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Grado en Química

TRABAJO FIN DE GRADO

Expression analysis of *fluG*, a developmental factor in *Aspergillus nidulans*.

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Abstract:

fluG (AN4819) is a gene present in *Aspergillus nidulans* that has been proved to be essential for the development of conidiophores. The FluG protein has been attributed to have enzymatic activity but no evidence is currently available on this aspect. Through bioinformatic analysis, two regions have been identified by sequence homology, and each region has been assigned an enzymatic function. On one hand, the N terminal region has been described as an amidohydrolase. On the other hand, the C terminus region has been described as a γ-glutamil ligase. Based on these hypothetical functions, a Q-PCR expression analysis study was performed with mutants affected in of both regions. The results show that disabling mutations increased expression. Assuming that substrates cause upregulation, and products, downregulation, the evidence supports a repressive effect by the product. Spectrofluorometry and western-blot techniques were also used to monitor protein levels and stability.

Resumen:

fluG, también conocido como AN4819, es un gen presente en *Aspergillus nidulans* que ha demostrado ser esencial para el desarrollo morfogénetico de conidióforos. A la proteína FluG se le atribuye actividad enzimática pero actualmente no hay evidencias acerca de este aspecto. A través de análisis bioinformáticos, se han identificado dos regiones por homología en la secuencia de dicha proteína y se le ha asignado una actividad enzimática a cada una. Por un lado, la región N terminal se ha descrito como una amidohidrolasa. Por otro lado, la región C terminal ha sido descrita como una γ-glutamil ligasa. Utilizando estas funciones hipotéticas como base, se realizó un análisis de expresión por Q-PCR con mutantes afectados en ambas regiones. Los resultados demostraron que aquellas mutaciones deshabilitadoras incrementaban la expresión. Partiendo de la suposición inicial de que los sustratos de las reacciones catalíticas de dicha proteína causan un aumento en la producción de la proteína y, los productos, una disminución, se corroboró en los resultados el efecto represivo de los productos. Por último, también se utilizaron técnicas de Espectrofluorometría y Western-Blot para controlar los niveles de proteína y su estabilidad.

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Abbreviations:

Ab	**Antibody**
ACM	Aspergillus complete medium
AMM	Aspergillus minimal medium
Bio	Biotin
BSA	Bovine serum albumin
cDNA	Complementary DNA
CDPs	Central developmental pathway genes
C-term	Strain with only C terminus region of *fluG*
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Nucleotide triphosphate
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescence
fluG	Gene *fluG*
FluG	Protein FluG
GABA	γ-aminobutiric acid
GFP	Green Fluorescent Protein
GSI	Type I glutamine synthetase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hx	Hypoxanthine
IgG	Type G Immunoglobulin
Kb	Kilobases
KDa	KiloDaltons

mRNA	Messenger RNA
NP-40	nonyl phenoxypolyethoxylethanol.
N-term	Strain with only the N terminus region of fluG
ON	Overnight
PBS-T	Phosphate buffered saline plus Tween
PCR	Polymerase Chain Reaction
PSA	Pressure sensitive adhesive
Pu	Putrescine
PVDF	Polyvinylidene fluoride
Q-PCR	Quantitative PCR
RNA	Ribonucleic acid
RT	Room Temperature
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide gel electrophoresis
Taq	*Thermus aquaticus*
TCA	Trichloroacetic acid
TEMED	Tetra methyl ethylenediamine
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
UA	Uric acid
UDAs	Upstream developmental activators
WB	Western-blot

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1. INTRODUCTION

1.1 *Aspergillus nidulans* as a species.

Aspergillus nidulans, also referred to as *Emericella nidulans*, is a eukaryotic filamentous fungus that belongs to the family Aspergillaceae, as part of the Ascomycota class (1). Its genome is well known, it consists of some 30 million base pairs and 10982 genes (2,3), and its sequence is easily accessible online in the Aspergillus Genome Database (http://www.aspgd.org/).

This species is considered a model organism for numerous reasons: for instance, it requires minimal nutrients to grow in either liquid or solid medium, which makes it very easy to grow and it is the first choice when carrying out experiments in genetics or molecular biology. Furthermore, it is related to species that are used in industrial applications, like fermentation processes or even in pharmacology to produce antibiotics (4).

The life cycle of *A.nidulans* can go through four different stages: vegetative growth, sexual development, parasexual development and asexual development (5). This project will focus on asexual development as well as vegetative growth, because it is the most common reproductive mode in this genus.

1.2 Vegetative growth.

The cycle begins with the germination of a spore (conidium), forming what is referred to as a germ tube, from which tubular branches (hyphae) emerge consisting in one or more cells. These expand radially and in polarized fashion via mitosis forming circular colonies (1). The collective body of hyphae is known as the mycelium, from which any of the other

three stages of the life cycle can start with the appropriate cell differentiation, depending on environmental conditions. Another phenomenon that can take place aside of the differentiation is autolysis which is a self-digestion that takes place to recycle nutrients in starvation conditions (6,7).

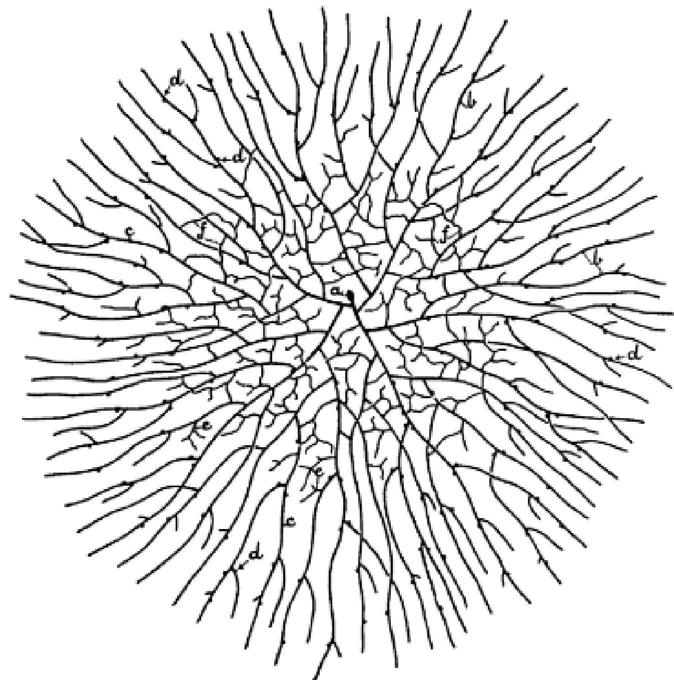

Figure 1. Schematic of a mycelium. Modified from (8).

1.3 Asexual development.

Vegetative hyphae normally extend away from the mycelium into the substrate, but the cells left behind the edge of the colony perform other functions, such as s nutrient stores, and communication pathways. Others, in turn, undergo a transition from vegetative growth to asexual development, which starts by the foot cell. The foot cell elongates perpendicularly to the substrate for some 100µm to form the stalk which then swells at the tip, generating a globular structure with a diameter of 10µm also known as the vesicle. The vesicle serves as platform for multiple budding of uni-nucleated cells or metulae, which undergo a specialized division generating two buds that will later become specialized conidia-

forming cells or phialides. characterized for having a dark green colour in the case of *A. nidulans*. Each phialide can produce up to 100 spores, coming up to 10,000 spores per conidiophore (6).

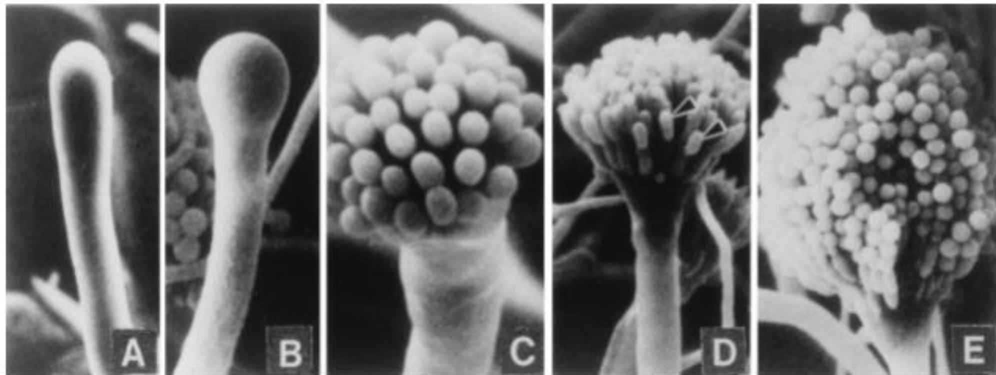

Figure 2. Scanning electron micrographs of the different morphological stages of conidiation. A) Formation of the stalk from the foot cell; B) Vesicle formation from the stalk; C) Metulae in development; D) Phialides in development; E) Conidium formed in a now mature conidiphore. (1)

This process is also known as conidiation (formation of conidiophores) and it can be induced by genetic or environmental factors, but also under conditions that are incompatible with growth, such as nutritional, osmotic or salt stress (6, 7).

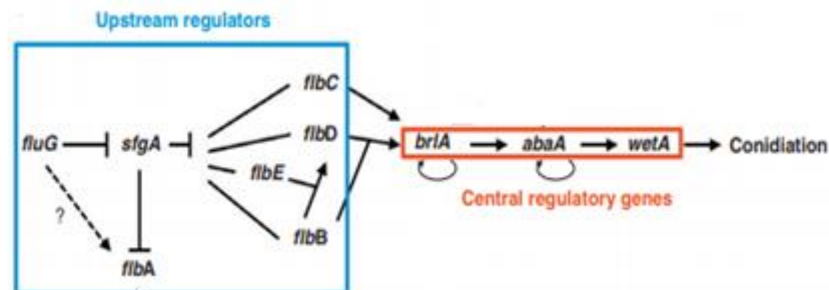

Figure 3. Schematic representation of the genes involved in the transition from vegetative growth to conidiation (UDAs in blue), as well as in conidiation (CDPs in red). [Modified from 9]

There are many genes involved in the conidiation process, the upstream developmental activators or UDAs and the CDPs or central developmental pathway genes (figure 3) (9). UDAs are expressed during vegetative growth and participate in the transition from vegetative growth to asexual development up until the formation of the vesicle during conidiation. CDPs, on the other hand, are expressed once the transition has been made and take care of forming the conidiophores starting from the

vesicle (figure 4) (6). In this work, we will focus on *fluG*, the first of the upstream developmental activators.

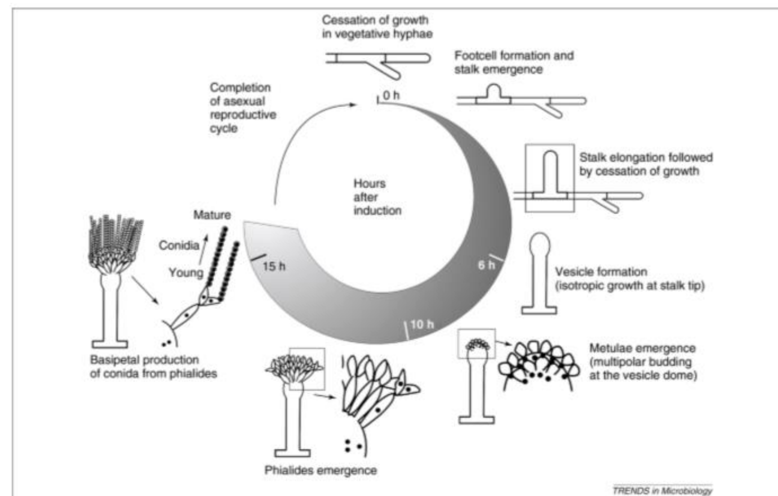

Figure 4. Morphological transformations during conidiation. Indicated time after induction at which the transformations mentioned in section 1.3 take place. (6)

1.4 *fluG* and its role in asexual development.

fluG (AN4819) is a 2.7 kb gene located in chromosome III of *A. nidulans*. It encodes a polypeptide consisting of 865 amino acids (96.5 kDa). The protein is present early in vegetative growth as well as in development and is primarily localized in the cytoplasm (10). There are two main regions in this polypeptide, the N terminus region, with a length of approximately 400 amino acids, and the C terminus region of approximately 465 amino acids of length. Both of these regions are joined together by a putative linker sequence (Mikel Iradi, personal communication).

FluG is essential for asexual development. Deletion of *fluG* (*ΔfluG* mutant strain) results in undifferentiated masses of vegetative cells, forming a cotton-like colony ("fluffy" phenotype). In addition, selected mutations or insertion of another gene inside of *fluG*'s sequence can have the same effect, and have been used to analyse the functionality of the protein (10).

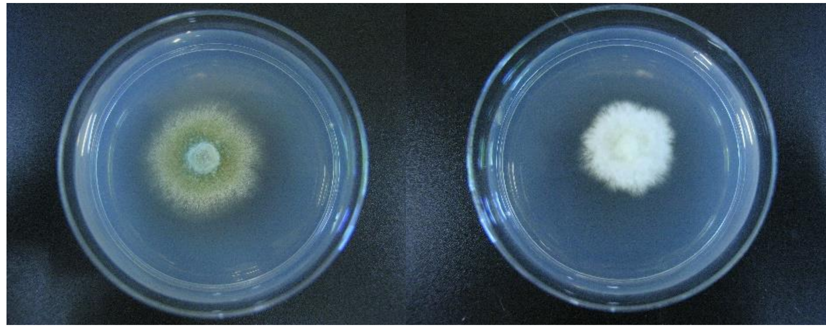

Figure 5. Agar plates of, on the left, a wild type conidiating strain and, on the right, a "fluffy" or aconidial strain.

Although *fluG* has been predicted to code for two enzymes, their function has not been proven. Nevertheless, it is known that wild type strains can transfer a chemical signal to *fluG* ⁻ mutants upon contact, restoring their sporulating capacity (1, 10, 11). Thus, the possibility remains that the compound responsible for the effect may, in fact be the product of the purported enzyme activity.

The substances that act as substrates and the products they are converted into by FluG remain unknown. Based on the hypothesized three-dimensional structure of the protein coded by *fluG* and on the key catalytic residues that will later be described on this study, an approach on the possible enzymatic activity of both regions of the protein was conducted. The N terminus region is believed to behave as an amidohydrolase, whose main function is to catalyse the hydrolysis of amide functional groups (12). On the other hand, the C terminus region was first considered as a type I Glutamine Synthetase due to the high sequence similarity with a prokaryotic GSI from *Bacillus subtilis*. This was later disproved, as FluG was shown not to complement a glutamine auxotrophy (13). Nevertheless, more recent unpublished work by doctorate student Mikel Iradi has suggested that this region functions as a γ-glutamyl ligase, an enzymatic family capable of linking the carboxylic group in γ position from the main carboxylic acid with an amine group of a different molecule, being the most common glutamate-receiving diamines (14).

Several studies completed on FluG concluded that the key activity required for conidiation lies mainly in the C terminus region, as a mutant only containing this region was capable of sporulating in absence of the N terminus region, while the *N-term* mutant could not sporulate (11).

Further studies in the laboratory (Mikel Iradi)lead to the identification of the key residues that could be involved in the biological function of the protein. So as to confirm this, point mutations of those residues were made and they all caused loss of function (aconidial phenotype).

Figure 6 shows the key residues that were identified in the N- and C-terminal region, which resulted in changes in function. Only the mutations in the C-terminal region resulted in an aconidial phenotype.

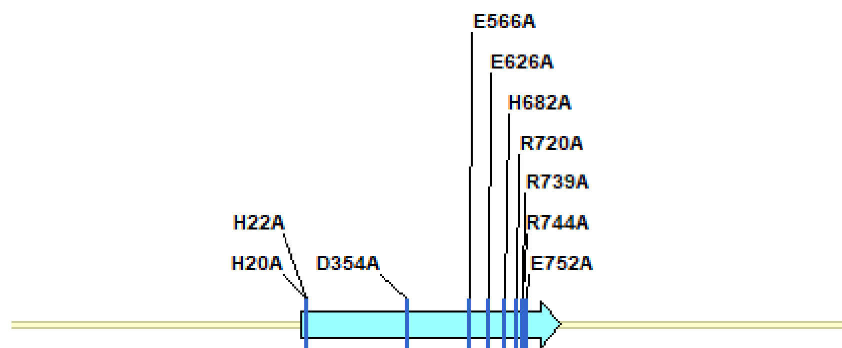

Figure 6. fluG with every point mutation indicated. Each mutation is expressed starting with the single-letter amino acid code for the original amino acid, followed by the position it occupies in the protein's sequence and the single-letter code for the amino acid it is substituted with. In this case, H stands for histidine, D for aspartic acid, E for glutamic acid, R for arginine and A for alanine.

1.5 Genetic expression in the cell.

The first step in genetic expression is transcription. In this process, a RNA strand is synthesized by polymerases using DNA as template. Three different types of RNA can be produced: ribosomal RNA, transfer RNA and messenger RNA. The first two, are functional molecules but the latter, must be processed to proteins in order to produce a functional molecule. The process of synthesizing proteins from messenger RNA is known as

translation, in which ribosomes follow the genetic code to generate the amino acidic sequence corresponding to the RNA sequence.

Figure 7. Schematic of gene expression process.

The fragments of DNA that are organized in genes generate messenger RNA and, therefore, a protein. That is why, to determine the expression levels of a particular gene there are two approaches: measure the amount of messenger RNA present in the cell at a particular time or measure the amount of protein that is being produced from that gene.

In this project, methods to measure expression levels with both RNA and protein were conducted. For RNA, Quantitative Polymerase Chain Reaction is proposed and, for protein, Fluorometry and Western-Blot.

1.6 Quantitative Polymerase Chain Reaction.

Polymerase Chain Reaction (PCR) is a technique developed in the 1980s by Kary Mullis based in the enzymatic activity of polymerases of amplifying nucleotide sequences yielding millions of copies. The basic principles of this technique summarized below are extensively explained in reference 17. Polymerases are capable of amplifying specific nucleotide sequences starting from a DNA sample as template, two primers (or oligonucleotides) to delimit the region to be amplified, and deoxyribonucleotide triphosphate or dNTPs, that act as substrate for the reaction.

Several variations of this technique have been described, one of them being Quantitative Polymerase Chain Reaction (Q-PCR), also known as Real Time Polymerase Chain Reaction. This technique allows quantification of amplicons during the progress of each reaction cycle. Its main advantage is the quantification with high sensibility, specificity and efficiency even with a small amount of starting material. The latter being either genomic deoxyribonucleic acid (DNA) or complimentary DNA (cDNA).

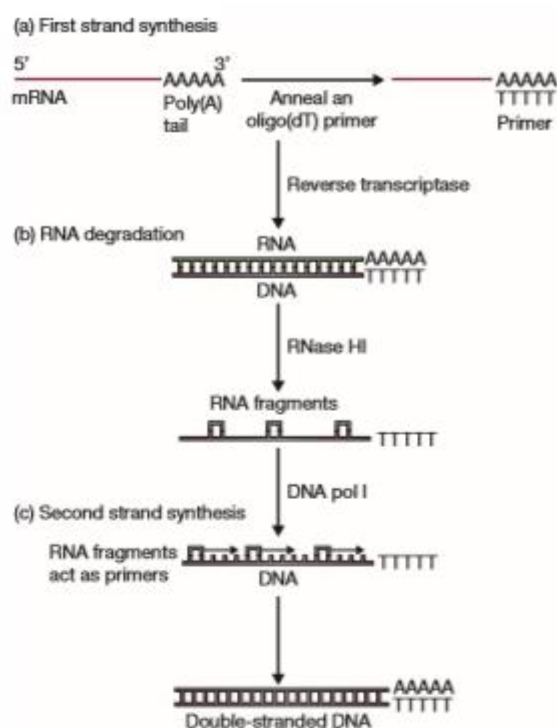

Figure 8. Scheme of cDNA synthesis process. In step a) The first strand of the DNA is synthetized starting from the corresponding RNA by a reverse transcriptase. In step b) the RNA strand used as template is broken down by a ribonuclease. And, finally, in step c) DNA polymerase synthetises the second strand of DNA using the first one as template. Modified from (18)

The main application of this technique is the detection and quantification of specific sequences of nucleotides by the use of fluorophores. These are usually fluorescent dyes that bind to the DNA strand once its doubled, these generate a signal proportional to the number of copies being produced on each cycle.

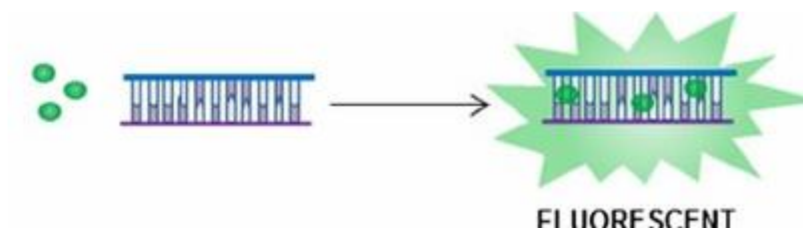

Figure 9. Dye-based detection method used in Quantitative Polymerase Chain Reaction. Modified from (19).

As in regular PCR, each cycle can be divided into three stages:

1. Denaturalization: is the process of separating the strands that constitute DNA's double helix. It requires high temperatures in order to break the hydrogen bonds that link both chains, that is why this step is performed at approximately 95℃. Still, the temperature can vary depending on the content of guanine a cytosine in the target sequence, this is due to the fact that guanine and cytosine are linked to each other by three hydrogen bonds, instead of two hydrogen bonds like adenine a thymine.

2. Annealing: this step consists in the annealing of single stranded primers to the chain that will act as template during the elongation. It is the most important step since it is necessary for the primers to fully anneal to their complementary sequence for the polymerase to perform correctly. Due to the importance of this stage, the primers need to be carefully designed, with a sequence complementary to the ends of the fragment to amplify but with not more than a 55% of content in guanine and cytosine, to avoid the formation of tertiary structures. Another determinant parameter on the success of this step is the annealing temperature also referred to as melting temperature of the primer-template complex. This temperature is usually 1 or 2 degrees below the melting temperature of the complex, to ensure a stable binding between primer and template.

3. Elongation: consists in the synthesis of DNA catalysed by the polymerase starting from the primer and adding to the chain those nucleotides complementary to the target sequence. The reaction

proceeds in a 5'-> 3' direction and, in the case of the Taq (*Thermus aquaticus*) polymerase used in this experiment, at 72℃.

These three steps are repeated for 40 cycles where temperature control is an important factor on the efficiency of amplification.

Starting from the quantity of amplicons obtained after completion of all the 40 cycles and through an algorithm integrated in the apparatus by the manufactures, the initial amount of target cDNA can be determined. The algorithm takes into account the standard kinetics of amplification to extrapolate the kinetic of the reaction to fluorescence values below the threshold (Figure 10) value of the detector.

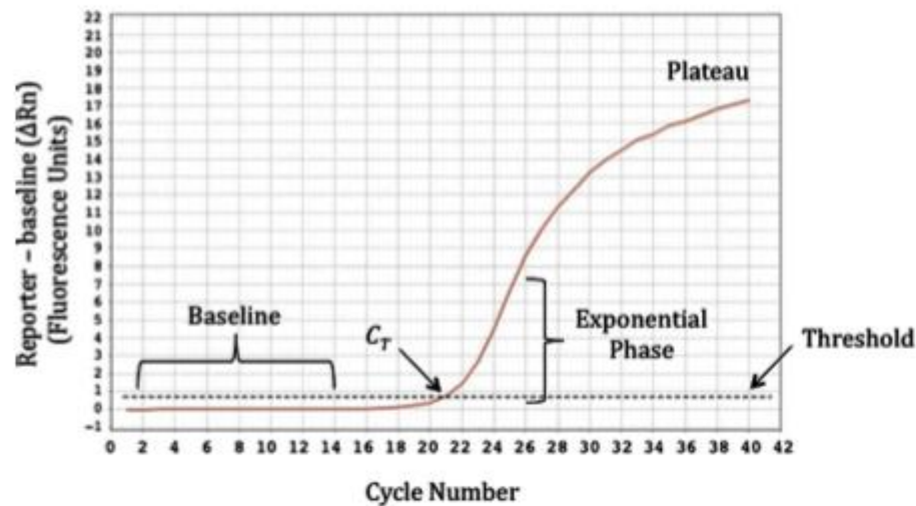

Figure 10. Example of a graph obtained on Q-PCR indicating the main parameters necessary for quantification. (17)

The initial amount of target cDNA is equivalent to the initial amount of target RNA, as observable on figure 8, when synthesizing a cDNA molecule the template RNA is degraded, yielding one molecule of cDNA per RNA molecule. That way, the Q-PCR experiment can be used to quantify the amount of RNA in one sample and, when carried out with different samples, to compare RNA levels between different samples, which is the objective in the present project.

1.7 Fluorometry.

Fluorescence spectroscopy is a technique widely used in Biochemistry and Biophysics for both quantification and qualification analysis. (20).

In the present project, this technique is proposed as a low-cost alternative to Q-PCR to determine the expression levels (protein) of *fluG*. It has been hypothesized that the product of FluG exerts a negative feedback inhibition effect on *fluG*'s expression, on the other hand, its substrate may affect the expression positively. In order to confirm this hypothesis, the fungus was given different substances as nitrogen sources, to be able to study the effect of each added substance in the activity of the promoter sequence of *fluG* and, therefore, in its expression, a reporter gene was used. This reporter gene was inserted after *fluG* so as to be expressed at the same time and quantity. In the present work, Green fluorescent protein (GFP), which codes for a fluorescent protein, was used as reporter.

Figure 11.Construction used in this experiment, GFP linked to fluG under the same promoter sequence.

1.8 Western-Blot.

Western blotting, also known as protein blotting or immunoblotting, is a powerful technique widely used in research to identify and quantify a specific protein in a complex mixture, as extensively explained in reference 21. To accomplish this task, Western-blot uses three different elements, the first one being the separation by molecular weight of the proteins present in the mixture. Then, transfer of the separated proteins to a solid

support, which usually is a nitrocellulose or a PVDF membrane. And, lastly, target proteins are marked using primary and secondary antibodies to visualize their presence.

The advantages Western blotting offers are manifold but, its sensitivity and specificity make it a very powerful technique, not only for research purposes but also for medicine applications, such as early diagnosis of several diseases.

- Step one: gel electrophoresis.

This blotting technique uses two different types of gels, the stacking gel and the resolving gel. The first one is slightly acidic (pH=6.8) and has a lower acrylamide concentration giving a porous gel that performs a very poor protein separation but allows the formation of thin, defined bands. On the contrary, the resolving gel is basic (pH=8.8) and has a higher concentration of acrylamide, generating narrower pores and, therefore, better separation. Protein samples have to undergo a heat treatment in order to denature their higher structure and release the negative charged amino acid side chains. These negative charges enable the protein to run through the gel when an electric field is applied.

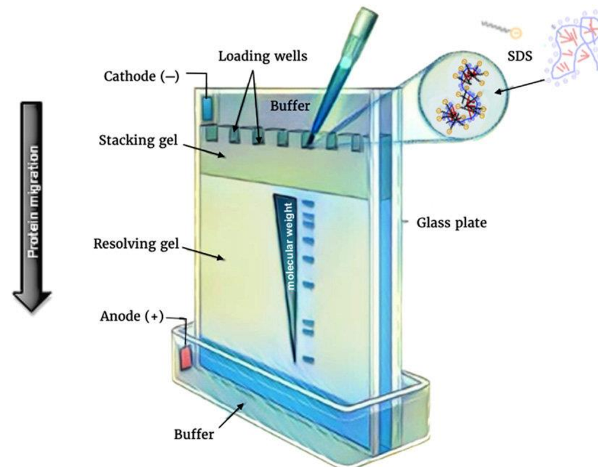

Figure 12. Schematic of gel electrophoresis (22).

- Step two: gel to membrane transfer.

There is more than one way to achieve protein transfer from the gel to the membrane, the most commonly used is the electrophoretic transfer in which the transfer is done by using an electric field oriented perpendicular to the surface of the gel. Complete contact between the gel and the membrane is required for correct transfer, that is why a sandwich-like construct is made. In this, the membrane is fixed on top of a fibre pad and filter papers soaked on transfer buffer, then the gel is placed on top of the membrane and, on top of the gel, filter papers and a fibre pad are deposited, also soaked in transfer buffer. Apart from the complete contact between the gel and the membrane, it is also important that the membrane is situated amid the gel and the positive electrode since, due to the negative charge of the protein, that is the direction in which the proteins will migrate under an electric field.

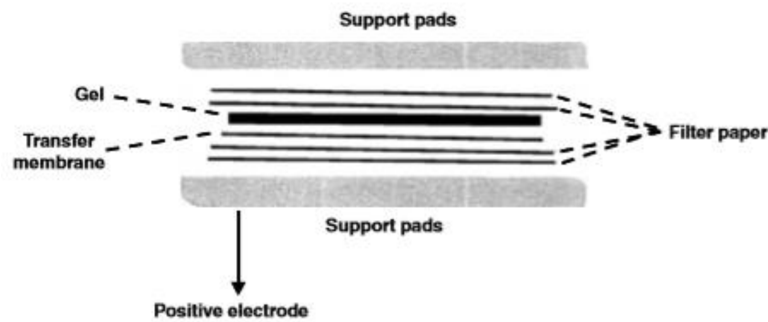

Figure 13. Gel-membrane sandwich. This is constructed using the Trans-blot TurboTM Transfer System Transfer Pack and is then introduced in the Trans-blot apparatus (23).

- Step three: protein immunodetection.

For detection of the target protein in the membrane there are two possibilities. The first possibility is the direct detection, which consists on using a labelled antibody specific for the target protein (antigen). The second possibility is the indirect detection that consists on using a primary antibody specific for the target protein and a secondary labelled antibody that binds to the primary antibody.

To avoid any nonspecific binding of the primary antibody to the membrane, a blocking step must be done. To this end, the membrane is incubated on a 5% bovine serum albumin (BSA) solution or on a nonfat dried milk solution using a buffer, such as PBS-T, as solvent.

The next step would be the incubation with each antibody, one at a time. These antibodies are usually dissolved in a non-fat dried milk solution since it allows to reuse this antibody in case a second test is necessary. Between the blocking step and the incubation is important to wash off any excess using a wash buffer, for instance, PBS-T.

To finally detect the spots where the antibodies have bound, the labelling in the antibody is used and, considering that the most common labelling is an enzyme, the membrane must be put in contact with the reactive of the reaction catalysed by that particular enzyme. The enzyme then generates a product that gives rise to a signal detectable by a molecular imager.

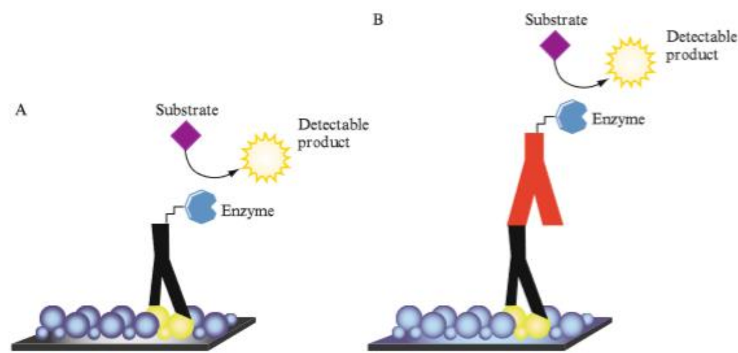

Figure 14. Schematic of the direct (A) and indirect (B) immunodetection methods for western-blotting. (24)

2. OBJECTIVES AND THEIR JUSTIFICATION.

The objectives pursued in this project were:

1. The transcriptional study of the expression pattern of *fluG* when selected point mutations which affect biological activity are introduced. Any change in expression observed would help determine whether the substrate or the product of the purported FluG enzyme is involved in expression.
2. To design a method to measure protein expression levels via spectrofluorometric detection of a reporter protein under the control of the *fluG* promoter.

3. MATERIALS AND METHODS.

3.1 Fungal strains.

The strains used in this study are presented in Table 1.

Table 1. Strains of A. nidulans used in this study with their genotype and source.

Strain	Genotype	Source
BD824	*pyrG89; pyroA4; ΔnkuA::bar; veA+*	Mikel Iradi
BD969	*pyrG89; ΔfluG::pyrG*$^{+}$*; pyroA; ΔnkuA::bar; veA*$^{+}$	Mikel Iradi
BD970	*pyrG89; fluG (1-406); pyroA4; ΔnkuA::bar; veA+*	Mikel Iradi
BD971	*pyrG89; fluG (427-865); pyroA4; ΔnkuA::bar; veA+*	Mikel Iradi
BD972	*pyrG89; fluG* $_{H20A;H22A}$*; pyroA4; ΔnkuA::bar; veA+*	Leire Tola
BD975	*pyrG89; fluG::GFP; ΔnkuA::bar; pyroA4; veA*$^{+}$	Mikel Iradi
BD1195	*pyrG89; fluG* $_{D354A}$*; pyroA4; ΔnkuA::bar; veA+*	Leire Tola
BD1196	*pyrG89; fluG* $_{H20A;H22A;D354A}$*;pyroA4; ΔnkuA::bar; veA+*	Leire Tola
BD1197	*pyrG89; fluG* $_{E566A}$*; pyroA4; ΔnkuA::bar; veA+*	Mikel Iradi
BD1198	*pyrG89; fluG* $_{E626A}$*; pyroA4; ΔnkuA::bar; veA+*	Mikel Iradi
BD1199	*pyrG89; fluG*$_{H682A}$*; ΔnkuA::bar; pyroA4; veA*$^{+}$	Mikel Iradi
BD1200	*pyrG89; fluG* $_{R720A}$*; pyroA4; ΔnkuA::bar; veA+*	Mikel Iradi
BD1201	*pyrG89; fluG* $_{R739A}$*; pyroA4; ΔnkuA::bar; veA+*	Mikel Iradi
BD1202	*pyrG89; fluG* $_{R744A}$*; pyroA4; ΔnkuA::bar; veA+*	Mikel Iradi
BD1203	*pyrG89; fluG* $_{E752A}$*; pyroA4; ΔnkuA::bar; veA+*	Mikel Iradi

2.2 Growth and culture conditions.

2.2.1 Medium for culture.

Table 2 shows the different media used to grow *A. nidulans* in the experiments carried out as part of this project.

Table 2. Culture medium used.

Medium	Preparation (per litre)
Aspergillus minimum medium liquid [AMM(l)]	20 mL Salt solution 50x 900 mL dH_2O Adjust pH=6,8 Sterilize in autoclave
Aspergillus minimum medium solid [AMM (s)]	MMA(l)+15 g bacteriologic agar

Preparation of this medium must be done in a sterile environment and inside of an extractor hood. Apart from the basic medium solutions (table 2), nutrients must be added for the fungi to grow. These nutrients consist on a nitrogen source, in our case ammonium tartrate, and a carbon source such as glucose. Both are necessary in certain amounts, that is why, in 1L of growth medium 100 mL of glucose 10x are added plus 10 mL of ammonium tartrate 100x.

When induction of sporulation is required, the most common way to achieve it is with sodium biphosphate (NaH_2PO_4) by addition of 100 mL of sodium biphosphate 5M per litre of growth medium.

Besides the main nutrients and components of the medium, supplements must be added according to the auxotrophies of the strain to be grown, as shown in table 4.

2.2.2 Solutions and supplements.

Table 3 indicates the different solutions needed to prepare the media previously mentioned.

Table 3. Solutions used for preparation of A. nidulans culture mediums.

Solutions	Preparation (per litre)
Salt solution 50x	26 g KCl 26 g $MgSO_4·7H_2O$ 76 g KH_2PO_4 25mLtrace elements solution Up to 1l with dH_2O Sterilize in autoclave

Trace elements 1000x	22 g $ZnSO_4 \cdot 7H_2O$ 11 g H_3BO_3 5 g $MnCl_2 \cdot 4H_2O$ 3,14 g $FeSO_4 \cdot 2H_2O$ 1,6 g $CoCl_2 \cdot 6H_2O$ 1,6 g $CuSO_4 \cdot 5H_2O$ 1,1 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 50 g Na_2EDTA Up to 1l with dH_2O Adjust pH=6,8 Sterilize in autoclave
Glucose 10x	200 g Glucose Up to 1l with dH_2O Sterilize in autoclave
Ammonium tartrate 100x [NH_4T]	92 g Ammonium tartrate Up to 1l with dH_2O Sterilize in autoclave
NaH_2PO_4 5 M [P]	599,9 g NaH_2PO_4 Up to 1l with dH_2O Sterilize in autoclave

Table 4 Illustrates the preparation and addition of the supplements needed to compensate the auxotrophies that the strains used have.

Table 4. Preparation of supplements and addition to the medium.

Auxotrophy	Supplements	Preparation (per litre)	Addition (per litre of medium)
PyroA4	Pyridoxine 1000x [pyro]	100 mg Pyridoxine Up to 1l with dH_2O Sterilize in autoclave	1 mL
- **	Biotin 10000x [bio]	100 mg Biotin Up to 1l with dH2O Sterilize by filtration	200µL
PyrG89	Uracil Uridine	Simple addition	0.56 g 1.22 g

**Biotin is added to the medium to help growth, not to compliment an auxotrophy.

2.3 Equipment.

Incubation on solid medium of each strain was done in a Sanyo MIR-153 refrigerated incubator. While incubation on liquid medium was done in a Shel-lab 1575R refrigerated shaking incubator.

Distilled water was obtained from an Elix 5 (Millipore) water purification system and milli-Q water to a Synergy UV (Millipore) water purification system.

Every solution in section 3.2.2, except biotin, was sterilized in a Presoclave 30 (selecta) autoclave.

For every centrifugation in the duration of this experiment, two different centrifuges were used. In the case of centrifuging Eppendorf tubes, Sorvall Legend Micro 21R (Thermo Scientific) centrifuge but, in case of Falcon tubes, Sorvall Biofuge primo R (Thermo Scientific) centrifuge.

For quantification of RNA and protein samples, NANODROP 200c spectrophotometer (Thermo Scientific) was used.

Photographs of agar plates or gels displayed in this paper were taken with a Nikon E8400 camera.

2.3.1 Q-PCR.

The thermocycler used to obtain cDNA for each strain was the T100™ Thermal Cycler Bio-Rad.

To perform the Q-PCR experiment, a 7500 Real Time PCR System (Applied Biosystems) was used.

2.3.2 Fluorescence measurements.

MiniBeadBeater -1 (Biospec product) was used to powder mycelium samples.

The Spectrofluorophotometer RF-540 (Shimadzu) with the data recorder DR-3 (Shimadzu) helped acquire the fluorescence values for each solution.

The lyophilisation process was completed using Cryodos (Telstar) freeze dryer.

2.3.3 Western-blot.

In order to run the samples through the gel a mini transfer-blot cell (Bio-rad)

was used to support the gel, and a power-pac basic (Bio-rad) provided the voltage necessary for the protein samples to move through the gel.

For transferring the samples from the gel to a membrane the Trans-blot Turbo Transfer System Transfer Pack was employed.

Membranes were visualized with the ChemiDoc XRS+ Molecular Imager (Bio-Rad).

2.4 Quantitative Polymerase Chain Reaction or Q-PCR.

Before carrying out the Q-PCR experiment, DNA samples had to be obtained for each studied strain. To do this, strains indicated in table 1 except for BD975, were inoculated in 100mL liquid medium at a concentration of $1x10^6$ spores/mL and incubated at 37℃ for 18 hours with

rotary shaking (at 200 rpm in a 250mL Erlenmeyer flask). The resulting mycelium was filtered from the Erlenmeyer flask used to grow each strain and submerged in liquid nitrogen. Starting from this, ribonucleic acid (RNA) was extracted using Nucleospin® RNA plant RNA extraction kit, Macherey-Nagel, ref: 740949.50. Followed by its conversion into cDNA using Primescript™ RT Reagent Kit, Takara, cat#: RR037A.

Once the starting genetic material was ready (cDNA), the next and last step was the Q-PCR reaction. The reactants and the quantity added to proceed with the reaction are shown In table 5. The reactant labelled as PyroTaq EvaGreen, is a Q-PCR reaction Mix produced by Cultek molecular bioline (Cmb™) and its composition is:

- PyroTaq HS (Hot Start DNA Polymerase).
- 5x EvaGreen® qPCR Buffer.
- 12.5 mM $MgCl_2$.
- dNTPs.
- EvaGreen® dye, DNA-binding dye.
- ROX dye, internal passive reference dye used to normalize the reporters signal.

Table 5. Reactants and quantity for Q-PCR mix.

Component	Volume
PyroTaq Evagreen	4μL
Primer forward	1 μL
Primer reverse	1 μL
DNA template	1.2 μL
MiliQ H_2O	Up to 20 μL (12.5 μL)
Total	20 μL

The reaction was performed in a 96-well microplate, using one well per reaction. During this reaction, the detection of the target gene is as easy as detecting fluorescence signal in the amplification process but the quantification of the target gene in the sample requires some sort of

reference. That is why, in this work constitutively expressed gene *benA* was used as a reference (25).

Four sets of primers (forward and reverse) were used in this experiment: one for *benA*, one for *fluG*, one of the N terminus region of *fluG* and one for the C terminus region. Due to the complexity of the experiment, to avoid contamination of one reaction mix with another one, a MasterMix for each target gene and each strain was made. This MasterMix included the reactants shown in table 5 in twice the volume displayed since two technical replicates were made to ensure a representative value.

Subsequently, once every reaction mix was in its corresponding well [Append 1], the microplate was taken to the themocycler with the setup shown in table 6.

Table 6. Temperature profile, duration and number of cycles of each reaction step.

Cycle Step	Temperature (℃)	Time	Number of cycles
Initial Denaturalization*	95	15 min	1
Denaturalization	95	15 s	
Annealing	60**	15 s	40
Elongation	72	30 s	

*Necessary to activate the polymerase, since it is a Hot Start Polymerase.

** This temperature depends on the primer used and its sequence.

After completion of every cycle, data was exported, processed and graphed using Excel.

2.5 Fluorescence assay.

To ensure having enough mycelium to continue with the experiment, instead of directly growing the fungus in the medium containing the alternative nitrogen source, it was first grown in 225mL of liquid medium with ammonium tartrate, as described in section 3.4.2. After 18 hours of culture, a 25mL aliquot was taken from the medium, mycelium was filtered, transferred into a 2 mL Eppendorf tube, submerged in liquid nitrogen and labelled as t_0, fraction extracted 0 hours after induction (the induction being the change of nitrogen source). Then, 40 mL aliquots were taken, mycelium filtered and cleaned with AMM to eliminate any remaining medium from the previous incubation. Once cleaned, mycelium was transferred to 100mL of the new medium using a spatula. Then, 25mL aliquots were extracted 2, 4 and 6 hours after induction and treated the same as sample t_0. The chosen times respond to the adaptation time required after transfer to the new nitrogen source. It is known that, after 6 hours of induction vesicle formation (figure 3), starts regulated by CDP genes, which can be interpreted as finalization of UDAs role, including fluG, and every change that might take place already did.

After extraction of every sample, the next step was overnight lyophilisation. Lyophilized samples are then treated for protein extraction. The first step is homogenising the mycelium, for that, one metal bead per Eppendorf was added and then shaken on a MiniBeadBeater in two intervals of 1 to 2 seconds each, introducing the sample on ice afterwards. 1mL of Dubrin (table 8) is added to each tube then mixed for 1 hour and 30 minutes at 4 °C. This buffer will release the proteins by lysis of the cells that constitute the mycelium, so that, after centrifugation for 30 min at 4°C and 14.000rpm, every protein extracted will remain in the supernatant. This supernatant was then separated and quantified using a NANODROP instrument.

After quantification, 2mL solutions with a final protein concentration of 1 mg/mL were prepared starting from each sample. [Append 2] Then fluorescence was measured on these solutions [Append 3].

Table 7.Concentration in final medium for each new nitrogen source.

Nitrogen source**	Number of N atoms per molecule	Final concentration in medium*	
		mM	g/L
Hypoxanthine (Hx, 136,11 g/mol)	4	2,5	0,34
Uric acid (UA, 168,11 g/mol)	4	2,5	0,42
Putrescine (Pu, 161,1 g/mol)[C]	2	5	0,81
GABA (GABA, 103,12 g/mol)	1	10	1,03

*These concentrations were chosen based on a phenotypic assay carried out by Leire Tola García, using these substances.

**The rest of the components are those that appear in tables 2 and 4, except for ammonium tartrate, which is substituted by the substrates on this table.

Table 8. Composition of the Dubrin buffer used during protein extraction.

Starting solution	Volume*	Final concentration
0.5M HEPES; pH=7.5	0.5 Ml	5mM
0.5M EDTA; pH=8	0.1 Ml	1mM
1M KCl	1 Ml	20mM
10% (w/v) NP-40	0.5 Ml	0.1% (w/v)
1M DTT	25 µL	0.5mM
100Mm Pfa-bloc**	0.5 mL	1mM
1mg/mL (in EtOH) Pepstatine**	34.3 µL	1µM
1mg/Ml (in H_2O) Leupeptine**	14.3 µL	0.6µM
MiliQ H_2O	Up to 50 mL	-

*Volumes listed to prepare 50mL of buffer.

** Protease inhibators , used to avoid denaturing of proteins.

2.6 Western-blot analysis.

Starting from the total protein extracts obtained for the fluorescence assay in section 3.7, proteins are precipitated to transfer them to a buffer appropriate for the electrophoresis. In the case of this experiment, calculations were made starting from the concentration data obtained through NANODROP (Append 2) to precipitate 300μg of total protein. The volume of total protein extract containing 300μg of protein was diluted to 100μL with cold miliQ H_2O and 10 μL of trichloroacetic acid (TCA) were added to cause protein precipitation by incubating the mixture for 10 minutes on ice. As soon as the 10 minutes passed, the samples were centrifuged for 30 minutes at 4℃ and 14000 rpm and the supernatant was discarded. Cleaning of the pellet formed during centrifugation was done resuspending the pellet, first on a mixture EtOH:Ether 1:1 and then on a mixture EtOH:Ether 1:3, centrifuging for 5 minutes in the same conditions as the first centrifuging step and discarding the supernatant. This process was executed with both of the mixtures and, after discarding the last supernatant, the Eppendorf tubes were left on ice with the lid open for 10 minutes, permitting the evaporation of any remaining EtOH:Ether mixture.

The last step of the sample preparation is the resuspension of the pellet in 40μL of rupture buffer (table 9) via vortexing and storage at -20℃ .

Table 9. Composition of rupture buffer.

Substance	Starting concentration	Quantity	Final concentration
Tris-HCl pH=6,8	0,5M	1.5Ml	625mM
SDS	10%	1.8mL	2%
Mercaptoethanol	5%	0.45mL	5%
Urea	-	3.24g	6M
MiliQ **H_2O**	Up to 9 mL	Up to 9mL	
Bromophenol blue*	Not specified		

*Tracking dye, provides the bands with colour to observe how far the samples have run through the gel.

As mentioned before, two gels must be prepared for gel electrophoresis, the stacking gel and the resolving. The set up for gel preparation consists of two glass plates clamped together designed to leave a hollow space in between them that allows the formation of a thin gel. Once the set was prepared, it was cleaned with water, which doubles as a test of leakage, and completely dried using Whatman paper. Then the stacking and resolving gels were prepared on a two separate PS tubes adding all the substances indicated in table 10 except for the polymerizing agents, that way both gels can be prepared at the same time. When the setup previously mentioned was dry and ready, the polymerizing agents were added to the resolving gel mixture and, with the help of a 1000ul Pipette, the latter was carefully inserted in between the glass plates up to the green bar holding the plates together, making sure no air bubbles formed inside. Immediately after addition, 75ul of isopropanol were added, once again with the help of a Pipette, drop by drop and uniformly on top of the resolving gel to ensure that the gel obtained was straight and uniform. After the addition of isopropanol, the gel was left to polymerize for about 20 minutes.

When the polymerization was completed, the isopropanol previously added was eliminated by tilting the assembling setup and with the help of a Whatman paper, with care of not touching the gel with the paper. The next and last step to gel preparation was the preparation of the stacking gel, for which the same procedure as in the preparation of the resolving gel was followed except for the addition of isopropanol. Before polymerization, a comb was inserted in the stacking gel to form the Wells and the mixture was left to polymerize. When completed the polymerization process and, therefore, the gel preparation, the comb was carefully pulled out of the setup and the gel was inserted in the mini Trans-blot cell, connected to a power supply and running buffer, in this case sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) buffer, was added up until the mark that indicates 2 gels, making sure that the gel is fully submerged in the buffer.

Table 10. Composition for both gels used in gel electrophoresis.

Resolving gel 10mL*		**Stacking gel 6mL ***	
Substance	**Volume**	**Substance**	**Volume**
30% acrylamide	3.3 Ml	**30% acrylamide**	1 mL
1M Tris-HCl pH=8.8	3.8 Ml	**0.5M Tris-HCl pH=6.8**	1.5 mL
10% SDS	0.1 Ml	**10% SDS**	0.06 mL
MiliQ H_2O	2.7 Ml	**MiliQ H_2O**	3.38 mL
10% PSA**	0.1 Ml	**10% PSA**	0.06 mL
TEMED**	4 μL	**TEMED**	6 μL

*Final volume of mixture, enough for two gels.

** Polymerizing agents.

With the complete setup prepared, samples stored at -20℃ underwent a treatment of 30 seconds vortexing, 95℃ incubation for three minutes and centrifuge at RT and 14000rpm for one minute. Wells were then loaded with 10 μL of sample, loading in the first well the molecular weight marker. Running of the gel was done at 30 mA until the samples passed the stacking gel and then at 60 mA. In this experiment, two gels were prepared, one for charge control and another one for protein detection but, in case of preparing just one gel, the voltage used for sample running must be 15 mA and then 30 mA.

After running, each gel was taken out of the electrophoresis tank, cleaned with distilled water (dH_2O) and the smallest of the glass plates was separated with help of a spatula. Then, they were again cleaned with dH_2O and the stacking gel was cut with the spatula, leaving only the resolving gel containing the protein samples. Then, each gel was put in a plastic container to be treated separately.

- Gel one, charge control: the molecular weight marker used in this case was the High-range Bio-Rad marker, diluting 0.5 µL in 10 µL of rupture buffer. After running, the gel was washed three times with dH2O 5 minutes on a shaker. Then incubated for 30-40 minutes with 40mL of Coomassie blue reagent on a shaker as in the washing steps. The Coomassie blue is a tinting agent that allows visualization of every band formed during electrophoresis. Finally washed 3 times with dH_2O and a Figure 19 was taken [Append 4].

- Gel two, protein detection: the molecular marker used was BlueStar prestained protein marker, and only 3 µL were added in the well. After running, gel-to-membrane transfer was performed using the Transfer Pack referred to in section 3.6.1. First, the bottom fibre pad with filter papers were placed on the Trans-Blot cassette, and the membrane was placed and placed on top, using a roller to eliminate any air bubbles formed that might endanger the transfer process. Subsequently the gel was situated on top the same way as the membrane and topped with filter papers and the fibre pad. The cassette was introduced in the Trans-Blot Turbo™ Transfer System and the transfer was completed with a 10-minute exposure to 1.3A (25V).

Another way to ensure that every well was charged equally is Ponceau Staining for which, the membrane was cleaned for 5 minutes 3 times with dH_2O after transfer and then incubated for 1 minute with shaking by hand with 40mL of Ponceau tinting agent (table 11, this tinting agent can be reused). A washing process with dH_2O was necessary to eliminate any excess of tinting agent and then Figure 20 was taken [Append 4]. To be able to continue with the protocol, is required to completely wash off the tinting agent, which was easily achieved with an acetonitrile-sodium hydroxide mixture (table 12). When the red bands left by the tinting agent faded away with this mixture, the membrane was

cleaned once again with dH_2O for 5 minutes and with Phosphate buffered saline x1 (PBSx1) for 10 minutes.

Table 11. Composition of Ponceau staining agent.

Ponceau staining 500 Ml	
0.1 % Ponceau S	0.5 g
5% glacial acetic acid	25 Ml
dH_2O	Up to 500 mL

Table 12. Composition of the acetonitrile-sodium hydroxide mixture.

Acetonitrile-NaOH mixture 50 Ml	
Acetonitrile	10 Ml
Sodium Hydroxide 1M	10 Ml
dH_2O	Up to 50 Ml

This test was followed by blocking of the membrane by incubation on a shaker with 0.75g of nonfat dried milk dissolved in 15 mL of PBSx1 for about 2 hours at room temperature. Membrane was washed for 10 minutes with PBS-T (1L of PBSx1 plus 500µL of tween 0.002%) three times and then was incubated ON at 4℃ with the primary antibody solution. Before incubating with the secondary antibody, the membrane was cleaned for 10 minutes with PBS-T 3 times and then incubated for one hour at RT for secondary-to-primary antibody binding. Antibody excess was washed off by incubation, first for 10 minutes 3 times with PBS-T and then once with PBSx1, to continue with membrane developing.

Every washing or incubating step mentioned was performed with enough volume of the washing agent to cover the membrane completely.

Table 13. Composition of Antibody solutions.

Primary antibody		Secondary antibody	
Ab α-GFP (mouse)	3 μL	Ab α-mouse IgG	4 μL
Nonfat dried milk	0.15 g	Nonfat dried milk	0.15 g
PBS-T	15 mL	PBS-T	15 mL

The membrane was then transferred to a flat surface such as the lid of the plastic container used during the whole process and the Enhanced Chemiluminescence mix (ECL mix; table 14) was uniformly distributed on top of the membrane with a pipette, letting it sit for 1 minute before visualization. The membrane is then placed inside of a plastic sheet to avoid direct light exposure and inserted in the molecular imager to obtained the desired results.

Table 14. Composition of ECL mix.

Substrate*	Volume
Luminol/enhancer solution	1 mL
Peroxide solution	1 mL

*Both substrates came in the Bio-Rad Clarity™ Western ECL kit.

3. RESULTS.

3.1 The effect of point mutations on fluG expression levels.

The expression pattern observed for the mRNA of *fluG* throughout the period of the wild type strain was found to be stable, in a manner which is consistent with previous reports (10). Figure 15 represents the expression levels of *fluG* in mutant strains containing selected the point mutations in Figure 6. The relative levels of expression were compared to those of *benA*, a constitutively expressed gene that codes for β-tubulin (25).

Those mutations introduced in the C terminus region (E566A to E752A), as well as the complete deletion of the C-terminus (NTERM) resulted in increases in expression that reached 9-fold or higher (up to 19-fold). On the other hand, mutations which affected the complete deletion of the N-terminus (CTERM) or the N-terminal region (H20H22A) presented no change, or increases no greater than 5-fold, respectively.

Thus, the pattern of responses observed clearly define a difference between the mutations affecting both defined regions of the protein. Of special interest is the clear increase in expression observed in mutants affected in the C-terminal region, suggesting that the product of this purported enzyme may exert a repressive effect on gene expression. This aspect will be further discussed in the following section:

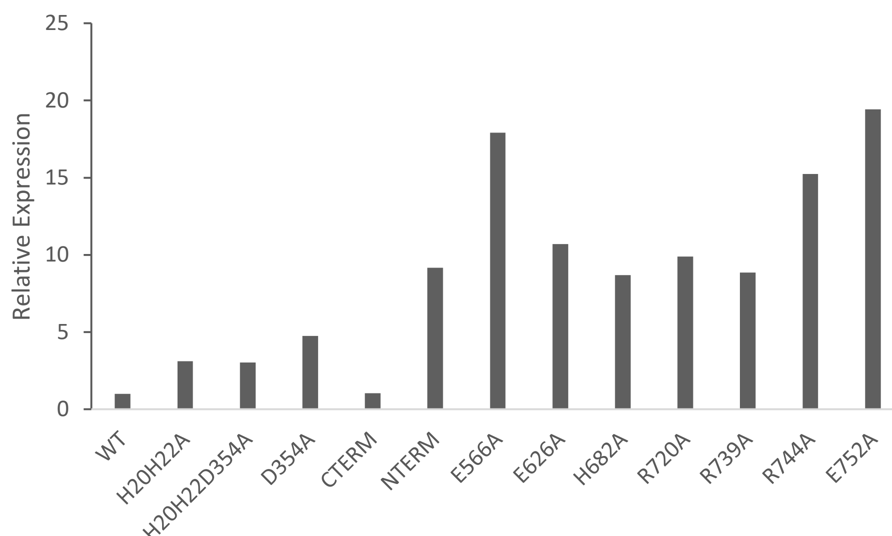

Figure 15. Bar chart representing fluG expression levels of each mutant strain. The results are expressed as the fold expression with comparison to the wild wild-type (WT), the value of which is arbitrarily chosen to be 1 Acronyms correspond to each mutant described in table 1 as follows: WT: Wild-type; H20A;H22A: Strain with mutation on H20 and H22 residues; H20A;H22A;D354A: Strain with mutation on residues H20, H22 and D354; D354; Strain with mutation on D354; C-TERM: Strain without the N terminus region of fluG; N-TERM: Strain without the C terminus region of fluG; E566A: Strain with a mutation on residue E566; E626A: Strain with a mutation on the residue E626; H682A: Strain with a mutation on the residue H682; R720A: Strain with a mutation on the residue R720; R739A: Strain with a mutation on the residue R739; R744A: Strain with a mutation on the residue R744; E752A: Strain with a mutation on the residue E752.

3.2 Gene expression analysis based on protein levels.

In order to acquire the skills to determine gene expression levels through protein levels, a method was devised to determine *fluG* expression by direct determination of GFP as a reporter protein. This was carried out using a mutant strain which contained the *GFP* sequence tagged to the C-terminal end of *fluG*. The rationale of this experiment is that a FluG:GFP protein would be produced, and that this protein could then be quantitatively determined through fluorescence spectroscopy techniques.

In order to apply this technique to the former experiment (Figure 15), *fluG::GFP versions of* each and every one of the mutants would have to be obtained, and this task was far beyond the scope of this project. Instead, a

different approach was adopted using the abovementioned *fluG::Gfp* strain.

A previous study carried out by members of the Biochemistry laboratory indicated that *fluG* mutants presented significant changes in the ratio of conidiospores formed per dry cell mass unit under different nitrogen source availabilities. Hence, a number of nitrogen sources were chosen as nitrogen source to determine whether they affected *fluG::Gfp* expression levels.

The experiment consisted in growing a *fluG-GFP* strain in MMA for 24 hours as explained in the Materials and Methods section, and then transferring the biomass to a new media in which four different nitrogen sources were present (Figure 16). The Biomass was then harvested at 2h intervals, and homogenates prepared for spectrofluorimetric analysis. The level of fluorescence was compared to that of a strain which did not express FluG::GFP (E 752A).

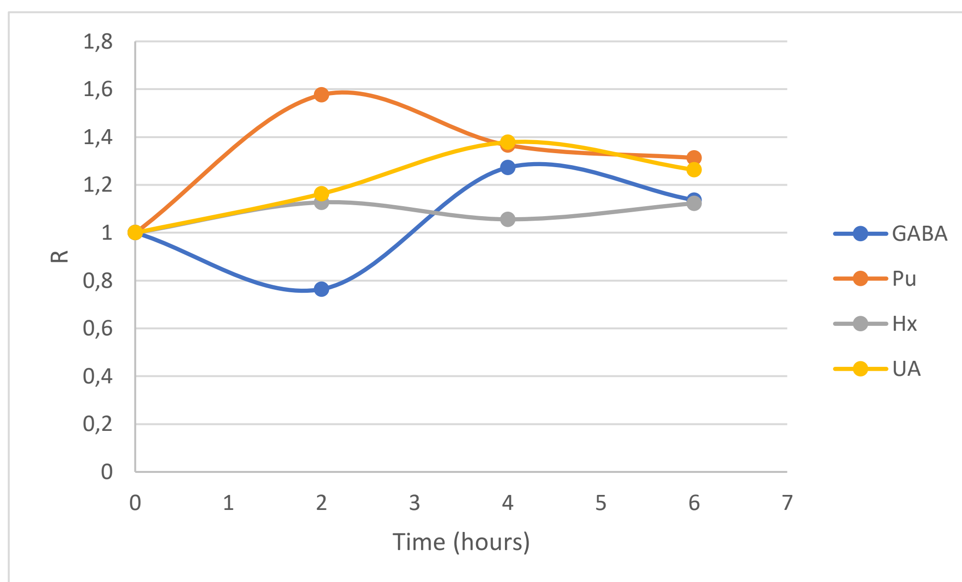

Figure 16. Graph representing the fluorescence variation caused by four different nitrogen sources and after 2, 4 and 6 hour of induction. R stands for: (FluG-GFP Signal/ E752 Signal) [tx] / (FluG-GFP Signal/ E752 Signal) [t0]

Since the *fluG-GFP* strain used has no functional activity (Mikel Iradi personal communication) it can be expected that the protein expression pattern would resemble that described for the non-functional mutants (Figure 12, NTERM-E752A). Thus, no great increases in fluorescence are expected, but if any of the nitrogen sources resembled the product of FluG, a transitory decrease could be expected. The results show that the different nitrogen sources resulted in changes in *fluG::Gfp* expression after 2 hours of incubation, with a relative increase in the case of Uric Acid, Putrescine and Hypoxanthine, and a relative decrease in the case of GABA.

The results show that the technique can be reliably used for the quantitative determination of protein expression at relatively short intervals, and with multiple samples subjected to different experimental conditions.

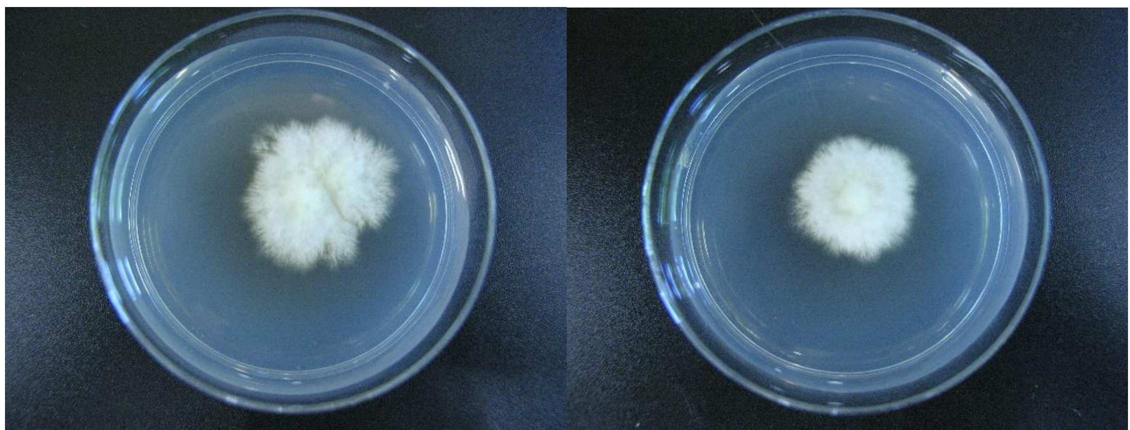

Figure 17. On the left, FluG-GFP and, on the right E752. Both strains present aconidial phenotype which means that both have indeed a non-functional FluG protein.

3.3 Assessment of FluG-GFP protein integrity by Western-Blot analysis.

After performing a quantification of *fluG::Gfp* expression, a Specific analysis to determine the integrity of the chimera protein was carried out. The purpose of this analysis is to verify how much of the full length polypeptide is present in the cell, as opposed to its constituent proteins (FluG and GFP). Since we disposed of a specific antibody against GFP, this protein could be detected by Western blot technique. By combining immune detection with electrophoretic separation, the molecular weight of the detected protein construct could be detected, as shown in Figure 18.

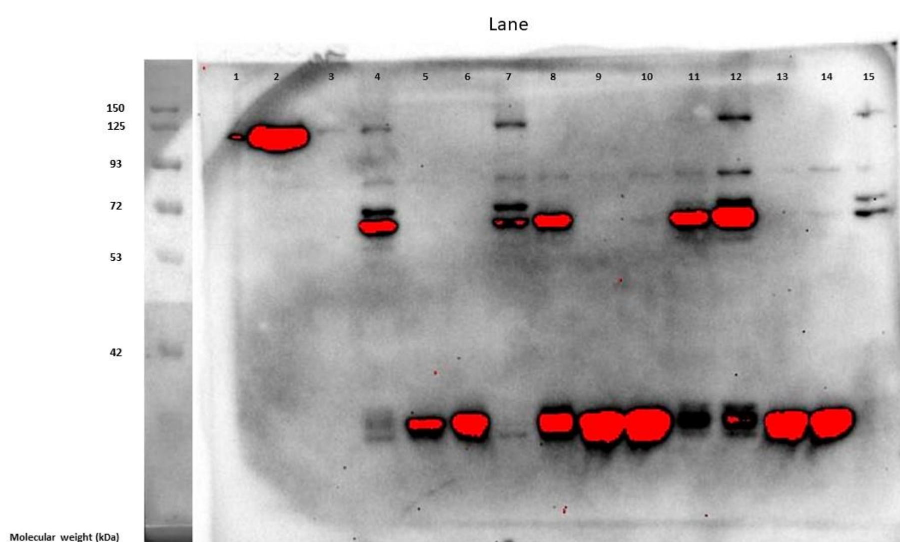

Figure 18. Western blot analysis of a membrane developed with an anti-GFP. The samples introduced on each lane were: 0) Protein marker; 2) Positive control of FluG-GFP supplied by the laboratory; 3) FluG-GFP strain at 0 hours of incubation; 4)FluG-GFP induced with GABA, 2 hours; 5) FluG-GFP induced with Putrescine, 2 hours; 6) FluG-GFP induced with Hypoxanthine, 2 hours; 7) FluG-GFP induced with Uric Acid, 2 hours; 8) FluG-GFP induced with GABA, 4 hours; 9) FluG-GFP induced with Putrescine, 4 hours; 10) FluG-GFP induced with Hypoxanthine, 4 hours; 11) FluG-GFP induced with Uric Acid, 4 hours; 12) FluG-GFP induced with GABA, 6 hours; 13) FluG-GFP induced with Putrescine, 6 hours; 14) FluG-GFP induced with Hypoxanthine, 6 hours; 15) FluG-GFP induced with Uric Acid, 6 hours.

Considering that the calculated molecular weight of FluG is 96.5 KDa, and that of GFP is 27 KDa, The combined FluG::GFP protein can be calculated to have a molecular weight of 123.5 KDa. This coincides with the molecular weight of the positive control for the protein obtained in the laboratory (lane 2). This molecular weight can also be detected in the mycelium incubated at time 0 (lane 3).

Two hours after incubation in GABA, a detectable amount of full protein can be detected, but some free GFP can be detected, as well as some bands representing partially degraded protein (lane 4). The other treatments show a heterogeneous pattern of degradation, including some samples in which no full-length protein can be detected, or practically all the detected protein is free GFP.

These results indicate that the FluG::GFP construct is not stable within the fungus, after it is synthetized, and this questions the functionality of the protein after synthesis, and this could affect the regulation of FluG expression, as will be further discussed in the following section.

4. Discussion.

FluG is a protein which has been attributed two catalytic activities based on bioinformatic analyses, as explained in the introduction. The main goal of this work was to better understand the function of this protein through the analysis of the transcriptional activation (expression) of the protein, and more specifically, to observe whether already characterized mutations could alter the expression.

The rationale followed in this project assumed that FluG has two catalytic activities that are coordinated, and that there is a substrate, a transitory product (product of the first catalytic activity and the substrate of the second one) and a final product. Following the general principles of gene expression for enzymes, accumulation of the substrate should

activate expression, whereas product accumulation should deactivate or even repress expression.

Considering that a number of point mutations are found in both hypothetical enzymes, any mutation that disables the production of the final product should result in an increase in expression of the protein. This effect has been clearly observed in mutations affecting the C-terminal region of the protein (Figure 15). On the other hand, mutations affecting the N-terminus only resulted in partial increases in expression.

Thus, the absence of the product from the C-terminal region appears to result in the greatest amount of expression. These results were in line with findings from the Biochemistry II lab, which indicate that the N-terminal region performs an ancillary role to the main function of the C-terminal region.

The abovementioned results have been obtained at the mRNA (transcriptional) level, but it would be desirable to also confirm whether the observed changes are also observable at the protein level. In order to determine a methodology that could report on protein expression levels, an attempt was conducted with an existing strain that expressed *fluG::Gfp* (Figure 16). This method showed promise in the multiple and repeated quantification of protein levels, but the results could not be tested in the same mutants as in the transcriptional experiments. Future work should most probably not be conducted using GFP. Small immunoactive peptide tags, such as Myc or HA, could prove more practical and efficient, as GFP has a rather high molecular weight, which represents 25% of the total FluG protein weight.

GFP tagging may have influenced protein stability, as evidenced by the degradation levels observed in Western Blot analysis, or the observed effect may have been due to the physiological state of cells after the switch to different nitrogen sources. In any case, the causes of this on pattern of degradation could be complex, and require a more detailed analysis, which falls beyond the scope of the project.

Considering the results obtained with different nitrogen sources, the expression levels measured by FluG-GFP protein fluorimetry yielded interesting results. It is known that an incubation period of approximately two hours is required after the medium switch before protein expression changes can be observed. Thus, the results obtained 2 hours after induction are the most relevant, and after this period, other metabolic adaptations may result, which could complicate the interpretation of the results. After two hours, with GABA, there is a decrease in the signal compared to the basal level, that might indicate that this substance may be related to the product of the reaction catalysed by FluG. Putrescine, on the other hand, produced an increase in expression, possibly indicating that it is either a substrate or a related derivative or precursor. Further experiments would be needed to confirm these findings, but the validity of protein tagging for the quantification of protein expression levels is confirmed as a valuable technique in this experimental system.

5. Conclusions.

Expression levels of the *fluG* gene were successfully measured using qPCR, indicating that those mutations which altered the activity of the C-terminus region of the protein resulted in notable increases in expression. Mutations in the N-terminus did not result in such large increases. The results indicate that the product of the C-terminal region may exert a repressive function in the expression of FluG.

The expression levels of FluG protein were studied using a FluG::GFP tagged chimera protein. Using four different nitrogen sources, it was shown that the expression levels of the protein fluctuated within two hours of the medium switch. Protein stability was irregular, however, and the cause of this effect could not be ascertained.

The use of smaller tags is suggested for future studies, or the use of GFP as a reported protein, under the control of the *fluG* promotor in a separate plasmid.

Conclusiones:

Los niveles de expression del gen *fluG* fueron medidos exitosamente por medio de Q-PCR, indicando que aquellas mutaciones que alteran la actividad de la región C terminal de la proteína resultan en incrementos notables en su expresión. Mutaciones en la región N terminal no resultan en incrementos tan significativos. Los resultados indican que el producto de la región C terminal ejerce un efecto represivo en la expresión de FluG.

Los niveles de expresión de la proteína FluG se estudiaron utilizando una proteína quimera etiquetada FluG::GFP. Utilizando cuatro fuentes de nitrógeno diferentes, se demostró que los niveles de expresión de la proteína fluctuaban durante las dos primeras horas tras el cambio de medio. Sin embargo, la estabilidad de la proteína se mostró irregular, no pudiéndose asegurar la causa de este efecto.

Se sugiere el uso de marcadores de menor tamaño para futuros estudios, o el uso de GFP como proteína reportera bajo el control del promotor de *fluG* en un plásmido separado.

6. References.

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7. Append.

7.1 Microplate setup.

In table 15 is shown the setup used to load the 96-wells microplate in the Q-PCR experiment.

7.2 Protein concentration for fluorescence assay.

To determine the range of concentrations on which GFP is detected by the spectrofluorophotometer, different solutions starting from a fluG-GFP protein extract were made resulting in the following fluorescence data:

Table 15. Fluorescence data and final concentrations of the solutions used for designing the fluorescence assay.

Strain	Starting concentration (mg/mL)	Volume used for dilution to 2 mL	Final concentration (mg/mL)	Fluorescence intensity
fluG-GFP t_0	33.6	25.0 µL	0.420	307
		50.0 µL	0.840	512
		100 µL	1.68	896
		200 µL	3.36	1608
fluG-GFP t_6	13.7	25.0 µL	0.170	214
		200 µL	1.37	800

Based on these results, final total protein concentration was set at 1 mg/mL for completion of this experiment.

Starting from the data in table 17, solutions of a final concentration of 1mg/mL were made, two for each sample.

Table 16. Microplate setup for Q-PCR experiment, at the top of each square indicated the strain except for the negative control (N) in which no cDNA was added. At the bottom indicated the gene amplified in each case, in green BenA and, in red, fluG.

N BenA	N fluG	WT BenA	WT BenA	WT fluG	WT fluG	H20A;H22A BenA	H20A;H22A BenA	H20A;H22A fluG	H20A;H22A fluG	H20A;H22A; D354A BenA	H20A;H22A; D354A BenA
H20A;H22A; D354A fluG	H20A;H22A; D354A fluG	D354A BenA	D354A BenA	D354A fluG	D354A fluG	Cterm BenA	Cterm BenA	Cterm fluG	Cterm fluG	R739A BenA	R739A BenA
R739A fluG	R739A fluG	Nterm BenA	Nterm BenA	Nterm fluG	Nterm fluG	E752A BenA	E752A BenA	E752A fluG	E752A fluG	E626A BenA	E626A BenA
E626A fluG	E626A fluG	E566A BenA	E566A BenA	E566A fluG	E566A fluG	R744A BenA	R744A BenA	R744A fluG	R744A fluG	H682A BenA	H682A BenA
H682A fluG	H682A fluG	R720A BenA	R720A BenA	R720A fluG	R720A fluG						

Table 17. Quantification results for each sample used in this experiment.

Strain	Time (hours)	Nitrogen source	Protein concentration (mg/mL)
FluG-GFP	**0**	**NH_4T**	**26.7**
	2	**GABA**	**12.4**
		Pu	**8.57**
		Hx	**13.1**
		UA	**21.6**
	4	**GABA**	**15.3**
		Pu	**11.5**
		Hx	**13.8**
		UA	**17.7**
	6	**GABA**	**14.6**
		Pu	**12.2**
		Hx	**17.7**
		UA	**6.62**
E752	**0**	**NH_4T**	**26.6**
	2	**GABA**	**14.3**
		Pu	**15.6**
		Hx	**16.8**
		UA	**18.3**
	4	**GABA**	**19.2**
		Pu	**18.2**
		Hx	**16.1**
		UA	**20.5**
	6	**GABA**	**20.3**
		Pu	**16.1**
		Hx	**25.4**
		UA	**24.0**

7.3 Fluorescence results.

The fluorescence values obtained for each sample appear represented in table 18 and 19.

Table 18. Fluorescence values measured for FluG-GFP strain.

Time (h)	Substrate	Solution number	Signal	Ordinate Scale (x_1)	Corrected Signal*	Mean value	Standard deviation
0	-	1	66.3	5	1058.6	1070.6	113.1
		2	67.8		1346.6		
2	GABA	1	39.7	5	633	662.6	41.9
		2	43.4		692.2		
	Pu	1	83.1	5	1327.4	1410.6	117.7
		2	93.5		1493.8		
	Hx	1	66.1	5	1055.4	1082.6	38.5
		2	69.5		1109.8		
	UA	1	56.9	5	908.2	917.8	13.6
		2	58.1		927.4		
4	GABA	1	67.4	5	1076.2	1029.8	65.6
		2	61.5		983.4		
	Pu	1	95.4	5	1524.2	1506.6	24.9
		2	93.2		1489		
	Hx	1	67.6	5	1079.4	1075.4	5.7
		2	67.1		1071.4		
	UA	1	68.3	5	1090.6	1080.2	14.7
		2	67.2		1073.0		
6	GABA	1	69.9	5	1116.2	1093.0	32.8
		2	67.0		1069.8		
	Pu	1	60.5	4	482.9	481.7	1.7
		2	60.2		480.5		
	Hx	1	66.0	5	1053.8	1093.0	55.4
		2	70.9		1132.2		
	UA	1	77.5	5	1237.8	1216.2	30.5
		2	74.8		1194.6		

*The ordinate scale implied a multiplication of the original signal by, in the case of X=5, 16 and, in the case of X=4, by 8. The background noise was substrated on each measurement.

Table 19. Fluorescence measurements for the E752 strain.

Time (h)	Substrate	Solution	Signal	Scale	Corrected Signal*	Mean Value	Standard deviation
0	-	1	84.3	5	1346.6	1426.6	17.0
		2	94.3		1506.6		
2	GABA	1	41.4	5	660.2	651.4	12.4
		2	40.3		642.6		
	Pu	1	42.4	5	676.2	671.4	6.8
		2	41.8		666.6		
	Hx	1	46.3	5	738.6	721.0	24.9
		2	44.1		703.4		
	UA	1	37.6	5	599.4	592.2	10.2
		2	36.7		585.0		
4	GABA	1	36.3	5	578.6	607.4	40.7
		2	39.9		636.2		
	Pu	1	53.2	5	849.0	827.4	30.5
		2	50.5		805.8		
	Hx	1	49.8	5	794.6	764.2	43.0
		2	46.0		733.8		
	UA	1	37.1	5	591.4	588.2	4.25
		2	36.7		585.0		
6	GABA	1	42.9	5	684.2	721.8	53.2
		2	47.6		759.4		
	Pu	1	35.8	4	285.3	275.3	14.1
		2	33.3		265.3		
	Hx	1	46.0	5	733.8	730.6	4.5
		2	45.6		727.4		
	UA	1	48.3	5	770.6	722.6	67.9
		2	42.3		674.6		

*The ordinate scale implied a multiplication of the original signal by, in the case of X=5, 16 and, in the case of X=4, by 8. The background noise was substrated on each measurement.

7.4 Charge control for Western-blot.

A factor that needs to be taken into account is the amount of protein that has been charged on each well. In this case, the objective was to load 300 µg of total protein on each well but, to take into account in the interpretation of the results, two different charge control methods were completed. That

way, it would be easily observable if by pipetting error one well was loaded more than another well.

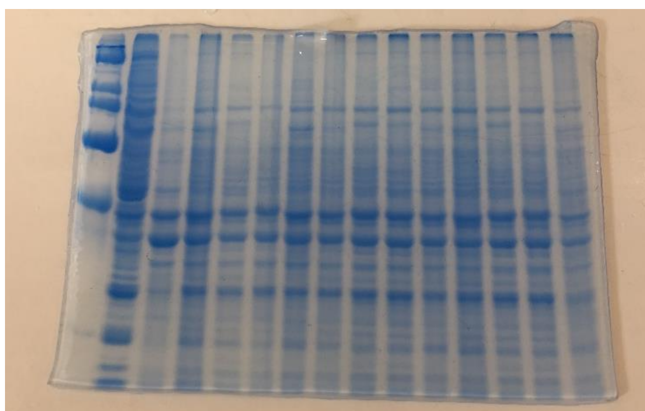

Figure 19. Commassie blue charge control.

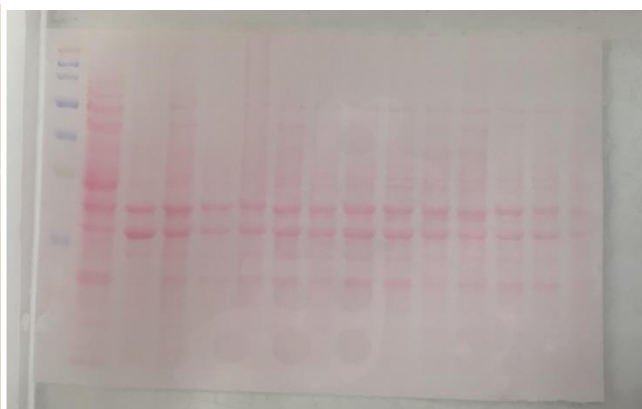

Figure 20. Ponceau staining charge control.