, P. (2017). *Turning the aquatic weed Azolla into a sustainable crop*. Utrecht University.

Brouwer, P., Bräutigam, A., Buijs, V. A., Tazelaar, A. O. E., van der Werf, A., Schlüter, U., Reichart, G. J., Bolger, A., Usadel, B., Weber, A. P. M., & Schluepmann, H. (2017). Metabolic adaptation, a specialized leaf organ structure and vascular responses to diurnal N2 fixation by nostoc azollae sustain the astonishing productivity of azolla ferns without nitrogen fertilizer. *Frontiers in Plant Science*. https://doi.org/10.3389/fpls.2017.00442

Brouwer, P., Bräutigam, A., Külahoglu, C., Tazelaar, A. O. E., Kurz, S., Nierop, K. G. J., van der Werf, A., Weber, A. P. M., & Schluepmann, H. (2014). Azolla domestication towards a biobased economy? *New Phytologist*. https://doi.org/10.1111/nph.12708

Brouwer, P., Nierop, K. G. J., Huijgen, W. J. J., & Schluepmann, H. (2019). Aquatic weeds as novel protein sources: Alkaline extraction of tannin-rich Azolla. *Biotechnology Reports*. https://doi.org/10.1016/j.btre.2019.e00368

Brouwer, P., Schluepmann, H., Nierop, K. G. J., Elderson, J., Bijl, P. K., van der Meer, I., de Visser, W., Reichart, G. J., Smeekens, S., & van der Werf, A. (2018). Growing Azolla to produce sustainable protein feed: the effect of differing species and CO2 concentrations on biomass productivity and chemical composition. *Journal of the Science of Food and Agriculture*. https://doi.org/10.1002/jsfa.9016

Brouwer, P., van der Werf, A., Schluepmann, H., Reichart, G. J., & Nierop, K. G. J. (2016). Lipid Yield and Composition of Azolla filiculoides and the Implications for Biodiesel Production. *Bioenergy Research*. https://doi.org/10.1007/s12155-015-9665-3

Bui, L. T., Cordle, A. R., Irish, E. E., & Cheng, C. L. (2015). Transient and stable transformation of Ceratopteris richardii gametophytes. *BMC Research Notes*. https://doi.org/10.1186/s13104-015-1193-x

Cai, Y., & Wolk, C. P. (1990). Use of a conditionally lethal gene in Anabaena sp. strain PCC 7120 to select for double recombinants and to entrap insertion sequences. *Journal of Bacteriology*. https://doi.org/10.1128/jb.172.6.3138-3145.1990

Crozet, P., Navarro, F. J., Willmund, F., Mehrshahi, P., Bakowski, K., Lauersen, K. J., Pérez-Pérez, M. E., Auroy, P., Gorchs Rovira, A., Sauret-Gueto, S., Niemeyer, J., Spaniol, B., Theis, J., Trösch, R., Westrich, L. D., Vavitsas, K., Baier, T., Hübner, W., De Carpentier, F., … Lemaire, S. D. (2018). Birth of a Photosynthetic Chassis: A MoClo Toolkit Enabling Synthetic Biology in the Microalga Chlamydomonas reinhardtii. *ACS Synthetic Biology*. https://doi.org/10.1021/acssynbio.8b00251

Davis, A. M., Hall, A., Millar, A. J., Darrah, C., & Davis, S. J. (2009). Protocol: Streamlined sub-protocols for floral-dip transformation and selection of transformants in arabidopsis thaliana. *Plant Methods*. https://doi.org/10.1186/1746-4811-5-3

De Vries, S., & De Vries, J. (2018). Azolla: A model system for symbiotic nitrogen fixation and evolutionary developmental biology. In *Current Advances in Fern Research*. https://doi.org/10.1007/978-3-319-75103-0_2

Elhai, J., Vepritskiy, A., Muro-Pastor, A. M., Flores, E., & Wolk, C. P. (1997). Reduction of conjugal transfer efficiency by three restriction activities of Anabaena sp. strain PCC 7120. *Journal of Bacteriology*. https://doi.org/10.1128/jb.179.6.1998-2005.1997

Elhai, J., & Wolk, C. P. (1988). Conjugal Transfer of DNA to Cyanobacteria. *Methods in Enzymology*. https://doi.org/10.1016/0076-6879(88)67086-8

Engler, C., Youles, M., Gruetzner, R., Ehnert, T. M., Werner, S., Jones, J. D. G., Patron, N. J., & Marillonnet, S. (2014). A Golden Gate modular cloning toolbox for plants. *ACS Synthetic Biology*. https://doi.org/10.1021/sb4001504

Faure, G., Shmakov, S. A., Yan, W. X., Cheng, D. R., Scott, D. A., Peters, J. E., Makarova, K. S., & Koonin, E. V. (2019). CRISPR–Cas in mobile genetic elements: counter-defence and beyond. *Nature Reviews Microbiology*. https://doi.org/10.1038/s41579-019-0204-7

Golzary, A., Abdoli, M. A., Yoshikawa, K., Khodadadi, A., & Karbassi, A. (2019). *Azolla as a Feedstock for Bio-Refinery: Cultivation, Conversion and Application*. https://doi.org/10.5339/qfarc.2016.eesp2082

González Linares, R. (2020). A CRISPR-associated transposase presents null cargo integration efficiency when targeting a transcriptionally highly active region. *TU Delft Education Repository*, 1–40.

Güngör, E. (2018). *Transformation and Genome Editing of Azolla Ferns* (Issue June). Utrecht University.

Hitchcock, A., Hunter, C. N., & Canniffe, D. P. (2020). Progress and challenges in engineering cyanobacteria as chassis for light-driven biotechnology. *Microbial Biotechnology*. https://doi.org/10.1111/1751-7915.13526

Kelly, C. L., Taylor, G. M., Hitchcock, A., Torres-Méndez, A., & Heap, J. T. (2018). A Rhamnose-Inducible System for Precise and Temporal Control of Gene Expression in Cyanobacteria. *ACS Synthetic Biology*. https://doi.org/10.1021/acssynbio.7b00435

Klompe, S. E., Vo, P. L. H., Halpin-Healy, T. S., & Sternberg, S. H. (2019). Transposon-encoded CRISPR–Cas systems direct RNA-guided DNA integration. *Nature*. https://doi.org/10.1038/s41586-019-1323-z

Lee, M. E., DeLoache, W. C., Cervantes, B., & Dueber, J. E. (2015). A Highly Characterized Yeast Toolkit for Modular, Multipart Assembly. *ACS Synthetic Biology*. https://doi.org/10.1021/sb500366v

Li, F. W., Brouwer, P., Carretero-Paulet, L., Cheng, S., De Vries, J., Delaux, P. M., Eily, A., Koppers, N., Kuo, L. Y., Li, Z., Simenc, M., Small, I., Wafula, E., Angarita, S., Barker, M. S., Bräutigam, A., Depamphilis, C., Gould, S., Hosmani, P. S., … Pryer, K. M. (2018). Fern genomes elucidate land plant evolution and cyanobacterial symbioses. *Nature Plants*. https://doi.org/10.1038/s41477-018-0188-8

Liu, M., Rehman, S., Tang, X., Gu, K., Fan, Q., Chen, D., & Ma, W. (2019). Methodologies for improving HDR efficiency. In *Frontiers in Genetics*. https://doi.org/10.3389/fgene.2018.00691

Maher, M. F., Nasti, R. A., Vollbrecht, M., Starker, C. G., Clark, M. D., & Voytas, D. F. (2020). Plant gene editing through de novo induction of meristems. *Nature Biotechnology*. https://doi.org/10.1038/s41587-019-0337-2

McArthur IV, G., Nanjannavar, P., Miller, E., & Fong, S. (2015). Integrative metabolic engineering. *AIMS Bioengineering*. https://doi.org/10.3934/bioeng.2015.3.93

Miranda, A. F., Biswas, B., Ramkumar, N., Singh, R., Kumar, J., James, A., Roddick, F., Lal, B., Subudhi, S., Bhaskar, T., & Mouradov, A. (2016). Aquatic plant Azolla as the universal feedstock for biofuel production. *Biotechnology for Biofuels*. https://doi.org/10.1186/s13068-016-0628-5

Moore, S. J., Lai, H. E., Kelwick, R. J. R., Chee, S. M., Bell, D. J., Polizzi, K. M., & Freemont, P. S. (2016). EcoFlex: A Multifunctional MoClo Kit for E. coli Synthetic Biology. *ACS Synthetic Biology*. https://doi.org/10.1021/acssynbio.6b00031

Murray, N. E., Brammar, W. J., & Murray, K. (1977). Lambdoid phages that simplify the recovery of in vitro recombinants. *MGG Molecular & General Genetics*. https://doi.org/10.1007/BF02425325

Okinawa Institute of Science and Technology. (2020). *Azolla and its cyanobacteria*. https://www.oist.jp/news-center/photos/azolla-and-its-cyanobacteria

Patron, N. J., Orzaez, D., Marillonnet, S., Warzecha, H., Matthewman, C., Youles, M., Raitskin, O., Leveau, A., Farré, G., Rogers, C., Smith, A., Hibberd, J., Webb, A. A. R., Locke, J., Schornack, S., Ajioka, J., Baulcombe, D. C., Zipfel, C., Kamoun, S., … Haseloff, J. (2015). Standards for plant synthetic biology: A common syntax for exchange of DNA parts. *New Phytologist*. https://doi.org/10.1111/nph.13532

Pattharaprachayakul, N., Lee, M., Incharoensakdi, A., & Woo, H. M. (2020). Current understanding of the cyanobacterial CRISPR-Cas systems and development of the synthetic CRISPR-Cas systems for cyanobacteria. *Enzyme and Microbial Technology*. https://doi.org/10.1016/j.enzmictec.2020.109619

Peters, J. E., Makarova, K. S., Shmakov, S., & Koonin, E. V. (2017). Recruitment of CRISPR-Cas systems by Tn7-like transposons. *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.1709035114

Ran, L., Larsson, J., Vigil-Stenman, T., Nylander, J. A. A., Ininbergs, K., Zheng, W. W., Lapidus, A., Lowry, S., Haselkorn, R., & Bergman, B. (2010). Genome erosion in a nitrogen-fixing vertically transmitted endosymbiotic multicellular cyanobacterium. *PLoS ONE*. https://doi.org/10.1371/journal.pone.0011486

Setiawati, M. R., Damayani, M., Herdiyantoro, D., Suryatmana, P., Anggraini, D., & Khumairah, F. H. (2018). The application dosage of Azolla pinnata in fresh and powder form as organic fertilizer on soil chemical properties, growth and yield of rice plant. *AIP Conference Proceedings*. https://doi.org/10.1063/1.5021210

Speelman, E. N., Van Kempen, M. M. L., Barke, J., Brinkhuis, H., Reichart, G. J., Smolders, A. J. P., Roelofs, J. G. M., Sangiorgi, F., De Leeuw, J. W., Lotter, A. F., & Sinninghe DamstÉ, J. S. (2009). The Eocene Arctic Azolla bloom: Environmental conditions, productivity and carbon drawdown. *Geobiology*. https://doi.org/10.1111/j.1472-4669.2009.00195.x

Strecker, J., Ladha, A., Gardner, Z., Schmid-Burgk, J. L., Makarova, K. S., Koonin, E. V., & Zhang, F. (2019). RNA-guided DNA insertion with CRISPR-associated transposases. *Science*, *364*(6448), 48–53. https://doi.org/10.1126/science.aax9181

Vasudevan, R., Gale, G. A. R., Schiavon, A. A., Puzorjov, A., Malin, J., Gillespie, M. D., Vavitsas, K., Zulkower, V., Wang, B., Howe, C. J., Lea-Smith, D. J., & McCormick, A. J. (2019). Cyanogate: A modular cloning suite for engineering cyanobacteria based on the plant moclo syntax. *Plant Physiology*. https://doi.org/10.1104/pp.18.01401

Vigil-Stenman, T., Larsson, J., Nylander, J. A. A., & Bergman, B. (2015). Local hopping mobile DNA implicated in pseudogene formation and reductive evolution in an obligate cyanobacteria-plant symbiosis. *BMC Genomics*. https://doi.org/10.1186/s12864-015-1386-7

Wagner, G. M. (1997). Azolla: A Review of Its Biology and Utilization. In *Botanical Review*. https://doi.org/10.1007/BF02857915

Wang, F., Gao, Y., & Yang, G. (2020). Recent advances in synthetic biology of cyanobacteria for improved chemicals production. In *Bioengineered*. https://doi.org/10.1080/21655979.2020.1837458

Wang, H., Wang, F., Wang, C., & Han, Y. (2019). Effects of floating Azolla on phosphorus fluxes and recovery from former agricultural lands in wetland microcosms. In *Soil Science and Plant Nutrition*. https://doi.org/10.1080/00380768.2018.1536387

Wang, M., Khan, M. A., Mohsin, I., Wicks, J., Ip, A. H., Sumon, K. Z., Dinh, C. T., Sargent, E. H., Gates, I. D., & Kibria, M. G. (2021). Can sustainable ammonia synthesis pathways compete with fossil-fuel based Haber-Bosch processes? *Energy and Environmental Science*. https://doi.org/10.1039/d0ee03808c

Watanabe, I., Roger, P. A., Ladha, J. K., & Van Hove, C. (1992). *Biofertilizer Germplasm Collections at IRRI* (Issue June 2014). http://www.ncbi.nlm.nih.gov/pubmed/15003161%5Cnhttp://cid.oxfordjournals.org/lookup/doi/10.1093/cid/cir991%5Cnhttp://www.scielo.cl/pdf/udecada/v15n26/art06.pdf%5Cnhttp:/

/www.scopus.com/inward/record.url?eid=2-s2.0-84861150233&partnerID=tZOtx3y1

Weber, E., Engler, C., Gruetzner, R., Werner, S., & Marillonnet, S. (2011). A modular cloning system for standardized assembly of multigene constructs. *PLoS ONE*. https://doi.org/10.1371/journal.pone.0016765

Werner, S., Engler, C., Weber, E., Gruetzner, R., & Marillonnet, S. (2012). Fast track assembly of multigene constructs using golden gate cloning and the MoClo system. *Bioengineered Bugs*. https://doi.org/10.4161/bbug.3.1.18223

Wolabu, T. W., Park, J. J., Chen, M., Cong, L., Ge, Y., Jiang, Q., Debnath, S., Li, G., Wen, J., & Wang, Z. (2020). Improving the genome editing efficiency of CRISPR/Cas9 in Arabidopsis and Medicago truncatula. *Planta*. https://doi.org/10.1007/s00425-020-03415-0

Zerulla, K., Ludt, K., & Soppa, J. (2016). The ploidy level of synechocystis sp. PCC 6803 is highly variable and is influenced by growth phase and by chemical and physical external parameters. *Microbiology (United Kingdom)*. https://doi.org/10.1099/mic.0.000264

Zhang, X., Henriques, R., Lin, S. S., Niu, Q. W., & Chua, N. H. (2006). Agrobacterium-mediated transformation of Arabidopsis thaliana using the floral dip method. *Nature Protocols*. https://doi.org/10.1038/nprot.2006.97