Gal4 based expression systems for *Penicillium chrysogenum*

Name: **Daniël-Morâh Meijer**

Student number: **s2395754**

Date: **23-09-2016**

Research group: **Molecular microbiology**

Supervisors: **Prof. Dr. A.J.M. Driessen, Y. I. Nygard & Z. Buttel**

Index

Abstract:

The production of secondary metabolites with micro-organisms has proven to be very useful for society. *Penicillium chrysogenum* is a fungus that is well known in the industry for the production of secondary metabolites. Engineering new secondary metabolite producing gene clusters will further help the industry in the production of useful secondary metabolites. UAS/Gal4 and QUAS/QF are transcription factor based expression systems that have been analyzed in several organisms.[16,18,22] Gal4 and QF can regulate the transcription of a specified gene downstream of the UAS or QUAS sequence.[16,18,22] In this research 12 UAS/Gal4 based expression systems have been assembled. In these constructs Gal4 will bind to the UAS sequence of a gene that will transcribe and translate for a fluorescent protein. This fluorescent protein can be used as a reporter to show that the construct works in *P. chrysogenum*. The UAS/Gal4 system is not able to negatively regulate the production of the fluorescent protein. The construct was assembled with the MoClo method. The fluorescence of *P. chrysogenum* was measured with a biolector after the construct was transformed and integrated into *P. chrysogenum*. At the end of the research all 12 constructs were assembled and 10 constructs were successfully transformed into *P. chrysogenum*.

1. Introduction:

Filamentous fungi are known for producing secondary metabolites and proteins.[3] *Penicillium chrysogenum* became famous for being the industrial producer of the first antibiotic penicillin.[3] *Penicillium chrysogenum* is an interesting organism to work with, since it has a long history of industrial use.[3] Filamentous fungi are known for containing a lot of silent secondary metabolite gene clusters, this was visible in all the in silico work that has been done in the recent years.[4,5] Most of these silent gene clusters can be activated by a certain environmental factor like communication between cells or the presence of certain compounds.[4,5] Some bacterial cells can activate a specific silent gene cluster when they are exposed to a certain antibiotic.[8] In certain cases when plant cells are exposed to a specific electric field they can also start producing extra secondary metabolites.[9] Both of these situations are not only changes in the environment, but are also a stress response from these organisms. Another way to activate silent gene clusters is by promoter exchange.[23,24] With promoter exchange the sequence of an inactive promoter (or one with low activity) can be recombined with the sequence of an active promoter.[23,24] The amount of promoters that can be used for promoter exchange is very low.[3] This is why a new transcription factor based expression system has been built that is potentially useful for promoter exchange.

1.1 Transcription factor

In a transcription factor based expression system a protein (a transcription factor) binds to the DNA in order to induce certain effects to the transcription of the DNA.[13] Gal4 and other transcription factors are able to bind to a specific upstream activation sequence (UAS) of the DNA.[12,13] A transcription factor can either induce or repress the recruitment of RNA-polymerase to the (core) promoter.[13,15] The (core) promoter is able to bind to RNA polymerase.[25] This way transcription factors can play a role in the regulation of the transcription of certain genes.[12]

1.2 Core promoter

A promoter is the sequence in the DNA that can be recognized by RNA polymerase.[25] The core promoter is the minimal sequence that is needed to direct and induce transcription of the DNA.[25] There is a high diversity of core promoters.[25] The core promoter is able to receive specific regulatory input and is able to use this input to precisely regulate transcription.[25] This indicates that every core promoter has their own level of regulation.[25]

1.3 Gal4 system

Gal4 is a protein that was originally found in yeast.[2] This protein is able to bind to the DNA in order to recruit RNA polymerase to a specific gene.[2] Gal4 binds only to a specific upstream activation sequence that originates from yeast.[2] Gal4 has a Zn_2-Cys_6 zinc finger.[11] This motif is required for Gal4 to bind to the UAS sequence of the DNA.[10,11]

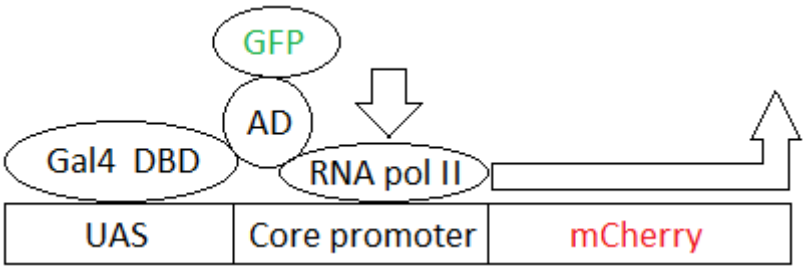
A system similar to UAS/Gal4 (the QUAS/QF system) has already been tested in *Drosophila melanogaster*.[16] This system has QF as the transcription factor and QUAS as the upstream activation sequence.[16] The QUAS/QF system that is introduced by Luo Liqun et al. also contains a transcription factor inhibitor QS.[16] Due to the additional use of QS the QUAS/QF system can be regulated by varying the quinic acid concentration.[16] Similar regulation might occur with the UAS/Gal4 system when Gal80 is also added to the construct.[17]

Inside yeast Gal4 is able to bind to the upstream activation sequence of the gene for Gal1.[21] Gal80 is able to bind to Gal4 in order to repress the Gal4 activity inside yeast.[22] When galactose is available for the cell then Gal3 will be able to bind to Gal80.[22] This causes that Gal4 can be released from gal80 binding and can bind to the UAS sequence of the Gal1 gene.[22] It might be interesting to use this type of regulation inside *P. chrysogenum* as well.

The UAS/Gal4 system is used in *D. melanogaster* to study certain changes inside the cells.[18] When a virus (an environmental factor) infects a cell in this organism then the Gal4 gene will be transcribed & translated.[18] When Gal4 is produced it can become visible in the infected cells by means of fluorescence.[18] Hultmark Dan et al. also states that the UAS/Gal4 system might eventually be usable to change the activity of certain genes inside the infected cells in order to give a better response to the infection.[18] The UAS/Gal4 system has also been used to map proteins that are involved in diseases with a genetic origin.[19,20]

In this research several plasmids have been built that are able to show if the Gal4 activity works properly in *Penicillium chrysogenum.* In this research we use Gal4, because it can specifically bind to a specific upstream activation sequence that (in this case) has been cloned upstream of a core promoter that will transcribe RNA for a red fluorescent protein (called mCherry). Figure 1 shows how Gal4 should work in our construct. The active domain that is presented in figure 1 can vary between Gal4AD and VP16AD. VP16AD is the active domain of the protein VP16 that originates from the Herpes simplex virus.[26] VP16AD has already shown to work as an active domain in the UAS/Gal4 system.[27]

Figure 1: The proposed reaction mechanism of the prepared constructs. Gal4 contains a varying active domain (either Gal4AD or VP16AD) and the fluorescent protein GFP which is used to localize Gal4 and to see if Gal4 is produced at all. Gal4 first binds to the UAS sequence. Then the active domain of the protein recruits RNA polymerase II to the core promoter. The RNA polymerase then transcribes RNA coding for mCherry. If mCherry is produced then red fluorescence should be visible in the cell.



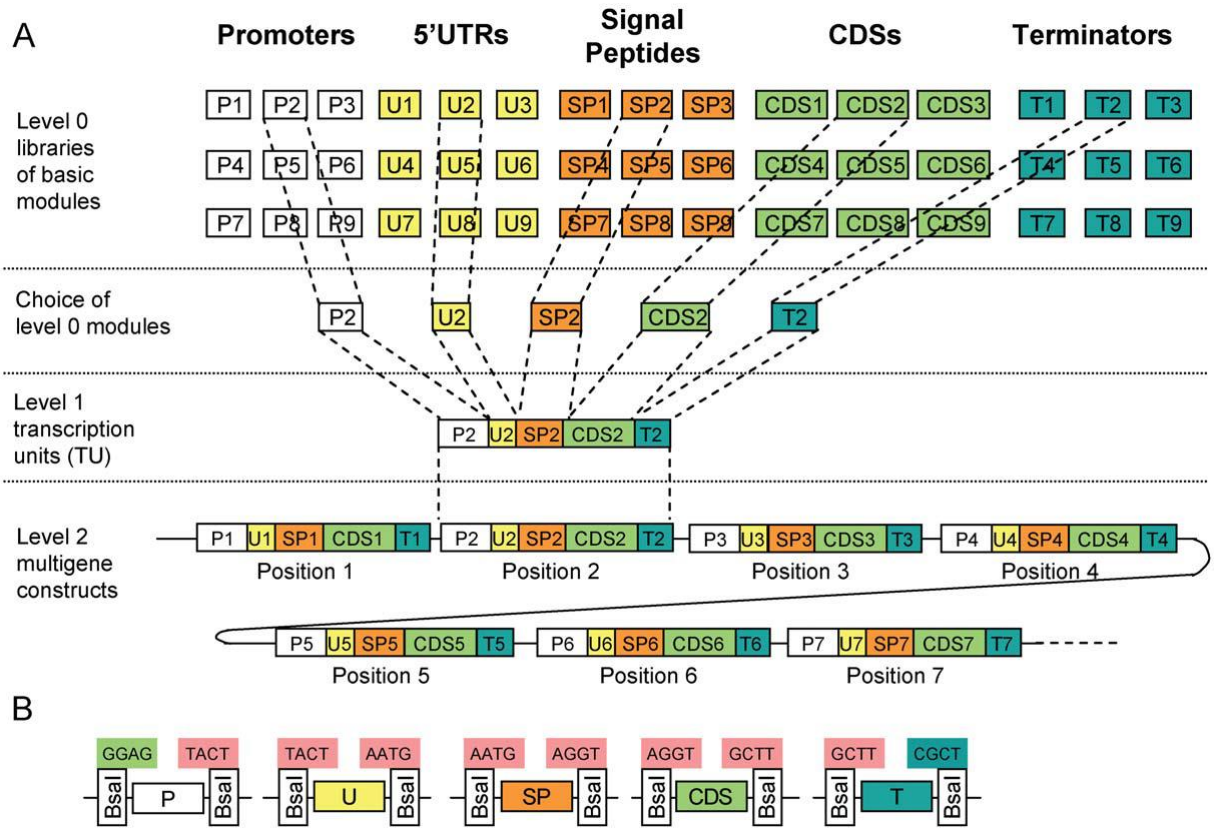
1.4 Golden gate cloning

The (DNA) assembly method that has been used in this research is called Golden gate cloning.[6] With Golden gate cloning one can incorporate genes into vectors which can be transformed into *E.coli*.[6] Golden gate cloning makes use of type IIS restriction enzymes.[6] Type IIS restriction enzymes are special restriction enzymes that are able to digest a piece of DNA outside of the recognition site.[6] These restriction enzymes can remove their own recognition site from varying pieces of DNA when the recognition site for these restriction enzymes is oriented correctly.[6] This way one can create different sticky ends while using the same restriction enzyme.[6] The MoClo assembly method defines a language to build up from simple genetic constructs (level 0) different transcriptional units (level 1), which then can be assembled into complex synthetic pathways (level 2) in a modular manner.[6]

1.5 MoClo assembly and transformation

With the MoClo method as shown in figure 2, first a level 0 plasmid is built (which contains a level 0 part).[6] This is done by amplifying a specified part of the DNA with a PCR using primers that will give the necessary restriction sites to the amplified construct, this is then digested (on both ends) with the type IIS restriction enzyme BpiI and then ligated into a plasmid in a one-pot reaction.[6] In order to make a level 1 construct several level 0 plasmids are digested with BsaI and the level 0 constructs are assembled by sticky end ligation.[6] After the digestion the first level 0 construct should contain sticky ends that are compatible with the sticky ends of the next level 0 construct and so on.[6] This is the case for all the level 0 constructs after digestion with BsaI and level 1 constructs after digestion with BpiI.[6] This way the level 0 or level 1 constructs are assembled in such a way that the sticky ends of the first and last construct fit onto the backbone that is used to make a level 1 (with level 0 constructs) or a level 2 (with level 1 constructs) plasmid. Every time a level 0, level 1 or level 2 plasmid is built, the plasmid is transformed into *E. coli.*[6] The *E. coli* cells are then selected on the antibiotic selection marker that comes with the plasmid (spectinomycin with level 0, ampicillin with level 1 and kanamycin with level 2).[6] The cells are selected on with a blue-white (LacZ + IPTG) screening (level 0 and level 1) or are selected on the production of canthaxanthin (level 2) (β-carotenoid pathway).[6]

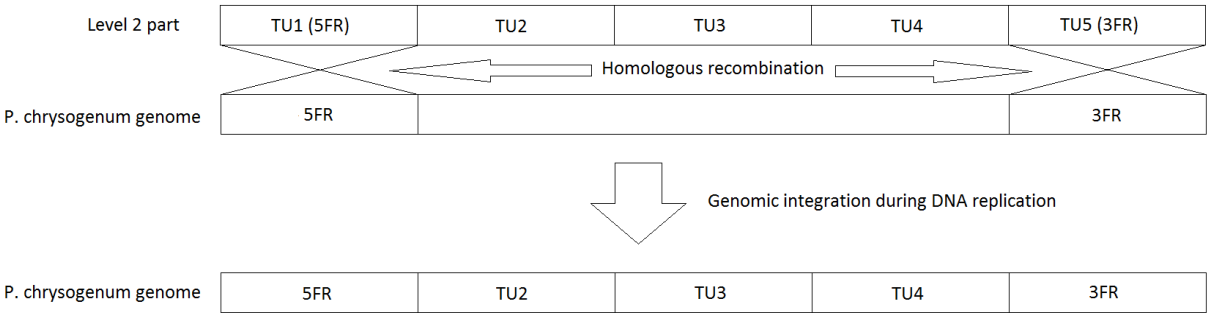
Figure 2[6]: Schematic overview of the MoClo method (Weber et al.). In section A is visible that several of the level 0 constructs are assembled into a level 1 construct. The level 1 constructs are then assembled into level 2 constructs. In section B is visible that every construct that is used for assembly has its own sticky ends. As a result of that the construct should assemble into the correct order and orientation.



When the final construct is assembled then it can be transformed into the destination organism (in our case *Penicillium chrysogenum*). The assembled plasmids contain DraIII restriction sites. When the plasmid is cut at these restriction sites the backbone of the level 2 plasmid will be removed. This will

make the construct linear. This will make it possible for the construct to be used for genomic integration with homologous recombination of the flanking regions. Figure 3 shows how the construct will be integrated into the genome of *P. chrysogenum*.

Figure 3: Schematic view of the genomic integration of a level 2 construct into the *P. chrysogenum* genome by homologous recombination.



1.6 Biolector analysis

The performance of the different constructs can be measured with a biolector.[3] This should happen after integrating the construct into the genome of *P. chrysogenum* and after purifying the strain. A biolector is able to monitor fermentation parameters like biomass formation, pH-values, O_2-concentration and fluorescence.[3] A biolector is also able to monitor the parameters of multiple cultures at the same time (max 48 cultures).[3]

2. Materials and methods:

2.1 Composition of the level 2 plasmids

Before this project, several constructs (promoters, open reading frames, terminators etc.) and transcriptional units (TUs) were made that can be used for the assembly of a final synthetic pathway. During the research 12 level 2 constructs were made by assembling several level 1 constructs (see figure 4 and table 1). All of these constructs contain an AMDS marker to select for the use of acetamide as a nitrogen source. These level 2 constructs are divided into two groups. The plasmid of one group contains information for a Gal4 DNA binding domain together with the Gal4 activation domain. The plasmid of the other group contains information for a Gal4 DNA binding domain together with the VP16 active domain. Varying the active domain of the RNA polymerase recruiting protein should lead to different transcriptional activity.[1] Also leaving out the upstream activation sequence should remove the transcriptional activity.[7] The constructs that have a removed upstream activation sequence are used as a control for the ones that contain an upstream activation sequence. The upstream activation sequence that is used in this research contains 5 Gal4 binding sites in order to amplify the Gal4 activity. Varying core promoters (namely Ura3p, NirAp or PcbCp) were used in order to see if they could give a different transcriptional activity.

Figure 4: This figure shows the full level 2 constructs that were made. It also shows the simple genetic parts that were assembled for the level 1 assembly of the transcriptional units. A total of 12 constructs were made. TU2 has either a Gal4 active domain or a VP16 active domain. TU4 either does or does not have an upstream activating sequence (UAS) and continues/ starts off with either an Ura3p, a NirAp or a PcbCp core promoter. The UAS sequence in these constructs contains 5 Gal4 binding sites. The Dra III restriction sites that are given at the ends of the construct are used for both the restriction analysis as well as for the digestion of the plasmid in order to integrate the construct into the genome.

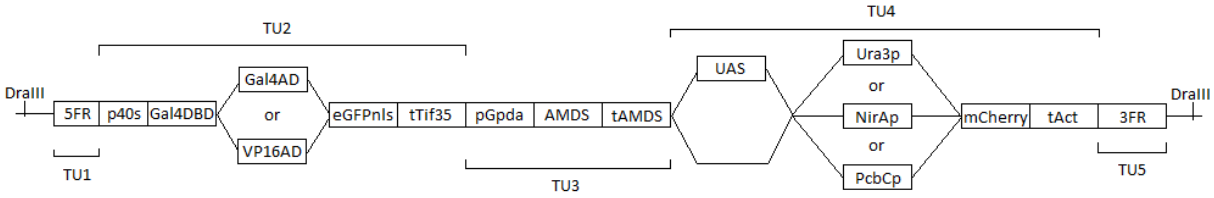


Table 1: The table below shows what the differences are between the constructs. The table also gives the length of each of the different constructs.

Construct	TU2 contains	TU4 contains	UAS	Length backbone with DraIII digestion (bp)	Length construct with DraIII digestion (bp)
A	Gal4AD	Ura3p	Yes	4813	10029
B	Gal4AD	NirAp	Yes	4813	9985
C	Gal4AD	PcbCp	Yes	4813	10091
D	Gal4AD	Ura3p	No	4813	9932
E	Gal4AD	NirAp	No	4248	9888
F	Gal4AD	PcbCp	No	4248	9994
G	VP16AD	Ura3p	Yes	4248	9822
H	VP16AD	NirAp	Yes	4248	9778
I	VP16AD	PcbCp	Yes	4248	9884
J	VP16AD	Ura3p	No	4248	9725
K	VP16AD	NirAp	No	4248	9681
L	VP16AD	PcbCp	No	4248	9787

2.2 Culture conditions:

The *E. coli* cells were grown on regular LB-medium or LB-agar plates containing either ampicillin (level 1 constructs) or kanamycin (level 2 constructs).
The AFF 407 strain was grown in YGG before protoplastation.

Preparation of T-agar:
Trace element solution, 10% acetamide solution and 1M of autoclaved phosphate buffer (pH=6.8) are prepared beforehand.

Table 2: Composition of T-agar media

	Concentration needed	Added weight
$FeSO_4$ x 7 H_2O	0.01 g/L	0.01 g
$MgSO_4$ x 7 H_2O	0.5 g/L	0.5 g
NaCl	3 g/L	3 g
$C_{12}H_{22}O_{11}$	342 g/L	342 g
$C_6H_{12}O_6$	10 g/L	10 g
Agar-Agar	15 g/L	15 g

After autoclaving add 10 mL of 10% acetamide solution, 10 mL of phosphate buffer & 2 mL of trace element solution to the medium before pouring the plates (**the trace element solution wasn't added to the plates during this research**).

Preparation of 1L R-agar:

Table 3: Composition of R-agar media

	Concentration needed	Added weight
Agar-Agar	15 g/L	15 g
Yeast extract	5 g/L	5 g
Sugar beet molasse	7.5 g/L	7.5 mL
Glycerol 85%	7 g/L	7 mL
$MgSO_4$ x 7 H_2O	0.05 g/L	0.05 g
NaCl	18 g/L	18 g
$CaSO_4$ x 2 H_2O	0.25 g/L	0.25 g
KH_2PO_4	0.06 g/L	0.06 g
$CuSO_4$ x 5 H_2O	0.01 g/L	0.01 mL
$NH_4Fe(SO_4)_2$ x 12 H_2O	0.16 g/L	0.16 mL

The sugar beet molasse, $CuSO_4$ x 5 H_2O & $NH_4Fe(SO_4)_2$ x 12 H_2O were already prepared at a proper concentration.

Preparation of S-agar:
Trace element solution, 10% acetamide solution and 1M of autoclaved phosphate buffer (pH=6.8) are prepared beforehand.

Table 4: Composition of S-agar media

	Concentration needed	Added weight
$FeSO_4$ x 7 H_2O	0.01 g/L	0.01 g
$MgSO_4$ x 7 H_2O	0.5 g/L	0.5 g
NaCl	3 g/L	3 g
$C_6H_{12}O_6$	10 g/L	10 g
Agar-Agar	15 g/L	15 g

After autoclaving add 10 mL of 10% acetamide solution, 10 mL of phosphate buffer & 2 mL of trace element solution to the medium before pouring the plates (**the trace element solution wasn't added to the plates during this research**).

Preparation of 500 mL YGG:
For the YGG medium 10 mL of autoclaved 10% yeast extract was prepared beforehand.
400 mL KCl-Glucose (needs to be autoclaved):

Table 5: Composition of the KCl-glucose solution

	Concentration needed	Added weight
KCl	10 g/L	4 g
$C_6H_{12}O_6$ x H_2O	20 g/L	8 g

100 mL 5x buffered YNB:

Table 6: Composition of the 5x buffered YNB

	Concentration needed	Added weight
YNB w/o amino acids	33.3 g/L	3.33 g
$C_6H_8O_7$ x H_2O	7.5 g/L	0.75 g
K_2HPO_4	30 g/L	3 g

Add the 100 ml of 5x buffered YNB to the KCl-glucose while filter sterilizing it. Add 10 mL of sterile 10% Yeast Extract afterwards.

2.3 Building level 2 constructs:

Figure 4 and table 1 (above) show the level 2 constructs. BpiI (Thermo Scientific) was used to cut the level 1 plasmids at both ends in order to perform the assembly reaction. The different transcriptional units were able to assemble by sticky end ligation with ligase (Thermo Scientific). Table 13 in the appendix shows which plasmids were used for each construct. 20 femto molar of each of the specified plasmids was taken in order to perform the reactions. The reactions were performed in a final volume of 15 µL. The ligase buffer that was used for the assembly reaction is from Thermo Scientific.

Table 7: An example of a reaction mixture for the assembly reaction of construct A when the plasmids would have a concentration of 20 nmol/L.

Plasmids or solutions	Reaction mixture
pAGM4723 cor	1 µL
pZB1_1_TU1_5FR	1 µL
pSD1_1_Donor_Gal4_TU2	1 µL
pYn1_65 AMDS (TU3)	1 µL
pSD1_3_Reciever_UAS_Ura3_mCherry (TU4)	1 µL
pZB1_2_TU5_3FR	1 µL
pICH41800	1 µL
MQ	2 µL
Ligase buffer	1.5 µL
Ligase	1 µL
BpiI	0.5 µL

2.4 Transformation and screening of *E. coli*:

All of the level 2 plasmids were transformed into chemically competent β-10 cells.
When the constructs were successfully transformed into *E.coli* they were screened with a colony PCR and a restriction analysis with DraIII. The reaction volume and incubation temperature of the colony PCR is visible in tables 8 and 9. Table 10 shows the reaction mixture of the restriction analysis. The colony PCR in *E. coli* was performed with primer pairs 99 & 146 and primer pairs 100 & 147 (see table 14 in the appendix). The phire green that is used for the amplification of the DNA is from Thermo Scientific.

Table 8: Reaction mixture for the colony PCR.

	1x reaction
Phire green	10 µL

MQ	9 µL
Primer fw	0.5 µL
Primer rev	0.5 µL

Table 9: Thermocycling conditions of the colony PCR.

Incubation time	Incubation temperature	Repeats
5 min	98 °C	1x
5 sec	98 °C	25x
5 sec	59 °C	
45 sec	72 °C	
1 min	72 °C	1x
Hold	12 °C	

Table 10: Reaction mixture for the restriction analysis. The restriction buffer and Dra III are from Thermo Scientific.

	Reaction mixture
Plasmid	150-250 ng
Restriction buffer	1 µL
Restriction enzyme (Dra III)	0.3 µL
MQ	Fill up to 10 µL

2.5 Transformation and screening of *P. chrysogenum*:

The constructs were transformed and integrated into the genome of *Penicillium*. The flanking regions of the construct (5FR and 3FR) are complementary to a part of the genome in the AFF407 strain. This will make it possible for the level 2 construct to integrate into the genome by homologous recombination (see figure 3). The transformation plate contains acetamide as its only nitrogen source. The AMDS selection marker is a gene that produces acetamidase. This gives *Penicillium* the ability to use acetamide from the plates as a nitrogen source. The transformations were performed according to Weber et al.[14] . Dra III was used to digest the plasmid in order to integrate the construct into the genome (see figure 4).

The transformed cells are screened with a colony PCR for containing either the wildtype copies of the chromosome, or the integrated construct or both (since these fungi may contain several copies of their nuclei). The reaction volume and incubation temperature of the colony PCR is shown in tables 11 & 12. A small piece of mycelium is first smashed in DNA dilution buffer (Thermo Scientific). The suspension is spun down, then this can be used as a template for the PCR reaction. Primers 28, 72, 110 & 111 were used for the colony PCR in *P. chrysogenum* (see table 14 in the appendix and figure 5). The phire polymerase, the PCR buffer and the DNA dilution buffer that were used here are from the Thermo Scientific phire plant direct PCR kit. Figure 5 shows how the colony PCR will proof that the construct should be correctly integrated into the genome.

Table 11: Reaction mixture for the colony PCR from *Penicillium*.

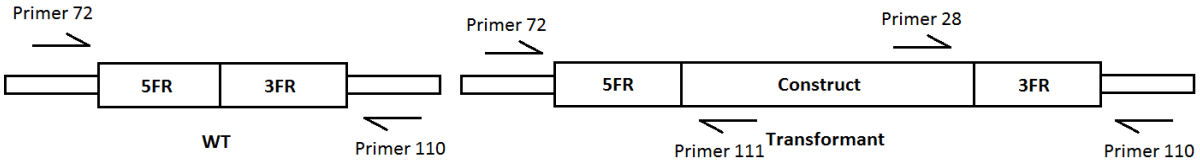
	1x reaction
Phire polymerase	0.4 µL
PCR buffer	10 µL
Primer fw	0.15 µL
Primer rev	0.15 µL
MQ	8.8 µL
Template	0.5 µL

Table 12: Incubation schedule of the colony PCR from *Penicillium*.

Incubation time	Incubation temperature	Repeats
5 min	98 °C	1x

20 sec	98 °C	35x
20 sec	62 °C	
2.5 min	72 °C	
10 min	72 °C	1x
Hold	4 °C	

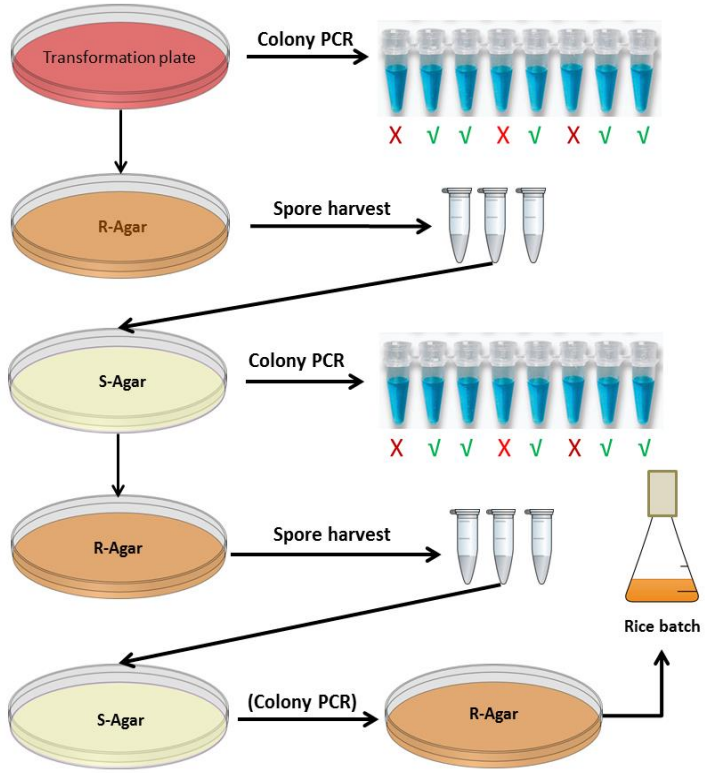
Figure 5: A schematic view of the primer position in the *P. chrysogenum* colony PCR.



2.6 Purification of *P. chrysogenum*

Figure 6 shows the steps that were performed for the purification. The colonies that have been proven to have the correct integration of the construct were plated out on R-agar medium for sporulation. The spores are then harvested with 50 µL of (autoclaved) spore harvest solution (0.8% NaCl & 0.05% Triton X-100 dissolved in MQ). This is pipetted twice on a sporulating colony. Approximately 50 µL of the harvested spores is then diluted in 450 µL sterile MQ (10x dilution). This solution is diluted 10x and 100x again and 100 µL of both dilutions (100x and 1000x) are then plated out on S-agar (selective medium). This medium contains acetamide as its only nitrogen source. The colonies on this plate are screened with a colony PCR and the colonies with the highest ratio of the correctly integrated construct compared to the wildtype sequence are plated out on R-agar. This cycle is repeated at least once in order to obtain cells that only appear to contain the integrated construct.

Figure 6: Schematic view of the purification of *P. chrysogenum*. (Carsten Pohl)



2.7 Analysis with the biolector

Mycelia was grown in a biolector for 5 days according to Polli et al.[3] . Fluorescence and biomass formation was monitored over time. The biolector data has been analyzed with TIBCO Spotfire software.

3. Results/discussion:

3.1 Restriction analysis:

All of the constructs have been built by using the MoClo system (see the results of figures 7, 8, 9 & 10). 10 out of 12 constructs were successfully integrated into the genome of *P. chrysogenum*. 6 of these strains were purified. The gels that are given in the figures below show a general outline of the results that have been acquired during this research.

Figure 7: A gel of the restriction analysis with DraIII (see figure 4) of constructs A, B, C and D (table 1 has additional information). This gel shows the results of the first successful MoClo assembly. The exact sizes of the constructs can be found in table 1. The construct is supposed to be the upper band (approximately 10000bp) and the backbone is supposed to be the lower band (4813bp). The correct band are indicated with an arrow.

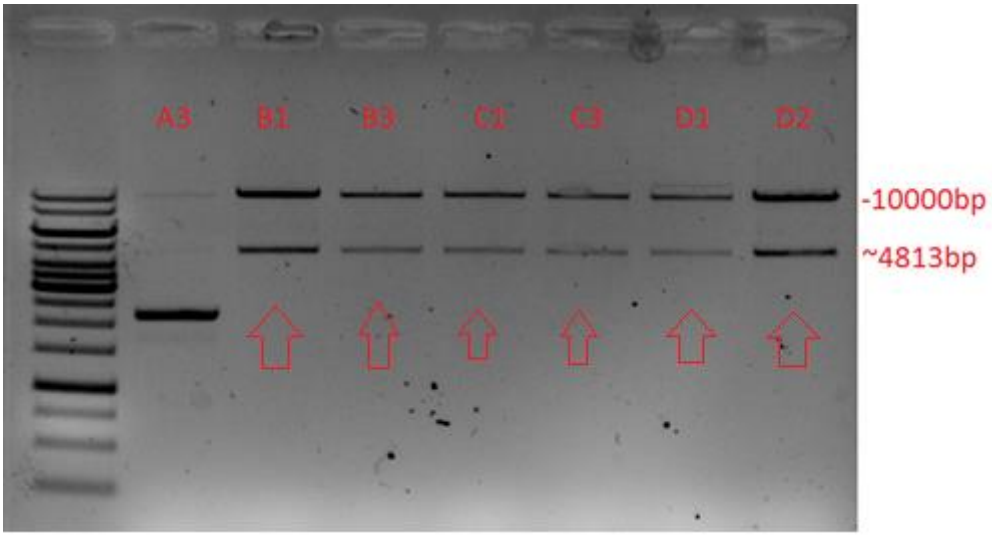


Figure 8: A gel of the restriction analysis with DraIII (see figure 4) of constructs E, G, H, I, K and L (table 1 has additional information). This gel shows the results of the second successful MoClo assembly. The exact sizes of the constructs can be found in table 1. The construct is supposed to be the upper band (approximately 10000bp) and the backbone is supposed to be the lower band(4248bp). The correct constructs are indicated with an arrow.

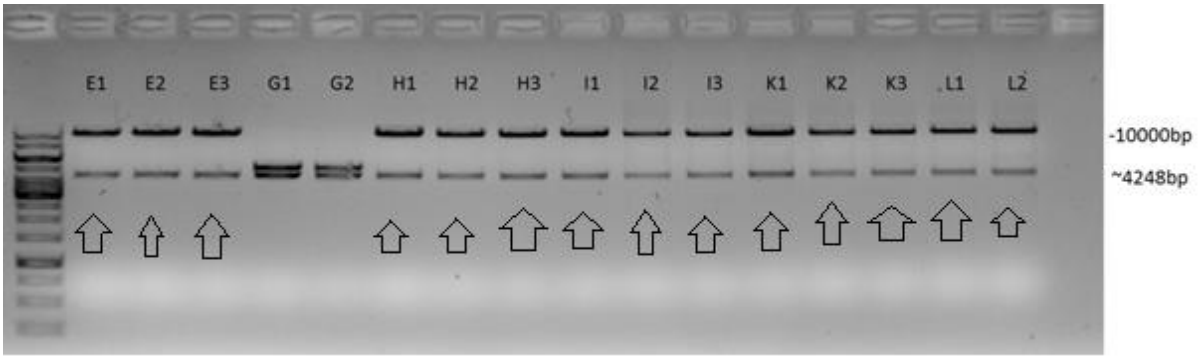


Figure 9: A gel of the colony PCR of A and the restriction analysis of construct F (table 1 has additional information). This gel shows another result of the second successful MoClo assembly. The exact sizes of the constructs can be found in table 1. The construct is supposed to be the upper band (9994bp) and the backbone is supposed to be the lower band (4248bp). The correct bands are indicated with an arrow.

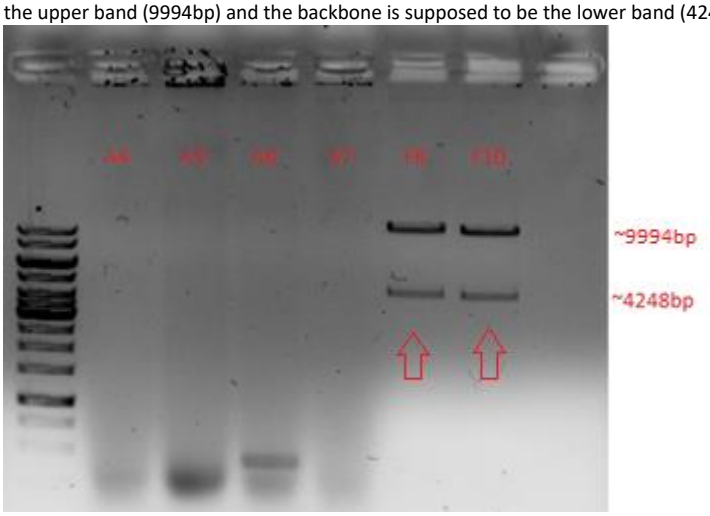
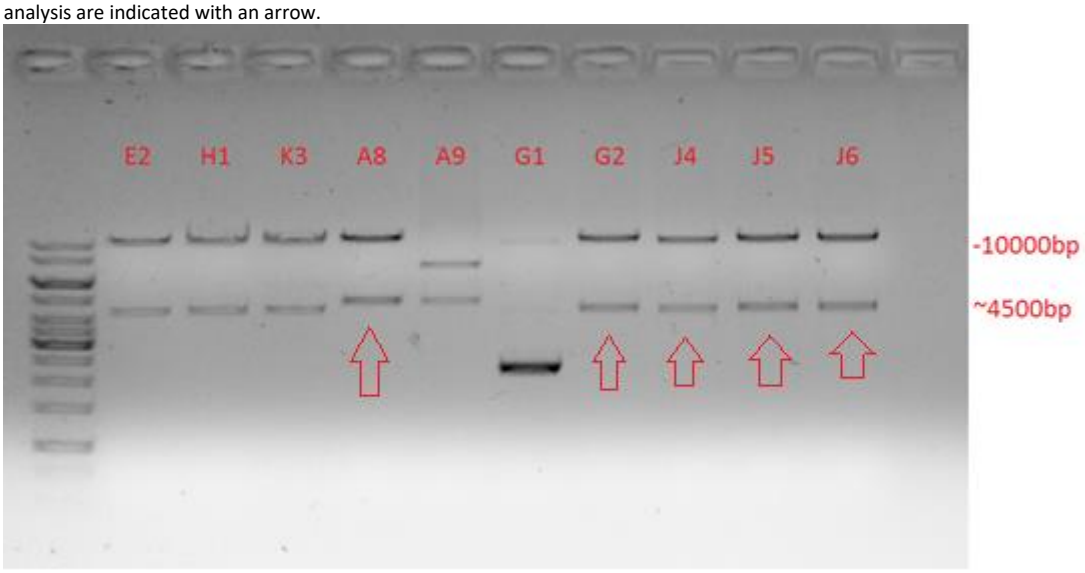


Figure 10: A gel of the digestion of constructs E, H and K and the restriction analysis with DraIII (see figure 4) of constructs A, G and J (table 1 has additional information). This gel shows the results of the first (construct A) and third (construct G and J) successful MoClo assemblies. The exact sizes of the constructs can be found in table 1. The construct is supposed to be the upper band (approximately 10000bp) and the backbone is supposed to be the lower band (approximately 4813bp or approximately 4248bp). The correct bands for the restriction analysis are indicated with an arrow.



With the first successful MoClo assembly constructs B, C and D (see table 1 and figure 7) are immediately found in the first few colonies. Construct A (see table 1 and figure 10) was found in later colonies from this assembly. Constructs E, F, H, I, K and L (see table 1 and figures 8 and 9) were prepared with the second successful MoClo assembly. Construct G and J (see table 1 and figure 10) were prepared with the third successful MoClo assembly. This data shows that 10 out of 12 of the constructs were already assembled during the first two successful MoClo assemblies. This shows that the MoClo assembly should be able to work in general.

3.2 Colony PCR of *P. chrysogenum*:

Figure 11: A gel of the colony PCR of some purified *Penicillium* colonies containing construct H (the other constructs are not fully purified here). The amplified size for the construct (lower row) is ~1550 base pairs. The amplified size for the wildtype (upper row) is ~3000 base pairs. The constructs that are supposedly pure enough are indicated with an arrow.

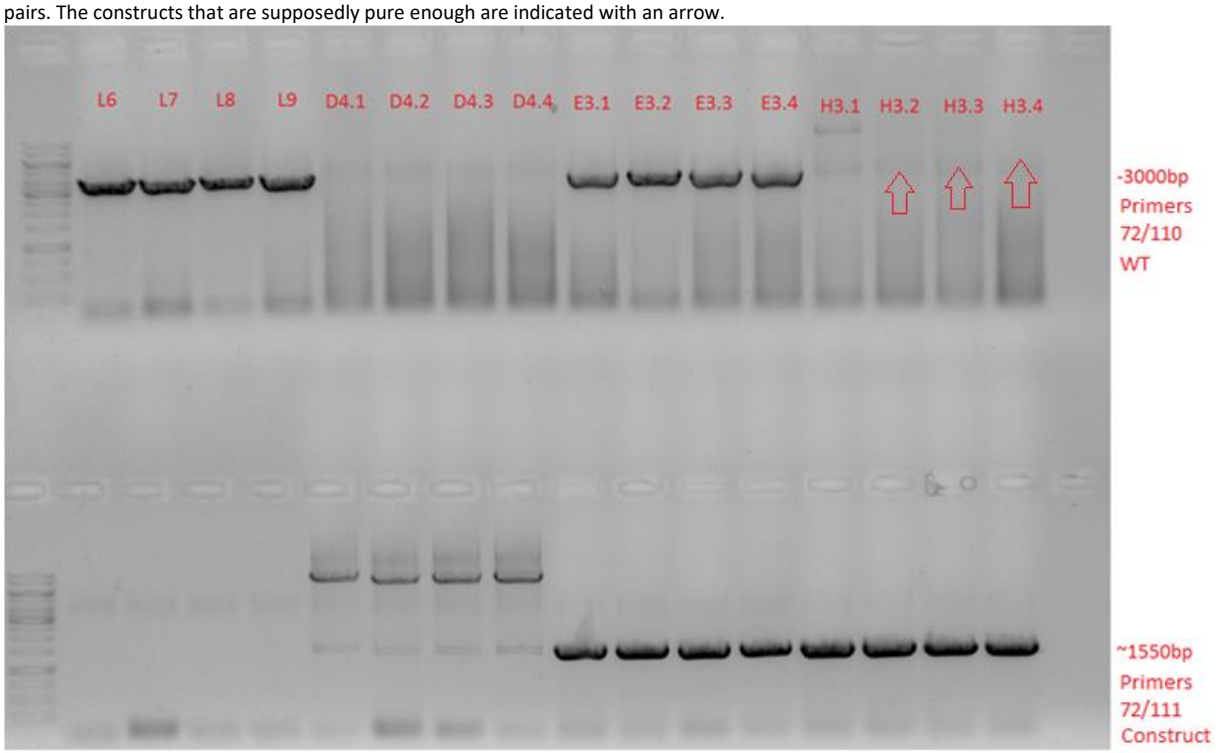


Figure 12 & 13: Two gels of the colony PCR of some purified *Penicillium* colonies containing construct D or E. The amplified size for the construct (upper row) is 3180 base pairs for D and 1550 base pairs for E. The amplified size for the wildtype (upper row) is ~3000 base pairs. The constructs that are supposedly pure enough are indicated with an arrow.

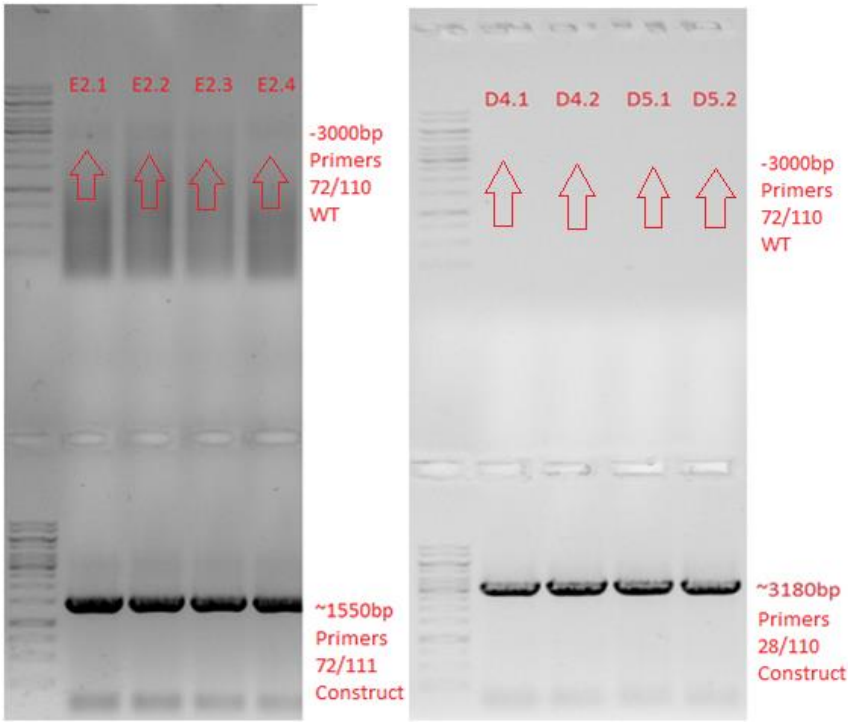


Figure 14: A gel of the colony PCR of some purified *Penicillium* colonies containing construct K. The amplified size for the construct (lower row) is ~3180 base pairs. The amplified size for the wildtype (upper row) is ~3000 base pairs. The constructs that are supposedly pure enough are indicated with an arrow.

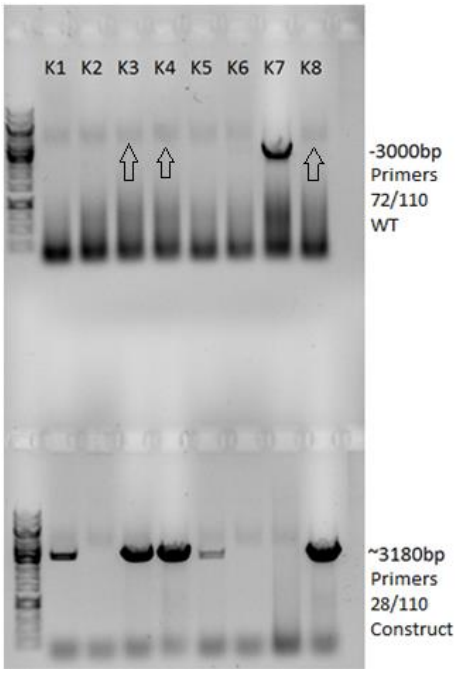
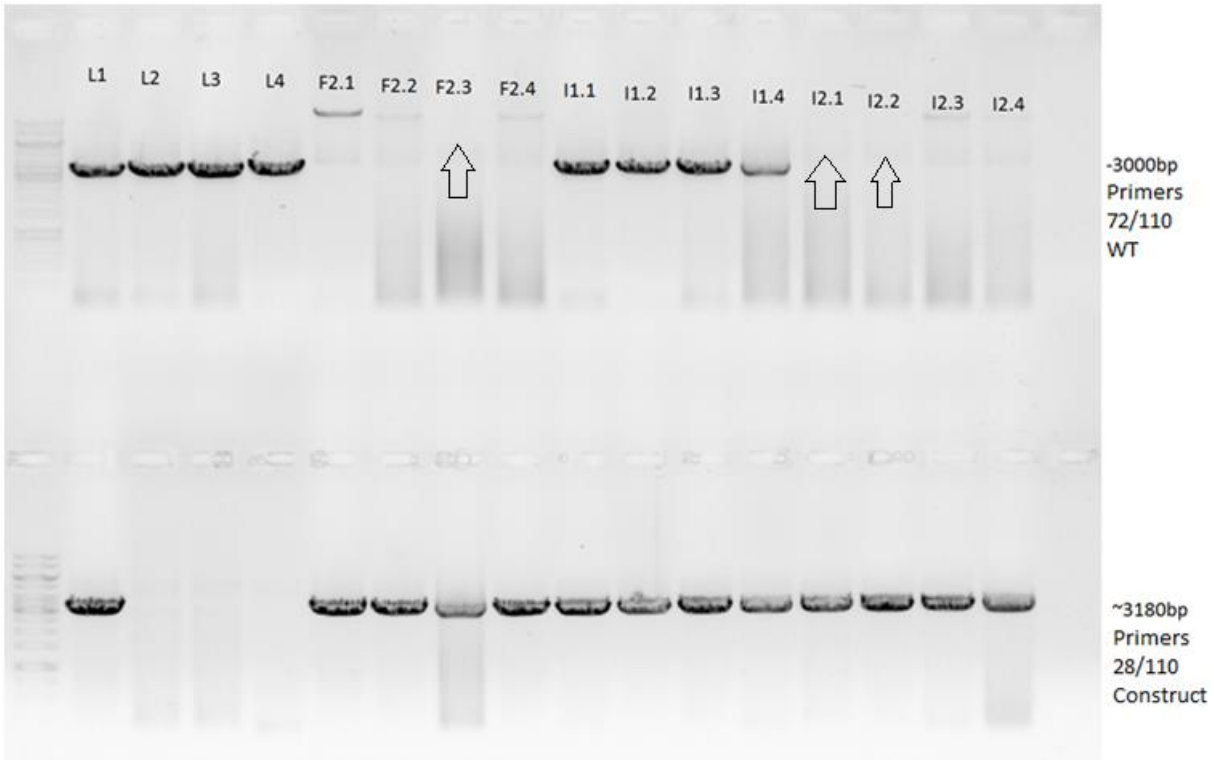


Figure 15: : A gel of the colony PCR of some purified *Penicillium* colonies containing constructs F or I. The amplified size for the construct (lower row) is ~3180 base pairs. The amplified size for the wildtype (upper row) is ~3000 base pairs. The constructs that are supposedly pure enough are indicated with an arrow.



The figures 11 t/m 15 show which strains are purified. If the gel does not show or barely shows any bands at the wildtype (WT) wells then the strain is pure enough to analyze it with the biolector. This does not mean that the strain is completely pure. There may still be a very low amount of wildtype present in the strain. Further purification of the strain is unnecessary, because it is not possible anymore to make any of these genetic changes visible.

3.3 Biolector data and analysis:

The strains can be analyzed with the biolector when the strains are pure enough. The strains containing constructs D, E and H (see table 1) were analyzed with the biolector. Figure 16, 17, 18 and 19 show the results of the most interesting parameters in this analysis.

Figure 16: Biolector data of the biomass of the D, E and H strains of *P. chrysogenum* and for comparison the wildtype. Each line in this graph represents two measurements of technical replicates. The error bars that are shown in this graph are therefore of only two measurements each. All of the constructs seem to grow at nearly the same pace. **The time here is given in hours.**

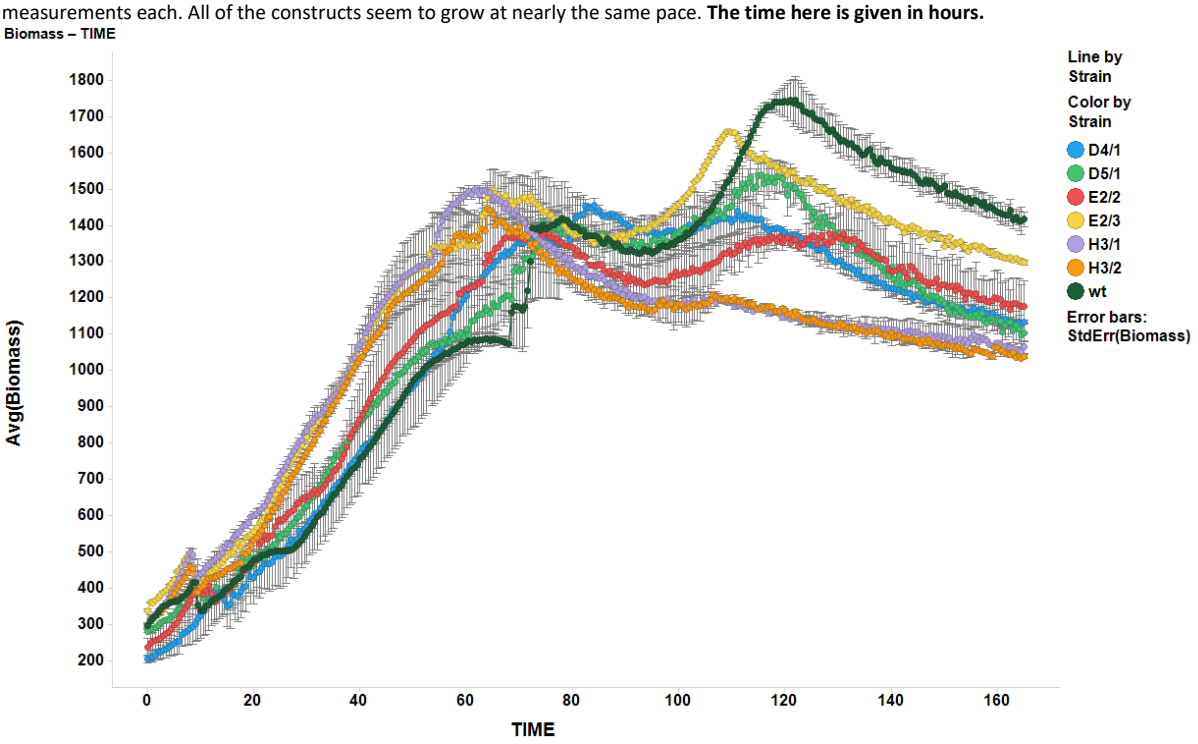


Figure 17: Biolector data of the GFP fluorescence of D, E and H strains of *P. chrysogenum* and for comparison the wildtype. Each line in this graph represents two measurements of technical replicates. The error bars that are shown in this graph are therefore of only two measurements each. Only construct H seems to have GFP production. **The time here is given in hours.**

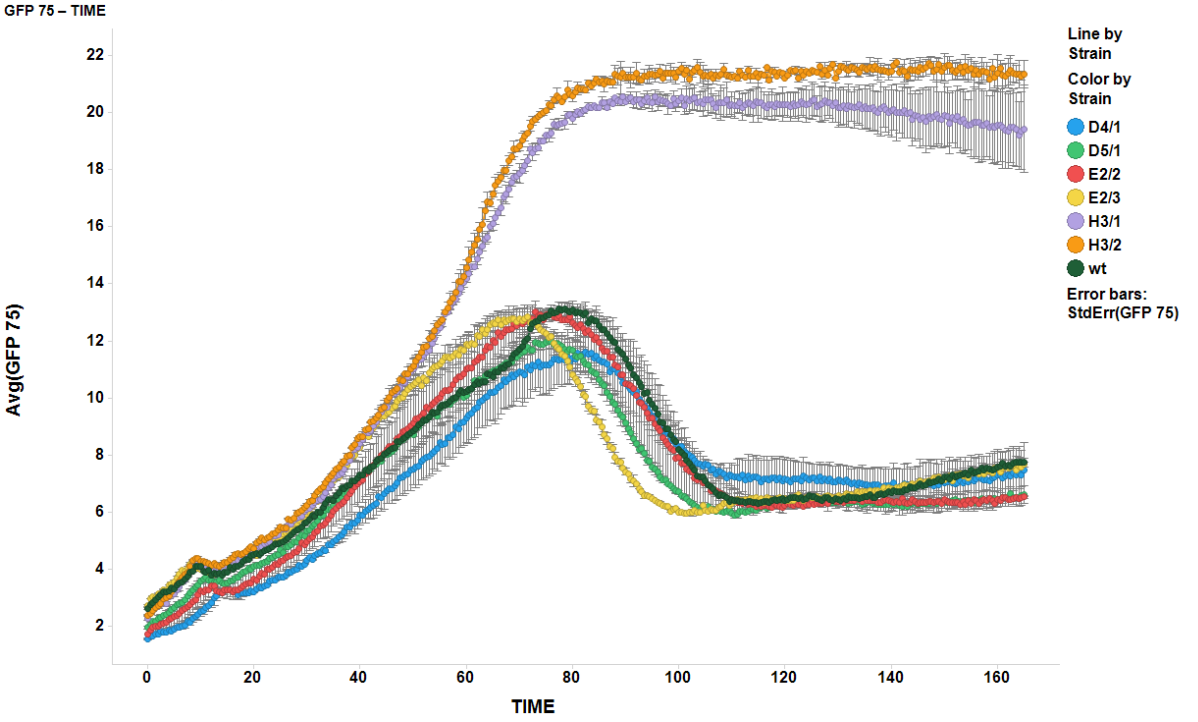
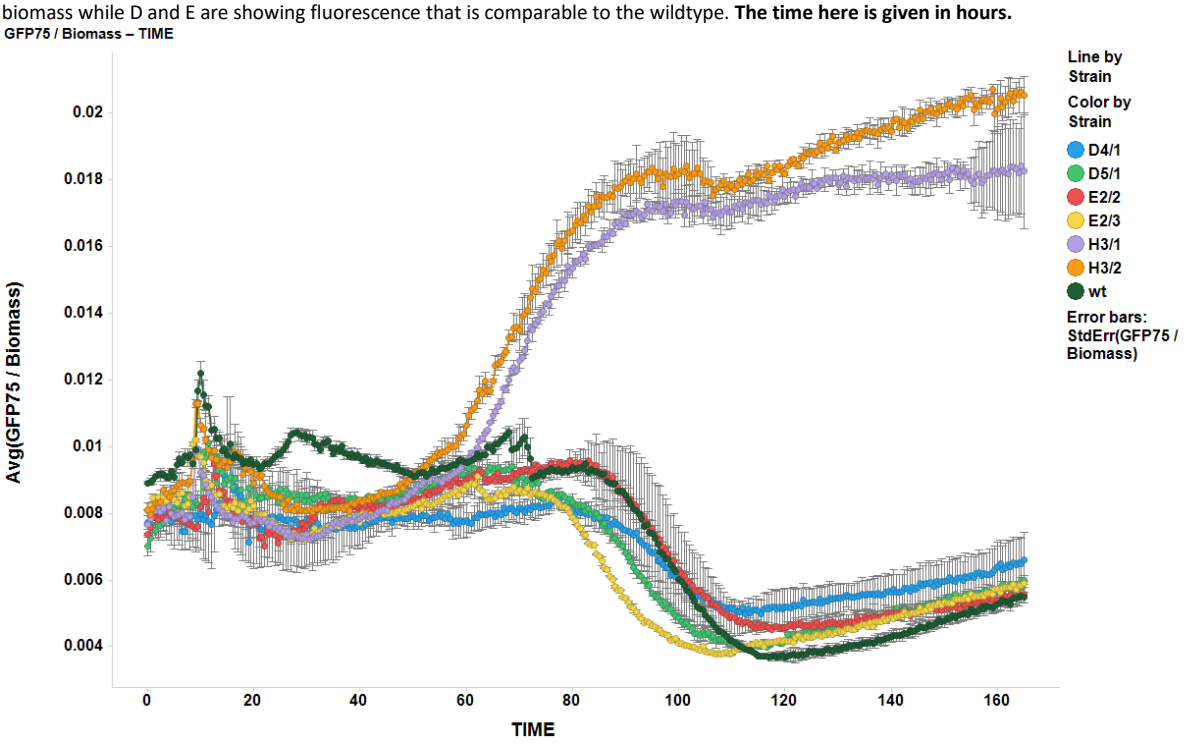
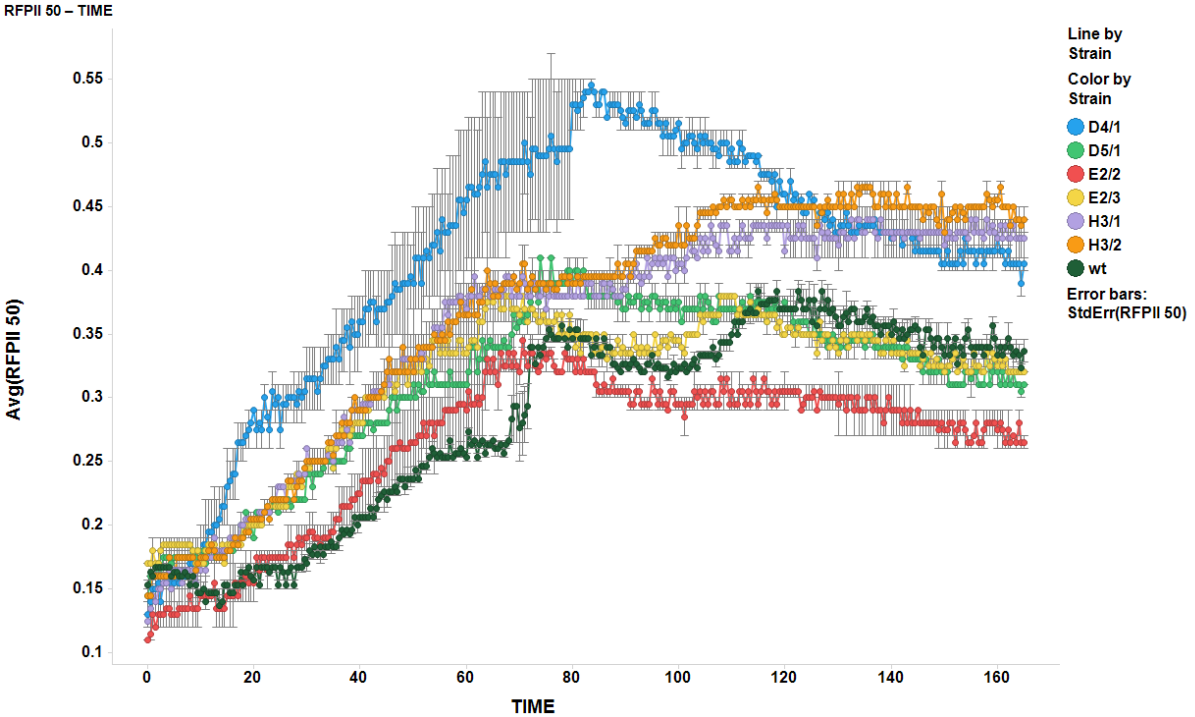


Figure 18: Biolector data about the fluorescence of GFP divided by the biomass of *P. chrysogenum* that contains either construct D, E or H and for comparison the wildtype. Each line in this graph represents two measurements of technical replicates. The error bars that are shown in this graph are therefore of only two measurements each. Only H seems to have an increase in fluorescence compared to the biomass while D and E are showing fluorescence that is comparable to the wildtype. **The time here is given in hours.**



Figures 16, 17 and 18 show that only strain H has an actual GFP (and therefore Gal4) production. Strain D and E seem to have no Gal4 production. This is certainly unexpected, since both strains should have the integrated construct according to figure 12 and 13. Figure 19 shows that strain H does not produce mCherry in a manner that would be expected with the Gal4 production in figures 16 and 18.

Figure 19: Biolector data about the fluorescence of RFP in *P. chrysogenum* that contains either construct D, E or H and for comparison the wildtype. Each line in this graph represents two measurements of technical replicates. The error bars that are shown in this graph are therefore of only two measurements each. The red fluorescence does not seem to correspond with the green fluorescence in figures 16 and 18. **The time here is given in hours.**



The UAS/Gal4 constructs that have been analyzed here are not yet working as expected in *P. chrysogenum*. The results that are given indicate that something may be wrong with the colonies that were used for the analysis. This may have happened if the integrated construct wasn't pure enough. Another reason for this could be that the colonies that were used for the colony PCR and for the analysis with the biolector were mixed up. It is recommended that this will be analyzed in further research.

4. Conclusion:

The gels of the restriction analysis in the results section show that the assembly reaction did assemble the constructs with a correct size. Also the colony PCR results show that the construct should be integrated at the right position in the *P. chrysogenum* genome. The biolector analysis pointed out that constructs D & E (see table 1) are not producing Gal4. It also shows that H could be producing Gal4 with the GFP tag, but we could not detect red fluorescence of the mCherry. A western blot of strain H could point out if Gal4 is produced completely by this strain and is (for example) not partially produced (with the GFP-tag). A colony PCR can be used to see if the strains that were analyzed with the biolector were the correct strains. Also several pieces of the integrated constructs will be sequenced in order to confirm whether the strains contain the correct constructs.

5. References:

1. Inducible, tightly regulated and growth condition-independent transcription factor in Saccharomyces cerevisiae. Ottoz, Diana S. M.; Rudolf, Fabian; Stelling, Joerg. Nucleic Acids Research 42(17). Oxford University Press 2014

2. A controllable on-off strategy for the reproductive containment of fish. Zhang, Yunsheng; Chen, Ji; Cui, Xiaojuan; Luo, Daji; Xia, Hui; Dai, Jun; Zhu, Zuoyan; Hu, Wei. Scientific Reports 5. Nature Publishing Group 2015

3. New promoters for strain engineering of *Penicillium chrysogenum.* Polli, Fabiola; Meijrink, Ben; Bovenberg, Roel A. L.; Driessen, Arnold J. M.. Fungal Genetics and Biology 89: page 62-71. Academic Press 2016-4

4. CASSIS and SMIPS: promoter-based prediction of secondary metabolite gene clusters in eukaryotic genomes. Wolf, Thomas; Shelest, Vladimir; Nath, Neetika; Shelest, Ekaterina. Bioinformatics 32(8): page 1138-1143. Oxford University Press 2016-4-15

5. Activation of a silent fungal polyketide biosynthesis pathway through regulatory cross talk with a cryptic nonribosomal peptide synthetase gene cluster. Bergmann, Sebastian; Funk, Alexander N.; Scherlach, Kirstin; Schroeckh, Volker; Shelest, Ekaterina; Horn, Uwe; Hertweck, Christian; Brakhage, Axel A.. Applied and Environmental Microbiology 76(24): page 8143-8149. American Society for Microbiology 2010-12

6. A Modular Cloning System for Standardized Assembly of Multigene Constructs. Weber, Ernst; Engler, Carola; Gruetzner, Ramona; Werner, Stefan; Marillonnet, Sylvestre. PLoS ONE 6(2). Public Library of Science 2011

7. An upstream activation sequence controls the expression of AOX1 gene in *Pichia pastoris*. Xuan, Yaoji; Zhou, Xiangshan; Zhang, Wenwen; Zhang, Xiao; Song, Zhiwei; Zhang, Yuanxing. Fems Yeast Research 9(8): page 1271-1282. Wiley 2009-12

8. A *Burkholderia thailandensis* acyl-homoserine lactone-independent orphan luxR homolog that activates production of the cytotoxin malleilactone. Truong, Thao T.; Seyedsayamdost, Mohammad; Greenberg, E. Peter; Chandler, Josephine R. Journal of Bacteriology 197(21): page 3456-3462. American Society for Microbiology 2015-11

9. Pulsed electric fields on phenylalanine ammonia lyase activity of tomato cell culture. Gürsul, Işil; Gueven, Alper; Grohmann, Anne; Knorr, Dietrich. Journal of Food Engineering 188: page 66-76. Elsevier 2016

10. Gal4 transcription factor is not a zinc finger but forms a ZN(II)2CYS6 binuclear cluster. Pan, Tao; Coleman, JE. Proceedings of the National Academy of Sciences of the United States of America 87(6): page 2077-2081. National Academy of Sciences 1990

11. Zinc finger transcription factors displaced SREBP proteins as the major Sterol regulators during Saccharomycotina evolution. Maguire, Sarah L.; Wang, Can; Holland, Linda M.; Brunel, Francois; Neuvéglise, Cécile; Nicaud, Jean-Marc; Zavrel, Martin; White, Theodore C.; Wolfe, Kenneth H.; Butler, Geraldine. PLoS Genetics 10(1). Public Library of Science 2014-1

12. High efficiency inducible gene expression system based on activation of a chimeric transcription factor in transgenic pine. Tang, Wei; Newton, Ronald J.; Charles, Thomas M.. Plant Cel. Reports 24(10): page 619-628. Springer Berlin Heidelberg 2005-12

13. Transcription factors: an overview. Latchman, David S.. International Journal of Biochemistry and Cell Biology, The 29(12): page 1305-1312. Pergamon 1997-12

14. Fungal ABC transporter deletion and localization analysis. Kovalchuk, Andriy; Weber, Stefan S.; Nijland Jeroen G.; Bovenberg, Roel A. L.; Driessen Arnold J. M.. Methods Mol. Biol. 835: page 1-16. 2012

15. Transcription of eukaryotic protein-coding genes. Lee, Tong Ihn; Young, Richard A.. Annual Review of Genetics 34: page 77-137. Annual Reviews 2000

16. Using the Q system in *Drosophila melanogaster*. Potter, Christopher J.; Luo, Liqun. NATURE PROTOCOLS 6(8): page 1105-1120. 2011-8

17. A flippase-mediated GAL80/GAL4 intersectional resource for dissecting appendage development in *Drosophila*. Smith, Brittany N.; Ghazanfari, Arash M.; Bohm, Rudolf A.; Welch, William P.; Zhang, Bing; Masly, John P.. G3 : Genes, Genomes, Genetics 5(10): page 2105-2112. Genetics Society of America 2015-10

18. A novel strategy for live detection of viral infection in *Drosophila melanogaster*. Ekström, Jens-Ola; Hultmark, Dan. Scientific Reports 6. Nature Publishing Group 2016

19. Mapping C-terminal transactivation domains of the nuclear HER family receptor tyrosine kinase HER3. Brand, Toni M.; Iida, Mari; Luthar, Neha; Wleklinski, Matthew J.; Starr, Megan M.; Wheeler, Deric L.. PLoS ONE 8(8). Public Library of Science 2013

20. *Drosophila* eye model to study neuroprotective role of CREB binding protein (CBP) in Alzheimer's disease. Cutler, Timothy; Sarkar, Ankita; Moran, Michael; Steffensmeier, Andrew; Puli, Oorvashi Roy; Mancini, Greg; Tare, Meghana; Gogia, Neha; Singh, Amit. PLoS ONE 10(9). Public Library of Science 2015

21. Construction of synthetic regulatory networks in yeast. Blount, Benjamin A.; Weenink, Tim; Ellis, Tom. FEBS Letters 586(15): page 2112-2121. Elsevier 2012-7-16

22. Self-association of the Gal4 inhibitor protein Gal80 is impaired by Gal3: evidence for a new mechanism in the GAL gene switch. Egriboz, Onur; Goswami, Sudip; Tao, Xiaorong; Dotts, Kathleen; Schaeffer, Christie; Pilauri, Vepkhia; Hopper, James E.. Molecular and Cellular Biology 33(18): page 3667-3674. American Society for Microbiology 2013-9

23. Yeast homologous recombination-based promoter engineering for the activation of silent natural product biosynthetic gene clusters. Montiel, Daniel; Kang, Hahk-Soo; Chang, Fang-Yuan; Charlop-Powers, Zachary; Brady, Sean F. Proceedings of the National Academy of Sciences 112(29): page 8953-8958. 2015-7-21

24. Simple "on-demand" production of bioactive natural products. Bode, Edna; Brachmann, Alexander O.; Kegler, Carsten; Simsek, Rukayye; Dauth, Christina; Zhou, Qiuqin; Kaiser, Marcel; Klemmt, Petra; Bode, Helge B.. ChemBioChem 16(7): page 1115-1119. Wiley 2015-5-4

25. Promoter architectures and developmental gene regulation. Haberle, Vanja; Lenhard, Boris. Seminars in Cell and Developmental Biology 57: page 11-23. Academic Press 2016-9

26. Proteomic analysis of the herpes simplex virus 1 virion protein 16 transactivator protein in infected cells. Suk Oh, Hyung; Knipe, David M.. PROTEOMICS 15(12): page 1957-1967. Wiley 2015

27. Establishment of a patterned GAL4-VP16 transactivation system for discovering gene function in rice. Liang, Dacheng; Wu, Changyin; Li, Caishun; Xu, Caiguo; Zhang, Jianwei; Kilian, Andrzej; Li, Xianghua; Zhang, Qifa; Xiong, Lizhong. Plant Journal, The 46(6): page 1059-1072. Wiley 2006

6. Appendix:

6.1 Table 13: Overview of the plasmids that were used for the assembly reaction and their respective sizes in base pairs.

Construct		Size (bp)
A	pAGM4723 cor (vector)	12919
	pZB1_1_TU1_5FR	5187
	pSD1_1_Donor_Gal4_TU2	7617
	pYn1_65 AMDS (TU3)	7544
	pSD1_3_Reciever_UAS_Ura3_mCherry (TU4)	6198
	pZB1_2_TU5_3FR	5208
	pICH41800 (linker)	3318
B	pAGM4723 cor (vector)	12919
	pZB1_1_TU1_5FR	5187
	pSD1_1_Donor_Gal4_TU2	7617
	pYn1_65 AMDS (TU3)	7544
	pSD1_4_Reciever_UAS_NirA_mCherry (TU4)	6154
	pZB1_2_TU5_3FR	5208
	pICH41800 (linker)	3318
C	pAGM4723 cor (vector)	12919
	pZB1_1_TU1_5FR	5187
	pSD1_1_Donor_Gal4_TU2	7617
	pYn1_65 AMDS (TU3)	7544
	pSD1_5_Reciever_UAS_pPcbC_mCherry (TU4)	6260
	pZB1_2_TU5_3FR	5208
	pICH41800 (linker)	3318
D	pAGM4723 cor (vector)	12919
	pZB1_1_TU1_5FR	5187
	pSD1_1_Donor_Gal4_TU2	7617
	pYn1_65 AMDS (TU3)	7544
	pSD1_6_Reciever_Ctrl_Ura3_mCherry (TU4)	6101
	pZB1_2_TU5_3FR	5208
	pICH41800 (linker)	3318
E	pAGM4673 cor (vector)	12919
	pZB1_1_TU1_5FR	5187
	pSD1_1_Donor_Gal4_TU2	7617
	pYn1_65 AMDS (TU3)	7544
	pSD1_7_Reciever_Ctrl_NirA_mCherry (TU4)	6057
	pZB1_2_TU5_3FR	5208
	pICH41800 (linker)	3318

F	pAGM4673 cor (vector)	12919
	pZB1_1_TU1_5FR	5187
	pSD1_1_Donor_Gal4_TU2	7617
	pYn1_65 AMDS (TU3)	7544
	pSD1_8_Reciever_Ctrl_pPcbC_mCherry (TU4)	6163
	pZB1_2_TU5_3FR	5208
	pICH41800 (linker)	3318
G	pAGM4673 cor (vector)	12919
	pZB1_1_TU1_5FR	5187
	pSD1_2_Donor_Gal4_ADVP_16TU2	7410
	pYn1_65 AMDS (TU3)	7544
	pSD1_3_Reciever_UAS_Ura3_mCherry (TU4)	6198
	pZB1_2_TU5_3FR	5208
	pICH41800 (linker)	3318
H	pAGM4673 cor (vector)	12919
	pZB1_1_TU1_5FR	5187
	pSD1_2_Donor_Gal4_ADVP_16TU2	7410
	pYn1_65 AMDS (TU3)	7544
	pSD1_4_Reciever_UAS_NirA_mCherry (TU4)	6154
	pZB1_2_TU5_3FR	5208
	pICH41800 (linker)	3318
I	pAGM4673 cor (vector)	12919
	pZB1_1_TU1_5FR	5187
	pSD1_2_Donor_Gal4_ADVP_16TU2	7410
	pYn1_65 AMDS (TU3)	7544
	pSD1_5_Reciever_UAS_pPcbC_mCherry (TU4)	6260
	pZB1_2_TU5_3FR	5208
	pICH41800 (linker)	3318
J	pAGM4673 cor (vector)	12919
	pZB1_1_TU1_5FR	5187
	pSD1_2_Donor_Gal4_ADVP_16TU2	7410
	pYn1_65 AMDS (TU3)	7544
	pSD1_6_Reciever_Ctrl_Ura3_mCherry (TU4)	6101
	pZB1_2_TU5_3FR	5208
	pICH41800 (linker)	3318

K	pAGM4673 cor (vector)	12919
	pZB1_1_TU1_5FR	5187
	pSD1_2_Donor_Gal4_ADVP_16TU2	7410
	pYn1_65 AMDS (TU3)	7544
	pSD1_7_Reciever_Ctrl_NirA_mCherry (TU4)	6057
	pZB1_2_TU5_3FR	5208
	pICH41800 (linker)	3318
L	pAGM4673 cor (vector)	12919
	pZB1_1_TU1_5FR	5187
	pSD1_2_Donor_Gal4_ADVP_16TU2	7410
	pYn1_65 AMDS (TU3)	7544
	pSD1_8_Reciever_Ctrl_pPcbC_mCherry (TU4)	6163
	pZB1_2_TU5_3FR	5208
	pICH41800 (linker)	3318

6.2 Table 14: Information on the primers which were used for the colony PCRs:

Name primer	Sequence primer
72_screening1fw	TGCAATCCAGAGGGTCTGGTCT
28_tAct1_Fwd	ACAGCGGAAGACAAGCTTGTGCTTCTAAGGTATGAGTCGCA
110_40Sp_Fwd1	TTGAAGACAAGGAGGAGTTATAGACGGTCCGGCATAGG
111_40Sp_Rev1	TTGAAGACAAGGTGTCGATCGGACGTATTGTCCAAG
99_Leve1_screening_fwd	CACATTGCGGACGTTTTTAATGTACTG
100_Level1_screening_rev	CCGCCAATATATCCTGTCAAACACTG
146_screening_rev_in eGFP	AACTTCAGGGTCAGCTTGCC
147_screening_fw_in Tamds	ACAGGTGACTCTGGATGGC