Development and implementation of (opto-) genetic tools in different eukaryotic organisms and application of synthetic biology approaches for studying plant signaling pathways

Inaugural-Dissertation

zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät
der Heinrich-Heine-Universität Düsseldorf

vorgelegt von

Lisa Cristin Hüsemann
aus Wesel

Düsseldorf, Juni 2020

aus dem Institut für Synthetische Biologie
der Heinrich-Heine-Universität Düsseldorf

Gedruckt mit der Genehmigung der
Mathematisch-Naturwissenschaftlichen Fakultät der
Heinrich-Heine-Universität Düsseldorf

Berichterstatter:

1. Prof. Dr. Matias Zurbriggen

2. Prof. Dr. Michael Feldbrügge

Tag der mündlichen Prüfung:

13.10.2020

EIDESSTATTLICHE ERKLÄRUNG

Ich versichere an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist.

_____ _____

Datum Unterschrift

Table of contents

DANKSAGUNG

„Der Beginn aller Wissenschaften ist das Erstaunen, dass die Dinge sind, wie sie sind.“ (Aristoteles)

Zunächst möchte ich mich bei Prof. Dr. Matias Zurbriggen für die Möglichkeit bedanken, meine Doktorarbeit an seinem Institut anzufertigen. Er hat mich stets unterstützt und mir bei jedem Anliegen beratend zur Seite gestanden. Unsere ertragreichen Diskussionen waren Inspiration und Motivation zugleich. Sie haben mich darin bestärkt mich in neue Themengebiete einzuarbeiten, Ergebnisse und Hypothesen kritisch zu hinterfragen und auch in schwierigen Phasen an den Erfolg der eigenen Arbeit zu glauben. Ich danke Prof. Zurbriggen auch für das hohe Maß an Vertrauen, das er mir stets entgegengebracht hat, und für die Möglichkeit, mein Themen- und Methodenspektrum in spannenden Kooperationsprojekten weiter auszubauen und zu vertiefen. Ich schätze es sehr, dass ich von Beginn an selbstständig und eigenverantwortlich die Planung meiner Projekte übernehmen durfte, wohlwissend, dass mir Prof. Zurbriggen bei Fragen und Problemen stets zur Seite stehen würde.

Darüber hinaus möchte ich mich bei Prof. Dr. Michael Feldbrügge für seine fachliche Expertise und seine wertvollen und hilfreichen Ratschläge bedanken.

Auch möchte ich mich bei Dr. Anita Loeschcke bedanken. Ihre fachliche und menschliche Unterstützung und Ihre aufmunternden Worte waren eine große Hilfe.

Des Weiteren gilt mein Dank unseren Kooperationspartnern Dr. Guido Grossmann und Anna Denzler vom COS in Heidelberg für die großartige Zusammenarbeit und die hoch interessanten und immer überaus angenehmen Meetings und Diskussionen.

Ich danke Nicole Heucken für unsere zahlreichen „fruitful discussions“! Wir haben uns einer großen Herausforderung gestellt und gemeinsam war es so viel besser. Ich werde sogar die Fahrten zu zahlreichen CombiCom und Focus Lab Meetings zu viert im Twingo ohne Klimaanlage bei 35 °C vermissen.

Ich danke Dr. Jennifer Andres für ihre freundschaftliche Unterstützung, ihre positive und herzliche Art und dafür, dass sie sich die Zeit genommen hat meine Arbeit immer und immer wieder Korrektur zu lesen. Das weiß ich wirklich sehr zu schätzen.

Reinhild Wurm, Michaela Gerads und Jessica Müller danke ich für die herausragende technische, organisatorische und menschliche Unterstützung. Ohne euch wäre es nicht möglich nach drei Jahren diese Ergebnisse zu präsentieren.

Danke auch an Kira Müntjes für die geduldige Einarbeitung in völlig neue Methoden und die tolle Unterstützung während der gesamten Zeit. Es hat einfach immer Spaß gemacht mit dir.

Ich danke all meinen Kollegen für die großartige Zusammenarbeit. Danke Tim Blomeier, dass du mir gefühlte tausend Mal das Mikroskop erklärt hast. Danke Patrick Fischbach, dass ich dir mit jedem IT-Problem auf die Nerven gehen durfte.

Danke Leonie Koch, für deinen Gerechtigkeitssinn und dass du dich immer für uns eingesetzt hast. Gracias Rocio Ochoa-Fernandez, für die vielen tollen Gespräche und die wertvollen Klonierungs-Tipps. Und ein ganz großes Dankeschön an Dr. Kun Tang, Estefania Pavesi, Dr. Sofia Romero, Pamela Molinari und Paula Vicino für eure Unterstützung, eure gute Laune und den großartigsten Junggesellinnenabschied aller Zeiten. Und vor allem für eure Freundschaft.
Ein besonderer Dank gilt hier meiner Familie. Vor allem meinen Eltern, meinen Großmüttern und meinem Bruder Thomas. Ihr habt in all der Zeit, während meines Studiums und meiner Promotion, hinter mir gestanden und mich unterstützt! Ihr hattet immer ein offenes Ohr für meine Sorgen und habt viel Geduld bewiesen. Dafür bin ich euch von Herzen dankbar.
Auch möchte ich mich bei Sonny bedanken. Deine stets fröhliche und positive Art haben es geschafft mich zum Lächeln zu bringen, auch wenn mir eigentlich nicht danach zumute war.
Am aller meisten möchte ich mich jedoch bei dem großartigsten Menschen bedanken den ich mir nur vorstellen kann: bei meiner Frau Judith. Wie sehr ich auch an mir gezweifelt habe, du hast es nie getan. Du siehst immer nur das Beste in mir. Ohne dich wäre so vieles nicht möglich gewesen. Danke!
Natürlich möchte ich allen danken die mich unterstützt haben, hier aber nicht explizit Erwähnung fanden.

FIGURES

ABBREVIATIONS

CRY	cryptochrome
ePDZ	engineered PDZ domain
E-protein	*E. coli* repressor protein
ET	E-protein transactivator
GAP	GTPase activating protein
GDP	guanosine diphosphate
GEF	guanine exchange factor
GFP	green fluorescent protein
GOI	gene of interest
GTP	guanosine triphosphate
HTH	helix-turn-helix
IRES	internal ribosome entry site
LOV domain	light-oxygen-voltage domain
nBDP	natural bidirectional promotor
ORF	open reading frame
Phy	phytochrome
PIP	pristinamycin-induced protein
ROP	Rho-like GTPase of plants
SEAP	secreted alkaline phosphatase
tTA	tetracycline-dependent hybrid transactivator
Tet	tetracycline
TetR	tetracycline repressor
UASG	upstream activating sequence for galactose
UTR	untranslated region
VP16	virus protein 16

SUMMARY

The field of synthetic biology offers a completely new approach to unravel complex cellular mechanisms as well as to develop urgently required biotechnological processes. For this purpose, engineering principles are applied to design and implement molecular tools for the assembly of complex synthetic switches and networks. Over the years, many of these tools and switches have been established in mammalian cell systems, plants, bacteria and yeast. The basidiomycete fungus *Ustilago maydis* was not among these organisms, even though it is of great interest for a broad spectrum of biotechnological applications, and for studying fundamental cellular principles.

To fill this gap, we implemented a synthetic biology toolbox, specifically designed to be applied in this microorganism. Therefore, we developed and tested tools for building multicistronic vectors, enabling the expression of more than one gene under control of a single promotor. Furthermore, we established different reporter genes and their corresponding assays to quantitatively analyze the functioning of all tools and systems, designed and constructed in the course of this work. These reporter genes can be applied for a multitude of current and future research attempts in this fungus. Having these basic tools at hand, we designed, built, implemented and characterized two chemically and three light-controllable gene expression systems in *U. maydis.* The tetracycline-regulated Tet-Off system that was established here, showed an unexpected reverse mode of function, converting the Off-system to an On-system. This Tet-On system showed low basal activity levels and a high induction fold, making it a promising tool for regulating the expression of genes in this organism. Additionally, we established a gene expression system which is negatively controllable in the presence of macrolide antibiotics. This system depends on the erythromycin-regulated binding of the E-protein from *Escherichia coli* to its operator sequence (ETR). Here we demonstrate a high level of controllability of gene expression in *U. maydis*, considering a low basal activity and a high reduction fold, provided by this E-Off system.

The utilization of light as a regulator of cellular events in synthetic biology approaches is one of the most outstanding accomplishments in this field. Optogenetic systems that are designed to integrate light signal inputs and generate an increased or reduced level of gene expression as an output, have been established in many eukaryotic organisms. In contrast to antibiotics or other drugs, the application of light as a regulating factor is offering a higher spatial and temporal control, causing less disadvantageous side effects. Here we report on the novel establishment of three blue light-controllable gene expression systems in *U. maydis*. These switches are based on the utilization of blue light-sensing light-oxygen-voltage (LOV) domains from *Avena sativa* and *Erythrobacter litoralis,* and show a high level of controllability.

The LOV domain von *A. sativa,* AsLOV2, was engineered to cage a peptide tag which is exposed upon illumination with blue light, making the peptide tag accessible for its binding partner ePDZ. This dimerization event leads to the induction of gene expression, which is fully reversible in the dark. The second blue light-regulated gene expression switch is utilizing the engineered EL222 transcription factor from *E. litoralis*, reduced to the minimal number of components needed for light-regulated activation: the LOV domain and a helix-turn-helix motif. Upon blue light illumination, EL222 dimerization and DNA binding are induced. This system was constructed as a blue-on and a blue-off system, by fusing either a VP16 transactivator or a Sql1 repressor to the EL222. The functionality of both versions of this system could be demonstrated here.

Besides the design and application of synthetic tools to implement highly controllable gene expression switches, another characteristic approach in synthetic biology is the usage of orthogonal systems to study complex signaling pathways without any interfering crosstalk of components present in the natural environment of the studied pathway. Here we report on the utilization of this approach to study the interaction of components involved in the formation of root hairs in *Arabidopsis thaliana*. Therefore, we heterologously expressed three key players of this pathway, GEF3, GEF4 and the small GTPase ROP2, in HeLa cells and microscopically analyzed the induced phenotype. The obtained results indicate an induction of polar cell outgrowth, induced by the interaction of plant GEF3, GEF4 and the small GTPase ROP2 with mammalian cell proteins regulating this process in mammals. These observations provide the basis for further orthogonal research on these cell outgrowth mechanisms, and their level of conservation among eukaryotes.

In the course of this work, a fully equipped synthetic biology toolbox for *U. maydis* was implemented, providing basic researchers and biotechnologist with highly versatile tools and systems for a multitude of applications in this organism. Additionally, we discovered strong evidence for an interaction of plant and mammalian key players of polar cell outgrowth processes, indicating a higher level of functional conservation than previously expected.

1 Introduction

1.1 Synthetic biology – the interface between engineering and life sciences

Although the field of synthetic biology is still lacking a uniform definition, the use of molecular tools and the modular reconstruction of biological pathways to engineer the behavior of single cells or whole organisms is what unifies the synthetic biology community.

The idea of directed manipulations of organisms with the purpose of studying cellular principles or engineering and optimizing biotechnological processes has its origin more than half a century ago (Cameron et al., 2014). But within the framework of the "genomic revolution" in the 1990s, a new scientific field at the interface between biology and engineering science arose, using precise genetic manipulations, thereby building new molecular devices to alter and study cellular processes (Cameron et al., 2014). This new branch of life science is constantly growing with a scope reaching into almost every research area in biological science.

1.1.1 The potential of synthetic biology approaches

The field of synthetic biology applies engineering principles to design and construct synthetic biological systems, like genetic switches, allowing the re-programming of signaling networks and metabolic pathways (Braguy and Zurbriggen, 2016). The idea is to manipulate synthesis pathways of valuable compounds, to increase the yield of a product or to heterologously express modified versions of certain products (Bailey, 1991; Brophy and Voigt, 2014a). A good example demonstrating the potential of metabolic engineering in synthetic biology is the biosynthesis of opioids in yeast (Galania et al., 2015).

This breakthrough in metabolic engineering impressively revealed the versatile opportunities of synthetic biology approaches for the production of pharmaceutical compounds. Besides this example there are various others demonstrating the broad spectrum of synthetic biology applications, from basic research and biomedicine, to drug discovery and biofuel production (Elowitz and Leibler, 2000; Gardner et al., 2000; Khalil and Collins, 2010; Lienert et al., 2014a).

1.1.2 Biology from an engineer's point of view

What makes the approaches in synthetic biology so unique, are the key characteristics of modularity, standardization and application of construction principles (Purnick and Weiss, 2009). As biological systems are built up in a modular way, it seems obvious to manipulate these systems in the same manner – by using standardized biological building blocks (Shetty et al., 2008; Khalil and Collins, 2010).

In the past, genetic manipulations were mainly limited to single genes or the insertion of single components followed by the observation of the induced effect (Purnick and Weiss, 2009).

Synthetic biology pursues a different goal: the idea is to implement new functions by combining a large variety of genetic building blocks in orthogonal biological or fully synthetic minimal systems (Kuruma et al., 2009).

A good example for such an approach is the implementation of chemically or light-inducible gene expression systems in microorganisms, mammalian cells or whole animals *in vivo* (Wang et al., 2012a; Abe et al., 2014). These systems are highly complex as they combine a large variety of different modules such as promotors, IRES sequences, photoreceptors, transactivators and others, originating from several different organisms (Wang et al., 2012a; Müller and Weber, 2013). These tightly controllable systems are exceedingly useful for answering basic research questions and as biotechnological tools for the production of pharmaceuticals or other biomedical applications (Müller and Weber, 2013).

1.2 Genetic tools in synthetic biology

The amount and diversity of molecular tools that are applied in synthetic biology is large and so is the number of applications. Some are used to control the expression of single or multiple genes by adding or detracting exogenous factors like antibiotics or light of a specific wavelength (Baron and Bujard, 2000; Weber et al., 2002a; Wang et al., 2012b; Müller and Weber, 2013; Lienert et al., 2014b; Braguy and Zurbriggen, 2016; Das et al., 2016). Some give us the opportunity to build multicistronic vectors, allowing the simultaneous expression of two or more proteins from one open reading frame (ORF) (Pelletier and Sonenberg, 1988; Mizuguchi et al., 2000; Fux and Fussenegger, 2003; Andersen et al., 2010; Liu et al., 2017; Öztürk et al., 2017). Others help us to quantitatively analyze the functionality of an implemented synthetic system (Gould and Subramani, 1988; Naylor, 1999; Schenborn and Groskreutz, 1999; Shifera and Hardin, 2010). The novel design, construction and implementation of such tools for organisms that have not been utilized for synthetic biological applications so far can be challenging, but it also offers a multitude of new opportunities. One of these organisms that is showing great potential for synthetic biology applications, is the basidiomycete fungus *Ustilago maydis.*

1.2.1 *Ustilago maydis* – a versatile platform for synthetic biology applications

What makes the implementation of synthetic molecular tools in *U. maydis* especially interesting, is its potential as a model organism for studying fungal dimorphism and pathogenesis (Bölker, 2001), as well as cell biological processes (Steinberg and Perez-Martin, 2008). Regarding these cellular processes, genome analyses have shown that this smut fungus is sharing numerous proteins with *Homo sapiens,* and that some fundamental cellular principles, like for instance long-distance transport, seem to be conserved between these two species (Steinberg and Perez-Martin, 2008).

Additional to these basic research potentials, *U. maydis* turned out to be a very promising candidate for a broad spectrum of biotechnological application. It has for example the ability to produce different glycolipids, that have a high potential value for pharmaceutical, cosmetic and food industry applications (Yang et al., 2013b). Furthermore, *U. maydis* synthetizes itaconate acid. This compound is interesting for the biofuel production, as it can easily be converted into a fuel with excellent combustion properties (Geiser et al., 2016). Due to these features, it appears to be highly beneficial to expand the application repertoire and the manipulation capabilities of *U. maydis* by implementing a synthetic biology toolbox for this organism. As the range of available genetic tools, and especially molecular switches, for this organism is extremely limited, such novel molecular tools will open up completely new perspectives in the utilization of *U. maydis* in basic research and biotechnology, and will crucially contribute to bringing forward the field of *U. maydis*-related research.

1.2.2 2A peptides

The implementation of highly complex synthetic molecular switches, consisting of multiple components, requires specific tools for the expression of two or more proteins from a single open reading frame. One tool that has already proven its applicability for this purpose are so called 2A peptides. These small peptides originally derive from RNA viruses (Szymczak-Workman et al., 2012), where they mediate the co-translational cleavage of polyproteins (Luke et al., 2008). Such polyproteins result from bicistronic open reading frames (ORFs) (Szymczak-Workman et al., 2012), which are typical genetic features of many viruses (Luke et al., 2008). By now, a multitude of 2A peptides from different mammalian or insect viruses are known and they all share a conserved motif comprising seven C-terminal residues and a N-terminal proline (-DxExNPG$^{\downarrow}$P-) (de Felipe, 2004). During their translation, 2A peptides are self-coordinating their interaction with the exit tunnel of a translating ribosome (ribosome skipping), thereby inducing the skipping of the synthesis of the last peptide bond between glycine and proline (de Felipe, 2004). This event produces an upstream protein with a C-terminal 2A peptide residue and a downstream protein with a N-terminal proline (Liu et al., 2017). The functionality of 2A peptides is further illustrated in Figure 1.

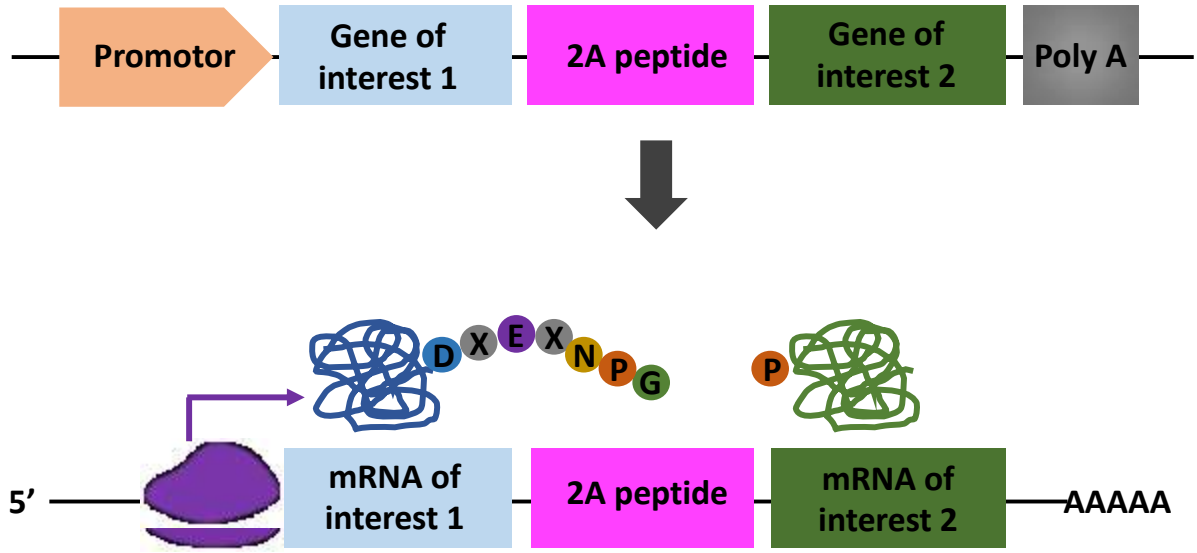

Figure 1: The application of 2A peptides leads to the expression of two or more genes from one ORF. For applications that require the construction of multicistronic vectors, 2A peptides can be utilized. During their translation, these small peptides are coordinating their self-cleavage at the last peptide bond between glycine and proline. This results in an upstream protein with a C-terminal 2A peptide residue consisting of a conserved DxExNPG motif, and a downstream protein with a N-terminal proline from the same motif (-DxExNPG↓**P**).

But not all of the 2A peptides that have been described and characterized in the last decades fulfill the requirements to become appropriate tools for synthetic biology application. The cleavage efficiency of the different 2A peptides varies, depending on the peptide itself, its position within a gene cluster, the combination with other 2A peptides and the organisms in which it is expressed (Mizuguchi et al., 2000). Due to this variability, it is necessary to thoroughly analyze the applicability of 2A peptides regarding its utilization as a tool in a specific organism and for a specific purpose.

1.2.3 Internal ribosome entry site (IRES)

Another commonly used synthetic tool for the expression of more than one protein under control of a single promotor are internal ribosome entry sites (IRES). These RNA elements enable the initiation of translation in a cap-independent manner (Mountford and Smith, 1995). The translation of eukaryotic mRNA usually depends on the association of the ribosome with the 5` cap structure. Subsequently, the ribosome starts scanning the mRNA transcript for the translation initiation site, the first AUG codon (Molla et al., 1992). This association and scanning model applies for most eukaryotic mRNA translation processes. Exceptions from this model can be found in mammalian (+)-strand RNA viruses and retroviruses (Mountford and Smith, 1995). These viruses produce RNA sequences with long 5` untranslated regions (UTRs) (Pelletier and Sonenberg, 1988). To ensure the translational efficiency of the transcripts, these UTRs comprise specific sequences, named internal ribosome entry sites (Mizuguchi et al., 2000).

These IRES sequences enable the ribosome to bind an IRES-internal AUG codon without scanning the 5` UTR of the RNA transcript (Pelletier and Sonenberg, 1988). This mechanism is shown in Figure 2.

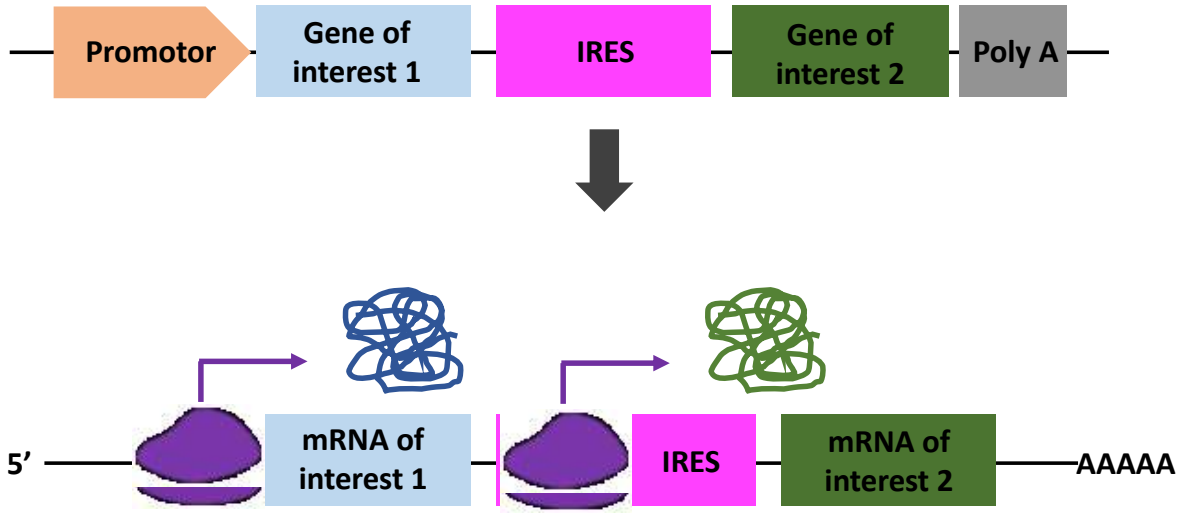

Figure 2: Internal ribosome entry sites (IRES) enable translation initiation in a 5` cap-independent manner. IRES sequences are a commonly used tool for the construction of bicistronic vectors. During the translation process, the ribosome binds to an IRES upstream of the second gene of a bicistronic ORF, initiating the translation of the encoded protein independent from the translation of the protein upstream of the IRES. This mechanism generates two fully separate proteins with no added amino acid residues.

In 1988, Pelletier and Sonenberg demonstrated the applicability of IRES sequences as an alternative to 5` cap dependent translation initiation. They inserted a poliovirus derived IRES in an artificial bicistronic mRNA and were able to observe an efficient translation of the downstream cistron even after blocking the cap-dependent translation of the upstream cistron (Pelletier and Sonenberg, 1988). Nowadays, IRES sequences are a widely used molecular tool for the construction of multicistronic vectors. Nevertheless, these sequences also have some characteristics that must be taken into consideration when using them as a molecular tool. It is for example known, that the expression of the gene downstream of an IRES sequence ranges from 6 to 100% compared to the cap-dependent expression of the upstream gene, depending on the cell line or organism (Mizuguchi et al., 2000).

1.2.4 Bidirectional promotors

Many synthetic biology applications require the expression of more than one heterologous protein at the same time. Besides 2A peptides and IRES sequences molecular biologists can resort to a tool that can be found in the genome of eukaryotic systems: bidirectional promotors. These promotors are controlling the transcription on both DNA strands and therefore in both directions. Many natural bidirectional promotors (nBDP) have been characterized and obtained data indicate a co-regulation of functionally related genes (Yang et al., 2013a).

This observation has inspired many bioengineers to utilize these natural bidirectional promotors or to engineer and implement synthetic bidirectional promotors as molecular tools to optimize heterologous gene expression in bacteria, yeast (Öztürk et al., 2017), plants (Xie et al., 2001) or mammalian systems (Fux and Fussenegger, 2003; Amendola et al., 2005; Andersen et al., 2010). A good example for the design and implementation of an engineered bidirectional promotor is the recombinant antibody expression under control of a bidirectional CMV promotor. The CMV promotor from human Cytomegalovirus is one of the strongest promotors for the expression of proteins in mammalian cells, and it is therefore especially interesting for the recombinant expression of antibodies (Andersen et al., 2010). In 2010, Andersen et al. designed a vector in which the expression of antibody genes was controlled by a promotor complex consisting of two CMV promotors arranged in opposite direction, their 5´ ends in close proximity to each other. Using this design, the expression level was significantly lower than the expression level of the individual CMV promotor. To overcome this shortcoming, a truncated version of the promotor complex with only one enhancer controlling the expression from two divergent minimal CMV promotors was designed and tested. These minimal promotors consist of the core polymerase II promotor, Exon 1 and Intron A of the major immediate-early promotor from human Cytomegalovirus. Additional to the truncation, the unique region upstream of the enhancer was removed. Using this construct, a 12-fold increased expression could be obtained. Still, the enhancer showed a preference for its natural orientation leading to a 2:1 expression ratio which has to be considered when applying this promotor complex (Andersen et al., 2010). The work of Andersen et al. generated highly interesting und useful fundamental information about design principles of bidirectional promotors. However, the design of such promotors, as of any molecular tool, always has to be adapted to individual characteristics of the expression host and the specific application.
The bidirectional promotor that was utilized for the construction of light-controllable gene expression systems established in this work, was designed, implemented and characterized by Nicole Heucken and is described in detail in her dissertation.

1.2.5 Reporter genes

The use of reporter genes to monitor cellular events, no matter if natural or synthetically implemented, has become relevant in the past two decades. These reporter genes give researchers a strong tool to study a nearly infinite diversity of biological processes, from expression and signaling events (Naylor, 1999) to the transport and translocation of proteins within single cells or whole organisms (Gould and Subramani, 1988; Schenborn and Groskreutz, 1999; Tannous et al., 2005; Badr et al., 2007). The range and diversity of applications is huge.

They are for instance applied for studying *cis*-acting elements like enhancers and promotors. Additionally, the attachment of reporters to specific response elements gives researchers the possibility to monitor the activation of pathways in response to intra- or extracellular stimuli, determine the efficiency of transient or stable transfection processes, and study the interaction of proteins (Schenborn and Groskreutz, 1999).

The variety of reporter genes that can be utilized for different purposes is quite diverse. They range from fluorescent proteins and luciferases to phosphatases and hormones. The different reporter genes and their corresponding assays come along with specific advantages and drawbacks that have to be considered when choosing a reporter (Naylor, 1999).

The reporter genes that should be discussed more detailed in this work are three luciferases, namely firefly, renilla and gaussia, as well as the alkaline phosphatase SEAP.

Luciferases are naturally occurring enzymes which are characteristically emitting light photons when catalyzing the turnover of their specific substrate (Hampf and Gossen, 2006). These enzymes are frequently used as alternatives to fluorescent or chemiluminescent reporters, mostly because of their high sensitivity and the possibility to combine different luciferases for multiplex analyses (Naylor, 1999). Another advantage of luciferases over fluorescent proteins, like for example GFP, is the lack of background signal in most cellular systems (Hampf and Gossen, 2006). However, for some applications the use of luminescent reporters can also be adverse, due to the dependence on cofactors and substrates.

The luciferase firefly is responsible for the bioluminescence of the eponymous firefly *Photinus pyralis.* It is one of the best studied luciferases, having a long history as a reporter gene in biological research, starting as a reporter in promotor studies (Gould and Subramani, 1988). In the presence of Mg^{2+}, ATP and O_2, the 62 kDa protein catalyzes the oxidation of D-luciferin, generating oxyluciferin and light photons with a high quantum yield of >88%. At a pH of 7-8 and at room temperature, the emitted light has a wavelength of 562 nm (Gould and Subramani, 1988). Renilla luciferase from the marine soft coral *Renilla reniformis* is a cofactor-less luciferase, often used for gene expression studies and biosensor applications (Woo and von Arnim, 2008). In *in vitro* assays, renilla catalyzes the oxidative decarboxylation of the renilla substrate coelenterazine with a low enzymatic turnover and quantum yield (5.5 %). This results in the emission of blue light with a wavelength of 480 nm and the production of oxyluciferin and CO_2 (Matthews et al., 1977). The active enzyme has a molecular weight of 35 kDa and is active without posttranslational modifications (Shifera and Hardin, 2010). Since the activities of the two luciferases firefly and renilla depend on different substrates, they are often used in dual assays with renilla acting as an internal control normalizing the experimental reporter firefly for variations (Sherf et al., 1996; Samodelov et al., 2016).

These variations in luciferase activities could be caused by transfection efficiencies, sample handling or cellular stress events triggered by internal or external factors (Hannah et al., 1996).

The characteristics of such a renilla/firefly-based normalization element are further explained and discussed under 5.1.3.7.

The use of other co-reporters, like for example GUS (β-glucuronidase), can be problematic due to differences in the assay procedure and the specific measurement features (Hannah et al., 1996).

A very useful characteristic of a reporter gene can be the secretion of the enzyme into the culture medium of cells or body fluids of living animals. Tannous et al. showed, that gaussia luciferase from the marine copepod *Gaussia princeps* is naturally secreted from mammalian cells (Tannous, 2009). Additionally, it was shown for different applications, that the corresponding assay detecting gaussia activity is highly sensitive (Tannous et al., 2005; Badr et al., 2007), and that the reporter level in the culture medium is linearly correlated to cell numbers, growth and proliferation (Tannous et al., 2005). This small (19.9 kDa) monomeric protein shows flash bioluminescence characteristics, typical also for other coelenterazine luciferases, and an emission peak at 480 nm. The luminescent signal intensity in cultured cells together with their immediate environment is over 1000-fold higher compared to humanized firefly and renilla luciferases (Tannous et al., 2005). As this luciferase is, other than firefly and renilla, secreted, it has to face the comparison with another secreted reporter gene: SEAP (secreted placental alkaline phosphatase). This reporter was first described by Berger et al. in 1988 (Berger et al., 1988). It provides several advantages when compared to other reporter genes. As recombinant SEAP originated from placental alkaline phosphatase is secreted from transfected cells (Hiramatsu et al., 2005), the preparation of cell lysates is not required. Therefore, it is possible to study the same culture for gene expression kinetic studies by collecting samples at different timepoints. And as the cells are not disturbed, they can also be used for further RNA or protein analyses. Additionally, SEAP has the unusual characteristic of being heat-stable and resistant to phosphatase-inhibitor L-homoarginine. Therefore, background signals can be prevented by eliminating endogenous phosphatases by heat treatment (65 °C) and inhibitor addition (Hiramatsu et al., 2005).

In summary, all four reporter genes come along with specific characteristics, which can be advantageous or disadvantageous. But it is obvious that the research perspectives that are opened up by the utilization of these reporters, from fluorescent proteins and luciferases to secreted phosphatases, are extensive. But just like for any molecular tool, their use needs careful evaluation regarding their applicability for a specific biological system or approach.

1.2.6 Tetracycline-regulated gene expression

The stringent control of gene expression is one of the major goals in synthetic biology and other biological fields. Therefore, the design and implementation of heterologous gene expression regulation systems are highly desirable. One of the most extensively studied chemically controllable gene expression systems was first described in 1992 by Gossen et al. and is based on the tetracycline repressor protein (TetR) from *Escherichia coli.*

The fusion of this repressor to the C-terminal domain of VP16 from the *Herpes simplex* virus generated a tetracycline dependent hybrid transactivator (tTA) (Gossen and Bujardt, 1992).

In *E. coli*, TetR negatively regulates the transcription of tetracycline resistance genes. In the presence of tetracycline, the repressor does not bind to its operator sequence within the promotor region of the tetracycline resistance gene operon (Tn*10*), thus enabling gene expression (Gossen and Bujardt, 1992). In a synthetic gene expression system (Figure 3), the tTA hybrid protein (TetR-VP16) stimulates a minimal promotor downstream of the TetR operating sequence $(tetO)_n$. The addition of tetracycline prevents the transactivator (tTA) from binding, leading to reduced gene expression (Baron and Bujard, 2000).

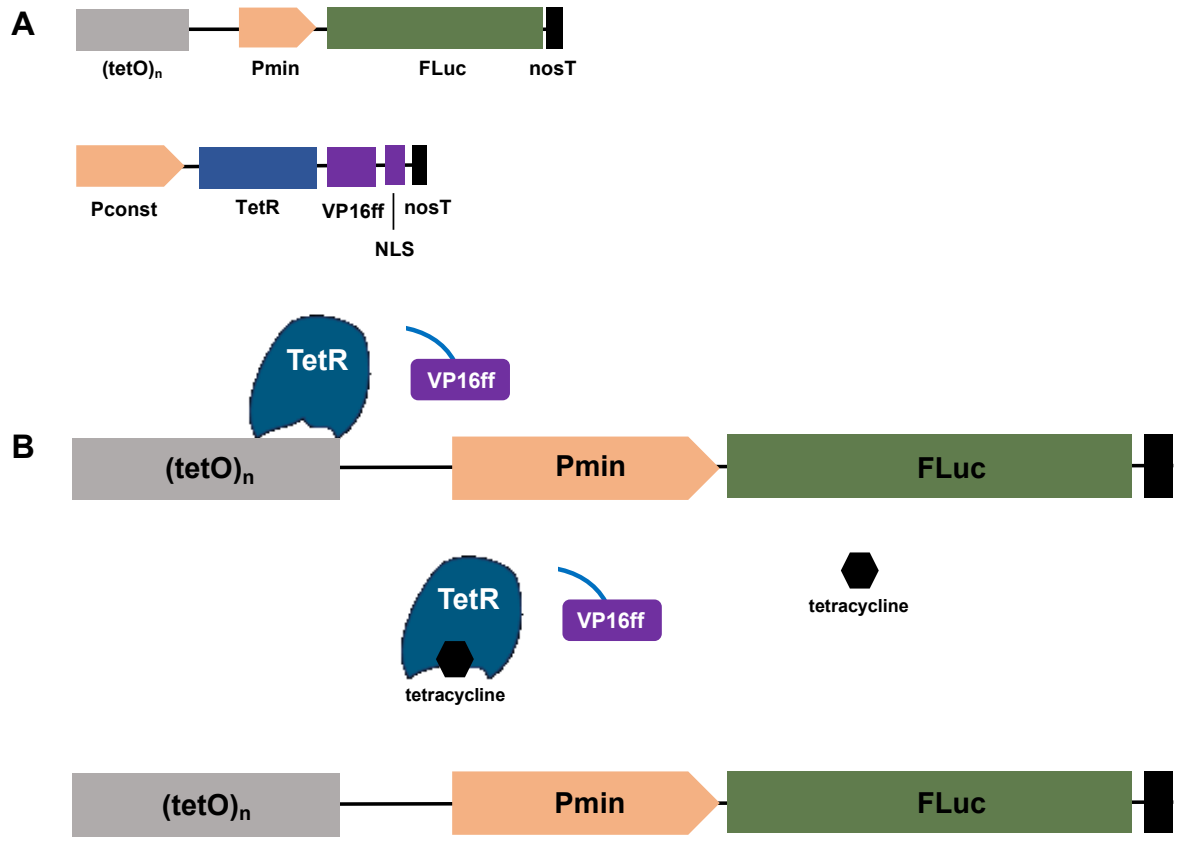

Figure 3: Tetracycline-controlled gene expression using a Tet-Off system. (A) This two-vector system consists of a reporter plasmid, containing several repeats of the TetR operator sequence (tetO) upstream of a minimal promotor (Pmin) controlling the expression of firefly (FLuc) as a reporter. The second vector comprises the tetracycline repressor protein TetR with a C-terminally fused VP16 transactivator from *Herpes simplex* and a nuclear localization sequence (NLS) for nuclear transport under control of a constitutive promotor (Pconst). (B) In the absence of tetracycline, TetR binds to the operator sequence $(tetO)_n$, inducing the expression of FLuc. Upon addition of tetracycline, the binding of TetR is inhibited, causing a decrease in reporter expression.

This Tet-Off system has proven its functionality in many eukaryotic systems, from cultured mammalian or plant cells to whole organisms like *Drosophila* or mice (Gossen et al., 1995). Nevertheless, there are some disadvantages of this negatively-regulated Tet-system.
Tetracycline or doxycycline have to be present continuously, except from the time of expression induction. This constant exposure to antibiotics and the fact, that the antibiotics have to be actively removed to activate gene expression, can be disadvantageous for many applications or biological systems.
To overcome these limitations, Gossen et al. developed a Tet-On system, allowing activation of gene expression by the addition of doxycycline (Gossen et al., 1995). To generate a TetR variant that functions in a reverse fashion, random mutagenesis followed by phenotype screening were performed. The generated variant (rTetR) differed from the original protein at four amino acid positions: E17K, D95N, L101S and G102D (Das et al., 2016). For both systems, the Tet-Off and the Tet-On system, it is desired to have a low background activity in the Off-status, and a high induction fold upon activation. For this purpose, the tetracycline controllable systems have undergone lots of optimizations to reach this goal, including minimal promotor studies and variations (Loew et al., 2010), and the design and implementation of new rTetR variants (Das et al., 2016). Beside tetracycline repressor-based gene expression systems, other chemically controllable systems are available, offering a multitude of opportunities for applications in different organisms.

1.2.7 E-protein-based gene expression system

In 2002, Weber et al. designed an erythromycin-adjustable gene expression system in mammalian cells. This system is based on the antibiotic-dependent interaction of the *E. coli* repressor protein (MphR(A)) and its operating sequence (ETR) (Weber et al., 2002). These components derive from the *E. coli* erythromycin-resistance operon which is negatively regulated in the presence of macrolide antibiotics like erythromycin, clarithromycin, and roxithromycin (Weber et al., 2002). The erythromycin-resistance operon regulates the transcription of the macrolide-inactivating 2'-phosphotransferase I gene (Noguchi et al., 2000). The expression of this gene is controlled by the repressor protein MphR(A), binding to an operating sequence of 35 bp length (ETR) (Noguchi et al., 2000). The binding of a macrolide antibiotic to MphR(A) results in a disruption of the MphR(A)-ETR interaction, and leads to a transcription stop of mph(A) (Weber et al., 2002).
The two-component synthetic macrolide-regulatable gene expression system consists of the E-protein transactivator (ET) hybrid protein, a fusion of the MphR(A) repressor and the VP16 transactivation domain from *Herpes simplex* virus, and the antibiotic-responsive promotor region, comprising the operator sequence ETR and a minimal promotor (Weber et al., 2002).

In the absence of macrolide, the hybrid protein binds to the operating sequence, which brings the VP16 transactivation domain in a close proximity to the minimal promotor to induce expression of a gene of interest (GOI). In the presence of antibiotics, ET binding to the ETR sequence is prevented, prohibiting GOI expression (Figure 4).

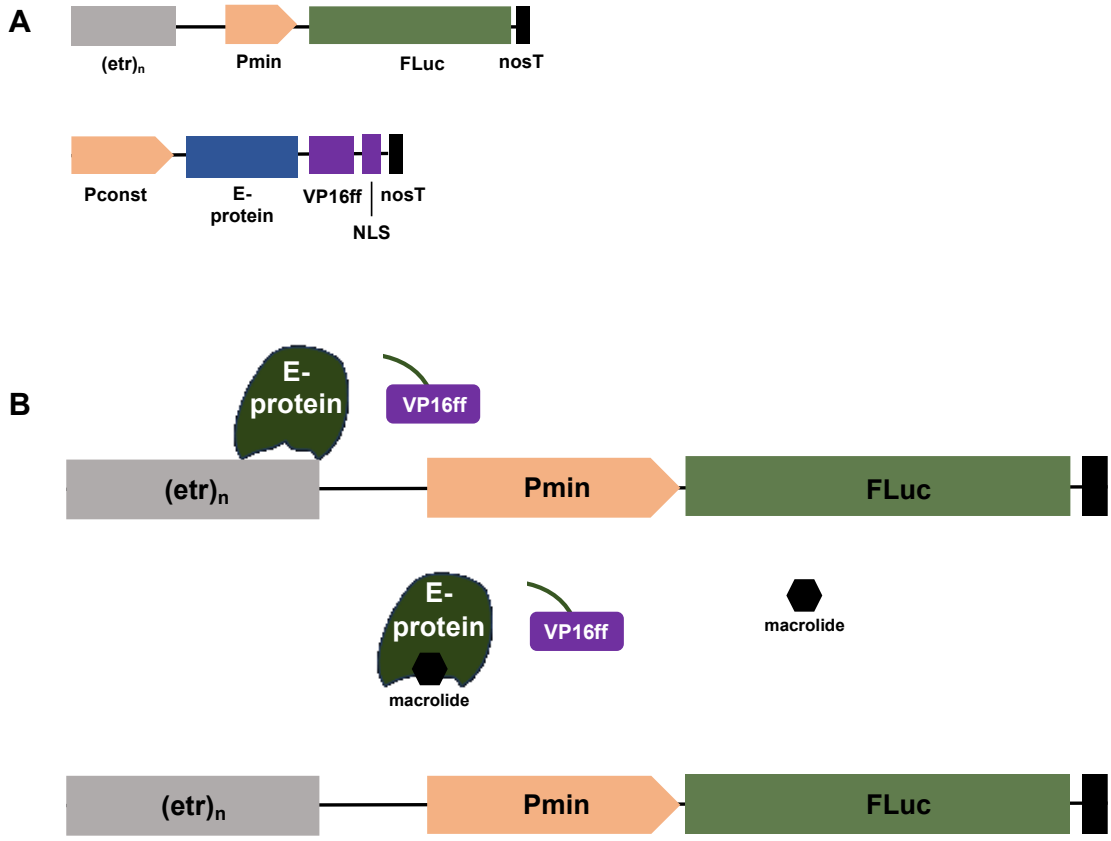

Figure 4: E-protein-based chemically controlled gene expression system. (A) The two-vector system comprises a firefly (FLuc) reporter plasmid with the E-protein operator sequence $(etr)_n$ upstream of a minimal promotor (Pmin), regulating the expression of firefly, and a plasmid containing the E-protein from *E. coli* with a C-terminally fused VP16 transactivation domain from *Herpes simplex* and a nuclear localization sequence (NLS) under control of a constitutive promotor (Pconst). (B) In the absence of a macrolide antibiotic, the E-protein binds to its operator sequence, inducing reporter gene expression. When a macrolide antibiotic is added to the system, it prevents the binding of the E-protein to the operator sequence, causing a decrease in reporter expression.

For this E-protein based gene expression system, Weber et al. introduced two variants: a repressible (E-Off) and an inducible (E-On) mammalian gene regulation system. The E-Off system functions like previously described, whereas the E-On system depends on binding of the E-protein to artificial ETR-derived operator sequences cloned upstream of a constitutive promotor (Weber et al., 2002). This leads to a repression of transcription. After addition of macrolides, gene expression is induced by dissociation of the E-protein repressor from the operating sequence (Weber et al., 2002).

It was shown, that this macrolide-controllable gene expression system is functionally compatible with other chemically regulated systems, like the Tet- or PIP-system, offering the possibility to engineer multigene interventions in mammalian cells or tissues (Müller et al., 2014c). In 2014, it was also demonstrated, that the E-protein based system can be applied very successfully in *Nicotiana tabacum* protoplasts.
Compared with other chemically controlled systems, it performed very favorable, regarding to expression and induction levels (Müller and Weber, 2013).
Müller et al. distinctly demonstrated how important the pre-testing of a synthetic molecular tool is, to determine its applicability for a specific biological system.

1.2.8 Utilization of “tunable, light-controlled interacting protein tags” (TULIPs) for optogenetic gene expression regulation

The term “optogenetics” mainly describes the combined use of optical and genetic components and methods (Müller and Weber, 2013). The aim is to build synthetic devices that can integrate optical signals and exhibit specific effector functions in response to these signals. One of the major advantageous of these optogenetic systems, in comparison to chemically controlled systems, is the high level of temporal and spatial control (Müller and Weber, 2013). As these light switches do not require the addition of chemicals, there is no perturbing and unwanted interference of these drugs with the studied system (Müller and Weber, 2013). As of today, a remarkable diversity of these optogenetic systems have been published. They differ in their molecular components, the color of light they are responding to, and the level of controllability they demonstrate in different organisms. As light is an essential environmental signal not only for photosynthetic, but also for non-photosynthetic organisms, a large diversity of sense and response systems have evolved in nature. Such light-sensing proteins and their corresponding response pathways can be found in prokaryotes (Losi and Gärtner, 2008), as well as in plants (Möglich et al., 2010) and mammals (Miyamoto and Sancar, 1998), and they can be utilized as control elements for synthetic biology applications. To maximize the control of a specific process, and to minimize the interference with the host organism, these systems should be orthogonal to the host environment (Müller and Weber, 2013). The most frequently used light-responsive systems in eukaryotes are based on LOV domains, cryptochromes and phytochromes. Phytochromes (Phy) are red/far-red absorbing photoreceptors occurring in plants, fungi and bacteria (Müller and Weber, 2013). One of the major advantageous of these phytochromes is, that they can be actively switched off by illuminations with far-red light (Ulijasz and Vierstra, 2011). What could be considered as a disadvantage for some applications is, that the phytochrome chromophore phytochromobilin is not present in orthogonal systems, and therefore has to be added to the culture medium as commercially available phycocyanobilin (Müller and Weber, 2013).

Cryptochromes, like Cryptochrome 2 (CRY2) from *Arabidopsis thaliana*, on the other hand are blue light-responsive receptors, utilizing FAD (flavin adenine dinucleotide), a chromophore which is available in orthologous system as well. By absorption of blue photons, these photoreceptors switch to their active conformation and passively return to the inactive state in the dark (Yu et al., 2010; Chaves et al., 2011). The third group of photoreceptors comprise the blue light sensing light-oxygen-voltage (LOV) domains, small light-sensing domains (~ 125 kDa) that can naturally be found in prokaryotes (Losi, 2004), fungi and plants (Müller and Weber, 2013).

These light-sensing domains use FMN (flavin mononucleotide) as a co-factor. Illumination with blue light leads to the formation of a covalent bond between FMN and a cysteine residue, causing a conformational change. Due to this change in conformation, a C-terminal α-helix (Jα) undocks from the protein core. This unfolding can then trigger different downstream effector functions, depending on the particular peptide which is fused the Jα-helix (Müller and Weber, 2013). Other LOV-proteins react to blue light-exposure via dimerization or rotation of subunits. In the dark, the flavin-cysteinyl bond is hydrolyzed causing the LOV protein to passively return to its inactive state (Strickland et al., 2012). One of the blue light-controllable gene expression systems that has been used in this work and should therefore be described more detailed here, is a blue light-inducible dimerization system which is utilizing the LOV2 domain from *Avena sativa* phototropin 1 (AsLOV2). It was published in 2012 by Strickland et al. and named TULIPs (tunable, light-controlled interacting protein tags) (Strickland et al., 2012). These light-inducible dimerization tags are based on the synthetic interaction of AsLOV2 and an engineered PDZ domain (ePDZ). Strickland et al. demonstrated the functioning of TULIPs by providing components of the yeast mating pathway with light sensitivity abilities and by directing the site of cell polarization via light-induced Rac1 recruitment (Strickland et al., 2012). The basic idea of the system is to fuse a peptide epitope to the C-terminus of the Jα helix. The LOV2-Jα interaction is thereby leading to a caging of the epitope tag preventing it from binding to a cognate partner. High affinity and specificity variants of the Erbin PDZ domain were used as such binding partners (Huang et al., 2009; Strickland et al., 2012). The interaction affinity of these clamshell-like ePDZ chimera variants and their cognate peptide vary from ~0.5 nM to > 10 µM (Huang et al., 2009; Strickland et al., 2012). For the implementation of the TULIP system, Strickland et al. tested five AsLOV2-peptide fusions with the peptide epitope appended to serial truncations of the Jα helix. One of these tested versions was further modified to increase the ePDZ binding affinity and the LOV-Jα docking. This further engineered version was named LOVpep (Strickland et al., 2012). Furthermore, the blue light-dependent recruitment capacities of this engineered synthetic system in yeast and mammalian cells were demonstrated (Müller et al., 2014a).

But by now, this system has also been adapted for light-tunable gene expression applications, alone or in combination with red and UV light controllable systems for triple-gene control in mammalian cells (Müller et al., 2014a). Such blue light-responsive gene expression systems combine the binding of the epitope tag of LOVpep to the ePDZ domain with split transcription factors, present in previously described chemically inducible gene expression systems. In 2014, Müller et al. used the GAL4/UAS system, consisting of the GAL4 yeast transcription factor and five repeats of its operator sequence UAS_G as a basis for an optogenetic gene expression system (Müller et al., 2014b).

The GAL4-LOVpep fusion protein is directed to a response construct consisting of five repeats of UAS_G upstream of a minimal promotor controlling the expression of SEAP as a reporter gene. The PDZ domain is fused to the VP16 transactivator domain from *Herpes simplex* virus. Upon illumination with blue light (450nm), the epitope tag is uncaged, resulting in the recruitment of PDZ-VP16 to induce expression of the SEAP reporter (Müller et al., 2014b). In this work, we demonstrate the novel implementation of such a LOVpep-ePDZ based blue-light responsive gene expression switch in the basiomycete fungus *Ustilago maydis.* This optogenetic switch is based on the split transcription factors PIP/$(Pir)_n$ from a Prestinamycin-controllable gene expression system (Figure 5). The implementation and characterization of the chemically-regulatable system was done by Nicole Heucken and is described in her dissertation.

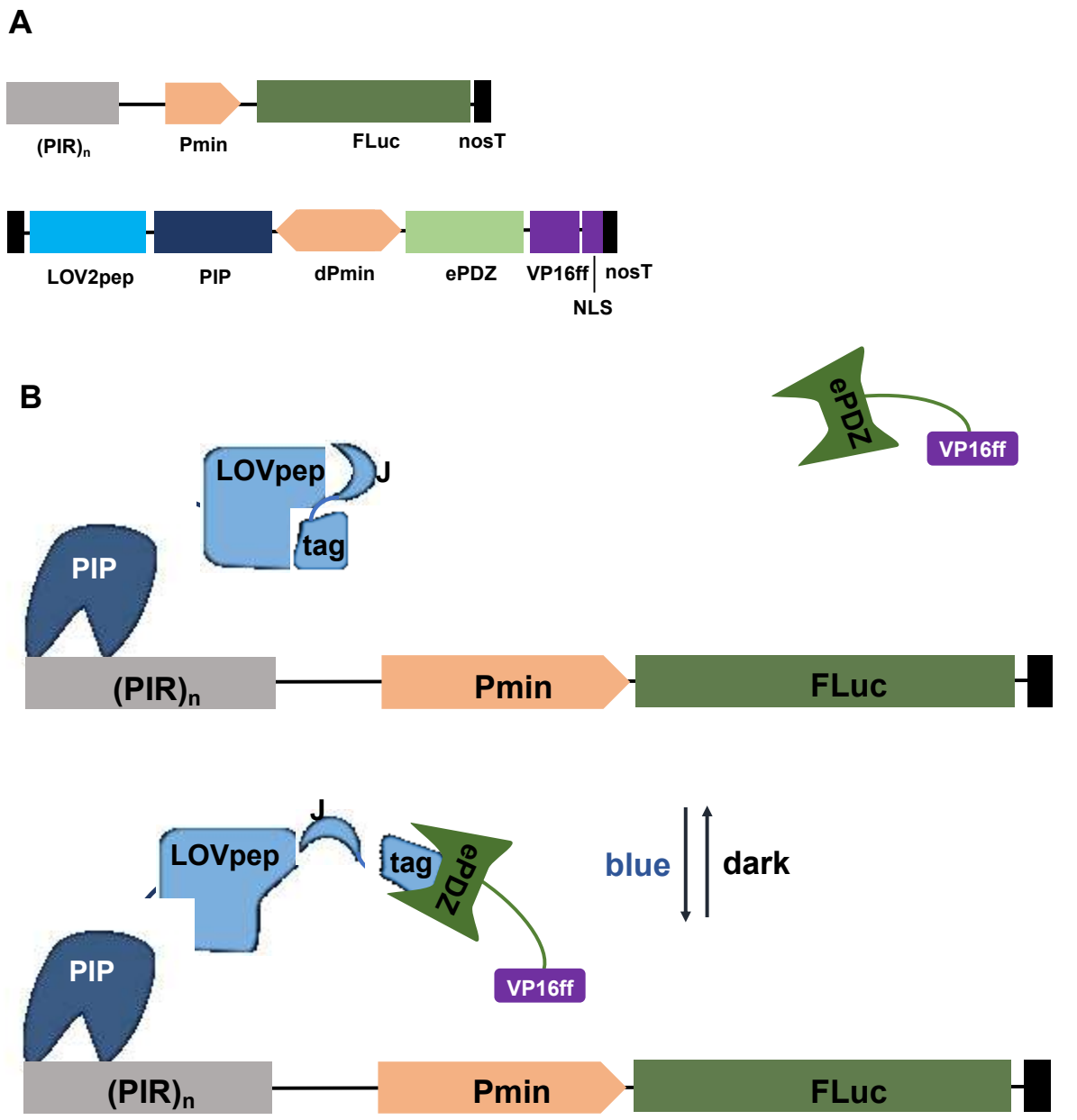

Figure 5: Design and functionality of a blue light-responsive gene expression system as it was constructed for implementation in *U. maydis*. (A) Two vector design of a LOVpep/ePDZ-based blue light-controllable gene expression system. The reporter plasmid comprises firefly (FLuc) as a reporter and the PIP operating sequence $(PIR)_n$ upstream of a minimal promotor (Pmin). The second vector consists of a bidirectional promotor (dPmin) controlling the expression of a LOVpep-PIP fusion and ePDZ with a C-terminally fused VP16 transactivator and a nuclear localization sequence (NLS). (B) Functionality of a LOVpep/ePDZ-based blue light-controllable gene expression system. The PIP-LOVpep fusion protein binds to its operator sequence $(PIR)_n$. Upon illumination with blue light, flavin mononucleotide (FMN) is covalently linked to LOVpep, causing a conformational change and leading to the uncaging of a peptide tag. This tag is now accessible for the interaction with an ePDZ domain. This interaction brings the fused VP16 transactivator in close proximity to the minimal promotor, thereby inducing the expression of firefly.

1.2.9 A blue light-inducible gene expression system based on an engineered version of the bacterial transcription factor EL222 from *Erythrobacter litoralis*

As optogenetic gene expression systems are offering a high spatial and temporal control, these systems are essential for a multitude of biological applications. Additional to the already described LOVpep-based blue light-controlled gene expression switch, another LOV domain related system, with rapid activation and deactivation kinetics, has been described by Motta-Mena et al. in 2014. For this system, an engineered version of the bacterial light-oxygen-voltage protein EL222 was used (Motta-Mena et al., 2014). Upon illumination with blue light, EL222 binds to DNA, thereby showing a large dynamic range of protein expression in different mammalian cell lines and zebrafish embryos (Motta-Mena et al., 2014).

The engineered EL222 transcription factor is reduced to the minimal number of components needed for light-responsive transcription activation: a LOV domain and a helix-turn-helix (HTH) DNA-binding motif (Nash et al., 2011). In the dark, the HTH motif is bound by the LOV domain, thereby caging the HTH 4α helix, which is needed for EL222 dimerization and DNA binding (Zoltowski et al., 2011). The blue light-triggered protein-flavin adduct formation disrupts the LOV-HTH interactions and enables EL222 dimerization and DNA binding (Zoltowski et al., 2011). In the dark, the system is rapidly reversed back into the inactive state within ~ 11s (Motta-Mena et al., 2014).

Using a minimally engineered EL222 version, Motta-Mena et al. report on a 200-fold upregulation and rapid activation (<10 s) and deactivation (<50 s) kinetic in HEK293T cells, compared to other LOV-based transcriptional systems (Motta-Mena et al., 2014).

To implement the bacterial transcription factor EL222 as an optogenetic expression regulating tool, an VP16 transactivation domain and a nuclear localization signal (NLS) have to be added to the N-terminus of the EL222 protein. A reporter vector containing the firefly luciferase reporter gene downstream of five repeats of the EL222 binding clone 1-20 bp $(C120)_5$ and a TATA box promotor are used to quantify the expression induction (Motta-Mena et al., 2014).

Upon blue light-illumination (465 nm), the HTH motif is activated and binds to the $(C120)_5$ sequence, thereby inducing expression of the reporter (Motta-Mena et al., 2014). As this blue light-regulated gene expression switch does not rely on the addition of co-factors, shows low toxicity, low basal transcriptional activity and high induction folds in different cell lines, it has a high potential for many synthetic biology applications. Therefore, we aimed to implement two versions of this system in *U. maydis:* a VP16 transactivator-based Blue-On, and a Sql1 repressor-based Blue-Off system for future utilization in biotechnological or basic research applications. The functionality of these systems is further illustrated in Figure 6.

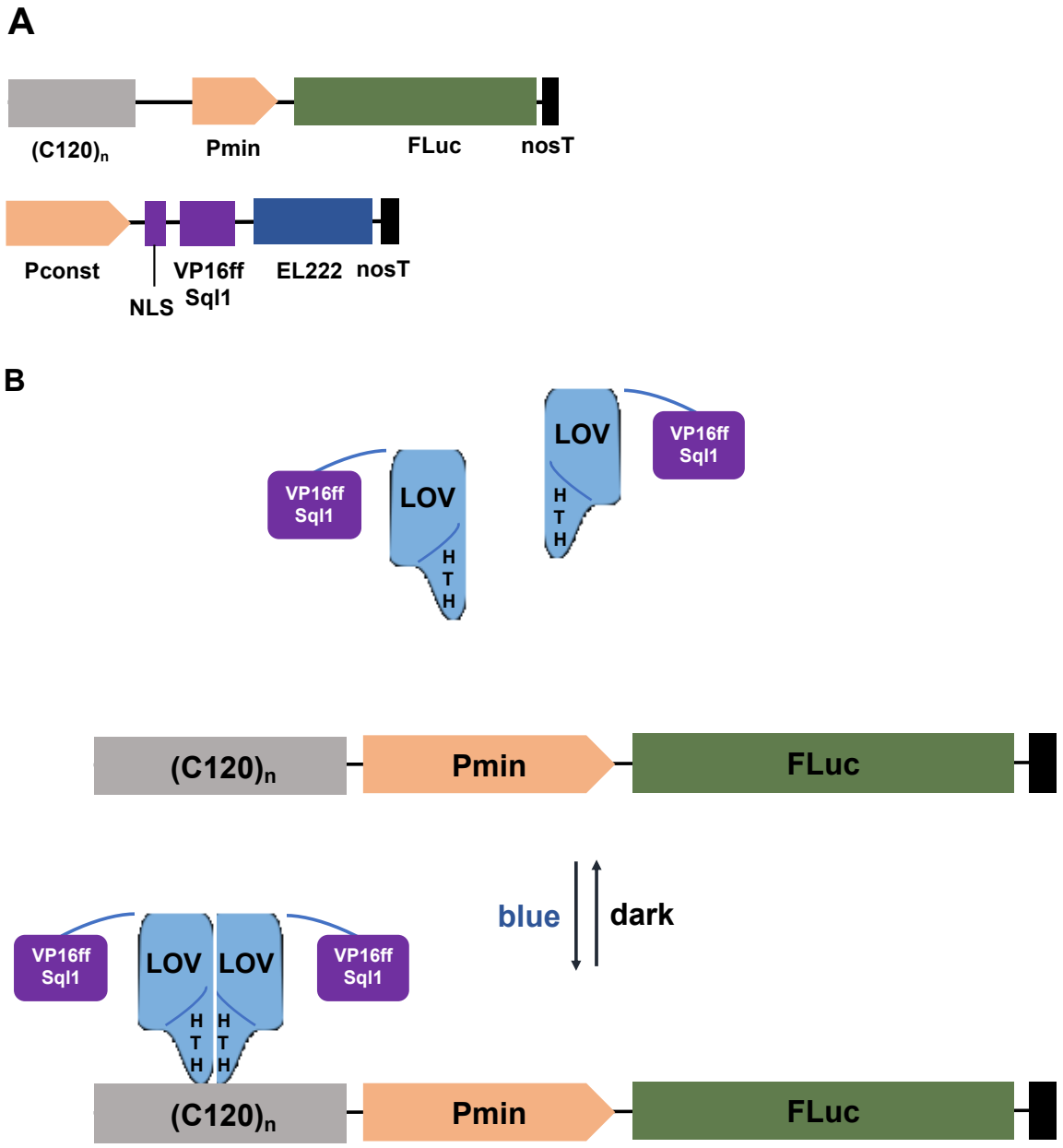

Figure 6: EL222-based blue light-controllable gene expression systems for the implementation in *U. maydis*. (A) Two vector design of an EL222-based blue light-controllable gene expression system in *U. maydis.* The reporter plasmid comprises firefly (FLuc) as a reporter and several repeats of the EL222 operating sequence (C120) upstream of a minimal promotor (Pmin). The second vector consists of a constitutive promotor (Pconst) controlling the expression of an EL222 with a N-terminally fused VP16 transactivator (Blue-On system) or an *U. maydis*-derived Sql1 repressor (Blue-Off system), and a nuclear localization sequence (NLS). (B) Functionality of an EL222-based blue light-controllable gene expression system. Upon illumination with blue light, the caged helix-turn-helix (HTH) motif of the EL222 enwinds, inducing EL222 dimerization and DNA binding. This interaction brings the fused transactivator/repressor in close proximity to the minimal promotor, thereby inducing/repressing the expression of firefly.

1.3 Reconstruction of plant signaling pathways in orthogonal cell systems

Vascular plants, as multicellular and sessile organisms, have developed unique and highly complex signal transduction pathways. The last decades of extensive research in this field have brought deep insight and an increased understanding of how plants perceive, integrate and transmit signals as a function of their developmental processes, and in response to environmental stimuli (Møller and Chua, 1999). It turned out, that plant signal transduction is not a linear cascade of events, but involves extensive cross-talk between different pathways. This crosstalk is likely to be important to integrate complex developmental events and to sense and respond to environmental cues (Møller and Chua, 1999; Mccarty and Chory, 2000). Many signaling pathways involved in plant development respond to endogenous signaling molecules like strigolactones, auxins, gibberellins and other phytohormones, but also to exogenous stimuli including temperature, salinity, drought (Liu et al., 1998) and light (Okada and Shimura, 1992). Due to the high complexity of the different signaling pathways in terms of interconnectivity and redundancy, and the intense cross-talk between different signaling cascades, it is difficult to study the function of single components within the signal perception and transduction machinery in their natural environment. This makes it necessary to isolate these single components from their plant environment and transfer them into fully orthogonal system, segregating them from the influence of interacting proteins or pathways. For this purpose, different orthogonal platforms have been established. From yeast cultures as appropriate and adaptable platforms for high-throughput protein-interaction screenings (Walhout and Vidal, 2001; Pruneda-Paz et al., 2014), to yeast-two-hybrid (Y2H) assays to monitor protein interactions (Mehla et al., 2015). But beside these yeast-based approaches, many other orthogonal platforms have been established for studying plant signaling pathways (reviewed by Braguy and Zurbriggen, 2016). One of them are mammalian cells, which are becoming more and more popular platforms for the reconstruction and analysis of plant signal transduction. What makes these mammalian systems so useful as orthogonal platforms, is the availability of plenty molecular switches to control gene expression, stability, or localization of proteins.

Additionally, different quantitative readout systems based on luciferase reporter gene assays and other molecular tools have been established in mammalian cells in the last decades (Brophy and Voigt, 2014b; Lienert et al., 2014b). Another advantage of these systems is, that normally a codon optimization of the used constructs is not necessary as plant proteins are readily expressed in most commonly used cell lines (Beyer et al., 2015b; Beyer et al., 2015a). As light and hormone signaling pathways of plants do not have homologous in mammalian cells, this platform is offering a strongly reduced interference when studying single components of these pathways.

Even though this high level of orthogonality can make it necessary to add certain components that are needed to achieve full functionality of the isolated compounds (Braguy and Zurbriggen, 2016), mammalian cells are offering a wide range of applications in the research field of plant signaling reconstruction when utilized as orthogonal platforms to study these complex signal transduction processes.

1.3.1 GTPases and guanine exchange factors as key regulators in eukaryotic signal transduction

GTPases, also known as G proteins, are conserved molecular switches in signal transduction processes of eukaryotic organisms (Li et al., 1998). These proteins cycle between an inactive GDP-bound and an active GTP-bound state, thereby acting as a binary switch controlling the transmission of extracellular signals to intracellular pathways (Gu et al., 2004). Their activation is catalyzed by guanine nucleotide exchange factors (GEFs), whereas the inactivation is regulated by GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs) (Gu et al., 2006) (Figure 7).

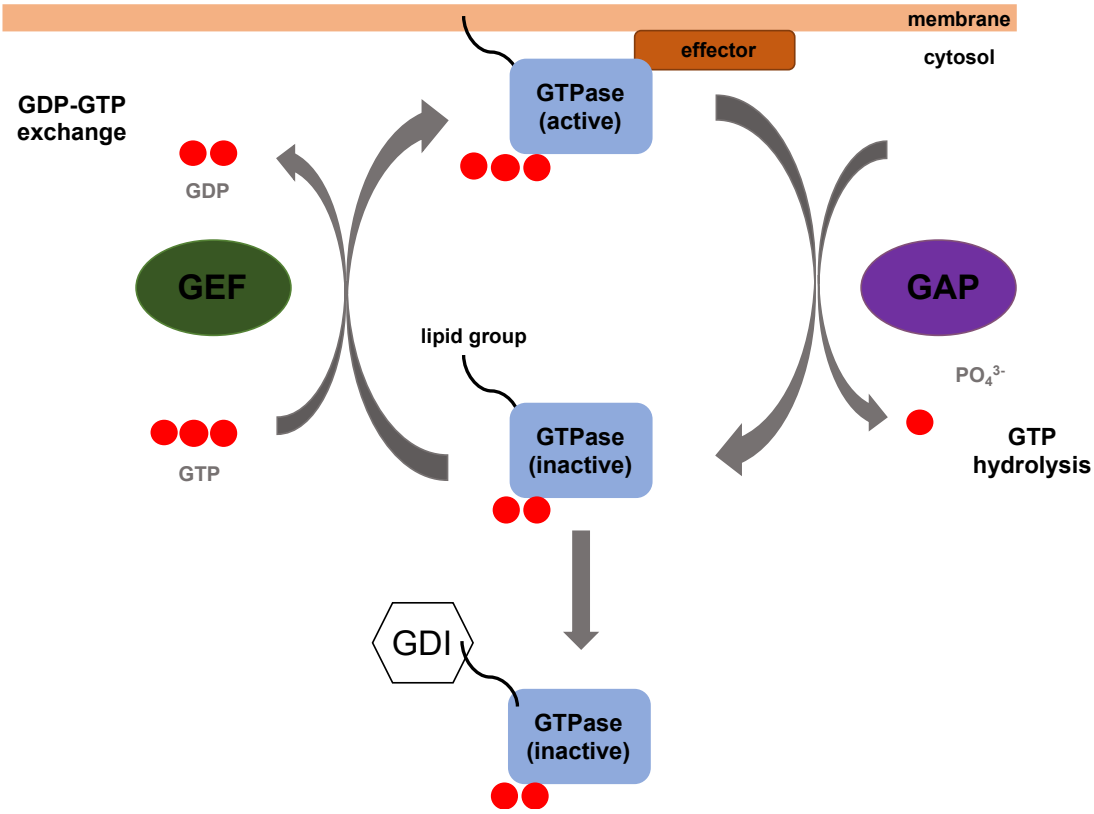

Figure 7: Activation/deactivation cycle of small GTPases. Small GTPases cycle between a GDP-bound inactive and GTP-bound active state. Activation and inactivation, as well as translocation to specific effectors are regulated by guanine nucleotide exchange factors (GEFs) catalyzing the exchange of GDP to GTP, guanosine-nucleotide dissociation inhibitors (GDIs) preventing the exchange of GDP to GTP, and GTPase-activating proteins (GAPs) that increase the rate of GTP hydrolysis to GDP. GTPases can interact with a variety of downstream effectors. Many small GTPases contain C-terminal sequences that are targets of proteolysis or post-translational modifications, or lipid groups, which are necessary for the binding of small GTPases to membranes and regulators, as well as for the activation of downstream effectors (modified from Bento et al., 2013).

G proteins can be divided into two classes: heterotrimeric G proteins and monomeric Ras/Ras-like small GTPases (Gu et al., 2004). While numerous of heterotrimeric G proteins with different combinations of 20 Gα, 5 Gβ and 12 Gγ type subunits can be found in mammals (Gu et al., 2004), only one Gα, one Gβ and two Gγ subunits are present in *Arabidopsis thaliana* (Assmann, 2002). In the superfamily of small GTPases, only Ras and Rho GTPases have been clearly shown to play a role in the transmission of extracellular signals (Gu et al., 2004), but these Ras GTPases do not seem to exist in plants (Yang, 2002; Vernoud et al., 2003). However, plants possess a unique subfamily of Rho GTPases, so called ROPs (Rho-type GTPases of plants) (Yang and Watsont, 1993; Li et al., 1998; Vernoud et al., 2003).
A wide range of Rho GTPase subfamilies has been identified in mammals and yeast (Johnson, 1999; Burridge and Wennerberg, 2004), but ROPs on the other hand are the only Rho GTPase subfamily that can be found in plants (Winge et al., 2000; Zheng and Yang, 2000; Yang, 2002). These small GTPases are known to control many cellular processes (Figure 8), especially those that are connected to the regulation of the cytoskeleton. This cytoskeletal regulation is essential for polar cell growth, which is a fundamental process in cell shape formation, root hair development and pollen tube elongation (Gu et al., 2004).

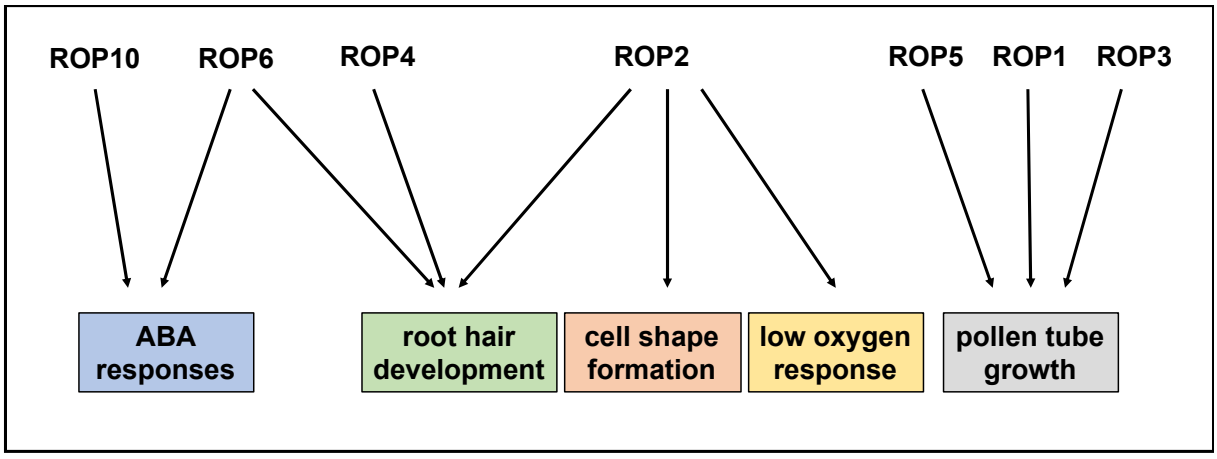

Figure 8: Functions of members of the ROP GTPases family. Loss-of-function and gain-of-function studies revealed that the function of individual ROPs are redundant or overlapping (modified from Gu et al., 2004).

The functional unity of these small GTPases might be linked to a shared common ancestor shared by the 11 ROPs in *A. thaliana* and Rho, Cdc42 and Rac GTPases from mammals (Li et al., 1998; Winge et al., 2000; Zheng and Yang, 2000; Vernoud et al., 2003).
As briefly mentioned in the beginning, these small GTPases are activated by guanine exchange factors (GEFs). In animals, these GEFs can be divided in two structural classes containing either a DH or a DOCKER catalytic domain (Gu et al., 2006), whereas the ~150 amino acid DH domain is the catalytic domain of RhoGEFs (Hoffman and Cerione, 2002). BLAST searches revealed no such DH domain-containing proteins in the genomes of *Arabidopsis thaliana* or *Oryza sativa* (Gu et al., 2006).

The first RopGEFs in *A. thaliana* have been described by Berken et al. in 2005 and in 2006, Gu et al. identified 14 different RopGEFs in *A. thaliana,* using yeast-two-hybrid screenings (Berken et al., 2005; Gu et al., 2006). These RopGEFs have a plant-specific ROP nucleotide exchanger (PRONE) domain. It catalyzes the multi-step reaction leading to an activation of ROP proteins (Thomas et al., 2009), and does not show any sequence homology to DH or DOCKER domains from animals.

The interaction of the different ROP GTPases and RopGEFs initiates the coordination of a multitude of downstream pathways, thereby generating a large output of possible responses to different stimuli in plants (Figure 8) (Gu et al., 2004). One of these outputs that is examined in more detail in this work is the ROP/GEF-mediated regulation of the outgrowth of root hairs. As they are exceptionally polarized structures, they provide a model system for cell polarity and polar growth research in other eukaryotes as well (Jones et al., 2002).

1.3.2 Interaction of RopGEFs and ROP GTPases initiates and promotes root hair outgrowth in *A. thaliana* trichoblasts

Root hairs are tubular structures emerging from the root epidermis. Their function is to anchor the plant root and increase the area of exploitable soil (Gilroy and Jones, 2000; Denninger et al., 2019). Their formation is subject to precise cell fate, specifying whether a cell is destined to form root hairs or not, and cell polarization to induce localized cell growth (Gilroy and Jones, 2000). In trichoblasts, the root hair generating cells, the formation of a root hair initiation domain (RHID) at the plasma membrane leads to a polarization of the growth machinery to this site, hereby inducing tip growth (Denninger et al., 2019). It is known, that ROPs play a central role in the determination of the RHID and the polar growth (Jones et al., 2002). The overexpression of ROP2 for instance results in a strong root hair phenotype, and overexpression of ROP7 in an inhibition of root hair tip growth (Jones et al., 2002). A lot of research has been done to further elucidate the molecular mechanisms leading to the RHID positioning (Ikeda et al., 2009; Kiefer et al., 2015) and the regulation of ROP activity (Fu et al., 2002; Carol et al., 2005; Zhang and Mccormick, 2007; Hwang et al., 2008; Yalovsky et al., 2008; Chang et al., 2013). It is however still not completely understood, which proteins are responsible for targeting ROPs to the RHID and which are stimulating cell outgrowth. In 2019, Denninger et al. showed that the site-specific positioning of the involved proteins and the outgrowth are temporally separate events, which are regulated by specific GEFs (Denninger et al., 2019). It was further demonstrated that trichoblast-specific GEF3 and GEF4 are essentially involved in these events: GEF3 as being crucial for the early formation of the RHID and the recruitment of ROP2, and GEF4 as a downstream activator of ROP2, triggering the polar cell outgrowth (Denninger et al., 2019). The interaction of GEF3/4 and ROP2 during the event of root hair formation in *A. thaliana* is illustrated in Figure 9.

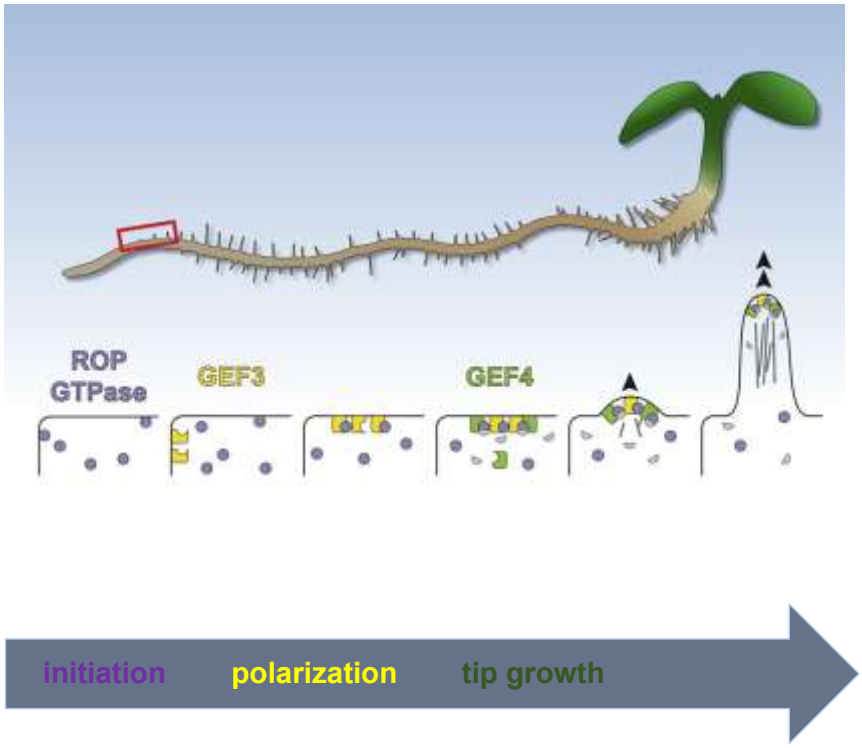

Figure 9: Temporal appearance and interaction of proteins involved in the formation of root hairs in *A. thaliana.* As root hairs are highly polarized cellular structures, they serve as a good model system to study cell polarity. They emerge from single epidermal root cells as a result of tip growth, an extreme form of polarized cell growth. During the initiation phase, proteins involved in the process are recruited to the site of future outgrowth. In this phase, no morphological changes can be observed. In the next step, the recruitment of ROP2 by GEF3 polarizes the cell, before GEF4 is activating ROP2, thereby initiating the polar outgrowth of the cell (modified from Denninger et al., 2019).

To deepen the understanding of these highly complex outgrowth mechanisms, and gain new insight into the interaction principles of different key players in this cellular event, we transferred *A. thaliana* GEF3, GEF4 and ROP2 into mammalian cell systems to study the interaction of the single components in an orthogonal environment.

1.4 AQUA 2.0: an update to AQUA cloning

AQUA cloning is an assembly cloning method that was published by Beyer et al. in 2015 (Beyer et al., 2015a). Like other assembly cloning approaches, it relies on homologous overhang pairing of assembled fragments. The novelty of AQUA cloning is, that it does not depend on the addition of enzymes but instead harnesses the intrinsic *in vivo* processing of linear DNA by *Escherichia coli.* AQUA cloning has proven over the years, to be a versatile, robust and fast cloning method, and it was mainly used for the construction of plasmids used in this work. Here, we demonstrate an upgrated version of this method, and demonstrate how AQUA can be utilized to add short sequences, too long to be included in a primer overhang, and too short to be effectively amplified *via* PCR, to a plasmid (see 7.1 Appendix).

2 Aims

The aims of this work are split in two parts, both related to the development and application of fundamental synthetic biology approaches. The first part comprises the design and implementation of a synthetic biology toolbox for the basidiomycete fungus *Ustilago maydis.* This toolbox includes different synthetic gene expression switches, and basic molecular tools for the construction and quantitative analysis of these switches.

These basic tools and switches comprise IRES sequences, bidirectional promotors, reporter genes and in total five chemically and light-controllable gene expression systems.

IRES sequences and bidirectional promotors are useful tools for the design of bicistronic plasmids, required for the construction of complex multi component regulatory systems. As the implementation and characterization of such synthetic systems require a quantitative read-out system to determine their functionality, we additionally established reporter genes and their corresponding assays to perform this task. After these basic tools were implemented and available in *U. maydis,* we designed two chemically and three optogenetic systems for transcriptional control of heterologous genes. The construction and implementation of such optogenetic switches in this microorganism is a novelty, and has never been done before. Here we report on the design of these systems, and demonstrate their functionality and applicability in *U. maydis*. This successful establishment of a synthetic toolbox, comprising basic tools and complex systems, will open up new perspectives and possibilities in different fields in *U. maydis*-related research.

In the second part of this work, we utilized mammalian cells as orthogonal platforms to reconstruct the plant signaling pathway regulating the formation of root hairs in *Arabidopsis thaliana.* Therefore, we transiently transfected HeLa cells with plasmids containing three key proteins of this pathway: the guanine-exchange factors GEF3 and GEF4 and the small GTPase ROP2 from plants. This resulted in an extensive lamellipodia-like structure formation, indicating an interaction of plant GEFs and ROP2 with mammalian cell proteins involved in the regulation of polar cell outgrowth. These observations provide a starting point for further mechanistical studies on this cell outgrowth events and on the degree of functional conservation, underlying this process.

3 Results and Discussion

This chapter contains data regarding the implementation of synthetic biology tools in *U. maydis* and the reconstruction of plant signaling pathways in mammalian cell systems, that have been obtained in the course of this work. The results in chapter 3.1.1, 3.1.2, 3.1.3 and 3.1.4 were generated together with Nicole Heucken. Due to time limitations, follow-up data will be generated and integrated in potential publications.

3.1 Construction and implementation of a synthetic biology toolbox in the basidiomycete fungus *Ustilago maydis*

3.1.1 Implementation and characterization of quantitative reporter gene assays

Reporter genes are a strong tool in synthetic biology approaches. They serve as a powerful instrument to quantitatively monitor the functionality and controllability of molecular tools, such as IRES sequences or bidirectional promotors, and chemically and light-controllable gene expression systems. Since quantitative reporter genes have not been established in *U. maydis* so far, we tested and characterized three different luciferases and the human alkaline phosphatase SEAP regarding their applicability as reporter genes in this organism. After their characterization and establishment, these reporter genes can be utilized to quantitatively determine the functionality of the synthetic molecular tools that were implemented in the course of this work. Furthermore, their establishment can be highly beneficial in other fields of *U. maydis*-related research, like for instance the quantitative tracking of infection events in plant-pathogen interaction studies. For implementation of the mentioned reporter genes, we generated plasmids containing the codon optimized reporter genes under control of the constitutive P_{O2tef} promotor, and generated four strains containing the three luciferases and the alkaline phosphatase SEAP, stably integrated in the cco1 locus. The correct integration of the constructs was confirmed via two rounds of antibiotic counter selection and southern blot analysis. The activity of the particular reporter was measured in the lysate and the supernatant. As depicted in Figure 10 A and B, firefly (FLuc) and renilla (RLuc) showed high activity levels in the cell lysate, and almost no activity in the supernatant, whereas the activity of renilla is approximately eight times as high as the activity of firefly. These high expression levels in the lysate accord with what is known about the expression of firefly and renilla in other eukaryotic systems, considering the applied protocols (Sherf et al., 1996; Naylor, 1999). Because of this, various attempts have been made to convert these luciferases into secreted reporters, using different strategies (Liu et al., 1997; Nazari and Hosseinkhani, 2011).

For the implementation of secreted firefly and renilla versions in *U. maydis,* the fusion of endochitinase Cts1 might be a promising strategy, as Cts1 functions as a carrier for the export of heterologous proteins, thereby avoiding disadvantageous N-glycosylation (Aschenbroich et al., 2019).

Different to firefly and renilla, the third luciferase gaussia shows a higher activity in the supernatant than in the lysate (Figure 10 C). This observation is corresponding to what is reported about the secretion of gaussia in mammalian cells (Tannous et al., 2005; Tannous, 2009). But the activity in the lysate is almost as high as in the supernatant. This observation could indicate a less efficient secretion of this luciferase in *U. maydis*, compared to mammalian cell systems. Comparing the activity of gaussia in the supernatant with activity of firefly and renilla in the lysate, it is ~40 times lower compared to firefly, and ~320 times lower compared to renilla. Nevertheless, the utilization of gaussia as a quantitative reporter can still be beneficial for approaches that require the secretion of a reporter gene into the culture medium, when high activity levels are not necessary. But ddditionally, the fusion of a Cts1 domain might again help to increase the secretion efficiency.

Testing the alkaline phosphatase SEAP, we could detect only very low activities in the supernatant as well as in the lysate, close to the background activity levels (Figure 10 D). Even though a multitude of publications have proven the applicability of SEAP as a powerful reporter gene in different eukaryotic systems (Berger et al., 1988; Cullen and Malim, 1992), SEAP does not seem to be functional in *U. maydis*, neither in the lysate, nor in the supernatant. There are different potential reasons for this, like e.g. misfolding or degradation events, but further research will be necessary to unravel these reasons.

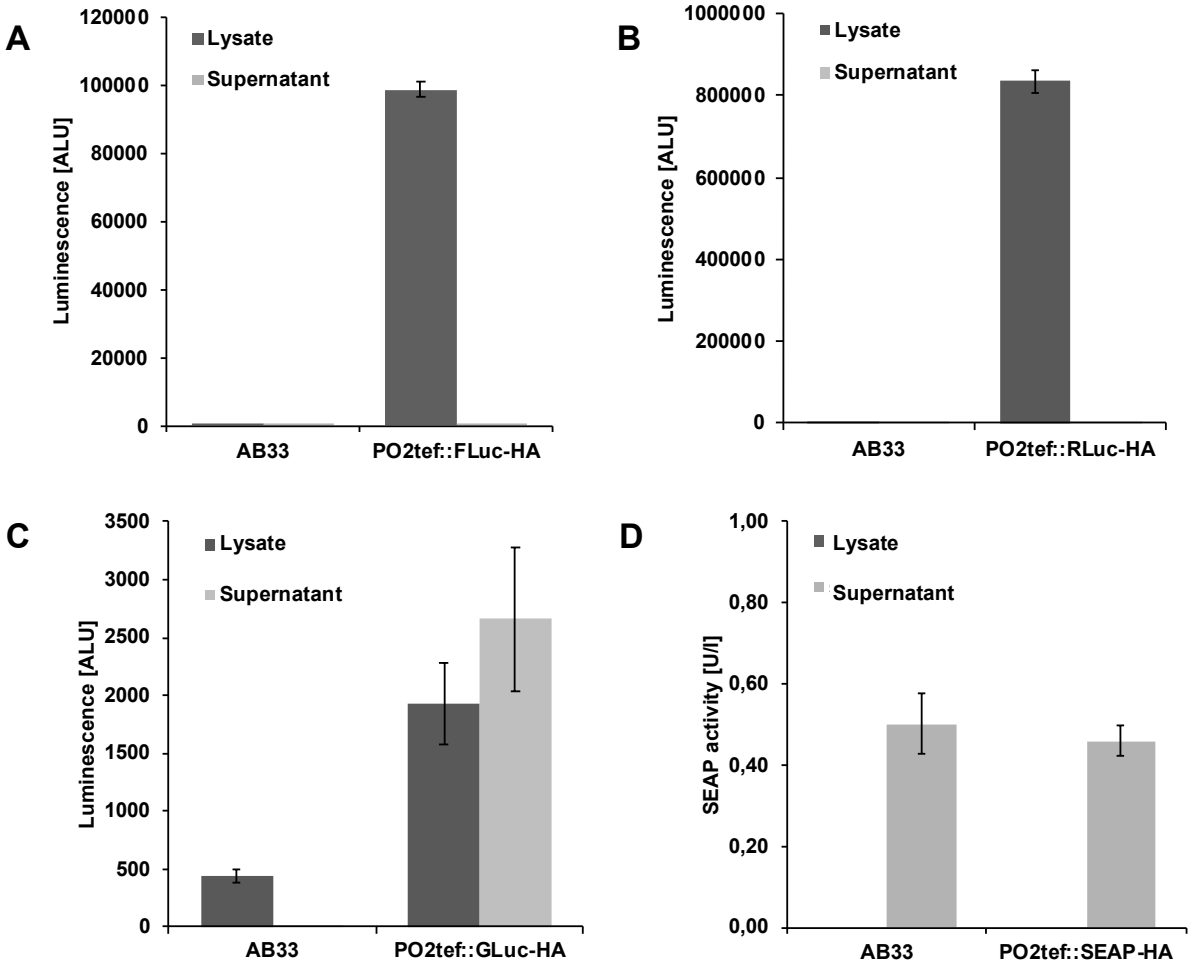

Figure 10: Quantitative characterization of reporter gene activities in *U. maydis*. Luciferase activities were measured in the lysate and the supernatant of *U. maydis* cultures after addition of the specific substrate. The reporter genes FLuc (firefly) (A), RLuc (renilla) (B), GLuc (gaussia) (C), and SEAP (D) were expressed under control of a constitutive promotor (P_{O2tef}). AB33 as the origin strain served as a negative control. Cultures were inoculated with the specific strain and grown in CM-medium. After 24 h, the cultures were adjusted to an OD_{600} of 0.5 and the cells were pelleted and lysed. For the measurement, the cell lysate and the supernatant were transferred to white 96-well plates and the substrate was added. The values are means of six biological and three technical replicates.

3.1.2 Development and characterization of a firefly-based “fast-screening platform”

One of the major methodological advantages of gaussia and SEAP assays, compared to firefly and renilla, is that the activity of these reporter genes can be measured in the supernatant, making cell lyses redundant. To overcome this limitation and combine the high enzymatic activity of firefly luciferase with the methodological advantages of other assays, we established a firefly-based fast-screening platform in *U. maydis.* This fast-screening method relies on the uptake of the firefly substrate luciferin by the cells, allowing the measurement of reporter activity directly in the culture. This makes cell lyses procedures and centrifugation steps dispensable, and provides a robust and very fast reporter gene-based screening method.

To test and characterize such a screening platform, we measured the firefly activity in the whole culture at different timepoints after addition of the substrate, and were able to detect high activity levels (~12,500 ALU) almost immediately after substrate addition (Figure 11).
The observation, that *U. maydis* quickly takes up the firefly substrate luciferin, provided the option for implementing a fast-screening platform. This platform served as a screening tool for testing the functionality of the numerous chemically and light-inducible gene expression systems. Without this method at hand, it would have been almost impossible to test the variety of systems that have been constructed in the course of this work in such a short time.

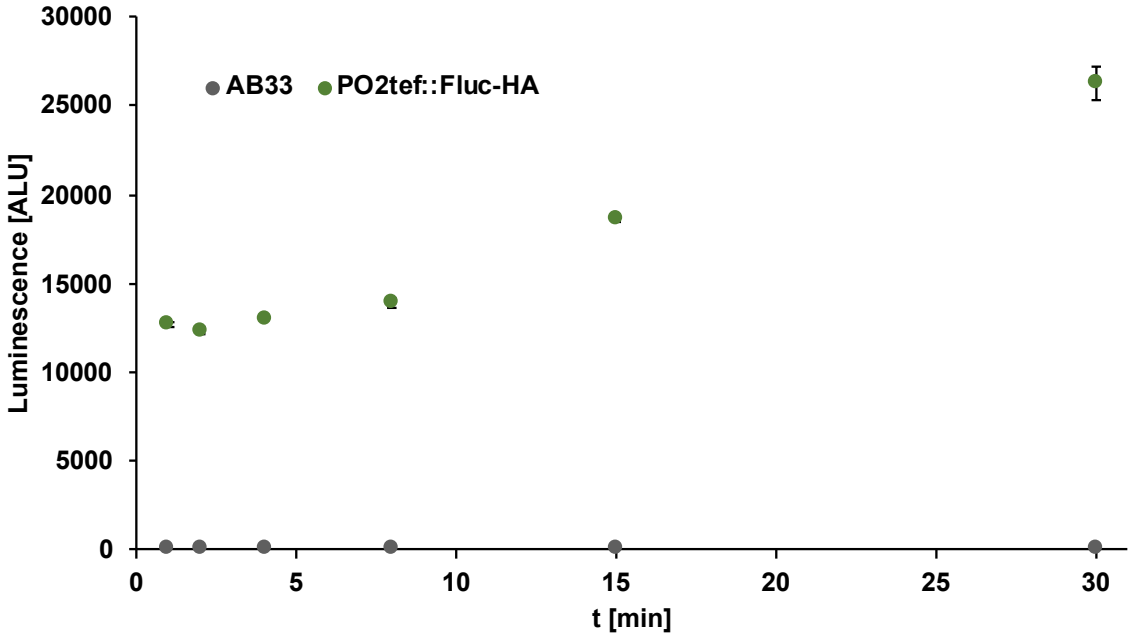

Figure 11: Implementation of a firefly-based fast screening platform in *U. maydis*. Measurement of firefly (FLuc) activity 1, 2, 4, 8, 15 and 30 min after substrate addition to the cultures. FLuc was expressed under control of the constitutive P_{O2tef} promotor and AB33 as the origin strain served as a negative control. For the reporter measurement, 80 µl of culture were transferred to 96-well assay plates and the firefly substrate was added directly to the culture. Values are normalized to an OD_{600} of 0.5. The data shown represent means of 6 biological and 3 technical replicates.

3.1.3 Implementation of a renilla/firefly normalization element

The primary purpose of normalization elements is to correct reporter gene data for factors other than those that are tested in the specific experiment. Such a normalization element is based on the combination of two reporter genes, like for instance firefly and renilla (Schenborn and Groskreutz, 1999). As the activity of these two luciferases depends on different substrates, it is possible to use them in dual assay with renilla as an internal control and firefly as the experimental reporter (Hampf and Gossen, 2006).

Renilla normalizes the firefly reporter for variations caused by cellular stress, sample handling or transfection efficiencies (Sherf et al., 1996; Samodelov et al., 2016).

Here we demonstrate, how such a renilla/firefly-based normalization element can be applied in *U. maydis.*

As the activity of promotors, and therefore the expression of proteins in *U. maydis* varies as a result of natural reactions to environmental cues, the absolute reporter values which determine e.g. the inducibility of a promotor, need to be corrected for environmental factors. For this purpose, we generated a strain containing renilla under control of the constitutive P_{O2tef} promotor, and firefly under control of an inducible P_{crg1} promotor. The induction of this promotor is triggered by shifting cultures from a CM-glucose to a CM-arabinose medium (Mahlert et al., 2006). The activity of constitutively expressed renilla was then used to normalize firefly activity data for variations. The obtained firefly/renilla ratio is therefore providing quantitative normalized data for the inducibility of the P_{crg1} promotor. Figure 12 is depicting this data, showing some variations in the renilla activity levels. Strains containing renilla alone under control of the constitutive promotor (green charts), or in combination with the inducible promotor (yellow and red charts) controlling the expression of firefly, show varying activity levels over a time period of 8 h. This variability can be caused by different extra- or intracellular stimuli, demonstrating the necessity of a normalizing reporter to provide robust data for an experimental factor, like the inducibility of the P_{crg1} promotor in *U. maydis.*

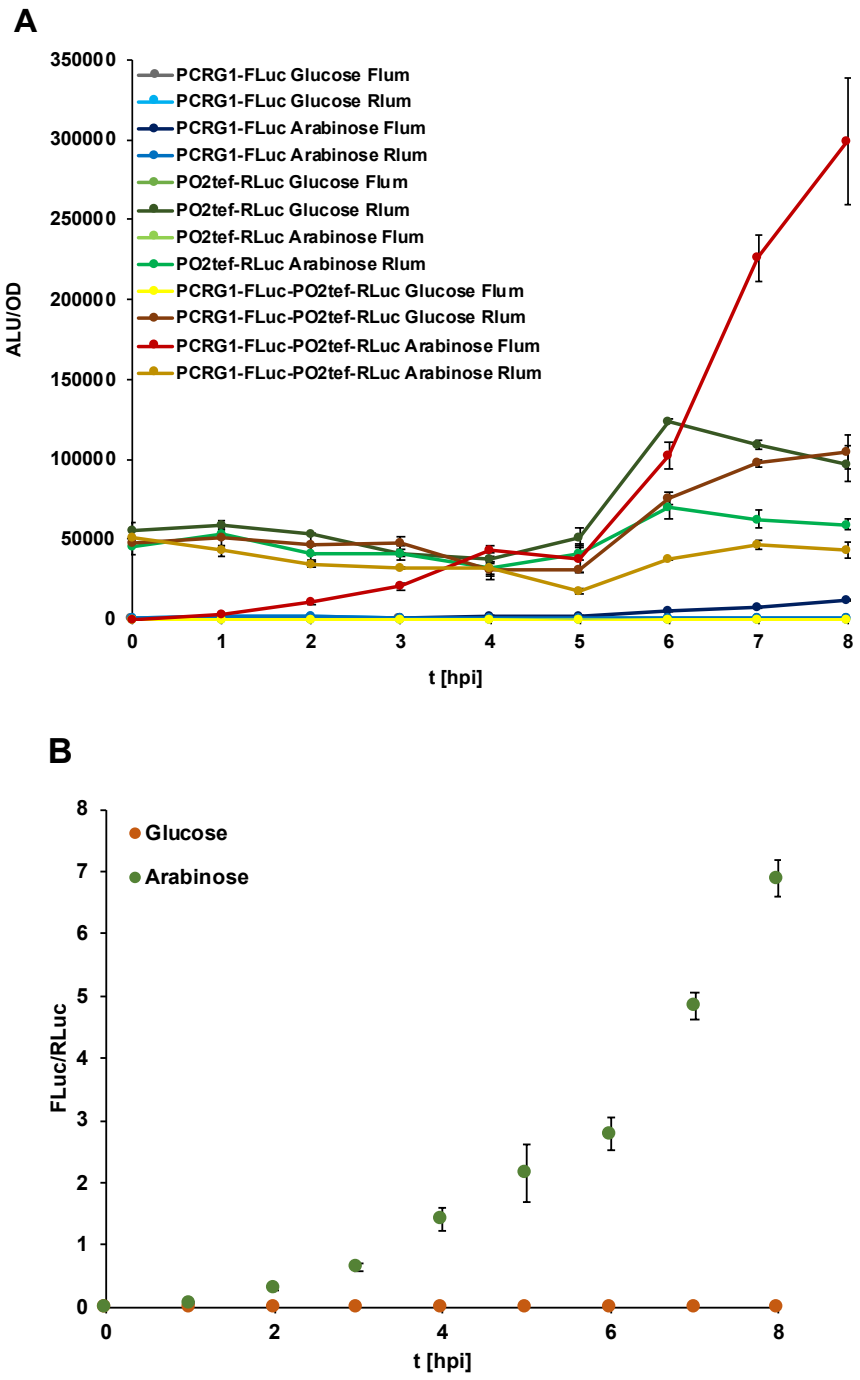

Figure 12: Implementation of a renilla/firefly-based normalization element in *U. maydis*. (A) Strains containing only one luciferase, firefly (FLuc) or renilla (RLuc) under control of a constitutive P_{O2tef} promotor or an inducible P_{crg1} promotor, and strains containing both luciferases, FLuc under control of the inducible promotor and RLuc under control of P_{O2tef}, were cultured in CM-medium supplemented with 1 % glucose or 1 % arabinose respectively. Firefly and renilla activities were measured in the lysate every hour for eight hours in total. (B) The obtained values for the normalization strain expressing FLuc under control of the P_{crg1} arabinose-inducible promotor, and renilla under control of the constitutive P_{O2tef} promotor were used to calculate the FLuc/RLuc ratio for cultures in CM-arabinose medium. All cultures were adjusted to an OD_{600} of 0.5. Shown data are means of three biological and three technical replicates.

3.1.4 Functionality testing and characterization of IRES sequences

The complexity of synthetic biology applications requires tools that give researchers the possibility to construct and implement multicistronic plasmids for transient or stable integration into host systems. For the implementation of such complex synthetic constructs in *U.* maydis, the use of multicistronic plasmids is especially necessary, as the number of loci available for stable genomic integration is strongly limited. Additionally, the transformation process itself and the verification of a correct insertion event can be a very difficult and time-consuming procedure. Therefore, it is highly desirable to keep the number of constructs that need to be included into the genome of *U. maydis* as small as possible.

One of these tools that can be extremely useful for this purpose are IRES sequences. These virus-derived internal ribosome entry sites enable translation in a cap-independent manner, and therefore expression of more than one gene under control of a single promotor (Mountford and Smith, 1995) (Figure 2). In mammalian cell systems, IRES sequences have already proven their applicability, for instance in the construction of light-controllable gene expression systems (Müller et al., 2014c).

In the course of this work, we aimed to implement such systems in *U.* maydis, but as the number of available loci for stable integration of DNA is limited in this organism, the utilization of tools for the construction of bicistronic vectors was necessary.

Therefore, we generated strains to test the functionality of IRES sequences in *U. maydis*. These strains contain fluorescent proteins or luciferases separated by three different IRES sequences: fIRES from *Foot-and-mouth disease virus*, pIRES from *Human poliovirus* and eIRES from *Encephalomyocarditis virus*.

The obtained data (Figure 13) indicate that none of the three IRES sequences is functional in *U. maydis,* as only very low fluorescent/luminescence levels could be detected for the first, and only basal levels for the second reporter. The observed low activity levels of the upstream reporter indicate a strongly reduced efficiency of the IRES-independent cap-mediated translation of the first reporter. This finding is rather surprising as it cannot be observed in other eukaryotic organisms (Hennecke et al., 2001).

The low activity of the second reporter on the other hand is a more expected observation, as it is known, that the IRES-dependent second gene expression is less efficient than the cap-dependent expression (Mizuguchi et al., 2000; Hennecke et al., 2001).

One hypothesis for the lack of function of the tested IRES sequences could be the large extension and flexibility of the IRES RNAs. The *Foot-and-mouth virus*-derived fIRES and the eIRES from *Encephalomycarditis virus* are considered to not fold into compact structures, retaining a certain conformational flexibility (Filbin and Kieft, 2009).

This largely extended structure might sterically perturb the binding of eukaryotic initiation factors (eIF) and the small (40S) subunit of the ribosome to the nucleotide cap on the 5´-end of the mRNA, causing a reduced translation efficiency of the first reporter.

As both IRESs cannot bind directly to the 40S subunit of the ribosome, they require several eIFs, like eIF4A, eIF5B and eIF3 and IRES *trans*-acting factors (ITAFs) in order to recruit the ribosome (Jackson, 2005). An absence of these factors in *U. maydis* might be responsible for a non-functioning of the tested IRES sequences.

Therefore, it would be interesting to test compactly folded IRES RNAs, like for instance (+) ssRNA Dicistroviridae intergenic region (IGR) IRESs, that fold into compact three-dimensional structures (Costantino and Kieft, 2005).

And as it was hypothesized that there is an inverse correlation between the dimension of inherent folded structures and the need for eIFs and ITAFs (Filbin and Kieft, 2009), this might also improve the translation efficiency of the second reporter.

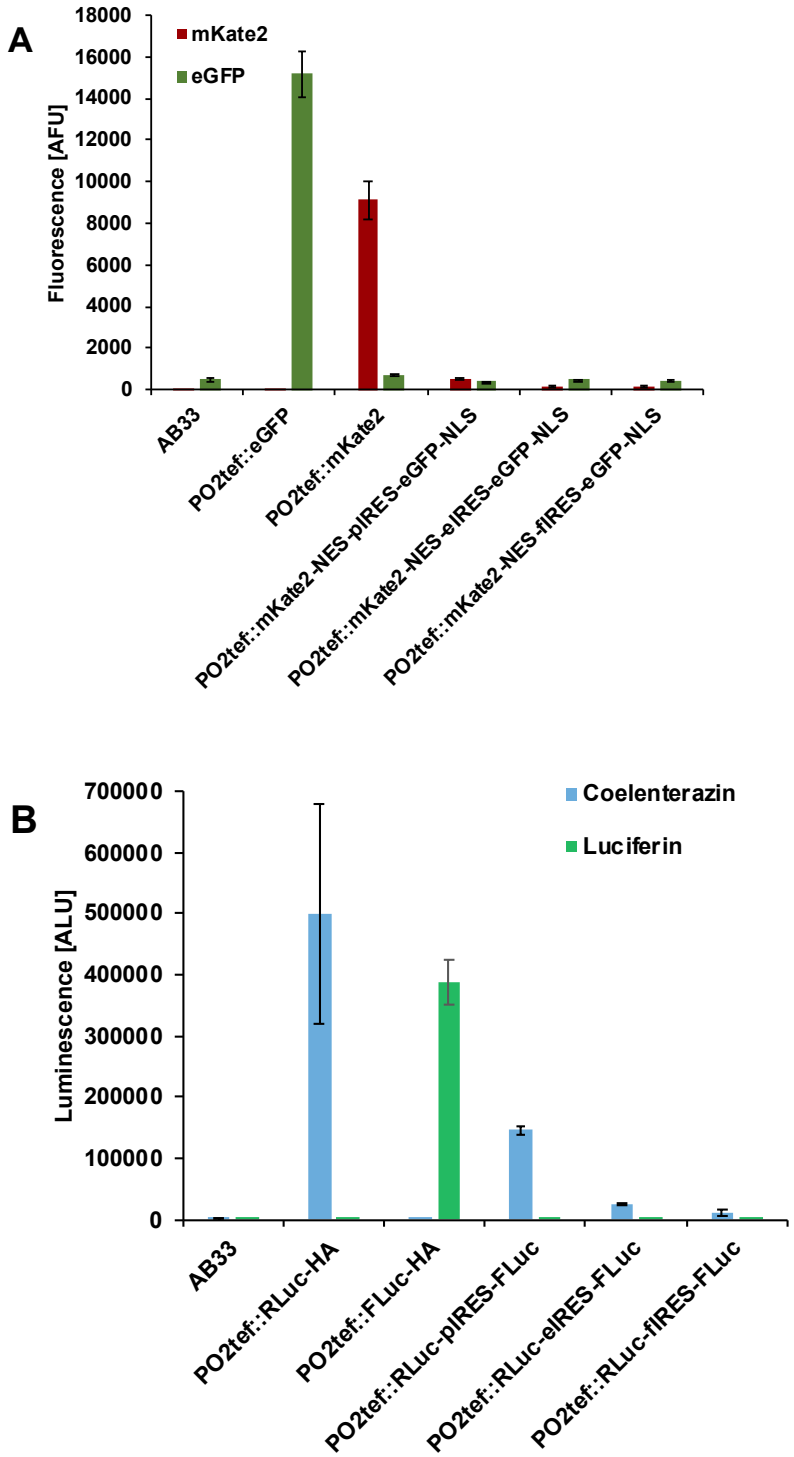

Figure 13: Quantitative analysis of IRES mediated bicistronic expression of reporter genes. (A) The fluorescent reporter genes mKate2 and eGFP were expressed under the control of the constitutive promotor P_{O2tef} and separated by pIRES, eIRES or fIRES sequences. Strains containing constitutively expressed eGPF or mKate2 served as positive controls, whereas the origin strain AB33 served as a negative control. The fluorescence activity was measured in cell lysates of *U. maydis* cultures adjusted to an OD_{600} of 0.5. (B) The luciferases renilla (RLuc) and firefly (FLuc) were separated by a pIRES, eIRES or fIRES and expressed under the control of a P_{O2tef} promotor. Strains expressing the luciferases alone served as positive controls, and AB33 as a negative control. The luciferase activities were measured in the cell lysate. All cultures were adjusted to an OD_{600} of 0.5. Shown data are means of six biological and three technical replicates.

3.1.5 Quantitative comparison of different combinations of operator sequences and minimal promotors in *U. maydis*

For the design and construction of chemical- or light-controllable gene expression systems, the combinatorial usage of operator sequences and minimal promotors is necessary. As a high level of controllability of these systems requires a low basal activity, namely a high dynamic range, we tested the leakiness of different combinations of the operator sequences $(tetO)_{13}$ (Tet system), $(etr)_8$ (E-protein system), and $(C120)_5$ (EL222-based blue light system), in combination with the two minimal promotors P_{hCMV} and P_{mfa1}.

As depicted in Figure 14, the P_{hCMV} minimal promotor shows a lower basal activity in combination with all three operator sequences than the P_{mfa1} promotor. As P_{mfa1} is an Ustilago-derived promotor driving the expression of the pheromone gene *mfa1*, this finding is rather expected and can be explained with the binding of endogenous transcription factors.

The lowest basal activity is achieved by the combination of $(etr)_8$ and the P_{hCMV} promotor, and the highest activity was measured for a combination of five repeats of the EL222 operator sequence C120 and the two minimal promotors. Here, the activity levels are even higher than in the positive control, constitutively expressing firefly under the P_{O2tef} promotor.

This might indicate that there is binding of endogenous transcription factors of *U. maydis* to the C120 operating sequence, thus activating the expression of the reporter gene.

Considering these results, we designed all gene expression systems that were established in this work, using the P_{hCMV} minimal promotor instead of P_{mfa1}.

Based on the high basal activity levels of the C120-P_{hCMV} combination, we extended the repertoire of inducible system with a Blue-Off optogenetic switch, based on EL222 light-responsive gene expression regulation. This novel Blue-Off system utilizes a Sql1 repressor protein from *U. maydis* (described in 3.1.6.2, Figure 6).

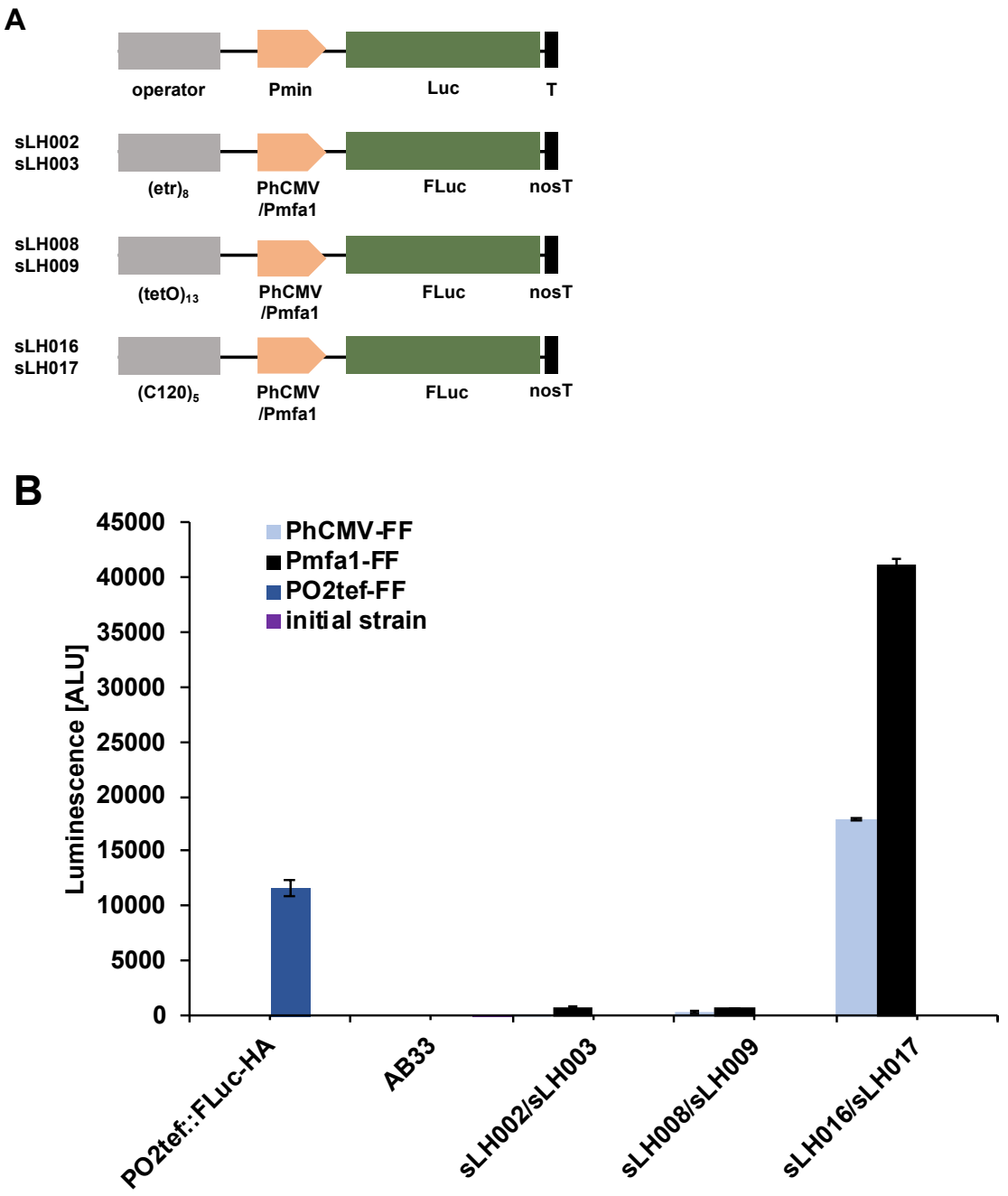

Figure 14: Comparison of basal activity levels for different combinations of operator sequences and minimal promotors. (A) The strains sLH002 and sLH003 contain the E-protein operator sequence $(etr)_8$ upstream of a P_{hCMV} or P_{mfa1} minimal promotor, controlling the expression of firefly (FLuc). The strains sLH008 and sLH009 contain the TetR operator sequence $(tetO)_{13}$, and the strains sLH016 and sLH017 the EL222 operator sequence $(C120)_5$. (B) Comparative analysis of reporter activities for different combinations of operating sequences and minimal promotors. Luminescence was measured in cultures, using the firefly-based fast screening method (see 3.1.2). The cultures were normalized to an OD_{600} of 0.5, and shown data represent means of three biological and three technical replicates.

3.1.6 Engineering and quantitative analysis of chemically controllable gene expression systems in *Ustilago maydis*

The implementation of controllable gene expression systems in *U. maydis* is of great interest for basic research as well as for biotechnological applications in this microorganism. Therefore, we aimed to establish two chemically regulated gene expression systems, based on the antibiotics tetracycline and the macrolide erythromycin. Both systems are designed to downregulate the expression of genes upon the addition of a specific antibiotic component (Off-systems).

3.1.6.1 Development, implementation and characterization of tetracycline-dependent gene expression system

The tetracycline repressor-regulated gene expression system is probably one of the best and most extensively studied chemically controllable gene expression systems. The functionality of this system relies on the binding of the tetracycline repressor protein (TetR) from *Escherichia coli* to a specific operating sequence, $(tetO)_n$, upstream of a minimal promotor regulating the expression of a gene of interest (Gossen and Bujardt, 1992).

As the tetracycline repressor is fused to the viral transactivator VP16, binding of this hybrid-protein to its operator sequence leads to an induction of gene expression (Gibson et al., 2009; Beyer et al., 2015a). Upon addition of tetracycline, the binding of TetR to its operator sequence is prevented, inducing a decrease in gene expression (Gossen and Bujardt, 1992) (Figure 3). This Tet-Off system has been modified to convert into a Tet-On system, allowing the activation of gene expression upon doxycycline addition. For this, random mutagenesis was performed, generating a variant with four mutated amino acids (rTetR) (Gossen et al., 1995).

Here, we designed and implemented the Tet-Off system, with a (tetO) operator sequence consisting of 13 repeats upstream of a P_{hCMV} minimal promotor, and TetR from *E. coli* fused to a short ff-version of the VP16 transactivator from *Herpes simplex.* As a reporter gene we used firefly to quantitatively determine the functionality of the system in firefly-based fast screening assays. As depicted in Figure 15, we observed, instead of the expected decrease, an 11-fold increase in firefly activity upon addition of 3 µg/ml tetracycline, and a 17-fold increase upon addition of 30 µg/ml. Comparing the utilized TetR sequence, which contains an additional NLS sequence and a sequence of 45 amino acids at the C-terminus, with the commercially available TetR version (tTA) (Figure 16), we identified a $S^2 \rightarrow A$ mutation. This mutation, together with the second NLS and the 45 additional amino acids, could be an explaination for the reversed mode of function of the implemented Tet-system.

The expected Tet-Off and the observed Tet-On mode of function are illustrated in Figure 17.

However, a more detailed analysis of the system, including the comparison of different versions of TetR, with and without the additional NLS and the 45 aa sequence of unknown function, has to be performed to give a final conclusion concerning the functionality of this system. However, the obtained data suggest that the system, as it was constructed in this work, might be a promising candidate for a Tet-On system in *U. maydis.*

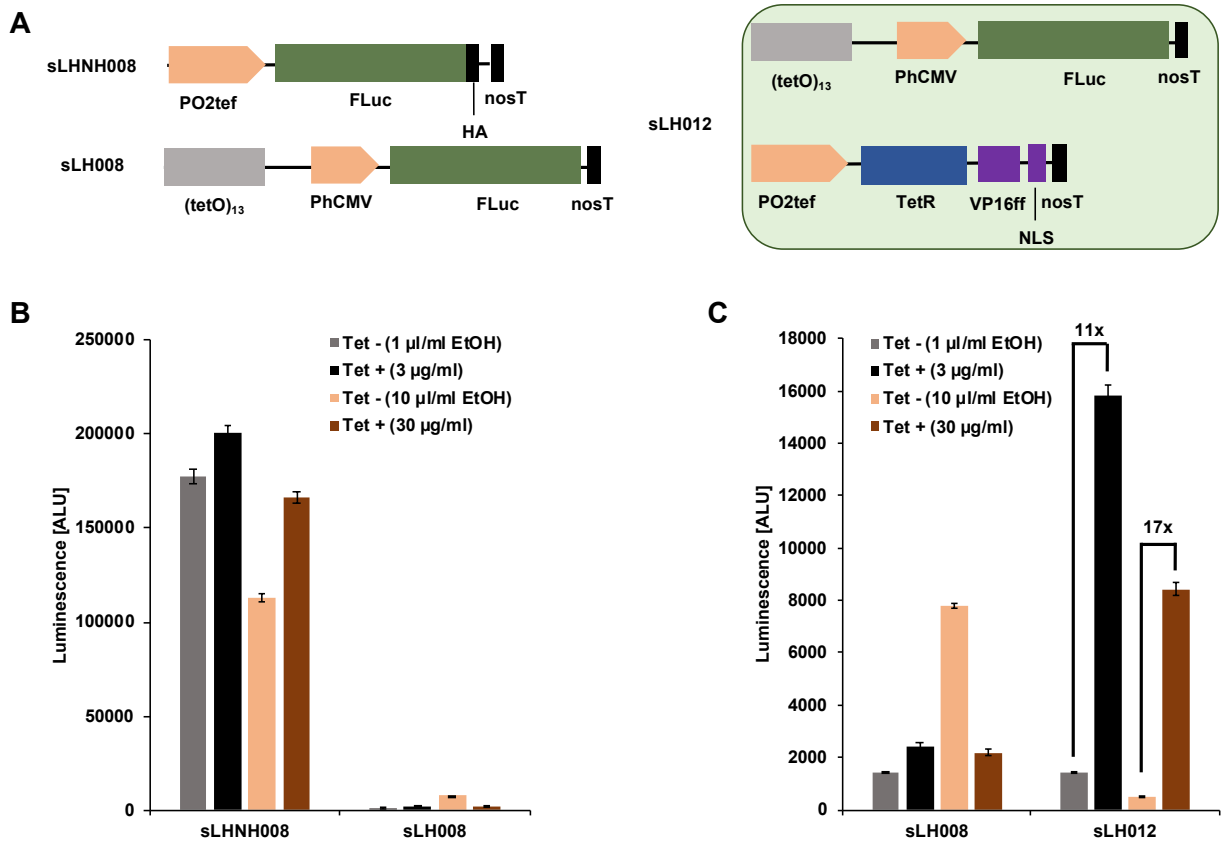

Figure 15: Tetracycline-controlled gene expression in *U. maydis.* (A) For tetracycline-controlled gene expression, strain sLH012 was generated. This strain contains the TetR operator sequence $(tetO)_{13}$ upstream of a P_{hCMV} minimal promotor, controlling the expression of FLuc as a reporter gene stably integrated in the upp3 locus. In the cco1 locus, the Tet-repressor (TetR) with a C-terminally fused VP16ff transactivation domain from *Herpes simplex* and a nuclear localization sequence (NLS) is integrated under control of the constitutive P_{O2tef} promotor. The strain sLH008 served as a negative control, containing only the operator sequence $(tetO)_{13}$ upstream of the P_{hCMV} minimal promotor and the firefly reporter. As a positive control sLHNH008 was used, expressing firefly constitutively under control of the P_{O2tef} promotor. (B) Tetracycline-controlled gene expression in control strain sLHNH008 and sLH008. Tetracycline was added at a concentration of 3 µg/ml or 30 µg/ml respectively. (C) Chemically controlled gene expression in the negative control strain (sLH008) and the Tet-system-containing strain sLH012. Tetracycline was added at a 3 µg/ml or 30 µg/ml concentration. The reporter activity in all strains was measured in cultures normalized to an OD_{600} of 0.5, performing the firefly-based fast screening method (see 3.1.2). The shown data are means of three biological and three technical replicates.

Figure 16: Comparison of the amino acid sequence of commercially available tTA and the utilized TetR for *U. maydis*. In position 2, a serine is changed to an alanine. Additionally, the *U. maydis* TetR comprises a C-terminal nuclear localization sequence (NLS) (PKKKRKV) and a 45 amino acid sequence of unknown function.

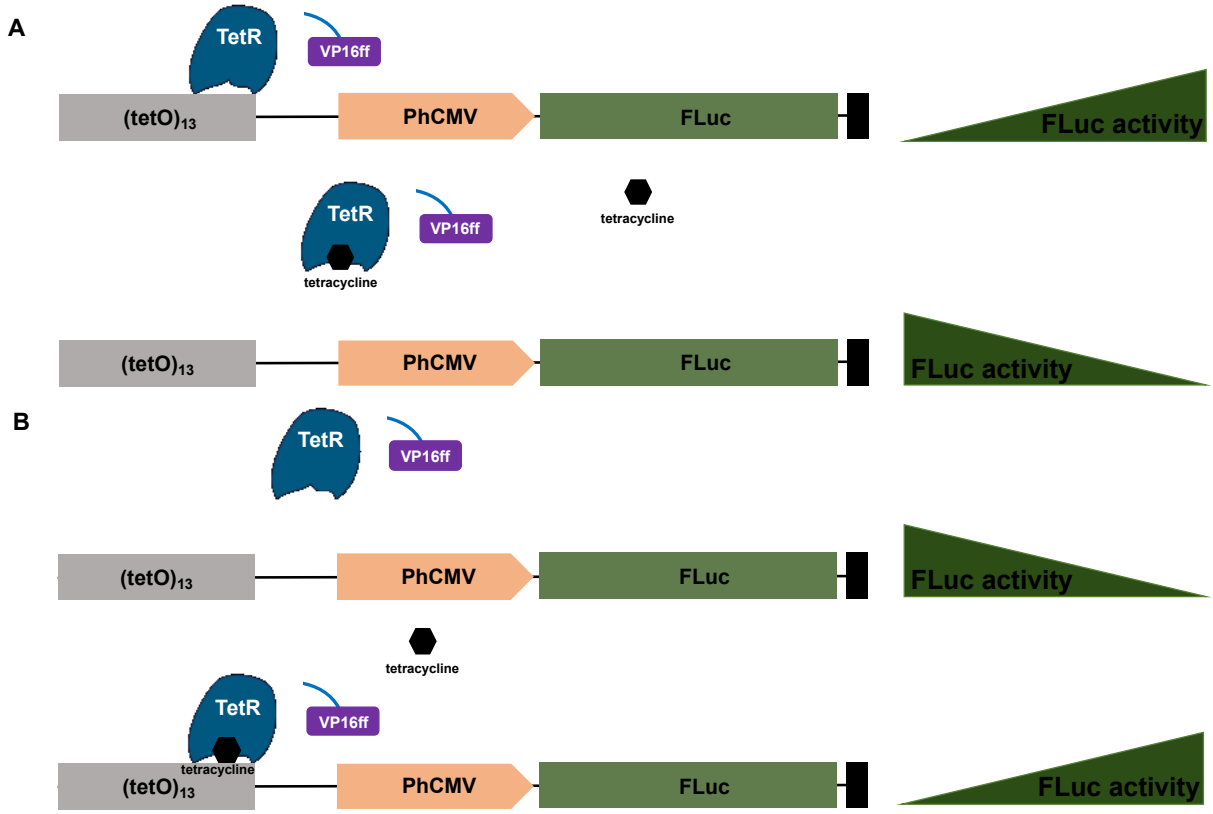

Figure 17: The Tet-Off and the Tet-On system function in a reverse manner in the presence of tetracycline. (A) Mode of function of the Tet-Off system, as it was expected. In this, the activity of the firefly reporter (FLuc) increases in the absence of tetracycline and decreases in the presence of the antibiotic. (B) Functionality of a Tet-On system. In the absence of tetracycline, TetR is not able to bind to its operator sequence $(tetO)_{13}$ and the activity of FLuc decreases. The addition of tetracycline induces the binding of TetR and consequently leads to an increase in reporter gene expression and activity.

3.1.6.2 Establishment of an E-protein-based, erythromycin-dependent transcriptional control system in *Ustilago maydis*

The functionality of chemically regulated gene expression systems is highly variable when compared in different eukaryotic systems. As we aimed to establish a system showing a high level of controllability upon drug addition and a low level of basal activity (leakiness), we compared two different system regarding these characteristics in *U. maydis.*

The second system we established in the course of this work, is an erythromycin-regulated gene expression system based on the *E. coli* repressor protein (E-protein) and its operator sequence (ETR). This erythromycin-controllable gene expression switch was first established in 2002 in mammalian cells (Weber et al., 2002), and in 2014 the functionality of the system in plants was demonstrated by Müller et al. (Müller et al., 2014c). We designed this system in the same manner as the Tet-system described in 3.1.5.1, with a short version of the VP16 transactivator fused to the E-Protein, and firefly as a quantitative reporter (Figure 4). In the absence of the macrolide antibiotic, the E-protein binds to its operator sequence $(etr)_8$, thereby inducing the expression of firefly. Upon addition of 10 µg/ml erythromycin, E-protein binding is prevented, leading to a decreased reporter expression (3-fold decrease). The collected data (Figure 18) suggest a high level of functionality of this system in consideration of controllability and leakiness. To further characterize this system in *U. maydis*, time- and dose-kinetics need to be performed to validate optimal conditions regarding specific applications of the system. As it is known that in plant cells, chlarithromycin performs much better than erythromycin as an inducer, perhaps because it diffuses through the cell wall and membrane more efficiently, it would be interesting to test this antibiotic as an inducer in *U. maydis* to improve the dynamic range of the system.

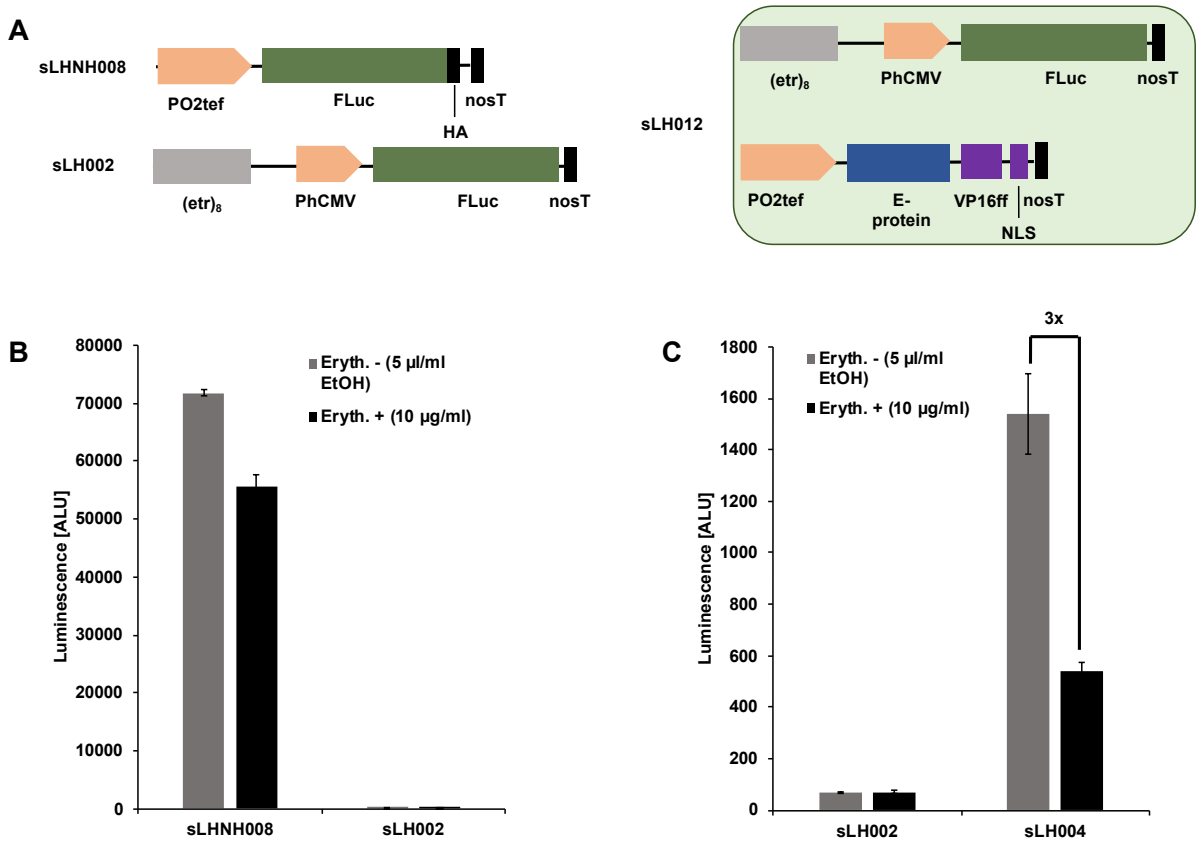

Figure 18: Erythromycin-controlled gene expression in *U. maydis*. (A) For erythromycin-controlled gene expression, strain sLH004 was generated. This strain contains the E-protein operator sequence $(etr)_8$ upstream of a P_{hCMV} minimal promotor, controlling the expression of firefly luciferase (FLuc) as a reporter gene, stably integrated in the upp3 locus. In the cco1 locus, the E-protein with a C-terminally fused VP16ff transactivation domain from *Herpes simplex* and a nuclear localization sequence (NLS) is integrated under control of the constitutive P_{O2tef} promotor. The strain sLH002 served as a negative control, containing only the operator sequence $(etr)_8$ upstream of the P_{hCMV} minimal promotor and the firefly reporter. As a positive control, sLHNH008 was used, expressing firefly constitutively under control of the P_{O2tef} promotor. (B) Erythromycin-controlled gene expression in control strain sLHNH008 and sLH002. Erythromycin was added in a concentration of 10 µg/ml. (C) Chemically controlled gene expression in the negative control strain (sLH002) and the E-protein-system-containing strain sLH004. Erythromycin was added in a 10 µg/ml concentration. The reporter activity in all strains was measured in cultures normalized to an OD_{600} of 0.5, using the firefly-based fast screening method (see 3.1.2). The shown data are means of three biological and three technical replicates.

3.2 Implementation of optogenetic gene expression systems in *Ustilago maydis*

3.2.1 Design, construction and quantitative characterization of blue light-regulatable gene expression systems in *U. maydis*

The utilization of light as a regulating factor to control cellular events is one of the most outstanding accomplishments of synthetic biology. What makes light, and its corresponding systems such a strong tool for the investigation and understanding of biological processes, is the high spatial and temporal control that these systems provide. In contrast to chemical compounds, the application of light can be controlled more precisely regarding a specific area or time period. These advantages make it strongly desirable to implement such light-regulated gene expression switches in the basidiomycete fungus *U. maydis.*

The establishment of these optogenetic switches gives researchers a strong and versatile tool for basic research or biotechnology approaches.

In the course of this work, we aimed to construct, implement and characterize two blue light-regulated systems to control the expression of heterologous genes in *U. maydis.*

3.2.1.1 Establishment and quantitative characterization of a LOVpep/ePDZ-based blue light inducible gene expression system

Blue light sensing light-oxygen-voltage (LOV) domains can be found in prokaryotes (Losi, 2004), fungi and plants (Müller and Weber, 2013), and haven been utilized as core components of different optogenetic tools for the regulation of translocation or gene expression events. Here, we demonstrate the applicability of a LOVpep/ePDZ-based blue light-controllable gene expression system in *U. maydis.* The functionality of this system is described in Figure 5. Therefore, we generated strains containing the AsLOV2 of phototropin 1 from *Avena sativa* fused to the bacterial PIP-repressor protein (Fussenegger et al., 2000) and an engineered ePDZ domain fused to the viral transactivator VP16ff integrated in the cco1 locus. The expression of both fusion-proteins is controlled by a bidirectional CMV promotor (Andersen et al., 2010). In the upp3 locus, three repeats of the operator sequence of PIP, (Pir), upstream of a P_{hCMV} promotor, and firefly as a reporter were stably inserted.

This PIP/$(Pir)_n$-based system was tested by Nicole Heucken and is described in her dissertation. It shows a strong binding of PIP to its operator sequence $(Pir)_3$ and almost no leakiness of $(Pir)_3$ in combination with the P_{hCMV} minimal promotor alone.

To determine the functionality of this optogenetic system, *U. maydis* cultures were illuminated with blue light for different durations and in the presence of 1 or 10 µM of the co-factor FMN. As a read-out system, firefly-based fast screening was applied.

The obtained data (Figure 19) show a strong increase in firefly activity upon blue light exposure compared to the dark control. After 24 h of illumination with 20 µE and in the presence of 1 µM FMN we observed a 16-fold induction of reporter activity, with 10 µM FMN the induction is increased to 30-fold. Even though a further characterization of the system will be beneficial for future application, these results clearly demonstrate the functionality of this blue light-controllable gene expression system in *U. maydis.*

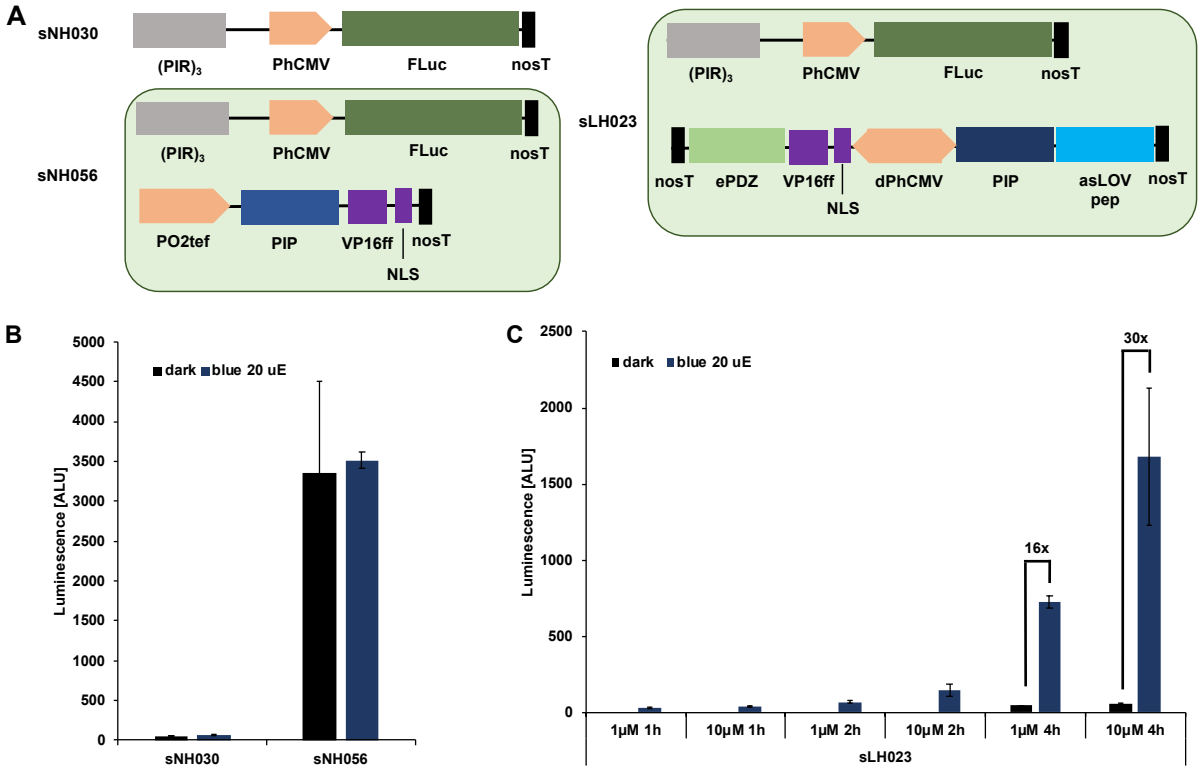

Figure 19: Blue light-controlled gene expression in *U. maydis.* (A) For blue light-controlled gene expression, strain sLH023 was generated. This strain contains the PIP operator sequence $(Pir)_3$ upstream of a P_{hCMV} minimal promotor, controlling the expression of firefly luciferase (FLuc) as a reporter gene, stably integrated in the upp3 locus. In the cco1 locus, a dP_{hCMV} bidirectional promotor is controlling the expression of an ePDZ-VP16-NLS-fusion in upstream direction, and a PIP-AsLOVpep-fusion in downstream direction. The strain sNH030 served as a negative control, containing only the operator sequence $(Pir)_3$ upstream of the P_{hCMV} minimal promotor and the firefly reporter (FLuc). sNH056 served as positive control for the system, containing $(Pir)_3$ upstream of the P_{hCMV} minimal promotor and firefly (FLuc) in the upp3 locus, and PIP-VP16-NLS under control of the constitutive P_{O2tef} promotor integrated in the cco1 locus. (B) Control strains sNH030 and sNH056 were illuminated with blue light for 4 h or kept in the dark. (C) Blue light-controlled gene expression in the LOVpep/ePDZ-system-containing strain sLH023. Cultures were either kept in the dark for 4 h or illuminated with blue light for 1, 2 and 4 h with an intensity of 20 µE. The LOV-domain co-factor FMN was added in a 1 µM or 10 µM concentration respectively. The reporter activity in all strains was measured in cultures normalized to an OD_{600} of 0.5, using the firefly-based fast screening method (see 3.1.2). The shown data are means of three biological and three technical replicates.

3.2.1.2 Implementation of a Sql1-EL222 Blue-Off and a VP16ff-EL222 Blue-On system

Many light-controllable gene expression systems depend on the pre-establishment of chemically-regulatable systems, based on a DNA-binding protein and its cognate DNA sequence.

In case of the LOVpep/ePDZ light switch for instance, the pristinamycin-controllable PIP/$(Pir)_n$ system was applied as a basis (see 3.2.1.1). To include a light-controllable switch in the *U. maydis* toolbox, which does not depend on such a base-system, we established EL222 Blue-On and Blue-Off switches, expanding the number and diversity of available optogenetic tools for this organism (Figure 6).

Therefore, we constructed strains comprising the EL222 domain from *Erythrobacter litoralis,* N-terminally fused to the VP16ff transactivator from *Herpes simplex,* or the Sql1 repressor from *Ustilago maydis* respectively (Loubradou et al., 2001). Additionally, these strains contain five repeats of the C120 operator sequence, $(C120)_5$, upstream of the P_{hCMV} minimal promotor and firefly as a reporter. As already demonstrated in this work (see 3.1.5), the basal activity of this system is very high, but still, the illumination with blue light for 6 or 24 h leads to a 1.5-fold or 2.5-fold increased reporter activity for the Blue-On system (Figure 20).

For the Blue-off-system, we could observe a 2.4-fold decrease after 6 h, and an 11-fold decrease after 24 h of blue light-exposure (Figure 20). With these data we could demonstrate the functionality and high level of controllability of a second blue light-inducible, and a novel blue light-repressible gene expression system in *U. maydis.*

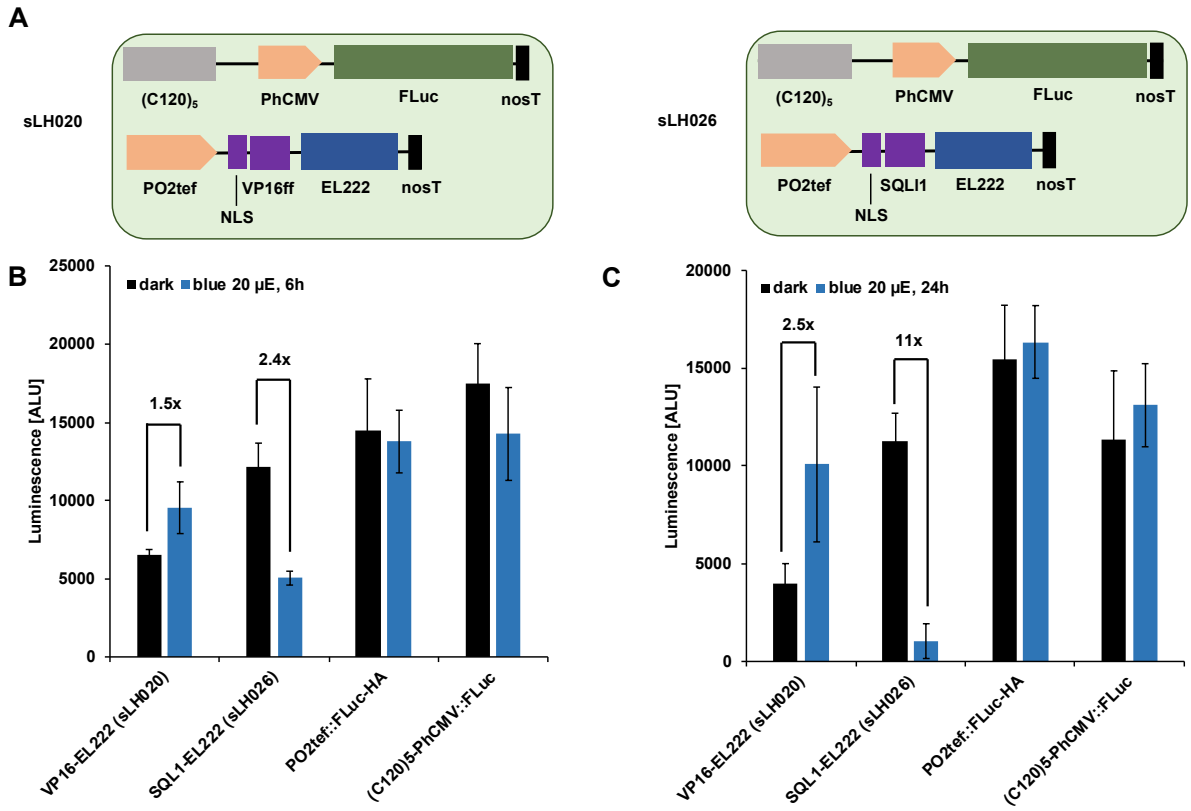

Figure 20: EL222-based blue light-controlled gene expression in *U. maydis*. (A) For blue light-controlled gene expression, strains sLH020 and sLH026 were generated, each containing two components: First, 5 repeats of the EL222 operator sequence (C120) upstream of a P_{hCMV} minimal promotor, controlling the expression of firefly luciferase (FLuc) as a reporter gene ("target component"), stably integrated in the upp3 locus. Second, a NLS-VP16ff-EL222-fusion (sLH020) or a NLS-Sql1-fusion (sLH026) controlled by a constitutive P_{O2tef} promotor ("switch component"). The Sql1 protein is a *U. maydis*-derived repressor protein. (B) Determination of reporter activity after sLH020 and sLH026 cultures were illuminated with blue light for 6 h with an intensity of 20 µE or kept in the dark. Strain sLH016, containing the $(C120)_5$ operator sequence upstream of the P_{hCMV} minimal promotor and the firefly reporter (FLuc) served as a negative control. The strain LHNH008, constitutively expressing firefly (FLuc), was used as a positive control. (C) Firefly (FLuc) activity of the sLH020 and sLH026 strains, measured after 24 h of illumination with 20 µE blue light (450 nm) or in the dark. The strains sLH016 and sLHNH008 served as negative and positive controls respectively. Cultures were normalized to an OD_{600} of 0.5. The shown data are means of three biological and three technical replicates.

3.3 Reconstruction of root hair formation-regulating plant signaling pathways in orthogonal mammalian cell systems

This work was done in a collaboration with Guido Grossmann and his PhD student Anna Denzler from the COS in Heidelberg.

The analysis of plant signaling events in their natural environment is extremely difficult, as the involved pathways are highly complex and strongly interconnected with other pathways and proteins. Therefore, the utilization of an orthogonal system gives researchers the possibility to study these signal transduction processes isolated from the influence of interacting proteins or pathways in their natural plant environment.

One of these processes that we aimed to reconstruct in a mammalian cell setting, to gain a better understanding of the interaction of the different players, is the formation of root hairs in *A. thaliana* (Figure 9). As some mechanisms of this root hair formation process resemble the establishment of cell polarity in other eukaryotes (Riquelme, 2013; Rounds and Bezanilla, 2013; Russell and Bashaw, 2017), the gained insights to this process could partly be extrapolated to other systems as well. To strongly reduce the complexity of the interplay of components involved in the root hair formation process, we aimed to isolate three key proteins that mediate this process in a recruitment- and activation-based manner: a small GTPase (ROP), and two guanine exchange factors (GEFs). It is known, that ROPs play a central role in the determination of the root hair initiation domain (RHID) and the polar cell outgrowth, but it was unclear for a long time, which proteins are responsible for targeting ROPs to the RHID and which molecular mechanisms are stimulating outgrowth induction. In 2019, Denninger et al. were able to demonstrate, that the site-specific positioning of involved proteins and the outgrowth are temporally separate events, and that these events are regulated by the guanine exchange factors GEF3 and GEF4 and the small GTPase ROP2 (Figure 9). To study the interaction of these key players in root hair formation in an orthogonal setting, we heterologously expressed these proteins in mammalian cells to analyze potential phenotypical changes.

3.3.1 Microscopic phenotype determination in plant GEF3, GEF4 and small GTPase ROP2 expressing HeLa cells

To develop a deeper understanding of how the interaction of GEF3, GEF4 and ROP2 leads to the induction of polar cell outgrowth, we transiently transfected HeLa cells with plasmids containing Lck-GEF3, Lck-GEF4 and ROP2-CAAX fused to different fluorescent proteins for microscopic cell localization studies. The Lck and CAAX domains were used for plasma membrane recruitment (Gao et al., 2009; Rawat et al., 2013). Cells were transfected with the three plasmids alone or in combination (Figure 21).

As a negative control, cells were transfected with a plasmid containing the red fluorescent protein mRuby tagged with a N-terminal Lck domain.

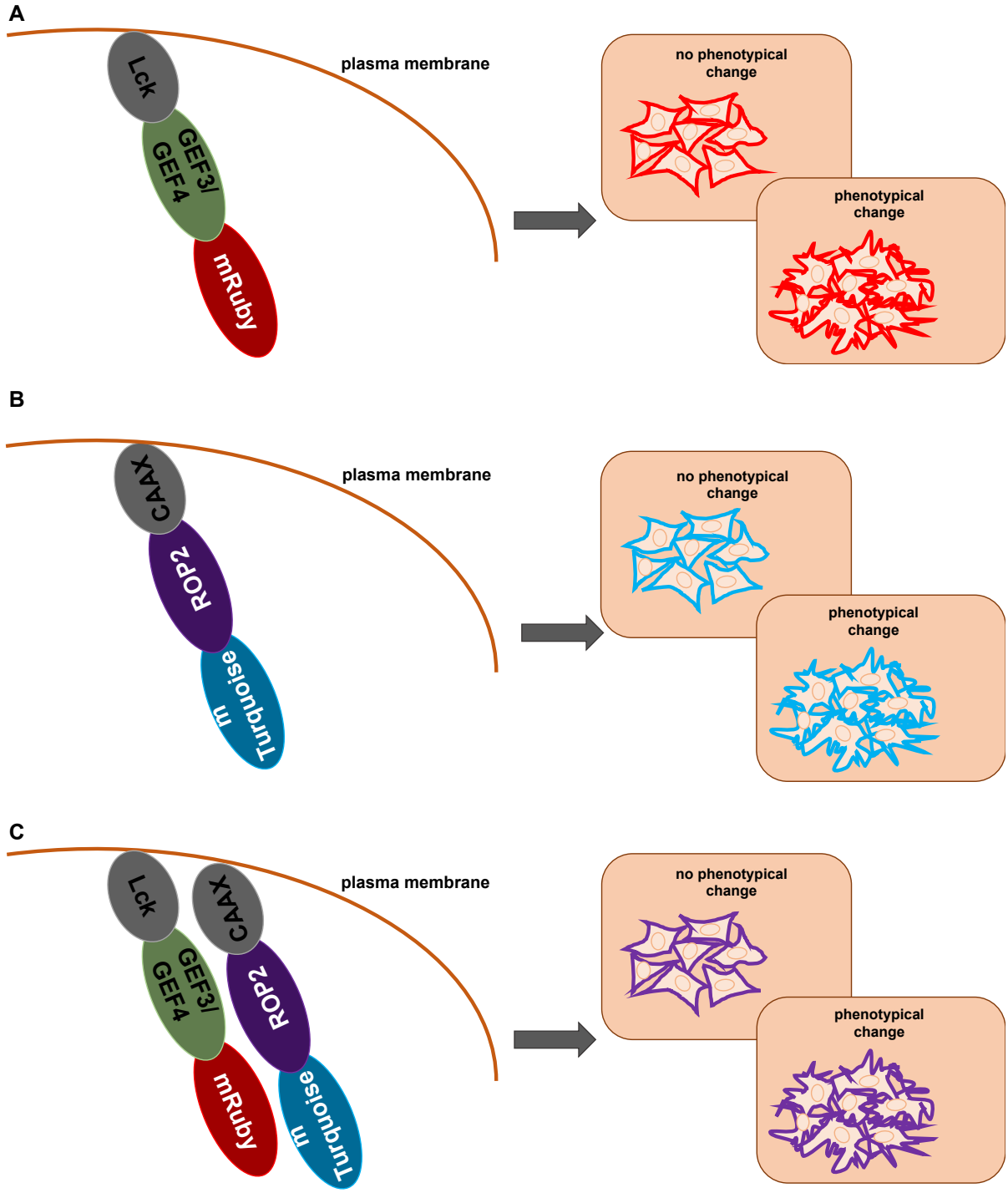

Figure 21: Experimental setup for the determination of phenotypical changes in response to the heterologous expression of plant GEF3 and 4 and the small GTPase ROP2 in mammalian cells. (A) HeLa cells were transfected with plasmids containing GEF3/GEF4 respectively with a N-terminally fused Lck domain and mRuby fused C-terminally, under control of P_{SV40}. After 24 h of incubation, the cells were fixed and the phenotype was analyzed under the microscope. (B) HeLa cells were transfected with mTurquoise-ROP2-CAAX constructs and the phenotype was microscopically determined. (C) HeLa cells were transfected with the three constructs in combination: Lck-GEF3-mRuby in combination with mTurquoise-ROP2-CAAX, and Lck-GEF4-mRuby in combination with mTurquoise-ROP2-CAAX.

The obtained results for the single transfections show a strong phenotype with extensive lamellipodia-like structure formations for both GEFs (Figure 22). In plants, the guanine exchange factors recruit and activate small GTPases within the cellular event of root hair formation. This process also serves as a model for polar cell outgrowth in other eukaryotes, and it leads to the suggestion, that the heterologously expressed plant GEFs are interacting with endogenous mammalian cell small GTPases, thereby inducing cytoskeletal remodeling and the formation of lamellipodia-like polar structures. These observations also imply, that both GEFs, GEF3 and GEF4, are able to activate small GTPases when they are isolated from their natural plant environment. This is different from what can be observed in plants. Here, GEF3 appears to be responsible for the recruitment of the small GTPase ROP2 within the process of root hair formation, whereas GEF4 is responsible for its activation (Denninger et al., 2019). This leads to the hypothesis, that the different roles of GEF3 and GEF4 in root hair formation are determined by their chronological appearance during this process, and that this temporal appearance is regulated by factors which are still unknown.

The finding, that plant GEFs are interacting with mammalian cell small GTPases is surprising because, even though these mechanisms are strongly conserved among eukaryotes, the 14 GEFs that have been identified in *A. thaliana* have a plant-specific ROP nucleotide exchanger (PRONE) domain, whereas GEFs from animals can be divided in groups containing either a DH or a DOCKER domain (Gu et al., 2006). Therefore, an interaction between plant GEFs and small GTPases from animals appears rather unlikely. However, an overexpression of these plant proteins seemed to induce cell outgrowth events in mammalian cells. The overexpression of plant ROP2 also caused a strong lamellipodia-forming phenotype (Figure 22), but this finding is rather expected, due to the high degree of conservation between ROPs and small GTPases from animals and yeast (Nagawa et al., 2010). When co-transfecting HeLa cells with plasmids containing GEF4 and ROP2, this results in the formation of GEF-aggregate-like structures and a weakened phenotype. This weakened phenotype could also be observed when co-transfecting GEF3 and ROP2 (Figure 23). This leads to the assumption, that ROP2 is the favored interaction partner of both GEFs, which is not surprising, but it also shows, that the activation of ROP2 by GEF3 and GEF4 is less efficiently inducing the formation of lamellipodia-like structures, than an interaction of plant GEFs and ROP2 with mammalian cell small GTPases and GEFs. Even though these plant proteins are capable of inducing this process, when expressed alone.

The results that were obtained in this work give a first impression of the role that plant GEFs play in the process of polar cell outgrowth in HeLa cells. But as a more quantitative approach to analyze the generated data, Anna Denzler from the group of Guido Grossmann at the COS in Heidelberg is analyzing images from this work to measure the cell surface increase upon overexpression of GEFs compared to the negative control.

Additionally, further experiments are planned to visualize the lamellipodia formation by applying actin filament staining. As a third approach, the application of small GTPase inhibitors could be of interests, to identify the mammalian cell interaction partner of the plant derived GEFs. Taken together, these current and future results will give novel insight into the molecular mechanisms coordinating the polar outgrowth of cells, and the level of conservation of these mechanisms among eukaryotes.

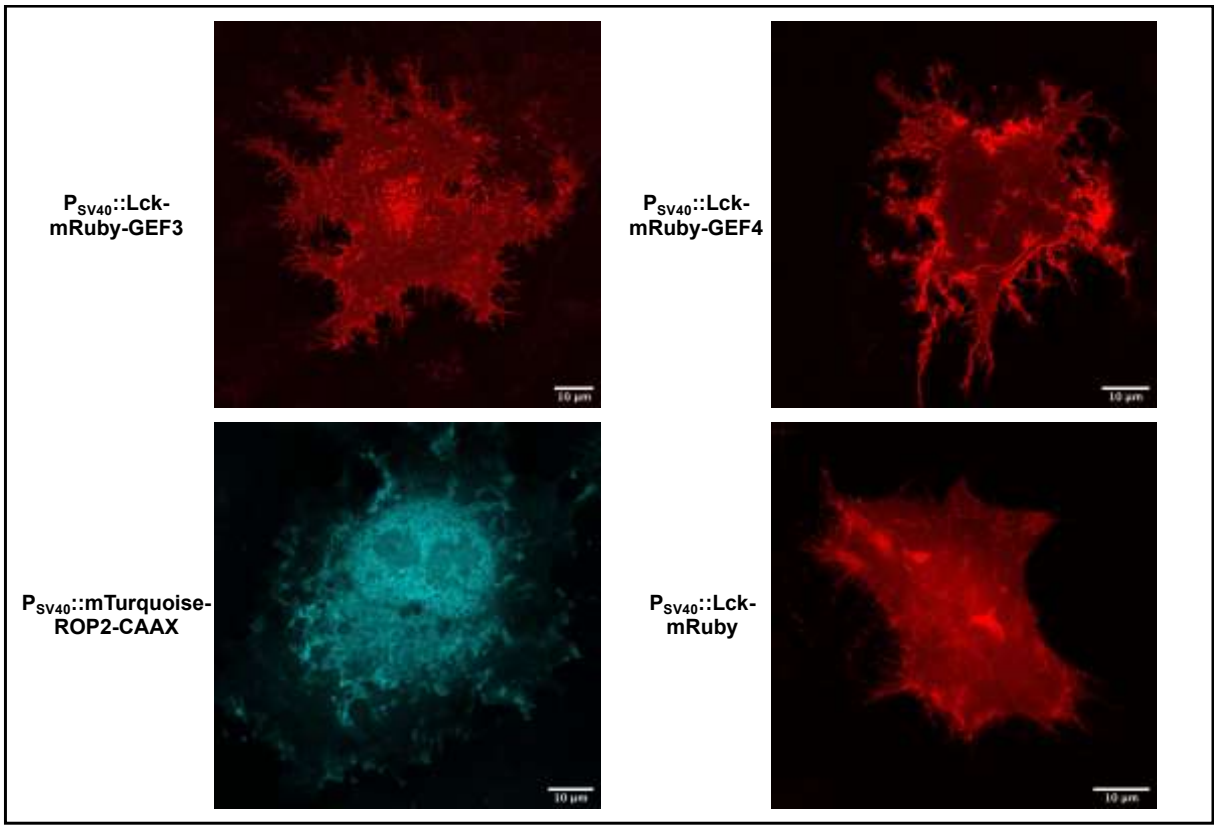

Figure 22: Overexpression of plant GEF3, GEF4 and ROP2 in HeLa cells. HeLa cells were transiently transfected with plasmids containing either GEF3 or GEF4, N-terminally fused to the fluorescent protein mRuby and a Lck sequence for plasma membrane recruitment, and plasmids with ROP2, fused N-terminally to the blue fluorescent protein mTurquoise, and C-terminally to a CAAX motif for plasma membrane recruitment of the fusion protein. In all constructs P_{SV40} is controlling the expression of the fusion proteins. Cells were fixed with paraformaldehyde 24 h post transfection.

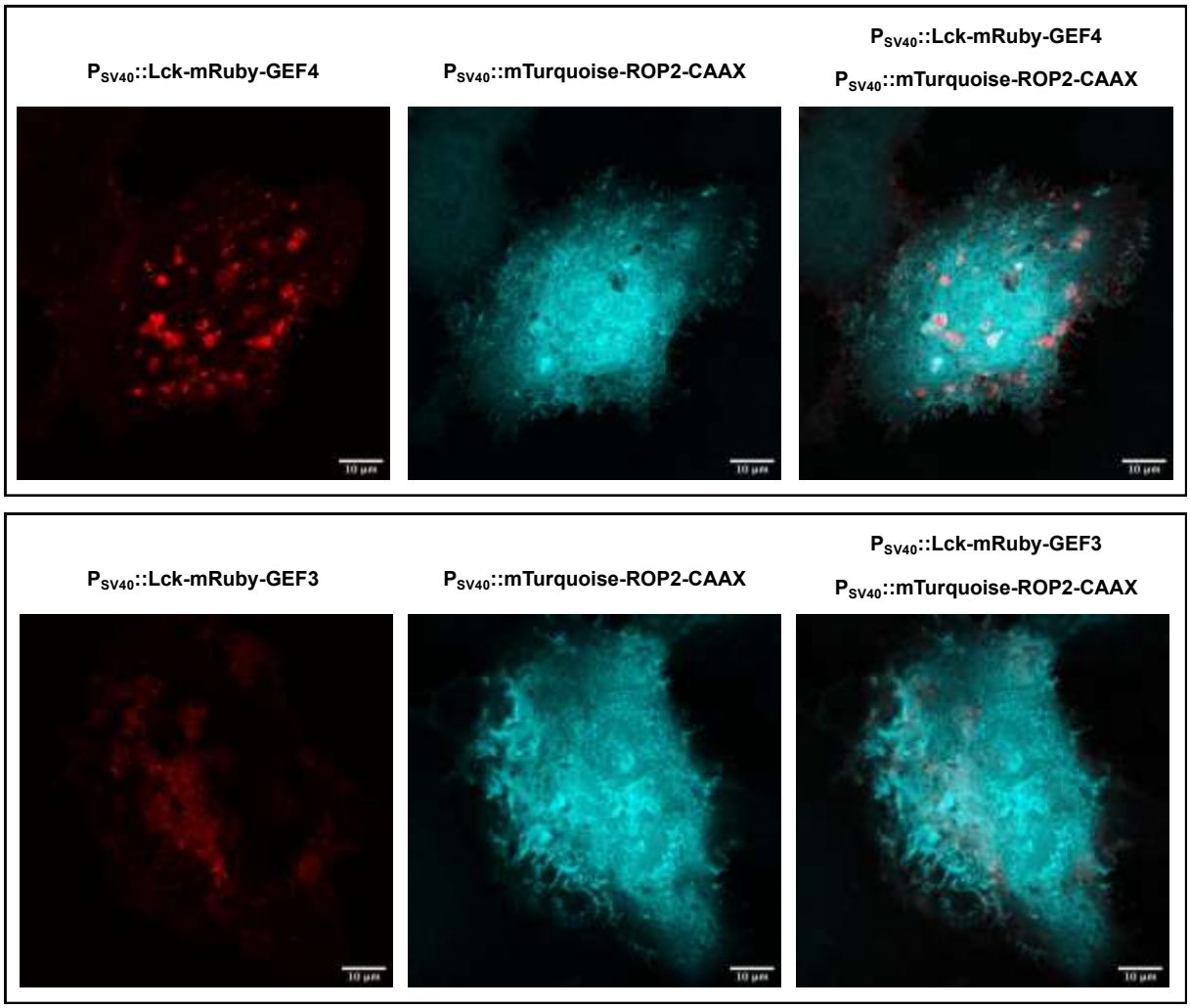

Figure 23: Simultaneous overexpression of plant GEFs and ROP2 in HeLa cells. HeLa cells were transiently co-transfected with plasmids containing either GEF3 or GEF4 and ROP2. For the construction of GEF-containing plasmids, GEFs were N-terminally fused to the fluorescent protein mRuby and a Lck sequence. ROP2 was fused to mTurquoise and a C-terminal CAAX motif. In all constructs P_{SV40} is controlling the expression of the fusion proteins. Cells were fixed with paraformaldehyde 24 h post transfection.

4 Conclusions

Summarizing this work and the obtained results, we can conclude, that a multitude of synthetic molecular tools and systems for the application in *Ustilago maydis* were designed and implemented, and that the utilization of an orthogonal mammalian cell platform was successfully applied to delivery new insights into conserved mechanisms controlling the polar outgrowth of cells in plants and animals.

In terms of implementing a synthetic biology toolbox in *U. maydis,* we tested and established different basic molecular tools, like IRES sequences and reporter genes, and implemented in total five controllable gene expression system. For a tetracycline repressor-based chemically controllable gene expression system, negatively regulated in the presence of tetracycline, we demonstrated a reverse mode of function, resulting in a Tet-On system with low basal activity and high induction folds upon antibiotic treatment. The second chemically controllable system implemented and tested in the course of this work, was the E-protein based gene expression system. This system was designed to show a reduction in gene expression upon erythromycin treatment. We established this system in *U. maydis,* demonstrating a high level of controllability, characterized by high expression levels in the absence, and strongly reduced expression levels in the presence of macrolide antibiotics.

As the application of light as a regulating factor for controlling gene expression provides a higher spatial and temporal control than the use of antibiotics or other drugs, we designed and implemented the first optogenetic tools ins *U. maydis,* namely three blue light-controllable gene expression systems. One is based on the utilization of the light-oxygen-voltage domain from *Avena sativa* (AsLOV2), and two are based on the EL222 domain from *Erythrobacter litoralis*. These EL222-based systems are designed as a Blue-On and a Blue-Off system, using either a VP16 transactivator or a Sql1 repressor respectively. For all three light-controllable systems we were able to demonstrate their functionality and high level of controllability in *U. maydis.* In summary, we accomplished the challenging and ambitious task to establish a synthetic biology toolbox in *U. maydis,* thereby providing highly versatile molecular tools and systems for a variety of applications in this promising microorganism.

The second aim that we followed in the course of this work, was the utilization of an orthogonal mammalian cell platform to study plant signaling pathways isolated from perturbing factors and unwanted cross-talk, present in their natural environment that preclude studying single components of these complex pathways. Using this synthetic biology approach, we aimed to gain a better understanding of the molecular mechanisms inducing and promoting the polar cell outgrowth in plant trichoblasts, leading to the formation of root hairs in *Arabidopsis thaliana*.

Therefore, we heterologously expressed key players of this pathway, namely GEF3, GEF4 and the plant small GTPase ROP2, in HeLa cells to study their interaction, and observed a strong phenotype with extensive lamellipodia-like structure formation even when the plant proteins are expressed separately.

These results interestingly indicate an interaction of plant GEFs and ROP2 with mammalian cell proteins, involved in polar cell outgrowth events in animal cells. As the plant specific interaction partners of GEF3, GEF4 and ROP2 are structurally highly diverse from their mammalian cell analogs, this finding is specifically interesting as it hypothesizes a strong degree of conservation underlying the process of polar cell outgrowth in eukaryotes.

5 Material and Methods

5.1 Synthetic biology toolbox implementation in *U. maydis*

5.1.1 Plasmid generation

Plasmids and oligonucleotides that were constructed in this work are listed and described in Table 2 and Table 3. Correctness of all plasmids was confirmed by sequencing.

All plasmids were generated with AQUA cloning or Gibson Assembly (Gibson et al., 2009; Beyer et al., 2015a). For all cloning procedures, *E. coli* Top10 cells were used. Transformation and plasmid isolation were performed using standard techniques. Heterologous genes were codon-optimized for expression in *U. maydis*.

5.1.2 Strain generation

Strains that were constructed in this work are listed and described in Table 4 and derive from the lab strain AB33 (Brachmann et al., 2001). Correctness of all strains was confirmed by southern blot analysis (Brachmann et al., 2004) or genotyping PCR.

Strains were generated by transforming progenitor strains with linearized plasmids using homologous recombination with the cco1 or upp3 locus. For sequence integration in the cco1 locus, plasmids were digested with the restriction enzyme SspI. For integration in the upp3 locus plasmids were digested with SwaI. Samples that were digested with SwaI were incubated at 65 °C for 20 min to inactivate the enzyme. For SppI digested samples the plasmid DNA was cleaned-up and isolated using a DNA clean up kit (NEB).

5.1.2.1 *U. maydis* protoplast preparation

The preparation of *U. maydis* protoplasts was performed as described in Bösch et al., 2016. Briefly, pre-cultures in 3 ml complete medium (CM) supplemented with 1 % glucose (0.25 % (w/v) casaminoacids (Difco), 0.1 % (w/v) yeast extract (Difco), 1.0 % (v/v) vitamin solution and 6.25 % (v/v) salt solution from Holliday (Holliday, 1974), 0.05 % (w/v) Deoxyribonucleic acid from herring sperm (Sigma Aldrich) and 0.15 % (w/v) NH_4NO_3 (Sigma) adjusted to pH 7.0 with NaOH) were diluted in 50 ml CM after 24 h incubation at 28 °C. Cells were grown to an OD_{600} of 0.6 to 1.0 and checked microscopely for contaminations. Cells were pelleted and resuspended in SCS buffer (solution 1 (20 mM tri-sodium citrate*2 H2O and 1 M sorbitol (Roth)) and solution 2 (20 mM citric acid*H2O and 1 M sorbitol) were prepared with ddH2O). Solution 2 was added to solution 1 until pH 5.8 was reached; autoclaved) and pelleted again. The pellet was resuspended in 2 ml protoplasting solution (100 mg/pellet Trichoderma lysing enzyme (Sigma Aldrich) solved in 4 ml SCS). After 20 to 30 min of incubation, cells were washed with cold (4 °C) SCS buffer.

After the last washing and centrifugation step, cells were resuspended in cold STC buffer (1 M sorbitol, 10 mM Tris-HCl pH 7.5, 100 mM $CaCl_2$; filter sterile), centrifuged again, and then resuspended in 1 ml cold STC. Protoplasts were stored in 100 µl aliquots at -80 °C.

5.1.2.2 *U. maydis* transformation

The transformation of *U. maydis* protoplasts with linearized plasmids was performed as described in Bösch et al., 2016. Briefly, RegLight bottom plates (1 % (w/v) yeast extract (BD), 0.4 % (w/v) bacto peptone (BD bioscience), 0.4 % (w/v) sucrose (Roth), 18.22 % (w/v) sorbitol (Roth), 1.5 % (w/v) agar (BD bioscience)) with selecting antibiotics (400 µg/ml hygromycine B-solution (Roth) and 300 µg/ml nourseothricin dihydrogen sulfate (Werner BioAgents)) were prepared. Protoplasts were thawed on ice and 1 µl heparin (15mg/ml) (Sigma Aldrich) and plasmid DNA (~ 1 µg) were added. During 10 min incubation on ice, RegLight top plates were prepared. 500 µl STC/PEG solution (40 % (v/v) polyethylene glycol PEG (Sigma Aldrich) in STC buffer) were added to the protoplasts and the mix was incubated on ice for another 15 min before carefully being distributed on two RegLight plates. Plates were incubated for 7 to 10 days at 28 °C. The obtained colonies were re-streaked on CM-antibiotic plates for counter-selection (200 µg/ml hygromycin, 150 µg/ml nourseothricin, 2 µg/ml carboxin (Sigma Aldrich) and 500 µg/ml Geneticin disulfate salt (G-418) (Sigma Aldrich)). For integration in the cco1 locus, hygromycin resistance was inserted, replacing the G-418 resistance gene. For the upp3 locus, nourseothricin resistance was replacing the carboxin resistance.

5.1.2.3 Genomic DNA (gDNA) preparation

For the preparation of gDNA, 3 ml CM-glucose cultures were inoculated with the strain that needs to be verified and incubated at 28 °C for 24 h. The next day, 2 ml of the cultures were transferred into reaction tubes and the cells were pelleted. The supernatant was discarded and 1 scoop of glass beads (~200 µl) and 500 µl of gDNA lysis buffer (10 mM Tris-HCl (pH 8.0), 10 mM NaCl, 1 % (w/v) SDS, 2 % (v/v) TritonX-100, 1 mM EDTA mixed with 1 x TE buffer (1.31 mM Tris-Base, 8.69 mM Tris-HCl, 10 mM Na2-EDTA*2H2O) in a 1:1 ration) were added to the pellet. The samples were shaken at 1500 rpm for 15 min. Afterward, the tubes were incubated at 65 °C for 20 min and then placed on ice for 5 min. Subsequently, 100 µl of 8 M potassium acetate were added, the tubes were inverted several times and then centrifuged at 12,000 *g* for 15 min. The supernatant (500 µl) was transferred into 1.5 ml reaction tubes containing 500 µl isopropanol. The tubes were inverted and centrifuged again (12,000 *g* for 5 min). The supernatant was soaked off completely and the pellet was dried at 50 °C for 4 min. Finally, 50 µl of TE/RNase (TE-buffer with 10 µg/ml RNaseA) were added and the samples were incubated at 50 °C and 400 rpm for 30 to 60 min.

5.1.2.4 Southern blot analysis for verification of successful genomic insertion

Southern blot analysis was performed as described in Brachmann et al., 2004; Bösch et al., 2016. Briefly, 15 µl of gDNA of mutant candidates and the progenitor strain were digested with restriction enzymes cutting outside the locus and in the integrated gene or resistance cassette. The obtained cutting pattern should be clearly distinguishable, with bands not larger than 10 kb. The upstream and downstream flanking sites were used as probes and labeled with a PCR DIG labelling Kit. The probes were mixed in a 1:1 ratio.

5.1.2.5 Genotyping PCR

For verification of a correct insertion into the *U. maydis* genome, genotyping PCR was performed according to the following protocol for one sample: 2.5 µl Taq polymerase buffer (Thermo Fisher), 0.25 µl Taq polymerase (Thermo Fisher), 1.5 µl dNTPs, 0.5 µl forward primer, 0.5 µl reverse primer, 1 µl $MgCl_2$, 1 µl DMSO, 16.75 µl ddH_2O and 1 µl gDNA (diluted 1:10). For amplification of a genomic sequence of 3,000 to 4,500 nucleotides, the following PCR program was used:

95 °C	2 min		
94 °C	20 s		
57 °C	20 s	ΔT -0.5 °C	10 x
72 °C	4 min		
94 °C	20 s		
52 °C	20 s		15 x
72 °C	4 min		
10 °C	∞		

Primers that were used for genotyping PCRs were designed to bind in the genome, upstream of the upstream flanking site (forward) and in the inserted sequence (reverse).

5.1.3 Luminescence determination and SEAP reporter assay

For all reporter gene assays, 5 ml CM-glucose cultures were inoculated with the reporter gene strains and incubated on a rotating wheel for 24 h at 28 °C.

5.1.3.1 *U. maydis* cell lysis for reporter gene assays

For lysis of *U. maydis* cells, cell pellets were resuspended in 1 ml lysis buffer supplemented with protease inhibitors (50 mM Tris-HCl pH 7,4, 150 mM NaCl, 0.5 mM EDTA pH 8.0, 0.5 % Nonident-P-40, 1mM PMSF, 1 mM DTT, 2.5 mM benzamidine and 200 µl complete protease inhibitor cocktail (Sigma Aldrich)). Afterwards, 2 scoops (~ 400 µl) glass beads were added and the samples were incubated at 4 °C and 1500 rpm for 20 min.

Then, the samples were centrifuged at 4 °C and 12,000 *g* for 5 min and the supernatant was transferred into 1.5 ml reaction tubes.

5.1.3.2 Firefly reporter assay

After 24 h of incubation, the cultures were adjusted to an OD_{600} of 0.5 in a total volume of 3 ml CM-glucose and then pelleted. Two times 1 ml of the supernatant were taken for luminescence determination in the supernatant, and for western blot analysis if intended, and then stored at -20 °C. The pellets were lysed (see 5.1.3.1) and the lysate was diluted 1:10 in lysis buffer. For determination of the luminescence in the lysate and the supernatant, 80 µl of diluted lysate or undiluted supernatant were pipetted in white 96-well assay plates. Before the measurement was started, 20 µl of firefly substrate (0.47 mM D-luciferin (Biosynth AG), 20 mM tricine, 2.67 mM $MgSO_4*7H_2O$, 0.1 mM $EDTA*2H_2O$, 33.3 mM dithiothreitol, 0.52 mM adenosine 5'-triphosphate, 0.27 mM acetyl-coenzyme A, 5 mM NaOH, 0.26 mM $MgCO_3*5H_2O$, in H_2O) was added to the samples. For determination of the firefly luminescence, a Berthold Technologies Centro XS^3 LB960 Microplate luminometer was used.

5.1.3.3 Renilla reporter assay

Renilla reporter gene assay was performed as described in 5.1.3.2. Instead of D-luciferin as a substrate, coelenterazine (472 mM coelenterazine stock solution in methanol) was used. The substrate was diluted in a 1:15 ratio in phosphate-buffered saline directly before use.

5.1.3.4 Gaussia reporter assay

Gaussia reporter gene assay was performed as described in 5.1.3.3.

5.1.3.5 SEAP reporter Assay

After 24 h of incubation, the cultures were adjusted to an OD_{600} of 0.5 in a total volume of 3 ml CM-glucose and then pelleted. Two times 1 ml of the supernatant were taken for luminescence determination in the supernatant, and for western blot analysis if intended. After the cell pellets were lysed (see 5.1.3.1), 100 µl of lysate and 100 µl of supernatant were pipetted in round bottom plates for heat inactivation of endogenous phosphatases at 65 °C for 1 h.
Afterward, 80 µl of the lysate and supernatant samples were transferred into a transparent 96 well assay plate, and mixed with 100 µl of SEAP buffer (20 mM L-homoarginine, 1mM MgCl2, 21 % (v/v) diethanolamine). Before the measurement was started, 20 µl of 120 nM para-Nitrophenylphosphate (pNPP, Sigma Aldrich) were added. The absorbance was measured in a Berthold technologies $Tristar^2S$ LB942 Multimode plate reader for 1 h at 405 nm.

To determine the SEAP activity [U/L], the following formula was used:

$$\frac{U}{L} = \frac{E}{\varepsilon * d} * 10^6 * \frac{200}{80}$$

E = increase in optical density/para-nitrophenolate per minute; ε = 18,600 M-1cm-1; d = length of the light path [cm], 0.6 cm; $\frac{200}{80}$ = amount of SEAP-containing sample/dilution factor of the sample

5.1.3.6 Firefly fast screening platform

For the establishment of a firefly luciferase based fast screening platform, 5 ml CM-glucose cultures of a firefly-containing strain were grown over 24 h at 28 °C. Afterward, 80 µl of the culture were transferred to white 96 well assay plates and 20 µl of firefly substrate were added. Then, the culture samples were incubated with the substrate for 1, 2, 4, 8, 15 and 30 minutes [addition of substrate to the 30 min-samples, 15 min later addition to the 15 min-samples, 7 min later addition to the 8 min-samples, 4 min later addition to the 4-min samples, 2 min later addition to the 2 min-samples and 1 min later addition to the 1 min-sample] before the measurement in a Berthold Technologies Centro XS^3 LB960 Microplate luminometer was started. After starting the measurement, the OD_{600} was measured, and the obtained values were calculated for an OD_{600} of 0.5.

5.1.3.7 Renilla/firefly normalization element

For establishment of a renilla/firefly normalization element luminescence assays were performed as described in 5.1.3.2 and 5.1.3.3. For induction of the Pcrg promotor the cells were shifted from CM-glucose to CM-arabinose medium.

5.1.4 Chemically controllable gene expression systems in *U. maydis*

The tetracycline (Tet-system) and erythromycin (E-protein-system) controllable gene expression systems were tested performing the firefly-based fast screening method described in 5.1.3.6. For the Tet-system, 4 ml CM-glucose cultures were supplemented with 3 µg/ml or 30 µg/ml tetracycline (3 mg/ml stock in ethanol (Sigma Aldrich)), for the E-protein-system, 4 ml cultures were supplemented with 10 µg/ml (2 mg/ml stock (Sigma Aldrich)).

To the negative control cultures, the same volume of ethanol was added, and the cultures were incubated at 28 °C for 24h.

5.1.5 Blue-light controllable gene expression systems in *U. maydis*

The functionality and controllability of blue light-regulated gene expression systems in *U. maydis* was determined using the firefly-based fast screening method described in 5.1.3.6.

5.1.5.1 Light boxes

The light boxes used in this work were constructed and used as described in Müller et al., 2014d.

5.1.5.2 Blue light treatment

For characterization of blue light-controllable gene expression systems in *U. maydis*, 3 ml Cm-glucose pre-cultures were inoculated and incubated at 28 °C for 24 h. The next day, 5 ml CM-glucose cultures in 6-well non-coated culture plates were inoculated with 2 µl of the pre-cultures. The cultures were supplemented with 1 µM or 10 µM flavin mononucleotide (FMN) and placed inside the light boxes. Here, the cultures were either kept in the dark or illuminated with blue light (460 nm, 20 µE) and incubated at 28 °C and 110 rpm.

5.2 Reconstruction of plant signaling pathways in orthogonal cell systems

For the reconstruction of plant signaling pathways human cervix carcinoma cells (HeLa) were used as an orthogonal mammalian cell system.

5.2.1 Plasmid generation

Plasmids and Oligonucleotides that were constructed in this work are listed and described in Table 2 and Table 3. Correctness of all plasmids was confirmed by sequencing.
All plasmids were generated with AQUA or Gibson cloning (Gibson et al., 2009; Beyer et al., 2015a). For all cloning procedures, *E. coli* Top10 cells were used. Transformation and plasmid isolation were performed using standard techniques.

5.2.2 Mammalian cell culture

HeLa cells were cultivated in DMEM medium (Dulbecco's modified Eagle's medium; PAN Biotech; cat. no. P04-03550). The culture medium was supplemented with FBS (10 % (v/v) tetracycline-free fetal bovine serum; PAN Biotech cat. no. P30-3602) and penicillin/streptomycin (1.4 % (v/v); PAN Biotech; cat. no. P06-07100).

5.2.3 Mammalian cell transfection

Prior to transfection, 30,000 HeLa cells/well were seeded in 500 µl DMEM cell culture medium on microscopy coverslips using 24-well plates (Corning). The cells were incubated at 37 °C and 5 % CO_2 for 24 h before being transfected with 0.75 µg DNA/well.

The DNA was diluted in 50 µl OptiMEM medium (Invitrogen) and 50 µl of PEI/OptiMEM mix (2.5 µl PEI solution (1 mg/ml; Polysciences Europe GmbH cat. no. 23966-1) in 50 µl OptiMEM) were added.
The transfection mixes were incubated at RT for 15 min before being added to the wells (100 µl/well). Afterward, the cells were incubated with the transfection mix for 4 h at 37 °C before the medium was exchanged to fresh DMEM cell culture medium.

5.2.4 Fixation of cells for microscopical analysis

For confocal imaging, cells were washed with phosphate-buffered saline (PBS) before fixation with paraformaldehyde (4 %, PFA) for 10 min on ice, and another 10 min at RT. Subsequently, the PFA was removed and the cells were washed once with PBS before each coverslip is placed upside-down onto a drop (8 µl) of Mowiol 4-88 (supplemented with DABCO, glycerol and Tris-HCl pH 8.5; for further information see Mowiol 4-88 cat. no. 0713 protocol by Carl Roth GmbH) on a microscopy slide. The slides were stored at 4 °C after being dried at 37 °C for 30 to 45 min.

5.2.5 Confocal imaging

For confocal imaging of fixed cell samples, a Nikon eclipse Ti confocal microscope was used. All images shown in this work were taken with a 100x objective and are representing multiple biological and technical replicates.

Table 1: Excitation and detection wavelengths

fluorescent protein	excitation wavelength [nm]	detection wavelength [nm]
mRuby	561	490 to 550
mTurquoise	405	410 to 470

5.2.6 Software

Geneious 10.2.2 for *in silico* cloning
Microsoft Exel 16.16.20 for graphs and statistical analysis
Microsoft PowerPoint 16.16.20 for graphical design

5.3 Plasmids

Table 2: Generation and description of plasmids used in this work. All plasmids were generated using AQUA cloning or Gibson Assembly (Gibson et al., 2009; Beyer et al., 2015a), **unless indicated otherwise.**

Plasmids	Description	Reference
pUMa047	**$(tetO)_6$-P_{mfa1}-eGFP-nosT** Vector encoding eGFP under control of TetR operator (tetO)6 and P_{mfa1} minimal promotor	Institute for Microbiology (University of Düsseldorf)
pUMa2055	**P_{tef}-TetR-NLS-VP16ff-nosT** Vector encoding TetR-NLS-VP16ff fusion under control of P_{tef} promotor	Institute for Microbiology (University of Düsseldorf)
pUMa2977	**mKate-HA** Vector encoding mKate-HA fusion	Institute for Microbiology (University of Düsseldorf)
pUMa3132	**P_{O2tef}-eGFP-nosT** Vector encoding eGFP under control of P_{O2tef} promotor	Institute for Microbiology (University of Düsseldorf)
pUMa3651	**P_{O2tef}-eGFP-nosT** Vector encoding eGFP under control of P_{O2tef} promotor	Institute for Microbiology (University of Düsseldorf)
pHB109	**5'LTR-psi-RRE-P_{hCMV}-PhyB(1-908)-L-mCherry-NES-3'LTR** Vector encoding PhyB(1-908)-linker-mCherry-NES fusion under control Rev Response Element (RRE) and P_{hCMV} minimal promotor	Hannes Beyer (University of Düsseldorf)
pKM006	**$(tetO)_{13}$-L-P_{hCMV}-SEAP-pA** Vector encoding SEAP under control of TetR operator $(tetO)_{13}$ and P_{hCMV} minimal promotor	(Müller et al., 2013)
pKM081	**$(etr)_8$-L-P_{hCMV}-SEAP-pA** Vector encoding SEAP under control of E-protein operator $(etr)_8$ and P_{hCMV} minimal promotor	(Müller et al., 2014)
pKM592	**$(C120)_5$-P_{hCMV}-Ubg-IaaH-IRES-PIP-KRAB-pA** Vector encoding Ubg-IaaH-IRES-PIP-KRAB under control of EL222 operator $(C120)_5$ and P_{hCMV} minimal promotor	University of Freiburg (Konrad Müller)
pLK002	**$(etr)_8$-P_{hCMV}-SEAP-BGHpA + P_{SV40}-RLuc-pA** Vector encoding SEAP under control of E-protein operator $(etr)_8$ and Renilla luciferase under control of P_{SV40}	Leonie-Alexa Koch (unpublished)

pMZ333	**P_{SV40}-PhyB(1-908)-L-mCherry-pA** Vector encoding PhyB(1-908)-linker-mCherry fusion under control P_{SV40} promotor	(Beyer et al., 2015)
pSW209	**P_{35S}-RLuc-2A-$(GA)_7$-FLuc-nosT** Vector encoding RLuc and FLuc separated by a 2A peptide under control of P_{35S}	(Beyer et al., 2015)
pPD0022	**SP6-mTurquoise-T7** vector encoding mTurquoise for *in vitro* expression under control of SP6 and T7 promotor	University of Heidelberg
pPD0095	**SP6-ROP2-T7** vector encoding ROP2 for *in vitro* expression under control of SP6 and T7 promotor	University of Heidelberg
pPD0293	**SP6-GEF4-T7** vector encoding GEF4 for *in vitro* expression under control of SP6 and T7 promotor	University of Heidelberg
pPD0331	**SP6-GEF3-T7** vector encoding GEF3 for *in vitro* expression under control of SP6 and T7 promotor	University of Heidelberg
pPD0358	**SP6-mRuby-T7** vector encoding mRuby for *in vitro* expression under control of SP6 and T7 promotor	University of Heidelberg
pNH009	**P_{O2tef}-mKate2-NES-pIRES-eGFP-NLS-nosT** Bicistronic vector encoding mKate2-NES and eGFP-NLS separated by a pIRES under control of P_{O2tef} promotor	Nicole Heucken
pNH010	**P_{O2tef}-mKate2-NES-eIRES-eGFP-NLS-nosT** Bicistronic vector encoding mKate2-NES and eGFP-NLS separated by an eIRES under control of P_{O2tef} promotor	Nicole Heucken
pNH011	**P_{O2tef}-mKate2-NES-fIRES-eGFP-NLS-nosT** Bicistronic vector encoding mKate2-NES and eGFP-NLS separated by a fIRES under control of P_{O2tef} promotor	Nicole Heucken
pNH023	**$(PIR)_3$-P_{hCMV}-FLuc-nosT** vector encoding firefly luciferase under control of the PIP operator sequence $(PIR)_3$ and P_{hCMV} minimal promotor	Nicole Heucken
pNH026	**P_{O2tef}-RLuc-pIRES-eGFP-FLuc-nosT** Bicistronic vector encoding renilla luciferase-NES and firefly luciferase-NLS separated by a pIRES under control of P_{O2tef} promotor	Nicole Heucken
pNH028	**P_{O2tef}-RLuc-eIRES-eGFP-FLuc-nosT** Bicistronic vector encoding renilla luciferase-NES and firefly luciferase-NLS separated by an eIRES under control of P_{O2tef} promotor	Nicole Heucken

pNH029	**P_{O2tef}-RLuc-fIRES-eGFP-FLuc-nosT** Bicistronic vector encoding renilla luciferase-NES and firefly luciferase-NLS separated by a fIRES under control of P_{O2tef} promotor	Nicole Heucken
pNH047	**nosT-AsLOV2pep-PIP-P_{hCMV}min-CMVenhancer(5'→3')-P_{hCMV}min-ePDZb-VP16ff-nosT** Bicistronic vector encoding AsLOV2pep-PIP and ePDZb-VP16ff under control of bidirectional P_{CMV} promotor	Nicole Heucken
pNH055	**P_{O2tef}-PIP-Sql1-NLS-nosT** Vector encoding PIP-Sql1-NLS fusion under control of P_{O2tef} promotor	Nicole Heucken
pLHNH001	**P_{O2tef}-GLuc-NLS-nosT** Vector encoding gaussia luciferase-NLS fusion under control of P_{O2tef} promotor	This work (cloned by Kira Müntjes)
pLHNH004	**P_{O2tef}-SEAP(1-221)-nosT** Vector encoding SEAP(1-221) under control of P_{O2tef} promotor. pLHNH001 was digested with MfeI/BglII. SEAP(1-221) was amplified from pLHNH020 with oNH048/oNH070.	This work
pLHNH005	**P_{O2tef}-FLuc-nosT** Vector encoding firefly luciferase under control of P_{O2tef} promotor. pLHNH001 was digested with MfeI/BglII. Firefly luciferase was amplified from pLHNH01 with oNH012/oNH067.	This work
pLHNH015	**P_{O2tef}-mKate2-HA-Pr2A-eGFP-L-NLS-nosT** Bicistronic vector encoding mKate2-HA and eGFP-linker-NLS separated by a Pr2A peptide under control of P_{O2tef}. pLHNH001 was digested with MfeI/PacI. Insert was amplified as fusion of mKate2-Pr2A and Pr2A-eGFP with oNH008 and oLH001. mKate2 was amplified from pUMa2977. eGFP was amplified from pUMa3132.	This work
pLHNH017	**geneArt (Invitrogen) synthesis of *U. maydis* codon optimized firefly luciferase**	This work
pLHNH018	**geneArt (Invitrogen) synthesis of *U. maydis* codon optimized gaussia luciferase**	This work
pLHNH019	**geneArt (Invitrogen) synthesis of *U. maydis* codon optimized renilla luciferase**	This work
pLHNH020	**geneArt (Invitrogen) synthesis of *U. maydis* codon optimized SEAP(1-221)**	This work
pLHNH021	**geneArt (Invitrogen) synthesis of *U. maydis* codon optimized E-protein and P65 transactivator**	This work
pLHNH023	**geneArt (Invitrogen) synthesis of *U. maydis* codon optimized EL222**	This work

pLHNH024	**geneArt (Invitrogen) synthesis of *U. maydis* codon optimized ePDZ and PIF6(1-100)**	This work
pLHNH028	**geneArt (Invitrogen) synthesis of *U. maydis* codon optimized PIP and LOVpep**	This work
pLHNH029	**P_{O2tef}-GLuc-L-NLS-nosT (geneArt (Invitrogen) synthesis)** vector encoding GLuc-NLS fusion under control of P_{O2tef} promotor	This work
pLHNH035	**geneArt (Invitrogen) synthesis of *U. maydis* codon optimized SEAP**	This work
pLHNH030	**P_{O2tef}-RLuc-HA-nosT** Vector encoding RLuc-HA fusion under control of P_{O2tef}. pLHNH001 was digested with MfeI/BglII. RLucwas amplified from pLHNH019 with oNH020/oNH116, adding HA-tag C-terminally to RLuc	This work
pLHNH031	**P_{O2tef}-GLuc-HA-nosT** Vector encoding GLuc-HA fusion under control of P_{O2tef}. pLHNH001 was digested with MfeI/BglII. GLuc was amplified from pLHNH018 with oNH016/oNH117, adding HA-tag C-terminally to GLuc	This work
pLHNH032	**P_{O2tef}-SEAP-HA-nosT** Vector encoding SEAP-HA fusion under control of P_{O2tef}. pLHNH001 was digested with MfeI/BglII. GLuc was amplified from pLHNH034 with oNH048/oNH132, adding HA-tag C-terminally to SEAP	This work
pLHNH033	**P_{O2tef}-FLuc-HA-nosT** Vector encoding FLuc-HA fusion under control of P_{O2tef}. pLHNH001 was digested with MfeI/BglII. FLuc was amplified from pLHNH017 with oNH012/oNH119, adding HA-tag C-terminally to FLuc.	This work
pLHNH034	**P_{O2tef}-SEAP-nosT** Vector encoding SEAP under control of P_{O2tef} promotor. pLHNH004 was digested with MfeI/BglII. SEAP was amplified from pLHNH035 with oNH048/oNH131.	This work
pLHNH100	**P_{35S}-RLuc-2A-$(GA)_7$-PEST-FLuc-nosT** Vector encoding RLuc and PEST-FLuc fusion separated by 2A peptide under control of P_{35S} promotor. pSW209 was digested with NheI. PEST sequence was introduced by assembly of 3 pre-annealed oligonucleotide paires (oLHNH001/oLHNH008, oLHNH002/oLHNH009, oLHNH003/oLHNH010).	This work

pLH100	**$(tetO)_{13}$-P_{hCMV}-SEAP(1-221)-nosT** Vector encoding SEAP(1-221) under control of TetR operator $(tetO)_{13}$ and P_{hCMV} minimal promotor. pLHNH004 digested with SbfI/MfeI. $(tetO)_{13}$ was amplified from pKM006 with oNH161/oLH028. PhCMV was amplified from pKM006 with oLH034/oNH091. $(tetO)_{13}$ and PhCMVmin were fused with oNH162/oNH085.	This work
pLH105	**P_{O2tef}-E-protein-VP16ff-NLS-nosT** Vector encoding E-protein-VP16ff-NLS fusion under control of P_{O2tef} promotor. pUMa3651 was digested with NcoI/AscI. E-Protein was amplified from pLHNH021 with oLH055/oLH042. VP16ff was amplified from pUMa2055 with oLH050/oLH058. E-Protein and VP16ff were fused with oLH041/oNH121.	This work
pLH106	**P_{O2tef}-TetR-P65-NLS-nosT** Vector encoding TetR-P65-NLS fusion under control of P_{O2tef} promotor. pUMa3651 was digested with NcoI/AscI. TetR was amplified from pUMa2055 with oLH056/oLH046. P65 was amplified from pLHNH021 with oLH049/oLH057. TetR and P65 were fused with oLH045/oNH120.	This work
pLH107	**P_{O2tef}-TetR-VP16ff-NLS-nosT** Vector encoding TetR-VP16ff-NLS fusion under control of P_{O2tef} promotor. pLH106 was digested with MfeI/AscI. TetR was amplified from pUMa2055 with oLH056/oLH110. VP16ff was amplified from pLH105 with oLH047/oLH158. TetR and VP16ff were fused with oLH045/oNH121.	This work
pLH110	**P_{O2tef}-NLS-P65-EL222-nosT** Vector encoding NLS-P65-EL222 fusion under control of P_{O2tef} promotor. pLH106 was digested with MfeI/AscI; EL222 was amplified from pLHNH023 with oLH145/oLH144. P65 was amplified from pLHNH021 with oLH151/oLH153. EL222 and P65 were fused with oLH155/oLH147.	This work
pLH111	**P_{O2tef}-NLS-VP16ff-EL222-nosT** Vector encoding NLS-VP16ff-EL222 fusion under control of P_{O2tef} promotor. pLH106 was digested with MfeI/AscI; EL222 was amplified from pLHNH023 with oLH146/oLH144; VP16ff was amplified from pUMa2055 with oLH152/oLH154; EL222 and VP16ff were fused with oLH156/oLH147.	This work
pLH112	**$(tetO)_{13}$-P_{hCMV}-FLuc-nosT** Vector encoding FLuc under control of TetR operator $(tetO)_{13}$ and P_{hCMV} minimal promotor. pLH100 was digested with MfeI/AscI. FLuc was amplified from pLHNH005 with oLH014/oLH018. FLuc with overhangs to pLH100 was amplified from FLuc PCR fragment with oNH184/oNH174.	This work

pLH113	**$(tetO)_{13}$-P_{mfa1}-FLuc-nosT** Vector encoding FLuc under control of TetR operator $(tetO)_{13}$ and P_{mfa1} minimal promotor. pLH112 was digested with MfeI/PacI. Pmfa1 was amplified from pUMa047 with oLH040/oLH037.	This work
pLH116	**$(etr)_8$-P_{hCMV}-FLuc-nosT** Vector encoding FLuc under control of E-protein operator $(etr)_8$ and P_{hCMV} minimal promotor. pLH112 was digested with SbfI/MfeI. $(etr)_8$ was amplified from pLK002 with oLH130/oLH131. $(etr)_8$ with overhang to P_{hCMV} was amplified from $(etr)_8$ PCR fragment with oLH130/oLH133. P_{hCMV} was amplified from pKM081 with oLH149/oLH054. $(etr)_8$ and P_{hCMV} were fused using oLH132/oLH031.	This work
pLH117	**$(etr)_8$-P_{mfa1}-FLuc-nosT** Vector encoding FLuc under control of E-protein operator $(etr)_8$ and P_{mfa1} minimal promotor. pLH116 was digested with MfeI/PacI. P_{mfa1} was amplified from pUMa047 with oLH150/oLH037.	This work
pLH120	**$(C120)_5$-P_{hCMV}-FLuc-nosT** Vector encoding FLuc under control of EL222 operator $(C120)_5$ and P_{hCMV} minimal promotor. pLH116 was digested with SbfI/PacI. $(C120)_5$ was amplified with overhangs including SbfI/PacI restriction sites from pKM592 with oLH140/oLH142. $(C120)_5$ PCR fragment was digested with SbfI/PacI. Parts were assembled via ligation with T4 ligase.	This work
pLH121	**$(C120)_5$-P_{mfa1}-FLuc-nosT** Vector encoding FLuc under control of EL222 operator $(C120)_5$ and P_{mfa1} minimal promotor. pLH117 was digested with SbfI/PacI. $(C120)_5$ was amplified with overhangs including SbfI/PacI restriction sites from pKM592 with oLH140/oLH142. $(C120)_5$ PCR fragment was digested with SbfI/PacI. Parts assembled via ligation with T4 ligase.	This work
pLH126	**P_{O2tef}-NLS-Sql1-EL222-nosT** Vector encoding NLS-Sql1-EL222 fusion under control of P_{O2tef}. pLH110 was digested with MfeI/PacI. Sql1 was amplified from pNH055 with oLH246/oLH247. Sql1 with overhangs to P_{O2tef} and EL222 was amplified from Sql1 PCR fragment with oLH248/oLH249.	This work
pLH400	**P_{SV40}-Lck-mRuby-LOVpep-pA** Vector encoding Lck-mRuby-LOVpep fusion under control of P_{SV40}. pMZ333 was digested with XhoI/XbaI. mRuby was amplified from pPD0358 with oLH166/oLH168. LOVpep was amplified from pKM292 with oLH169/oLH192. mRuby and LOVpep were fused with oLH167/oLH170.	This work

pLH403	**P_{SV40}-mTurquoise-SspBnano-pA** Vector encoding mTurquoise-SspBnano fusion under control of P_{SV40}. pMZ333 was digested with XhoI/XbaI. mTurquoise was amplified from pPD0022 with oLH163/oLH183. SspBnano was amplified from pMZ1274 with oLH186/oLH185. mTurquoise and SspBnano were fused with oLH182/oLH187.	This work
pLH406	**P_{SV40}-Lck-mRuby-GEF4-pA** Vector encoding Lck-mRuby-GEF4 fusion under control of P_{SV40}. pLH400 was digested with SbfI/XbaI. GEF4 was amplified from pPD0293 with oLH201/oLH202.	This work
pLH407	**P_{SV40}-Lck-mRuby-GEF3-pA** Vector encoding Lck-mRuby-GEF3 fusion under control of P_{SV40}. pLH400 was digested with SbfI/XbaI. GEF3 was amplified from pPD0331 with oLH203/oLH204.	This work
pLH409	**P_{SV40}-mTurquoise-ROP2-CAAX-pA** Vector encoding mTurquoise-CAAX fusion under control of P_{SV40}. pLH403 was digested with SbfI/XbaI. ROP2-CAAX was amplified from pPD0095 with oLH207/oLH208.	This work
pLH415	**P_{SV40}-Lck-mRuby-pA** Vector encoding Lck-mRuby fusion under control of P_{SV40}. pMZ333 was amplified with oLH239/oLH240. mRuby was amplified from pPD0358 with pLH238/oLH194	This work

The plasmids pNH009 – pNH055 were designed and cloned by Nicole Heucken.

The plasmids pLHNH001-pLHNH100 were designed and cloned together with Nicole Heucken.

5.4 Oligonucleotides

Table 3: Olidonucleotides used in this work

Oligo	Sequence (5'→3')	Description
oTB047	AAAGGGAATAAGGGCGAC	Fw AmpR
oROF299	GCATTCTAGTTGTGGTTTGTCC	Rev PSV40
oROF322	CCAGAAGTAGTGAGGAGGCTT	Fw PSV40
oNH008	CGGGATCCCCCGGGCTGCAGGAATTCGATCCCCAATTG ATGGTGTCGGAGCTCAT	Fw mKate2
oNH009	CAATAAAGGGCGCTGTCT	Fw PO2tef
oNH010	GTATAATTGCGGGACTCTAATCA	Rev nosT
oNH012	CGGGATCCCCCGGGCTGCAGGAATTCGATCCCCAATTG ATGGAGGACGCCAAGAA	Fw FLuc
oNH016	CGGGATCCCCCGGGCTGCAGGAATTCGATCCCCAATTG ATGGGCGTCAAGGTG	Fw GLuc
oNH020	CGGGATCCCCCGGGCTGCAGGAATTCGATCCCCAATTG ATGACCAGCAAGGTCTAC	Fw RLuc
oNH048	CGGGATCCCCCGGGCTGCAGGAATTCGATCCCCAATTG ATGGTGCTCGGTCCTT	Fw SEAP
oNH049	CCAGAAGTAGTGAGGAGGCTT	Fw PSV40
oNH067	GCCGGGCGGCCGGCGCGCCGGCCGCTAGATCTTTAGA CGGCGATCTTGC	Rev FLuc
oNH070	GCCGGGCGGCCGGCGCGCCGGCCGCTAGATCTTTAGT CGATGTCCATGTTCG	Rev SEAP
oNH085	AGCAGCATGCAAGGACCGAGCACCATCAATTGAGGCTG GATCGGTCC	Rev PhCMV
oNH091	AGGCTGGATCGGTCC	Rev PhCMV
oNH093	GTATTGGCATCAACATTCTGAATC	Fw upp3
oNH117	GCCGGGCGGCCGGCGCGCCGGCCGCTAGATCTTTAGG CGTAGTCGGGCACGTCGTAAGGGTAGAGCGGACCCTGG TCACCACCGGCAC	Rev GLuc
oNH119	GCCGGGCGGCCGGCGCGCCGGCCGCTAGATCTTTAGG CGTAGTCGGGCACGTCGTAAGGGTAGAGCGGACCCTGG ACGGCGATCTTGCC	Rev FLuc
oNH120	TGTTTGAACGATCGCCGGGCGGCCGGCGCGCCTTAGAC CTTTCTCTTCTTTTTTGGAGGCGCTTTCTTGTCGTCGTCG TCC	Rev P65
oNH121	TGTTTGAACGATCGCCGGGCGGCCGGCGCGCCTTAGAC CTTTCTCTTCTTTTTTGGAGGCGCTTTCAGCATATCCAGG TCGAAG	Rev VP16ff
oNH131	CGCCGGGCGGCCGGCGCGCCGGCCGCTAGATCTCTAT CCAGGGTGGGCG	Rev SEAP
oNH132	GCCGGGCGGCCGGCGCGCCGGCCGCTAGATCTTTAGG CGTAGTCGGGCACGTCGTAAGGGTAGAGCGGACCCTGC TATCCAGGGTGGGCG	Rev SEAP
oNH161	GTCGACGATATCCCTCGA	Fw $(tetO)_{13}$
oNH162	TCACCATAGCAGGCCTAGATGGCCCCTGCAGGGTCGAC GATATCCCTCGA	Fw $(tetO)_{13}$
oNH174	TTGCCAAATGTTTGAACGATCGCCGGGCGGCCGGCGCG CCGGCCGCTTTAGACGGCGATCTTGC	Rev FLuc
oNH184	CTCCATAGAAGACACCGGGACCGATCCAGCCTCAATTGA TGGAGGACGCCAAGAA	Fw FLuc

oNH731	CTTCGACGATGCTGTTCGTCGC	Fw upstream cco1
oNH733	CACGAGGTGATGCAGCGTCATTG	Fw upstream upp3
oLHNH001	TGCCGGGGCAGGCGCTGGCGCTAGCAAGCTCTCTCATG GATTCCCGCCAGCTGTAGCCGCTCAGGACGATGGA	Fw PEST
oLHNH002	TAGCCGCTCAGGACGATGGAACCCTACCCATGAGCTGC GCGCAAGAATCTGGCATGGATCGACATCCTGCAGC	Fw PEST
oLHNH003	ATGGATCGACATCCTGCAGCCTGCGCTTCCGCAAGGATT AACGTGGGCGCGCCATGGAAGACGCCAAAAACAT	Fw PEST
oLHNH008	CGCTGGAGCCGGTGCCGGGGCAGGCGCTGGCGCTAGC AAGCTCTCTCATGGATTCCCGCCAGCTGTAGCCGCTCAG GACGATGGAACCC	Rev PEST
oLHNH009	GCCAGCTGTAGCCGCTCAGGACGATGGAACCCTACCCA TGAGCTGCGCGCAAGAATCTGGCATGGATCGACATCCT GCAGCCTGCGCTT	Rev PEST
oLHNH010	TGGCATGGATCGACATCCTGCAGCCTGCGCTTCCGCAA GGATTAACGTGGGCGCGCCATGGAAGACGCCAAAAACA TAAAGAAAGGCCC	Rev PEST
oLH001	GCTTTGGCACCCGAGGCCGAGCCTTTAATTAACTTGTAC AGCTCGTCCATG	Rev eGFP
oLH014	ATGGAGGACGCCAAGAA	Fw FLuc
oLH018	TTAGACGGCGATCTTGC	Rev FLuc
oLH028	GCTAGCTCTTGAAGTTGGC	Fw $(tetO)_{13}$
oLH031	TTCTTGATGTTCTTGGCGTCCTCCATCAATTGAGGCTGG ATCGGTCC	Rev PhCMV
oLH034	GAACGGCATCAAGGCCAACTTCAAGAGCTAGCTTAATTA ACCTATATAAGCAGAGCTCGTTTAG	Fw PhCMV
oLH037	TTCTTGATGTTCTTGGCGTCCTCCATCAATTGGTGATAGA AGTAAGGTAGTTGATTTG	Rev Pmfa1
oLH040	GAACGGCATCAAGGCCAACTTCAAGAGCTAGCTTAATTA ACTAGTATTAGCAAGGCCTTTCC	Fw Pmfa1
oLH041	CCGGGCTGCAGGAATTCGATCCCCAATTGCATATGCCG CGTCCCAAG	Fw E-protein
oLH042	CGAGGCACCCGAACCGGCCGAGTAGGCCGAGGC	Rev E-protein
oLH045	CCGGGCTGCAGGAATTCGATCCCCAATTGCATATGGCG CGTCTCGAC	Fw TetR
oLH046	GAGACCCATCGCTCGG	Rev TetR
oLH047	GATGGGTCTCTTAATTAACTCCCCCGCCGATG	Fw VP16ff
oLH049	TTCACCAAGGGCGACGCCGAGCGATGGGTCTCTTAATTA ACCAGTACCTGCCCGAC	Fw P65
oLH050	GCCGGTTCGGGTGCCTCGTTAATTAACTCCCCCGCCGAT G	Fw VP16ff
oLH053	GTGATAGAAGTAAGGTAGTTGATTTG	Rev Pmfa1
oLH054	AGGCTGGATCGGTCC	Rev PhCMV
oLH055	ATGCCGCGTCCCAAG	Fw E-protein
oLH056	ATGGCGCGTCTCGAC	Fw TetR
oLH057	TTACTTGTCGTCGTCGTC	Rev P65
oLH058	TTACAGCATATCCAGGTCGAAG	Rev VP16ff
oLH110	GTTAATTAAGAGACCCATCGCTCGG	Rev TetR
oLH130	GAAGCATTTATCAGGGTTATTGTCTCATG	Fw $(etr)_8$

oLH131	CGGCAACTACAAGACCCG	Rev $(etr)_8$
oLH132	TCACCATAGCAGGCCTAGATGGCCCCTGCAGGGAAGCATTTATCAGGGTTATTGTCTCATG	Fw $(etr)_8$
oLH133	CTAAACGAGCTCTGCTTATATAGGTTAATTAACGGCAACTACAAGACCCG	Rev $(etr)_8$
oLH140	TCACCATAGCAGGCCTAGATGGCCCCTGCAGGGTGTGAATCGATAGTACTAAC	Fw $(C120)_5$
oLH142	GAGGAAAGGCCTTGCTAATACTAGTTAATTAAAAGCTTCATGGACTAAAG	Rev $(C120)_5$
oLH144	GATACCGGCCTCGACGG	Rev EL222
oLH145	ACTACAAGGACGACGACGACAAGTTAATTAATGGTGCCGACGACACG	Fw EL222
oLH146	ATGACTTCGACCTGGATATGCTGTTAATTAATGGTGCCGACGACACG	Fw EL222
oLH147	TTGAACGATCGCCGGGCGGCCGGCGCGCCTTAGATACCGGCCTCGACGG	Rev EL222
oLH148	CCTATATAAGCAGAGCTCGTTTAGTG	Fw PhCMV
oLH149	TCGGCGCGGGTCTTGTAGTTGCCGTTAATTAACCTATATAAGCAGAGCTCGTTTAGTG	Fw PhCMV
oLH150	TCGGCGCGGGTCTTGTAGTTGCCGTTAATTAACTAGTATTAGCAAGGCCTTTCCTC	Fw Pmfa1
oLH151	CAGTACCTGCCCGAC	Fw P65
oLH152	TCCCCCGCCGATG	Fw VP16ff
oLH153	TCGACGCGCGTGTCGTCGGCACCATTAATTAACTTGTCGTCGTCGTCC	Rev P65
oLH154	TCGACGCGCGTGTCGTCGGCACCATTAATTAACAGCATATCCAGGTCGAAGTC	Rev VP16ff
oLH155	CGGGATCCCCCGGGCTGCAGGAATTCGATCCCCAATTGATGAAAGCGCCTCCAAAAAAGAAGAGAAAGGTCCAGTACCTGCCCGAC	Fw P65
oLH156	CGGGATCCCCCGGGCTGCAGGAATTCGATCCCCAATTGATGAAAGCGCCTCCAAAAAAGAAGAGAAAGGTCTCCCCCGCCGATG	Fw VP16ff
oLH158	CAGCATATCCAGGTCGAAGTC	Rev VP16ff
oLH163	ATGGTGAGCAAGGGCG	Fw mTurquoise
oLH164	CTTGTACAGCTCGTCCATGC	Rev mTurquoise
oLH166	ATGGTGTCTAAGGGCGAAGAG	Fw mRuby
oLH167	AGGCTTTTGCAAAAAGCTCCCTCGAGATGGGGTGCTGGTGCAGTTCAAACCCAGAGGACGACATGGTGTCTAAGGGCGAAG	Fw mRuby
oLH168	GCCAATCCTGCAGGGCCGCTCAGATCAATGCCCTTGTACAGCTCGTCCATG	Rev mRuby
oLH169	ACAAGGGCATTGATCTGAGCGGCCCTGCAGGATTGGCTGCTGCACTTGAAC	Fw LOVpep
oLH170	GATCGAAGCTTGGGCTGCAGGTCGACTCTAGATTACACCCAGGTATCCACCG	Rev LOVpep
oLH182	AGGCCTAGGCTTTTGCAAAAAGCTCCCTCGAGATGGTGAGCAAGGGCG	Fw mTurquoise
oLH183	CGTTTCGGGGAGCTGAATTCCTGCAGGGTCAGCTTGTACAGCTCGTCCATGC	Rev mTurquoise
oLH185	TTAACCAATATTCAGCTCGTCATAGATTTC	Rev SspB
oLH186	GGATCACTCTCGGCATGGACGAGCTGTACAAGCTGACCCTGCAGGAATTCAG	Fw SspB

oLH187	GATCGAAGCTTGGGCTGCAGGTCGACTCTAGATTAACCAATATTCAGCTCGTCATAGATTTC	Rev SspB
oLH192	TTACACCCAGGTATCCACCG	Rev LOVpep
oLH194	GATCGAAGCTTGGGCTGCAGGTCGACTCTAGATTACTTGTACAGCTCGTCCATGC	Rev mRuby
oLH201	ACAAGGGCATTGATCTGAGCGGCCCTGCAGGAATGGAGAGTTCTTCGAATTCCG	Fw GEF4
oLH202	GATCGAAGCTTGGGCTGCAGGTCGACTCTAGACTAATCATCTCTGTTTCTCACTGTTCTG	Rev GEF4
oLH203	ACAAGGGCATTGATCTGAGCGGCCCTGCAGGAATGGAGAATTTATCGAATCCAGATGAAAAC	Fw GEF3
oLH204	GATCGAAGCTTGGGCTGCAGGTCGACTCTAGATTATTCACTACCTCTCATGGTTTTGTCTAC	Rev GEF4
oLH207	TCGGCATGGACGAGCTGTACAAGCCTGCAGGAATGGCGTCAAGGTTTATAAAGTGTG	Fw ROP2
oLH208	GATCGAAGCTTGGGCTGCAGGTCGACTCTAGATCACAAGAACGCGCAACG	Rev ROP2
oLH238	ATGGGGTGCTGGTGCAGTTCAAACCCAGAGGACGACATGGTGTCTAAGGGCGAAG	Fw mRuby
oLH239	TTGGTGGTGGCATGGACGAGCTGTACAAGTAATCTAGAGTCGACCTGCAGC	Fw pMZ333
oLH240	GTCGTCCTCTGGGTTTGAACTGCACCAGCACCCCATGGTGGCGCCTCCTG	Rev pMZ333
oLH246	ATGCCGCCTTCAGCTC	Fw Sql1
oLH247	GGCGTGATTGGGATTTTCAGC	Rev Sql1
oLH248	CGGGATCCCCCGGGCTGCAGGAATTCGATCCCCAATTGATGAAAGCGCCTCCAAAAAAGAAGAGAAAGGTCATGCCGCCTTCAGCTC	Fw Sql1
oLH249	GGCTGCACCTCGACGCGCGTGTCGTCGGCACCATTAATTAAGGCGTGATTGGGATTTTCAGC	Rev Sql1

5.5 Strains

Table 4: Strains generated and used in this work

Strain	Description	Reference
AB33	Pnar bW1, Pnar bE1/2	(Brachmann et al., 2001)
pUMa2549	AB33_cco1D::G-418R_upp3D::CbxR Strain encoding	Kira Müntjes (University of Düsseldorf)
sNH001	AB33_upp3D::P_{O2tef}::RLuc-pIRES-FLuc-nosT-NatR	Nicole Heucken
sNH003	AB33_upp3D::P_{O2tef}::RLuc-eIRES-FLuc-nosT-NatR	Nicole Heucken
sNH004	AB33_upp3D::$_{PO2tef}$::RLuc-fIRES-FLuc-nosT-NatR	Nicole Heucken
sNH036	AB33_upp3D::$_{PO2tef}$::mKate2-pIRES-eGFP-nosT-NatR	Nicole Heucken
sNH037	AB33_upp3D::P_{O2tef}::mKate2-eIRES-eGFP-nosT-NatR	Nicole Heucken
sNH038	AB33_upp3D::P_{O2tef}::mKate2-fIRES-eGFP-nosT-NatR	Nicole Heucken
sNH039	AB33_upp3D::P_{crg}::FLuc-PO2tef::RLuc-nosT-NatR	Nicole Heucken
sNH023	AB33_upp3D::$(PIR)_3$-P_{hCMV}::Firefly-nosT-NatR	Nicole Heucken
sLHNH005	AB33_upp3D::P_{O2tef}::RLuc-HA-nosT-NatR	This work
sLHNH006	AB33_upp3D::P_{O2tef}::GLuc-HA-nosT-NatR	This work
sLHNH007	AB33_upp3D::P_{O2tef}::SEAP-HA-nosT-NatR	This work
sLHNH008	AB33_upp3D::P_{O2tef}::FLuc-HA-nosT-NatR	This work
sLH001	AB33_cco1D::P_{O2tef}::E-Protein-VP16ff-NLS-nosT-HygR	This work
sLH002	AB33_upp3D::$(etr)_8$-P_{hCMV}::Firefly-nosT-NatR	This work
sLH003	AB33_upp3D::$(etr)_8$-P_{mfa1}::Firefly-nosT-NatR	This work
sLH004	AB33_cco1D::P_{O2tef}::E-Protein-VP16ff-NLS-nosT-HygR_upp3D::$(etr)_8$-P_{hCMV}::Firefly-nosT-NatR	This work
sLH007	AB33_cco1D::P_{O2tef}::TetR-VP16ff-NLS-nosT-HygR	This work
sLH008	AB33_upp3D::$(tetO)_{13}$-P_{hCMV}::Firefly-nosT-NatR	This work
sLH009	AB33_upp3D::$(tetO)_{13}$-P_{mfa1}::Firefly-nosT-NatR	This work
sLH012	AB33_cco1D::P_{O2tef}::TetR-VP16ff-NLS-nosT-HygR_upp3D::$(tetO)_{13}$-P_{hCMV}::Firefly-nosT-NatR	This work
sLH016	AB33_upp3D::$(C120)_5$-P_{hCMV}::Firefly-nosT-NatR	This work
sLH017	AB33_upp3D::$(C120)_5$-P_{mfa1}::Firefly-nosT-NatR	This work
sLH020	AB33_cco1D::P_{O2tef}::VP16ff-EL222-nosT-HygR_upp3D::$(C120)_5$-P_{hCMV}::Firefly-nosT-NatR	This work
sLH023	AB33_cco1D::nosT-ePDZ-VP16ff-NLS::dP_{hCMV}::PIP-LOVpep-nosT-HygR_upp3D::$(PIR)_3$-P_{hCMV}::Firefly-nosT-NatR	This work
sLH026	AB33_cco1D::P_{O2tef}::NLS-Sql1-EL222-nosT-HygR_upp3D::$(C120)_5$-P_{hCMV}::Firefly-nosT-NatR	This work

The strains sNH001 – sNH023 were constructed by Nicole Heucken.

The strains sLHNH005 – sLHNH008 were constructed together with Nicole Heucken.

6 References

Abe K, Miyake K, Nakamura M, Kojima K, Ferri S, Ikebukuro K, Sode K (2014) Engineering of a green-light inducible gene expression system in *Synechocystis sp*. PCC6803. Microbial Biotechnology **7**: 177–183

Aschenbroich J, Hussnaetter KP, Stoffels P, Langner T, Zander S, Sandrock B, Bölker M, Feldbrügge M, Schipper K (2019) The germinal centre kinase Don3 is crucial for unconventional secretion of chitinase Cts1 in *Ustilago maydis*. BBA – Preoteins and Proteomics 1867(12)

Amendola M, Venneri MA, Biffi A, Vigna E, Naldini L (2005) Coordinate dual-gene transgenesis by lentiviral vectors carrying synthetic bidirectional promoters. Nature Biotechnology **23**: 108–116

Andersen CR, Nielsen LS, Baer A, Tolstrup AB, Weilguny D (2010) Efficient expression from one CMV enhancer controlling two core promoters. Molecular Biotechnology **48**: 128–137

Assmann SM (2002) Heterotrimeric and unconventional GTP binding proteins in plant cell signaling. Plant Cell. doi: 10.1105/tpc.001792

Badr CE, Hewett JW, Breakefield XO, Tannous BA (2007) A highly sensitive assay for monitoring the secretory pathway and ER stress. PLoS ONE. doi: 10.1371/journal.pone.0000571

Bailey JE (1991) Toward a Science of Metabolic Engineering. Science **252**: 1668–1675

Baron U, Bujard H (2000) Tet repressor-based system for regulated gene expression in eukaryotic cells: Principles and advances. Methods in Enzymology. doi: 10.1016/S0076-6879(00)27292-3

Bento CF, Puri C, Moreau K, Rubinsztein DC (2013) The role of membrane-trafficking small GTPases in the regulation of autophagy. Journal of Cell Science **126**: 1059–1069

Berger J, Hauber J, Hauber R, Geiger R, Cuilen BR (1988) Secreted placental alkaline phosphatase: a powerful new quantitative indicator of gene expression in eukaryotic cells. Surgical Clinic Innenstadt 66:

Berken A, Thomas C, Wittinghofer A (2005) A new family of RhoGEFs activates the Rop molecular switch in plants. Nature **436**: 1176–1180

Beyer HM, Gonschorek P, Samodelov SL, Meier M, Weber W, Zurbriggen MD (2015a) AQUA cloning: A versatile and simple enzyme-free cloning approach. PLoS ONE. doi: 10.1371/journal.pone.0137652

Beyer HM, Juillot S, Herbst K, Samodelov SL, Müller K, Schamel WW, Römer W, Schäfer E, Nagy F, Strähle U, et al (2015b) Red Light-Regulated Reversible Nuclear Localization of Proteins in Mammalian Cells and Zebrafish. ACS Synthetic Biology **4**: 951–958

Bölker M (2001) *Ustilago maydis* - a valuable model system for the study of fungal dimorphism and virulence. Microbiology **147**: 1395–1401

Bösch K, Frantzeskakis L, Vraneš M, Kämper J, Schipper K, Göhre V (2016) Genetic manipulation of the plant pathogen *Ustilago maydis* to study fungal biology and plant microbe interactions. Journal of Visualized Experiments. doi: 10.3791/54522

Brachmann A, König J, Julius C, Feldbrügge M (2004) A reverse genetic approach for generating gene replacement mutants in *Ustilago maydis*. Molecular Genetics and Genomics **272**: 216–226

Brachmann A, Weinzierl G, Kämper J, Kahmann R (2001) Identification of genes in the bW/bE regulatory cascade in *Ustilago maydis*. Molecular Microbiology **42**: 1047–1063

Braguy J, Zurbriggen MD (2016) Synthetic strategies for plant signalling studies: molecular toolbox and orthogonal platforms. The Plant journal for cell and molecular biology **87**: 118–138

Brophy J, Voigt CA (2014) Principles of genetic circuit design. Nature Methods **11**: 508–520

Burridge K, Wennerberg K (2004) Rho and Rac Take Center Stage. Cell **116**: 167–179

Cameron DE, Bashor CJ, Collins JJ (2014) A brief history of synthetic biology. Nature Reviews Microbiology **12**: 381–390

Carol RJ, Takeda S, Linstead P, Durrant MC, Kakesova H, Derbyshire P, Drea S, Zarsky V, Dolan L (2005) A RhoGDP dissociation inhibitor spatially regulates growth in root hair cells. Nature **438**: 1013–1016

Chang F, Gu Y, Ma H, Yang Z (2013) AtPRK2 promotes ROP1 activation via RopGEFs in the control of polarized pollen tube growth. Molecular Plant. doi: 10.1093/mp/sss103

Chaves I, Pokorny R, Byrdin M, Hoang N, Ritz T, Brettel K, Essen LO, van der Horst GTJ, Batschauer A, Ahmad M (2011) The Cryptochromes: Blue Light Photoreceptors in Plants and Animals. Annual Review of Plant Biology **62**: 335–364

Costantino D, Kieft JS (2005) A preformed compact ribosome-binding domain in the cricket paralysis-like virus IRES RNAs. RNA **11**: 332–343

Cullen BR, Malim MH (1992) Secreted placental alkaline phosphatase as a eukaryotic reporter gene. Methods in Enzymology. doi: 10.1016/0076-6879(92)16033-G

Das AT, Tenenbaum L, Berkhout B (2016) Tet-On Systems For Doxycycline-induced Gene Expression. Bentham Science **16**: 156–167

Denninger P, Reichelt A, Schmidt VAF, Mehlhorn DG, Asseck LY, Stanley CE, Keinath NF, Evers JF, Grefen C, Grossmann G (2019) Distinct RopGEFs Successively Drive Polarization and Outgrowth of Root Hairs. Current Biology **29**: 1854-1865.e5

Elowitz MB, Leibler S (2000) A synthetic oscillatory network of transcriptional regulators. Nature **403**: 335–338

de Felipe P (2004) Skipping the co-expression problem: The new 2A “CHYSEL” technology. Genetic Vaccines and Therapy. doi: 10.1186/1479-0556-2-13

Filbin ME, Kieft JS (2009) Toward a structural understanding of IRES RNA function. Current Opinion in Structural Biology. doi: 10.1016/j.sbi.2009.03.005

Fu Y, Li H, Yang Z (2002) The ROP2 GTPase controls the formation of cortical fine F-actin and the early phase of directional cell expansion during Arabidopsis organogenesis. Plant Cell **14**: 777–794

Fussenegger M, Morris RP, Fux C, Rimann M, von Stockar B, Thompson CJ, Bailey JE (2000) Streptogramin-based gene regulation systems for mammalian cells. Nature Biotechnology 18:

Fux C, Fussenegger M (2003) Bidirectional expression units enable streptogramin-adjustable gene expression in mammalian cells. Biotechnology and Bioengineering **83**: 618–625

Galania S, Thodey K, Trenchard IJ, Filsinger Interrante M, Smolke CD (2015) Complete biosynthesis of opioids in yeast. Science **349**: 1095–1100

Gao J, Liao J, Yang G-Y (2009) CAAX-box protein, prenylation process and carcinogenesis. Am J Transl Res **1**: 312–325

Gardner TS, Cantor CR, Collins JJ (2000) Construction of a genetic toggle switch in Escherichia coli. Nature **403**: 339–342

Geiser E, Przybilla SK, Friedrich A, Buckel W, Wierckx N, Blank LM, Bölker M (2016) Ustilago maydis produces itaconic acid via the unusual intermediate trans-aconitate. Microbial Biotechnology **9**: 116–126

Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA, Smith HO (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. Nature Methods **6**: 343–345

Gilroy S, Jones DL (2000) Through form to function: Root hair development and nutrient uptake. Trends in Plant Science **5**:

Gossen M, Bujardt H (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Cell Biology **89**: 5547–5551

Gossen M, Freundlieb S, Bender G, Müller G, Hillen W, Bujard H (1995) Transcriptional activation by tetracyclines in mammalian cells. Science **268**: 1766–1769

Gould SJ, Subramani S (1988) Firefly Luciferase as a Tool in Molecular and Cell Biology. ANALYTICAL BIOCHEMISTRY **175**: 5–13

Gu Y, Li S, Lord EM, Yang Z (2006) Members of a novel class of Arabidopsis Rho guanine nucleotide exchange factors control Rho GTPase-dependent polar growth. Plant Cell **18**: 366–381

Gu Y, Wang Z, Yang Z (2004) ROP/RAC GTPase: An old new master regulator for plant signaling. Current Opinion in Plant Biology **7**: 527–536
Hampf M, Gossen M (2006) A protocol for combined Photinus and Renilla luciferase quantification compatible with protein assays. Analytical Biochemistry. doi: 10.1016/j.ab.2006.04.046
Hannah R, Sherf BA, Navarro SL, Hannah RR, Wood K v (1996) Dual-Luciferase TM Reporter Assay: An Advanced Co-Reporter Technology Integrating Firefly and Renilla Luciferase Assays. Promega Notes Magazine **57**: 2
Hennecke M, Kwissa M, Metzger K, Oumard A, Kröger A, Schirmbeck R, Reimann J, Hauser H (2001) Composition and arrangement of genes define the strength of IRES-driven translation in bicistronic mRNAs. Nucleic Acids Research **29**: 3327–3334
Hiramatsu N, Kasai A, Meng Y, Hayakawa K, Yao J, Kitamura M (2005) Alkaline phosphatase vs luciferase as secreted reporter molecules *in vivo*. Analytical Biochemistry. doi: 10.1016/j.ab.2005.01.023
Hoffman GR, Cerione RA (2002) Signaling to the Rho GTPases: networking with the DH domain. FEBS letters **513**: 85–91
Holliday R (1974) *Ustilago maydis*. R.C. King (Ed.), Bacteria, Bacteriophages, and Fungi, Springer, US. pp 575–595
Huang J, Makabe K, Biancalana M, Koide A, Koide S (2009) Structural Basis for Exquisite Specificity of Affinity Clamps, Synthetic Binding Proteins Generated through Directed Domain-interface Evolution. Journal of Molecular Biology. doi: 10.1016/j.jmb.2009.07.067
Hwang JU, Vernoud V, Szumlanski A, Nielsen E, Yang Z (2008) A Tip-Localized RhoGAP Controls Cell Polarity by Globally Inhibiting Rho GTPase at the Cell Apex. Current Biology. doi: 10.1016/j.cub.2008.11.057
Ikeda Y, Men S, Fischer U, Stepanova AN, Alonso JM, Ljung K, Grebe M (2009) Local auxin biosynthesis modulates gradient-directed planar polarity in Arabidopsis. Nature Cell Biology **11**: 731–738
Jackson RJ (2005) Alternative mechanisms of initiating translation of mammalian mRNAs. Biochemical Society Transactions **33**: 1231–1241
Johnson DI (1999) Cdc42: An Essential Rho-Type GTPase Controlling Eukaryotic Cell Polarity. MICROBIOLOGY AND MOLECULAR BIOLOGY REVIEWS **63**: 54–105
Jones MA, Shen JJ, Fu Y, Li H, Yang Z, Grierson CS (2002) The Arabidopsis Rop2 GTPase is a positive regulator of both root hair initiation and tip growth. Plant Cell **14**: 763–776
Khalil AS, Collins JJ (2010) Synthetic biology: Applications come of age. Nature Reviews Genetics **11**: 367–379
Kiefer CS, Claes AR, Nzayisenga JC, Pietra S, Stanislas T, Hüser A, Ikeda Y, Grebe M (2015) Arabidopsis AIP1-2 restricted by WER-mediated patterning modulates planar polarity. Development (Cambridge) **142**: 1022
Kuruma Y, Stano P, Ueda T, Luisi PL (2009) A synthetic biology approach to the construction of membrane proteins in semi-synthetic minimal cells. Biochimica et Biophysica Acta - Biomembranes. doi: 10.1016/j.bbamem.2008.10.017
Li H, Wu G, Ware D, Davis KR, Yang Z (1998) Arabidopsis Rho-Related GTPases: Differential Gene Expression in Pollen and Polar Localization in Fission Yeast 1. Plant Physiology **118**: 407–417
Lienert F, Lohmueller JJ, Garg A, Silver PA (2014a) Synthetic biology in mammalian cells: Next generation research tools and therapeutics. Nature Reviews Molecular Cell Biology **15**: 95–107
Lienert F, Lohmueller JJ, Garg A, Silver PA (2014b) Synthetic biology in mammalian cells: Next generation research tools and therapeutics. Nature Reviews Molecular Cell Biology **15**: 95–107
Liu J, O'Kane DJ, Escher A (1997) Secretion of functional *Renilla reniformis* luciferase by mammalian cells. Gene. doi: 10.1016/S0378-1119(97)00505-2

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1998) Two Transcription Factors, DREB1 and DREB2, with an EREBP/AP2 DNA Binding Domain Separate Two Cellular Signal Transduction Pathways in Drought-and Low-Temperature-Responsive Gene Expression, Respectively, in Arabidopsis. The Plant Cell **10**: 1391–1406

Liu Z, Chen O, Wall JBJ, Zheng M, Zhou Y, Wang L, Ruth Vaseghi H, Qian L, Liu J (2017) Systematic comparison of 2A peptides for cloning multi-genes in a polycistronic vector. Scientific Reports. doi: 10.1038/s41598-017-02460-2

Loew R, Heinz N, Hampf M, Bujard H, Gossen M (2010) Improved Tet-responsive promoters with minimized background expression. BMC Biotechnology. doi: 10.1186/1472-6750-10-81

Losi A (2004) The bacterial counterparts of plant phototropins. Photochemical and Photobiological Sciences **3**: 566–574

Losi A, Gärtner W (2008) Bacterial bilin- and flavin-binding photoreceptors. Photochemical and Photobiological Sciences **7**: 1168–1178

Loubradou G, Brachmann A, Feldbru M, Kahmann R (2001) A homologue of the transcriptional repressor Ssn6p antagonizes cAMP signalling in *Ustilago maydis*. Molecular Microbiology **40**: 719–730

Luke GA, de Felipe P, Lukashev A, Kallioinen SE, Bruno EA, Ryan MD (2008) Occurrence, function and evolutionary origins of “2A-like” sequences in virus genomes. Journal of General Virology **89**: 1036–1042

Mahlert M, Leveleki L, Hlubek A, Sandrock B, Bölker M (2006) Rac1 and Cdc42 regulate hyphal growth and cytokinesis in the dimorphic fungus *Ustilago maydis*. Molecular Microbiology **59**: 567–578

Matthews JC, Hori K, Cormier MJ (1977) Purification and Properties of *Renilla reniformis* Luciferase. Biochemistry **16**: 324–332

Mccarty DR, Chory J (2000) Conservation and Innovation in Plant Signaling Pathways. Cell **103**: 201–209

Mehla J, Caufield JH, Uetz P (2015) The yeast two-hybrid system: A tool for mapping protein-protein interactions. Cold Spring Harbor Protocols **2015**: 425–430

Miyamoto Y, Sancar A (1998) Vitamin B 2-based blue-light photoreceptors in the retinohypothalamic tract as the photoactive pigments for setting the circadian clock in mammals. PNAS **95**: 6097–6102

Mizuguchi H, Xu Z, Ishii-Watabe A, Uchida E, Hayakawa T (2000) IRES-Dependent Second Gene Expression Is Significantly Lower Than Cap-Dependent First Gene Expression in a Bicistronic Vector. Molecular Therapy. doi: 10.1006/mthe.2000.0050

Möglich A, Yang X, Ayers RA, Moffat K (2010) Structure and Function of Plant Photoreceptors. Annual Review of Plant Biology **61**: 21–47

Molla A, Jang SK, v. Paul A, Reuer Q, Wimmer E (1992) Cardioviral internal ribosomal entry site is functional in a genetically engineered dicistronic poliovirus. Nature **356**: 255–257

Møller SG, Chua NH (1999) Interactions and intersections of plant signaling pathways. Journal of Molecular Biology. doi: 10.1006/jmbi.1999.2992

Motta-Mena LB, Reade A, Mallory MJ, Glantz S, Weiner OD, Lynch KW, Gardner KH (2014) An optogenetic gene expression system with rapid activation and deactivation kinetics. Nature Chemical Biology **10**: 196–202

Mountford PS, Smith AG (1995) Internal ribosome entry sites and dicistronic RNAs in mammalian transgenesis. Trends in Genetics. doi: 10.1016/S0168-9525(00)89040-X

Müller K, Engesser R, Timmer J, Zurbriggen MD, Weber W (2014a) Orthogonal optogenetic triple-gene control in mammalian cells. ACS Synthetic Biology **3**: 796–801

Müller K, Siegel D, Rodriguez Jahnke F, Gerrer K, Wend S, Decker EL, Reski R, Weber W, Zurbriggen MD (2014b) A red light-controlled synthetic gene expression switch for plant systems. Molecular BioSystems **10**: 1679–1688

Müller K, Weber W (2013) Optogenetic tools for mammalian systems. Molecular BioSystems **9**: 596–608

Müller K, Zurbriggen MD, Weber W (2014c) Control of gene expression using a red- and far-red light-responsive bi-stable toggle switch. Nature Protocols **9**: 622–632
Nagawa S, Xu T, Yang Z (2010) RHO GTPase in plants: Conservation and invention of regulators and effectors. Small GTPases **1**: 78–88
Nash AI, McNulty R, Shillito ME, Swartz TE, Bogomolni RA, Luecke H, Gardner KH (2011) Structural basis of photosensitivity in a bacterial light-oxygen-voltage/helix-turn-helix (LOV-HTH) DNA-binding protein. Proceedings of the National Academy of Sciences **108**: 9449–9454
Naylor LH (1999) Reporter gene technology: The future looks bright. Biochemical Pharmacology. doi: 10.1016/S0006-2952(99)00096-9
Nazari M, Hosseinkhani S (2011) Design of disulfide bridge as an alternative mechanism for color shift in firefly luciferase and development of secreted luciferase. Photochemical and Photobiological Sciences **10**: 1203–1215
Noguchi N, Takada K, Katayama J, Emura A, Sasatsu M (2000) Regulation of Transcription of the mph(A) Gene for Macrolide 2-Phosphotransferase I in *Escherichia coli*: Characterization of the Regulatory Gene mphR(A). JOURNAL OF BACTERIOLOGY **182**: 5052–5058
Okada K, Shimura Y (1992) Aspects of Recent Developments in Mutational Studies-of Plant Signaling Pathways Minireview. Cell **70**: 369–372
Öztürk S, Ergün BG, Çalık P (2017) Double promoter expression systems for recombinant protein production by industrial microorganisms. Applied Microbiology and Biotechnology **101**: 7459–7475
Pelletier J, Sonenberg N (1988) Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. Nature **334**: 320–325
Pruneda-Paz JL, Breton G, Nagel DH, Kang SE, Bonaldi K, Doherty CJ, Ravelo S, Galli M, Ecker JR, Kay SA (2014) A Genome-Scale Resource for the Functional Characterization of Arabidopsis Transcription Factors. Cell Reports. doi: 10.1016/j.celrep.2014.06.033
Purnick PEM, Weiss R (2009) The second wave of synthetic biology: From modules to systems. Nature Reviews Molecular Cell Biology **10**: 410–422
Rawat A, Harishchandran A, Nagaraj R (2013) Fatty acyl chain-dependent but charge-independent association of the SH4 domain of Lck with lipid membranes. Journal of Biosciences **38**: 63–71
Riquelme M (2013) Tip Growth in Filamentous Fungi: A Road Trip to the Apex. Annual Review of Microbiology **67**: 587–609
Rounds CM, Bezanilla M (2013) Growth Mechanisms in Tip-Growing Plant Cells. Annual Review of Plant Biology **64**: 243–265
Russell SA, Bashaw GJ (2017) Axon Guidance Pathways and the Control of Gene Expression. Developmental Dynamics **247**: 571–580
Samodelov SL, Beyer HM, Guo X, Augustin M, Jia K-P, Baz L, Ebenhöh O, Beyer P, Weber W, Al-Babili S, et al (2016) StrigoQuant: A genetically encoded biosensor for quantifying strigolactone activity and specificity. Science Advances 2(11)
Schenborn E, Groskreutz D (1999) Reporter Gene Vectors and Assays. Molecular Biology **13**: 29–44
Sherf BA, Navarro SL, Hannah RR, Wood K v (1996) Dual-LuciferaseTM Reporter Assay: An Advanced Co-Reporter Technology Integrating Firefly and Renilla Luciferase Assays. Promega Notes Magazine Number **57**: 2
Shetty RP, Endy D, Knight TF (2008) Engineering BioBrick vectors from BioBrick parts. Journal of Biological Engineering. doi: 10.1186/1754-1611-2-5
Shifera AS, Hardin JA (2010) Factors modulating expression of Renilla luciferase from control plasmids used in luciferase reporter gene assays. Analytical Biochemistry **396**: 167–172
Steinberg G, Perez-Martin J (2008) Ustilago maydis, a new fungal model system for cell biology Introduction: Fungal model systems in cell biology. Trends Cell Biol **18**: 61–67

Strickland D, Lin Y, Wagner E, Hope CM, Zayner J, Antoniou C, Sosnick TR, Weiss EL, Glotzer M (2012) TULIPs: Tunable, light-controlled interacting protein tags for cell biology. Nature Methods **9**: 379–384
Szymczak-Workman AL, Vignali KM, Vignali DAA (2012) Design and construction of 2A peptide-linked multicistronic vectors. Cold Spring Harbor Protocols **7**: 199–204
Tannous BA (2009) Gaussia luciferase reporter assay for monitoring biological processes in culture and in vivo. Nature Protocols **4**: 582–591
Tannous BA, Kim DE, Fernandez JL, Weissleder R, Breakefield XO (2005) Codon-optimized gaussia luciferase cDNA for mammalian gene expression in culture and in vivo. Molecular Therapy. doi: 10.1016/j.ymthe.2004.10.016
Thomas C, Fricke I, Weyand M, Berken A (2009) 3D structure of a binary ROP-PRONE complex: The final intermediate for a complete set of molecular snapshots of the RopGEF reaction. Biological Chemistry **390**: 427–435
Ulijasz AT, Vierstra RD (2011) Phytochrome structure and photochemistry: Recent advances toward a complete molecular picture. Current Opinion in Plant Biology. doi: 10.1016/j.pbi.2011.06.002
Vernoud V, Horton AC, Yang Z, Nielsen E (2003) Analysis of the small GTPase gene superfamily of Arabidopsis. Plant Physiology **131**: 1191–1208
Walhout AJM, Vidal M (2001) High-throughput yeast two-hybrid assays for large-scale protein interaction mapping. Methods. doi: 10.1006/meth.2001.1190
Wang X, Chen X, Yang Y (2012) Spatiotemporal control of gene expression by a light-switchable transgene system. Nature Methods **9**: 266–269
Weber W, Fux C, Daoud-El Baba M, Keller B, Weber CC, Kramer BP, Heinzen C, Aubel D, Bailey JE, Fussenegger M (2002) Macrolide-based transgene control in mammalian cells and mice. Nature Biotechnology **20**: 901–907
Winge P, Brembu T, Kristensen R, Bones AM (2000) Genetic Structure and Evolution of RAC-GTPases in Arabidopsis thaliana. Genetics **156**: 1959–1971
Woo J, von Arnim AG (2008) Mutational optimization of the coelenterazine-dependent luciferase from Renilla. Plant Methods. doi: 10.1186/1746-4811-4-23
Xie M, He Y, Gan S (2001) Bidirectionalization of polar promoters in plants. Nature Biotechnology **19**: 677–679
Yalovsky S, Bloch D, Sorek N, Kost B (2008) Regulation of Membrane Trafficking, Cytoskeleton Dynamics, and Cell Polarity by ROP/RAC GTPases. Plant Physiology **147**: 1527–1543
Yang S, Sleight SC, Sauro HM (2013a) Rationally designed bidirectional promoter improves the evolutionary stability of synthetic genetic circuits. Nucleic Acids Research. doi: 10.1093/nar/gks972
Yang XL, Awakawa T, Wakimoto T, Abe I (2013b) Induced production of the novel glycolipid ustilagic acid C in the plant pathogen *Ustilago maydis*. Tetrahedron Letters 3655–3657
Yang Z (2002) Small GTPases: Versatile signaling switches in plants. Plant Cell. doi: 10.1105/tpc.001065
Yang Z, Watsont JC (1993) Molecular cloning and characterization of rho, a ras-related small GTP-binding protein from the garden pea. Proc Natl Acad Sci USA **90**: 8732–8736
Yu X, Liu H, Klejnot J, Lin C (2010) The Cryptochrome Blue Light Receptors. The Arabidopsis Book **8**: e0135
Zhang Y, Mccormick S (2007) A distinct mechanism regulating a pollen-specific guanine nucleotide exchange factor for the small GTPase Rop in *Arabidopsis thaliana*. PNAS **104**: 18830–18835
Zheng Z-L, Yang Z (2000) The Rop GTPase: an emerging signaling switch in plants.
Zoltowski BD, Nash AI, Gardner KH (2011) Variations in protein-flavin hydrogen bonding in a light, oxygen, voltage domain produce non-Arrhenius kinetics of adduct decay. Biochemistry **50**: 8771–8779

7 Appendix: Manuscripts

Manuscript accepted for publication

Nicole Heucken*, Lisa C. Hüsemann* and Matias D. Zurbriggen. AQUA 2.0: an update to AQUA cloning

Contribution: *Design, performance, and analysis of all experiments. Preparation of all figures and writing of the manuscript.*

**equal contribution*

7.1 AQUA 2.0: an upgrate to AQUA cloning

AQUA 2.0: an upgrade to AQUA cloning

Nicole Heucken, Lisa C. Hüsemann and Matias D. Zurbriggen

Key words assembly cloning, plant synthetic biology, *Arabidopsis thaliana*

Abstract

Assembly cloning methods like Gibson and AQUA (advanced quick assembly), are increasingly replacing conventional restriction enzyme and DNAligase-dependent cloning methods for reasons of efficiency and performance. AQUA Cloning harnesses intrinsic in vivo processing of linear DNA fragments with short regions of homology of 16 to 32 bp mediated by *Escherichia coli*. Here, we describe an update to AQUA and demonstrate the possibility of integrating short DNA sequences encoding e.g. for signal peptides into existing vectors. This is achieved by assembly of several pre-annealed oligonucleotide pairs with the digested vector backbone. In this protocol the integration of a PEST sequence into an already existing vector, it's transformation into *Arabidopsis thaliana* protoplasts and a subsequent Luciferase assay enables the determination of the potential induction-fold for sensor modules used in the reconstruction of plant hormone signaling pathways.

Introduction

The implementation of synthetic biology approaches requires complex combinations of a wide variety of proteins and genetic tools, including synthetic protein modules, reporter genses, promotors, and many others. The assemly of such complex constructs makes it necessary to simplify the cloning process and make it more efficient by inventing new methods that are flexible, fast and cheap. One of such methods is AQUA [1]. It has already proven to be a versatile, robust and, compared to other commonly used cloning methods, cheap and fast cloning approach. It fully relies on homologous overhang pairing and is therefore completely independent of the addition of enzymes. Beyer et al. already exemplified the applicability of AQUA cloning for various application. What we want to present here is an update of this list of prooven applications. Therefore, we demonstrate how AQUA cloning can be used to add short sequences, too long to be included in a primer overhang, and too short to be effectively amplified via PCR, to your plasmid.

Here we cloned a PEST sequence of 126 bp into a plasmid containing Firefly and Renilla luciferases separated by a 2A peptide (see fig. 2). In this experimental setup, the PEST sequence is the product of three forward and their complementary reverse primers. These oligonucleotides are assembled to double stranded DNA fragments via primer annealing (see fig. 1). The idea is to tag this sequence to Firefly luciferase leading to a degradation of the protein. The successful inclusion of the sequence and its functionality are verified by Firefly/Renilla assays (see fig. 3).

Materials

All solutions should be prepared using double distilled water and p.a. purity grade chemicals. For all plant growth and protoplast isolation media we recommend to use plant cell culture tested reagents. The reagents must be prepared and stored at 4 °C unless indicated otherwise.

2.1 Plant Growth

1. SCA (Seedling Culture Arabidopsis) (modified from [2]): 0,32 % (w/v) Gamborg B5 basal salt powder with vitamins (bioWORLD), 4 mM $MgSo_4 \cdot 7H_2O$, 43.8 mM sucrose, and 0,8 % (w/v) phytoagar. Mix and adjust to pH 5.8 and autoclave. Add 0,1 % (v/v) Gamborg B5 Vitamin Mix (bioWORLD) and 1:2000 ampicillin and pour 50 ml of the medium into 12-cm^2 plates (Greiner Bio-One).
2. Seed sterilization solution for *A. thaliana* (modified from [3]): 5 % (w/v) calcium hypochloride, 0,02 % (v/v) Triton X-100 in 80 % (v/v) EtOH. Combine all chemicals and mix for a few hours at room temperature. Let the formed precipitate settle and store the solution at 4 °C. Do not agitate the bottle before use.
3. Parafilm
4. Syringe and 22 µm filter
5. Ampicillin stock (100 mg/ml)

2.2 Protoplast Isolation and PEG Mediated Protoplast Transformation

1. MMC (MES, Mannitol, Calcium) [2]: 10 mM MES, 40 mM $CaCl_2 \cdot H_2O$, add mannitol until obtaining an osmolarity of 550 mOsm (ca. 85 g/l). Adjust to pH 5.8 and filter sterilize.
2. Enzyme solution stock 5 % (10x concentrated): cellulase Onozuka R10 and macroenzyme R10 (SERVA Electrophoresis GmbH, Germany) in MMC. Add 10 g of cellulase and 10 g of macroenzyme and dissolve in preheated (37 °C) MMC to a total volume of 200 ml H_2O. Sterile filter the solution with a bottle-top filter and make aliquots of 2 ml. Store aliquots at -20 °C and avoid any thaw-freeze cycles.
3. MSC (MES, Sucrose, Calcium) [2]: 10 mM MES, 0.4 M sucrose, 20 mM $MgCl_2 \cdot 6H_2O$, add mannitol until you obtain an osmolarity of 550 mOsm (ca. 85 g/l). Adjust to a pH of 5.8 and filter sterilize.

4. W5 solution (modified from [4]): 2 mM MES, 154 mM glucose. Adjust to pH 5.8 and filter sterilize.

5. MMM (MES, Mannitol, Magnesium) [2]: 15 mM $MgCl_2$, 5 mM MES, mannitol up to 600 mOsm (ca. 85 g/l). Adjust to a pH of 5.8 and filter sterilize.

6. PEG solution: freshly made for each experiment. Mix 2.5 ml of 0.8 M mannitol, 1 ml of 1 M $CaCl_2$, 4 g PEG_{4000} and 3 ml H_2O. Do not filter. Use directly after placing the tube at 37 °C for dissolution of PEG.

7. PCA (Protoplast Culture Arabidopsis) (modified from [2]): 0.32 % (w/v) Gamborg B5 basal salt powder with vitamins (bioWORLD), 2 mM $MgSO_4 \cdot 7H_2O$, 3.4 mM $CaCl_2 \cdot 2H_2O$, 5 mM MES, 0.342 mM l-glutamine, 58.4 mM sucrose, glucose 550 mOsm (ca. 80 g/l), 8.4 µM Ca-panthotenate, 2 % (v/v) biotin from a biotin solution 0.02 % (w/v) in H_2O (biotin solution should be warmed up to dissolve). Adjust the pH to 5.8 and filter sterilize. Add 0.1 % (v/v) Gamborg B5 Vitamin Mix and 1:2000 ampicillin to the PCA before use.

8. Scalpel

9. Disposable 70 µm pore size sieve (Greiner bio-one international, Germany)

10. Petri dish 94 x 16 mm

11. Parafilm

12. 200 µl and 1 ml large orifice pipette tips

13. Round-bottom 15 ml Falcon tubes

14. Rosenthal cell counting chamber

15. Nontreated 6-well plates

2.3 Luminescence Reporter Assay

1. Costar® 96-well flat-bottom white plate

2. Firefly luciferase substrate: 20 mM tricine, 2.67 mM $MgSO_4 \cdot 7H_2O$, 0.1 mM EDTA·$2H_2O$, 33.3 mM DTT, 0.52 mM ATP, 0.27 mM acetyl-CoA, 0.47 mM d-luciferin (Biosynth AG), 5 mM NaOH, 264 µM $MgCO_3 \cdot 5H_2O$, in H_2O. Prepare a beaker with a magnetic stirrer and add the components in the order as above. Then add the luciferin and H_2O and mix the solution. Finally add the NaOH and the $MgCO_3 \cdot 5H_2O$. Adjust the solution to a pH of 8. Make aliquots in precooled black Falcon tubes and store them at -80 °C.

3. Renilla luciferase substrate (Coelenterazine): 472 mM coelenterazine stock solution in methanol, diluted with PBS directly before use.

2.4 Plasmid generation and purification

1. Plasmid digestion: 2 – 5 µg of plasmid, 5 µl of 10 x CutSmart® buffer (NEB), 17 u of restriction enzyme, fill up to 50 µl with ddH_2O. Before loading on gel add 10 u of CIP and incubate for 1 h at 37 °C.
2. TAE buffer (50 x): 242 g Tris base in water, add 57.1 ml glacial acetic acid, and 100 ml of 500 mM EDTA (pH 8.0) solution. Bring the solution to a final volume of 1 l.
3. Plasmid purification: 0.8 % agarose gel (0.8 g/100 ml TAE 1x) with 1 µg/ml ethidium bromide.
4. Plasmid gel extraction: QIAquick Gel Extraction Kit (QIAGEN); DNA is eluted in 20 µl ddH_2O.
5. Gel electrophoresis chambers
6. Heating block with shaking function

2.5 AQUA cloning

1. ddH_2O
2. Oligonucleotides (Sigma; stock 100 µM)
3. Annealing buffer (1x): 10 mM Tris (pH 7.5 -8), 50 mM NaCl, 1 mM EDTA

2.6 *E. coli* transformation

1. TOP10 (Invitrogen) *E. coli* strain prepared for chemical competency
2. LB liquid medium

Methods

3.1 AQUA cloning

1. digest the vector plasmid (pSW209) with NheI for 2 h at 37°C. Afterwards add CIP and incubate for another 30 min.
2. Load the digest on a 0.8 % agarose gel and let it run for 20 min.
3. Extract the DNA from the gel using the QIAquick Gel Extraction Kit (QIAGEN) and following the protocol of the manufacturer.
4. dilute oligonucleotides 1:10 in annealing buffer; mix 5 µl of each forward primer with 5 µl of the complementary reverse primer in 1.5 ml reaction tubes (reaction 1 - 3); put the 3 reactions to 95 °C for 5 min; let cool down to room temperature;

mix 3 µl of each reaction and 1 µl of vector plasmid in a 1.5 ml reaction tube; incubate for 1 h at room temperature.

5. Transform 10 µl into chemically competent *E. coli* TOP10 cells and incubate on ice for 30 min.

6. Heat shock at 42 °C for 45 sec.

7. Add 250 µl LB medium and incubate on a shaker at 37 °C and 700 rpm for 1 h.

8. Plate the whole reaction on LB-ampicillin plates and incubate at 37 °C over night.

9. Single out colonies on new LB-ampicillin plates and incubate at 37 °C over night.

10. Perform a miniprep and test digest the plasmids. Positive clones should be sequenced.

11. Inoculate 100 ml of LB medium with antibiotics in shaking flasks with plasmid containing *E. coli* cells and incubate shaking over night at 37 °C.

11. Perform a midiprep. Make a 1:10 dilution and load the following mixture on an 1 % agarose gel to determine the quality of the plasmid preparation: 3 µl plasmid dilution, 7 µl H2O and 2 µl loading dye.

3.2 Seed Sterilization and Plant Material

1. The sterilization of *A. thaliana* (Wild type, Columbia-0) seed should be done in a sterile working hood in 1.5 ml tubes. The maximum filling volume of a single tube should not exceed 250 µl, otherwise the sterilization efficiency may vary.

2. Rinse seed multiple times with 80 % (v/v) ethanol until all large dirt and other particles are removed.

3. sterilize the seeds of *A. thaliana* sterilization solution for 10 min under agitation.

4. Remove the solution and add 1 ml of 80 % (v/v) ethanol and incubate for 5 min under agitation.

5. Repeat step 4 with an incubation time of 2 min.

6. Replace the solution with 1 ml absolute Ethanol and incubate for 1 min under agitation.

7. Remove the ethanol and let seeds dry completely.

8. Add autoclaved water and plate the seeds in a line on autoclaved filter paper strips (200 – 300 seeds/strip) placed on 12 cm square plates containing SCA medium. Seal the plates with parafilm.

9. Place the prepared plates in a growth chamber with a 16 h light regime at 22 °C. The seedlings should be 2 – 3 weeks to be used for protoplast isolation.

3.3 Protoplast isolation and Polythylene Glycol-Mediated Transformation

A. thaliana protoplast isolation and transformation were performed as described in [2] and [5] with a few alterations. For any pipetting, only wide open orifice tips were used to avoid damaging the protoplasts. Use medium acceleration and lowest deceleration settings for the centrifugation steps (140 s acceleration, and 300 s deceleration according to DIN58970).

1. Slice the plant leaves of *A. thaliana* with a scalpel in 2 ml of MMC.
2. Transfer cut leaf material into a new petri dish containing 7 ml of MMC.
3. Add 1 ml of 10 x enzyme stock solution to start the enzymatic digestion (final concentration of each enzyme: 0.5 %)
4. Seal the dish with parafilm and cover it with aluminum foil. Incubate the dish over night (12 – 16 h) in the dark at 22 °C.
5. Homogenize (carefully) the leaf material to release the protoplasts by pipetting the mixture up and down.
6. Pass the mixture through a disposable 70 µm pore size sieve.
7. Transfer the filtered protoplast solution to 15 ml round bottom Falcon tubes. Use one tube for each plate of digested leaf material, and complete all remaining steps in these tubes.
8. Centrifuge the filtered protoplasts solution at 100 x g for 10 – 20 min for sedimentation of the protoplasts. Remove the supernatant and resuspend the protoplasts in 10 ml MSC.
9. Carefully overlay the protoplast solution with 2 ml of MMM.
10. Centrifuge for 10 min at 80 x g for accumulation of the protoplasts at the interface of MSC and MMM.
11. Collect the protoplasts from the interphase and transfer them into a new Falcon tube containing 7 ml of W5 solution. Prepare two W5-filled collection Falcon tubes for each floatation tube. Multiple rounds of protoplast collection can be done.
12. Centrifuge the protoplasts for 10 min at 100 x g to pellet and resuspend in 10 – 15 ml of W5 for counting.
13. Determine the density using a Rosenthal cell counting chamber.

14. Centrifuge for 5 min at 80 x g to sediment the protoplasts. Remove the supernatant and adjust the density to 5 x 10^6 cells/ml with MMM solution.

15. For the transformation of *A. thaliana* protoplasts, prepare 15 – 30 µg of DNA in H_2O (mentioned DNA amounts are total amounts of DNA. When more than one plasmid is used, the amounts of DNA must be adjusted proportionally. Before transformation the plasmid DNA must be purified using a midiprep kit, and the quality of the plasmid DNA must be checked by agarose gel electrophoresis) adjusted to a maximum volume of 20 µl with MMM solution. Transfer the 20 µl DNA solution to the rim of a well of a 6-well culture plate. Dispense 100 µl of protoplast solution to each well with DNA and mix gently by pipetting. Incubate the mixture for 5 min.

16. Gently shake the 6-well plate to distribute the protoplasts and DNA along the rim before directly adding 120 µl of PEG_{4000} dropwise (tip-in-tip). Do not mix after addition of PEG. Incubate for 8 min and quickly add 120 µl of MMM and, directly afterwards, 1.2 ml of PCA. Gently mix by tilting the plate.

17. If only one condition is to be tested, leave the protoplast suspension in the 6-well plate.

3.4 Reporter Assay

1. To determine reporter expression, gently mix the protoplast suspension and transfer 80 µl (25,000 protoplasts) into one Costar® 96-well flat bottom white plate for Firefly assay, and into one for Renilla assay, including 4 – 6 replicates for each.

2. Add 20 µl of firefly luciferase (final concentration of 131 µg/ml) and 20 µl of coelenterazine (472 mM coelenterazine stock solution in methanol, diluted directly before use, 1:15 in cooled phosphate-buffered saline) and monitor the luminescence in a plate reader. The following program is advisable: 10 s of shaking plate for homogeneous substrate availability and direct luminescence measurement for 20 min with an interval of 2 min.

References

1. Beyer H, Gonschorek P, Samodelov SL, Meier M, Weber W, Zurbriggen MD (2015) AQUA Cloning: A versatile and simple enzyme.free cloning approach. PLoS ONE 10(9): e0137652

2. Dovzhenko A, Bergen U, Koop HU (1998) Thin-alginate-layer technique for protoplast culture of tobacco leaf protoplasts: shoot formation in less than two weeks. Protoplasma 204(1-2):114–118

3. Luo Y, Koop H-U (1997) Somatic embryogenesis in cultured immature zygotic embryos and leaf protoplasts of Arabidopsis thaliana ecotypes. Planta 202(3):387–396

4. Menczel L, Galiba G, Nagy F, Maliga P (1982) Effect of radiation dosage on effi ciency of chloroplast transfer by protoplast fusion in nicotiana. Genetics 100(3):487–495

5. Koop H-U, Steinmüller K, Wagner H, Rößler C, Eibl C, Sacher L (1996) Integration of foreign sequences into the tobacco plastome via polyethylene glycol-mediated protoplast transformation. Planta 199(2):193–201

6. Wend S, Bosco CD, Kämpf MM, Ren F, Palme K, Weber W, Dovzhenko A, Zurbriggen MD (2013) A quantitative ratiometric sensor for time-resolved analysis of auxin dynamics. Sci Rep 3:2052

Figure legends

Fig. 1 AQUA 2.0 Cloning work-flow. (1) DNA parts are generated by Oligo pre-annealing and PCR amplification or restriction digest. (2) Vector backbone is purified by gel-electrophoresis. (3) pre-annealed oligos and digested plasmid are mixed and incubated in water prior to transformation into chemically competent E. coli Top10 cells for in vivi assembly. (4) Finally, obtained colonies are confirmed for correct assembly by standard methods such as analytical PCR, restriction digest, or comprehensive sequencing.

Fig. 2 Design of the pifold (potential-induction-fold-determination) gene expression system in plants. (a) Configuration of the vectors. (b) Mode of function. Pifold construct expressing a renilla luciferase (RLuc; blue) connected via a 2A peptide to the degradation module (PEST) fused to a firefly luciferase (FLuc; green), under the control of a constitutive 35S promoter. The 2A peptide in the synthetic construct leads to stoichiometric coexpression of RLuc (normalization element) and PEST-FLuc. PEST-FLuc becomes degraded, whereas RLuc expression remains constant, leading to a decrease in the FLuc/RLuc ratio.

Fig. 3 Potential-induction-fold-determination for biosensors in *Arabidopsis thaliana* mesophyll protoplasts. Protoplasts were isolated from WT seedlings and transformed with the respective plasmid. Twenty-four hours after transformation, luciferase activity was determined. Results are averaged FF/REN ratios, normalized to the sample without PEST sequence. The data shown correspond to one representative experiment. Error bars represent SEM from the individual experimental data shown. n = 12.

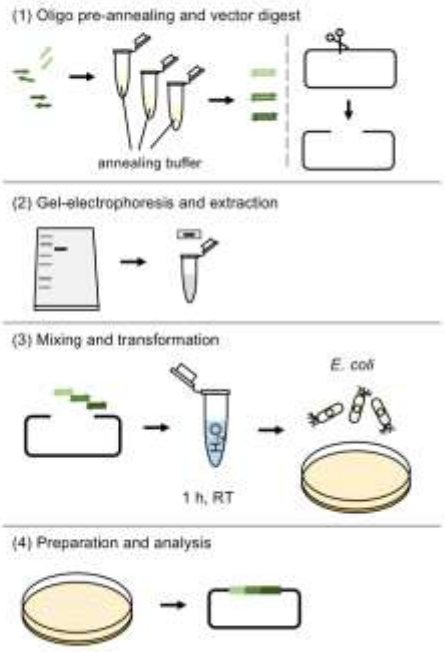
(1) Oligo pre-annealing and vector digest
annealing buffer
(2) Gel-electrophoresis and extraction
(3) Mixing and transformation
E. coli
1 h, RT
(4) Preparation and analysis

Fig. 1

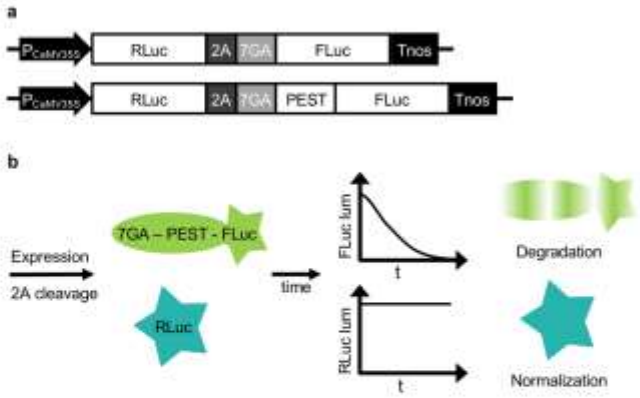
a
RLuc
2A
7GA
FLuc
Tnos
RLuc
2A
7GA
PEST
FLuc
Tnos
b
Expression
2A cleavage
7GA – PEST - FLuc
RLuc
time
FLuc lum
t
RLuc lum
t
Degradation
Normalization

Fig.2

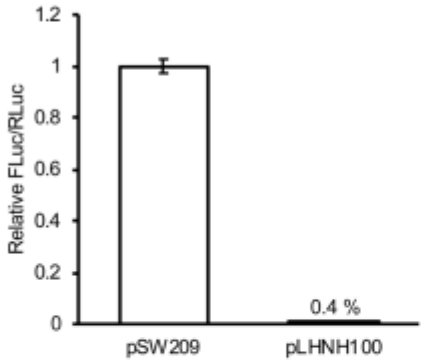

Fig. 3

Table 1
Description of the plasmids CtrlQuant and Pifold for plant use

Vector	Description	Reference
SW209	$P_{CaMV35S}$-*At*RLuc-2A-$(GA)_7$-*At*FLuc-Tnos Vector encoding firefly luciferase (FLuc), a 2A-peptide, a small repeated GA sequence and renilla luciferase (RLuc) under the controle of the Cauliflower mosaic virus (CaMV) 35S promoter. (1)	[6]
pLHNH100	$P_{CaMV35S}$-*At*RLuc-2A-$(GA)_7$-PEST-*At*FLuc-Tnos Vector encoding firefly luciferase (FLuc), a 2A-peptide, a small repeated GA sequence, a PEST sequence and renilla luciferase (RLuc) under the controle of the Cauliflower mosaic virus (CaMV) 35S promoter. The PEST sequence was introduced by assembly of 3 pre-annealed Oligonucleotide paires (oLHNH001 + oLHNH008, oLHNH002 + oLHNH009 and oLHNH003 + oLHNH010) of 73 bp in length each and NheI + CIP digested pSW209 as the backbone.	This work

Table 2
Oligonucleotides used in this work

Oligo	Sequence (5'→ 3')
oLHNH001	TGCCGGGGCAGGCGCTGGCGCTAGCAAGCTCTCTCATGGATTCC CGCCAGCTGTAGCCGCTCAGGACGATGGA
oLHNH002	TAGCCGCTCAGGACGATGGAACCCTACCCATGAGCTGCGCGCAAG AATCTGGCATGGATCGACATCCTGCAGC
oLHNH003	ATGGATCGACATCCTGCAGCCTGCGCTTCCGCAAGGATTAACGTG GGCGCGCCATGGAAGACGCCAAAAACAT
oLHNH008	TCCATCGTCCTGAGCGGCTACAGCTGGCGGGAATCCATGAGAGAG CTTGCTAGCGCCAGCGCCTGCCCCGGCA
oLHNH009	GCTGCAGGATGTCGATCCATGCCAGATTCTTGCGCGCAGCTCATG GGTAGGGTTCCATCGTCCTGAGCGGCTA
oLHNH010	ATGTTTTTGGCGTCTTCCATGGCGCGCCCACGTTAATCCTTGCGGA AGCGCAGGCTGCAGGATGTCGATCCAT