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Functional and expression analyses of the ribosomal RNA methyltransferase NSUN5 upon heavy metals and trace elements

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Resum

Introducció: L'addició d'un grup metil al substrat d'una molècula d'RNA és coneguda per estar involucrada en la regulació de l'expressió gènica i la translació. *Nsun5* és un membre d'una família de proteïnes que treballen com metil transferases. Estan incloses a un grup d'enzims anomenats rARN citosina metil transferasa (RMC1 a llevats o *nsun5* a humans o plantes) que catalitza la metilació d'una citosina de l'ARN ribosòmics 25S. A la planta *Arabidopsis thaliana*, el mutant *nsun5-2* té una inserció T-ADN al primer exó i *nsun5-1* al setè exó. La metilació sobre la mostra control és ja coneguda, essent un 66 %, d'un 29% a *nsun5-2* i d'un 2 % a *nsun5-1* (Burgess A.L et al 2015).

Objectius: Analitzar l'expressió NSUN5 a la mostra control i a dos al·lels mutants sota diferent estressos de metalls pesats i elements traça.

Materials i mètodes: Primerament les insercions T-ADN a ambdós mutants van ésser confirmades pel genotipat. Seguidament l'expressió del gen NSUN5 va ésser quantificada a la mostra control i a ambdós mutants. Per a determinar si el gen NSUN5 esta involucrat a la resposta per metalls pesats i elements traça, diversos assaigs van ser duts a terme amb ambdós mutants en comparació amb la mostra control. Per tant, diferents medis que contenen metalls pesats com cadmi, zinc, plom, níquel, coure o manganès van ser preparats i les llavors van ésser germinades i cultivades durant dotze dies a la cambra de creixement. Amb l'ajuda del programa Image J, la longitud de l'arrel va ser mesurada a diferents dies després de la germinació i va ésser comparada amb la mostra control i entre diferents metalls pesats. Per a determinar l'expressió de *NSUN5* a la mostra control i als mutants, fins a quatre qPCR a temps real van ser dutes a terme amb les llavors cultivades amb medi control i altres suplementades amb níquel. Tres diferents gens constitutius *TUBULINA-9, UBIQUITINA 5* i la *PROTEINA FOSFATASA 2* van ser utilitzats per normalitzar la informació d'expressió obtinguda.

Resultats: El genotipat va demostrar que totes les plantes que vaig rebre de la universitat d'Adelaide eren homozigots per al mutants *nsun5-1* i *nsun5-2*. Encara que la variació entre els diferents assaigs de creixement de l'arrel era gran, el mutant *nsun5-1* va ésser híper sensible per a cadmi i manganès a dos experiments independents. Resultats conflictius van ser obtinguts per a níquel quan *nsun5-1* a un experiment era més tolerant i a un altre era més sensible. El mutant *nsun5-2* va tindre una alta tolerància a cadmi, plom i zinc en un experiment. L'expressió del gen *NSUN5* a *nsun5-2* era només més alta que a la mostra control mentre que l'expressió de *nsun5-1* no podia ser detectada. El resultat indica que l'al·lel *nsun5-1* es un vertader mutant noqueig mentre que l'al·lel *nsun5-2* sobre-expressa el gen *NSUN5*. La anàlisis de l'expressió a temps real per a mesurar els efectes del níquel a la expressió gènica de NSUN5 no són concloents perquè mentre a un experiment níquel redueix l'expressió en un altre experiment indueix l'expressió de *NSUN5* a la mostra control.

Conclusions: Encara que els experiments mostren resultats variables, *NSUN5* mostra unes primeres evidències que els metalls pesats i els elements traça no solament afecten l'expressió de *NSUN5* si no que la planta necessita el gen *NSUN5* funcional per a respondre a aquests causants d'estrès abiòtics.

Resumen

Introducción: La adición de un grupo metilo en el sustrato de una molécula de ARN es conocida por estar involucrada en la regulación de la expresión génica y la translación. *Nsun5* es un miembro de una familia de proteínas que trabajan como metiltransferasas. Está incluida en un grupo de enzimas llamadas rARN citosina-metiltransferasa l (RCM1 en levadura o *nsun5* en humanos y plantas) que catalizan la metilación de una citosina en el ARN ribosómico 25S. En la planta *Arabidopsis thaliana*, el mutante *nsun5-2* tiene una inserción T-ADN en el primer exón y *nsun5-1* en el séptimo exón. La metilación en la muestra control es ya conocida, siendo de un 66%, de un 29% para *nsun5-2* y de un 2% para *nsun5-1*(Burgess A.L et al 2015).

Objetivos: Analizar la expresión de *NSUN5* en la muestra control y en dos alelos mutantes bajo diferentes estreses con metales pesados y elementos traza.

Materiales y métodos: Primero las inserciones T-ADN de ambos mutantes son confirmadas con el genotipado. Seguidamente la expresión del gen *NSUN5* es cuantificada en la muestra control y en ambos mutantes. Para determinar si el gen *NSUN5* está envuelto en la respuesta con metales pesados y elementos traza, diversos ensayos de crecimiento son hechos con ambos mutantes y comparados con la muestra control. Por ello diferentes medios que contienen metales pesados como cadmio, zinc, plomo, níquel, cobre o manganeso fueron preparados y las semillas germinadas y cultivadas durante doce días en la cámara de crecimiento. Con la ayuda del programa Image J, la longitud de las raíces fue medida en diferentes días y comparada la muestra control con la de los diferentes metales pesados. Para determinar la expresión de *NSUN5* en la muestra control y en ambos mutantes, cuatro qPCR a tiempo real son hechas con semillas que habían sido cultivadas en medio control y otras suplementadas con níquel. Hasta tres diferentes genes constitutivos TUBULIN9, UBIQUITIN 5 y la PROTEINA FOSFATASA2 fueron utilizados para normalizar los datos de la expresión obtenidos.

Resultados: El genotipado demostró que todas las plantas que recibí de la universidad de Adelaide eran homocigotas para el mutante *nsun5-1* y *nsun5-2*. Aunque la variación entre los tres ensayos de crecimiento de raíz era alta, el mutante *nsun5-1* era hipersensible al cadmio y al manganeso en dos experimentos independientes. Resultados opuestos fueron obtenidos para el níquel donde *nsun5-1* en un experimento era tolerante y en otro era sensible. El mutante *nsun5-2* tuvo una mayor tolerancia al cadmio, plomo y al zinc. La expresión del gen *NSUN5* en *nsun5-2* era solamente más alta que la muestra control cuando la expresión de *nsun5-1* no podía ser detectada. El resultado indica que el alelo *nsun5-1* es un verdadero mutante noqueo mientras que el alelo *nsun5-2* sobre expresa el gen *NSUN5*. El análisis de la expresión a tiempo real para medir los efectos del níquel en la expresión del gen *NSUN*5 no fue muy concluyente puesto que mientras en un experimento reduce la expresión, en el otro experimento induce la expresión de *NSUN5* en la muestra control.

Conclusiones: Aunque los experimentos muestran resultados variables, *NSUN5* muestra primeras evidencias que los metales pesados y los elementos traza afectan no solamente a la expresión de *NSUN5* si no que la planta necesita el gen funcional *NSUN5* para responder a estos causantes de estrés abióticos.

Abstract

Introduction: The addition of a methyl group on a substrate in a RNA molecule is known to be involved in the regulation of gene expression and translation. *Nsun5 is* a member of the protein family that works as methyltransferases. They include a group of enzymes called rRNA cytosine methyltransferase l (*RCM1* in yeast or *nsun5* in human and plant) that catalyzes the methylation of one cytosines of the 25S ribosomal RNA. In the model plant *Arabidopsis thaliana,* the mutant *nsun5-2* has a T-DNA insertion in the first exon and *nsun5-1* in the seventh exon. The methylation from wild type in both mutants is already known, being 29% from *nsun5-2* and 2% from *nsun5-1*. From wild type is up to 66 % (Burgess A.L et al 2015).

Aims: Analyze NSUN5 expression in wildtype and two mutant alleles under different heavy metal and trace elements stresses.

Materials and methods: First the T-DNA insertions of the two *nsun5* mutants were confirmed by genotyping. Second the expression of the *NSUN5* gene was quantified in wildtype and the two mutants. To determine if the *NSUN5* gene is involved in heavy metal and trace element responses, growth assays were performed with the two nsun5 mutants in comparison to wildtype. Therefore medium containing different heavy metals like cadmium, zinc, lead, nickel, copper and manganese was prepared and seeds were germinated and seedlings cultivated for twelve days in the growth chamber. With the help of the program ImageJ root length was measured at different days after germination and compared to wildtype and between different heavy metals. To determine the expression of *NSNU5* in wildtype and the mutants four real time qPCR were performed the seedlings cultivated on control medium and supplemented with nickel. Three different housekeeping genes TUBULIN9, UBIQUITIN 5 and a PROTEIN PHOSPHATASE2 were used to normalize the expression data.

Results: Genotyping demonstrated that all the plants I received from the University of Adelaide were homozygous for the mutant *nsun5-1* and *nsun5-2*. Although the variation between the three different root growth assays was high the *nsun5-1* mutant was in two independent experiments hypersensitive to cadmium and manganese. Conflicting results were obtained for nickel where *nsun5-1* was in one experiment more tolerant and in another more sensitive. The *nsun5-2* mutant had in one experiment a higher tolerance to cadmium, lead and zinc. The expression of the NSUN5 gene in the *nsun5-2* background was only higher than in wildtype while the expression in the *nsuns5-1* background could not be detected. The result indicates that the *nsun5-1* allele is a real knock-out mutant while the *nsun5-2* allele even overexpresses the NSUN5 gene. The real-time expression analyses to measure the nickel effects on NSUN5 gene expression were not conclusive since in one experiment nickel reduced the expression and in the other experiment induced the expression of NSUN5 in wildtype.

Conclusions: Even though the experiments showed variable results NSNU5 there are first evidences that heavy metals and trace elements affect not only *NSUN5* expression but also plants need a functional *NSNU5* to respond to these abiotic stressors.

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Chapter 1: Introduction and aims

1.1 Arabidopsis thaliana

Arabidopsis thaliana is a plant native to Europe, Asia and northwestern of Africa although it has also been introduced and naturalized worldwide. Up to 700 natural varieties of *Arabidopsis thaliana* have been found around the world. Each variety show several differences dependent the part of the world they are found but it has been suggested that all the varieties could have a common ancestor from they have adapted evolutionary over time (TAIR- about *Arabidopsis*, 2010). This leads us to deduce as it has been already demonstrated, the high variability of *Arabidopsis thaliana* to be adapted to a large number of different environments.

The small size of its genome, about 135 mega base pairs and just five chromosomes makes *Arabidopsis thaliana* an useful plant for genetic mapping and sequencing which is maintained updated by Arabidopsis information Resource (TAIR-Genome assembly, 2010)

Its short life cycle (about 5 weeks), small genome size, efficient chemical and radiation mutagenesis and a low content of repeated DNA make *Arabidopsis thaliana* often chosen as a model for plant molecular biology research.

Nowadays, it is widely used for studying plant sciences, including genetics, population genetics, plant development and evolution. Even though is considered suitable for the agriculture has been mostly used to build a model to understand the genetics, cellular and molecular biology of flowering plants.

Arabidopsis thaliana is, nowadays a really popular model organism in plant biology and genetics, thus it was the first plant to have its whole genome sequence in the called *Arabidopsis genome sequence* in the year 2000 (Feldmann, K. A., & Goff, S. A. 2014)

A list of all genes is no more than a list of a genetic map that it has to be decoded knowing their interactions and functions to be able to understand the whole. One of many approaches to find out several of this interactions and functions is the called forward genetics.

Picture 1: Leaves from a *Nsun5-2 mutant plant. From: Alice Louise Burgess, Rakesh David and Iain Robert Searle (2015), Conservation of tRNA and rRNA 5-methylcitosine in the kingdom Plantae.*

1.2 Heavy metals and trace elements

Forwards genetics starts by observing the phenotype of a plant and wondering the reason behind, the genotype. Genotype is one of three factors that determine the phenotype of a plant, the other two are carried by epigenetic factors which are the results from environmental factors that switch on and off the genes and affect the way they are expressed.

Within all the environmental factors that could lead into a change of the genotype on a plant we find heavy metals. Heavy metals are natural constituents on the soil and some of them are also needed by the plants, but have been altered their geochemical cycles and biochemical balance of human waste of resources. This results in accumulation of metals in plant that could cause the elimination or modification of the ecosystem of an area and thus the plants and animals that live in it. Prolonged exposure to high concentrations of heavy metals such as cadmium, copper, lead, nickel, and zinc can cause deleterious health effects in plants. Molecular understanding of plant metal accumulation has numerous biotechnological implications also, the long term effects of which might not be yet known.

We consider a contaminant species any metal if it occurs where it is unwanted, or in a concentration or form that causes a detrimental environmental effect. Heavy metals and trace elements included in this project are lead (Pb), cadmium (Cd), copper (Cu), nickel (Ni), manganese (Mn) and zinc (Zn). Other less common metallic contaminants include aluminum (Al), cesium (Cs) and cobalt (Co), among others.

In spite of this, several physiological plant functions still require different amounts of heavy metals such as copper, iron, manganese, molybdenum, nickel and zinc, among others, the called trace elements or micronutrients.

Another interesting point about heavy metals is that several plants, algae's and fungi's are also known to extract and accumulate heavy metals, as led or mercury, from the soil and the reason is still unknown.

The metabolic functions of the plant could be modified in few different ways:

- Inhibition of the growth (roots or rest of the organs)
- Affect some enzymes involved in Calvin's cycle.
- Delay or inhibit germination of the seeds.
- Decrease of chlorophyll content and leaf water potential.
- Stomatal closure that implies the consequent lack of CO2 to carry out a proper photosynthesis.

Plants experience oxidative stress upon exposure to heavy metals that leads to cellular damage.

1.3 T-DNA Mutagenesis

One way in which the genotype can be modified is by T-DNA transfer method. The insertional mutagenesis not only creates mutations in the gene sequence but it also labels the gene of interest, thus allowing for its isolation and further study. T-DNA insertional mutagenesis, which is the insertion of foreign DNA into the gene of interest, not only changes the expression of the gene but also behaves as genomic marker that allows the researcher to identify the mutation within the gene sequence. To characterize the function of a gene, insertional mutagenesis has been widely used for cloning genes in *Arabidopsis thaliana*.

Polymerase chain reaction (PCR) methods have been developed that allow isolate individual plants which carry a particular T-DNA mutation of interest. In this project, two different mutants are carried in the gene AT5g26180 (*nsun5-1* and *nsun5-2*). This is an advantage because T-DNA insertions will not transpose subsequent to integration within the genome and are therefore chemically and physically stable through multiple generations. (Krysan,P.J et al 1998) Therefore, they can be located and isolated easily.

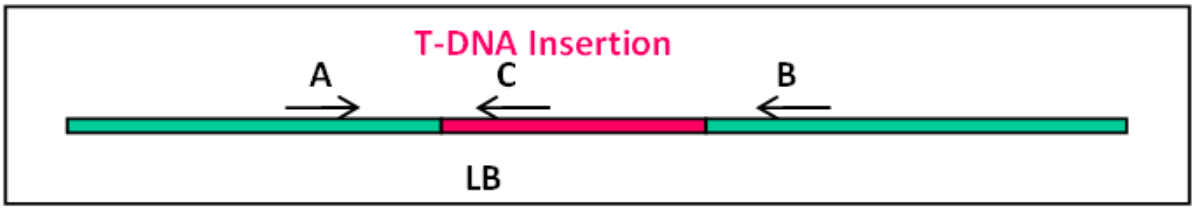


Figure 1: ***How foreign DNA of interest is introduced into the gene sequence using a T-DNA insertion using the left borders approach to locate it.***

To prove that the mutants are expressed in the T-DNA sequence of the plants several primers, which are present in the exons of the gene sequence, are tested (figure 2); the gene sequence was obtained from the website TAIR (figure 3).

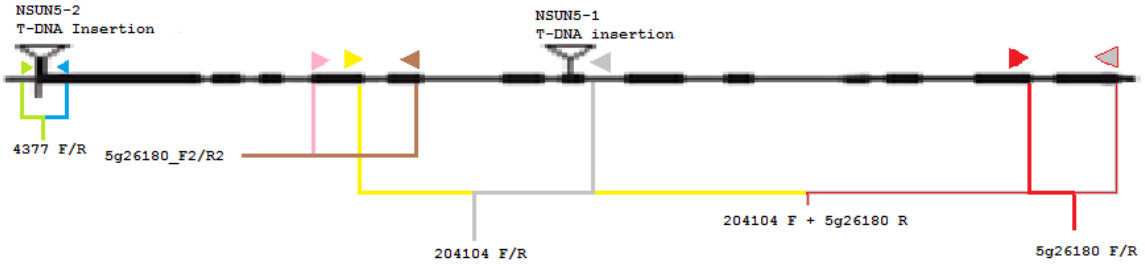


Figure 2: ***Presentation of all the primers present in the exons of the gene sequence, nsun5-2 is inserted in the first exon and nsun5-1 in the seventh exon.***

Figure 3: ***Shows the primers position and the within the gene AT5g26180 sequence. Capital letters represent exons and lower case letters represent introns.***

1.4 Ribosomal RNA Methylation

Methylation is the addition of a methyl group on a substrate such as a DNA or RNA molecule. In biological systems, methylation is catalyzed by enzymes regulates the gene expression. *Nsun5-1* and *nsun5-2* are in this group of enzymes called rRNA cytosine methyltransferase l (*RCM1* in yeast or *nsun5* in human and plant) that catalyzes the methylation of one cytosine on the 25S rRNA in *Arabidopsis thaliana*. (Alice Louise Burgess et al 2015).

Unlike methylation of DNA, RNA methylation is less known and researched from several kinds of plants. First of all, RNA molecule is known to be involved in the regulation of gene expression and translation. To know how it can be regulated firstly we need to know that several types of RNA exist even though you can mainly classify them in three different main groups:

Messenger RNA or mRNA is the responsible for transcript the genetic code from DNA in the nucleus into a form in the cytoplasm that can be read to synthesize proteins. M-RNA is the one who carries the genetic information.

Transfer RNA or tRNA is also involved in the protein synthesis bringing or transforming amino acids in the cellular cytoplasm to the ribosome, which is the protein synthesis machine. Once the proteins are in the ribosome, the y can be used to make polypeptides and proteins.

Ribosomal RNA or rRNA is the main component together with protein of the ribosome.

Ribosomal RNA form two different subunits, the large subunit (LSU) and the small subunit (SSU). The LSU is composed of three rRNA species in eukaryotes while SSU only contains one rRNA species.

Now the ribosome, which is a complex molecular machine that performs the translation, is found both in eukaryotes and prokaryotes cell, its function, is the creation of a chemical bond between two amino acids guided by mRNA in a specific order and the tRNA brought inside the ribosome.

It is clear that the ribosomal RNA has an important role in the protein synthesis and a better understanding of its complete function could lead us to the removal of any formation of abnormal protein synthesis that cause several problems in living organisms.

Furthermore, rather than merely being a molecular machine executing translation, the ribosome also shows functional diversity by the modification of a single rRNA nucleotide resulting of an alteration by a stress response, for example heavy metal medium, into an unknown physiological behavior of the organism.

As a post-translational modification, RNA methylation plays a significant role as an epigenetic mechanism. 5-methylcytosine (m^5C) is a common methylation modification in RNA molecules present in eukaryotes and may affect the regulation of some biological processes in plants.

The post transcriptional modification 5 methylcytosine can be detected in transcripts from all three organelles: nuclear, mitochondrial and chloroplast and up to 7 different m^5C sites are found in the nuclear LSU 25s rRNA, chloroplast SSU 16s, LSU 23S and mitochondrial SSU 18s and LSU 26S rRNAs

Nuclear LSU 25S contains two different m^5C sites, C 2268 and C 2860 (Figure 4). In this project I will focus on the nuclear large subunit 25S rRNA in position C2268 rRNA which requires the conserved RNA methyltransferase nsun5 (Alice Louise Burgess et al 2015).

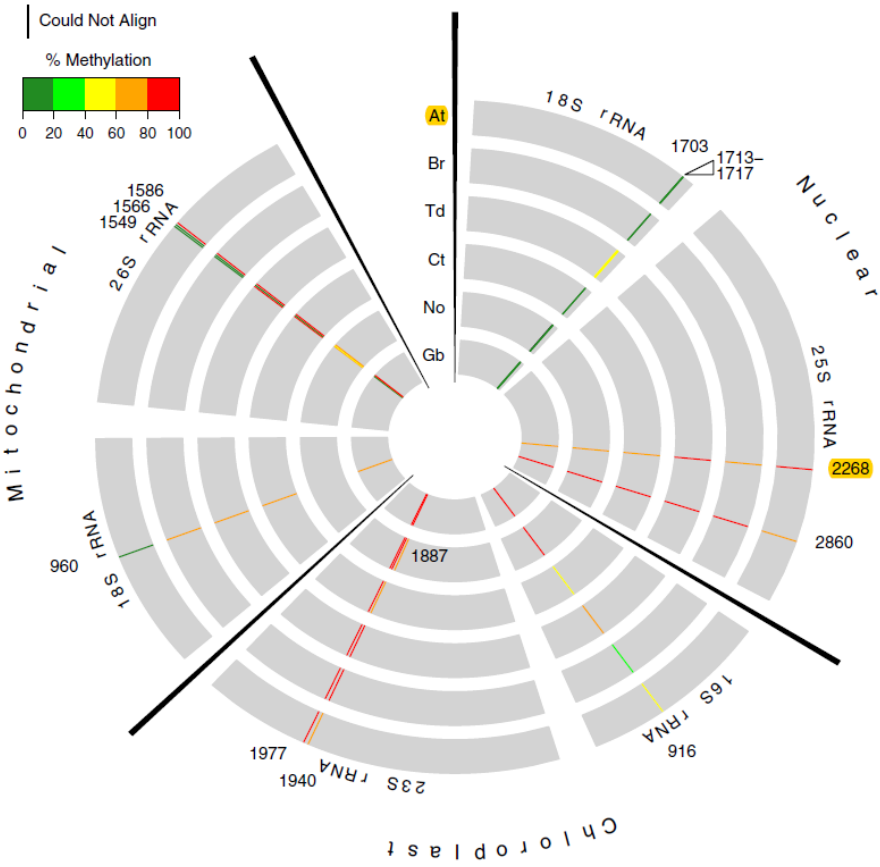


Figure 4*: Representation of the three sections of nuclear, chloroplast and mitochondrial rRNA sequences that an m^5C sites have been found in different plants. At is the abbreviation of Arabidopsis thaliana and the Cytosine that is located in the nuclear 25S LSU in the position 2268 represents the aim of my work. From: Alice Louise Burgess, Rakesh David and Iain Robert Searle (2015), Conservation of tRNA and rRNA 5-methylcitosine in the kingdom Plantae.*

1.5 Aims of this project

My project is based in the work of Ms. Alice Louise Burgess, Mr. Rakesh David and Mr. Iain Robert Searle from the school of Biological Sciences at the University of Adelaide in Australia and their publication on the conservation of tRNA and rRNA 5-methylcytosine in the Kingdom Plantae (Burges et al,2015).

They demonstrate that the methyltransferase, *NSUN5,* is required for the 5-methylcytosine modification of C2278 of the 25S ribosomal RNA.

They identified two mutant alleles and show that in wildtype 66% of the C2278 is methylated and this methylation is reduced to 2% in the *nsun5-1* mutant. They also found that in the second independent allele, *nsun5-2*, the methylation was reduced up to 29 %.

At the beginning of my thesis a few questions were formulated and sought to be answered.

Confirmation of the genotypes of mutant alleles, *nsun5-1* and *nsun5-2*

Before starting the experiments the mutant lines from University of Adelaide were genotyped to proof their identity and their homozygosity. To determine if the two mutants are real knock-out alleles the expression of the NSNU5 gene in the two mutants should be quantified by real-time PCR.

Is *NSNU5* involved in heavy metal and trace element responses?

In previous experiment of the laboratory of Marie-Theres Hauser it was shown that heavy metals and trace elements affect the efficiency of translation. The hypothesis of this thesis was that epigenetic regulation of the C2278 by *NSUN5* might be involved in protein synthesis regulation upon heavy metal and trace element exposure. To test this hypothesis a genetic approach was applied which used the available mutants and tested their root growth behavior on media supplemented with heavy metals and trace elements. A different root growth of the *nsun5* mutants in comparison to wildtype would indicate a function of *NSUN5* in the response to the tested stress. Based on the previous results on the influence of heavy metals and trace elements on translation the mutant root growth responses was evaluated on cadmium, copper, nickel, zinc, lead and manganese.

Does heavy metals and trace elements influence the expression of NSUN5?

Altered gene expression is often used to infer a functional relevance of a gene upon different stressors or during development. Therefore the second aim was to quantify the influence of heavy metals and trace elements on the expression of *NSUN5* by quantitative real time qPCR.

In addition if there is any difference between starter material with leaves or seeds will be also tested.

Chapter 2: Materials

2.1 Primers

Here is the list of all the primers used in the project.

TUB BETA-9_R	5'-GTTCTGGACGTTCATCATCTGTTC-3'
TUB BETA-9_F	5'-GTACCTTGAAGCTTGCTAATCCTA-3'
5g26180 _R	5'-ACAACCATTACGTGATAACAGGA-3'
5g26180 _F	5'-GGTCTCCCTGTATTTGCAGGA-3'
SALK_004377	*5'-GGCTTGAACCACAATTTGTTG-3'*
SALK_004377_RP	*5'-TGCTTCTCTTCTGGCGTAGAG-3'*
SALK_204104_LP	*5'-GAGCTGGAGAAGCATTACACG-3'*
SALK_204104_RP	*5'-AAATGAATGCGAGAACTGTGG-3'*
SALK_LBC1	TGGACCGCTTGCTGCAACTCT
SIGNAL_LBB1	GCGTGGACCGCTTGCTGCAACT
SIGNAL_LBA1	TGGTTCACGTAGTGGGCCA
25S_RRNA_F	5'-GGTGGGAGTAATTATGATTTTTTTAAGGGAGT-3'
25S_RRNA_R	5'-CTTTCCCCACTAATTCTACCAAACC-3'
5g26180_F2	5'-GTCATTTGAAACCACGGTATG-3'
5g26180_R2	5'-CGTCCATTCGCAACCAAACG-3'

2.2 Materials

- RNA extraction buffer

Containing *2% [w/v] hexadecyltrimethylammonium bromide, 2% [w/v] polyvinylpyrrolidone, 100 mM Tris /HCl pH 8, 0,25mM EDTA, 2M NaCl, 0.5 g/L spermidine and 2, 7% [v/v] 2-mercaptoethanol]*

- Chloroform

Mixture of chloroform 99 % and Isoamyl alcohol 99 % (ratio 24:1)

- 10 M Lithium Chloride (LiCl)

50 mL Falcon tube [4.239 g 10 M LiCl + Fill up with high quality water (double distilled) to 10 mL]

- 75 % Ethanol

Containing ¾ parts of 96-100% EtOH and ¼ part of RNAse free double-distilled water

- 96-100 % Ethanol
- 100 base pairs marker [Concentration 50 µg /ml]
- Ethidium Bromide (EtBr)

- X1 TAE buffer

TAE buffer has to be prepared freshly and used as 1x but the starter dilution usually is 50x. TAE buffer is prepared as a 50X stock solution for laboratory use. A 50X stock solution is prepared by dissolving 242g Tris base in water, adding 57.1 mL glacial acetic acid, and 100mL of 500mM EDTA (pH 8.0) solution, and bringing the final volume up to 1 liter. This stock solution can be diluted 50:1 with water to make a 1X working solution. This 1X solution will contain 40mM Tris, 20mM acetic acid, and 1mM EDTA.

- Agar plant powder
- Low melting agar
- 5%-Na-Hipoclorite
- Polysorbate 20 (Tween 20)
- X2 CTAB Buffer

100 mL of 2xCTAB buffer contains 2 g of 2% CTAB (w/v), 10 mL of 100 mM TRIS (8.00 pH), 4 mL of 20 mM EDTA (8.00 pH), 8,12 f of1.4 M NaCl with a molar weight of 58 g/mol and 1 g of 1% PVP].

- 10x buffer (25 mM $MgCl_2$)

100 µL 1M Tris/HCl pH 8.50 1mM, 500 µL 500 mM KCl, 25 µL 25 mM $MgCl_2$, 15 µL 100 %triton and fill it up to 1mL with ddH_2O

- 10x buffer (30 mM $MgCl_2$)

100 µL 1M Tris/HCl pH 8.50 1mM, 500 µL 500 mM KCl, 30µL 25 mM $MgCl_2$, 15 µL 100 %triton and fill it up to 1mL with ddH_2O

- 2 mM Deoxynucleotides (dNTP)

$2\ mM * 1000\ \mu L = 100\ mM * X$

$x = 20\ \mu L$

100 mM dATP	20 µL
100 mM dCTP	20 µL
100 mM dGTP	20 µL
100 mM dTTP	20 µL
H2O	920 µL
	1000 µL

Split the final volume in 5 different tubes of 200 µL each and store it at -20 ºC.

- Oil sigma
- Loading dye
- Homemade Taq DNA polymerase
- Phusion™ Master mix[Containing taq DNA polymerase, dNTP and 25 mM $MgCl_2$]
- Dream taq DNA polymerase
- Heavy metal mediums

Heavy Metal	Molarity	Molarity per plate	100 mL medium
Control	0	0	0
$ZnSo_4$ x 7 H20	1 M	100 µM	10 µL
$CdCl_2$ x 2,5 H20	100 mM	10 µM	10 µL
$Pb(NO3)_2$	1 M	100 µM	10 µL
$NiSo_4$ x 6 H2O	200 mM	15 µM	7,5 µL
$CuSo_4$ x 5 H2O	100 mM	10 µM	10 µL
$MnSo_4$ x H2O	1 M	500 µM	50 µL

- QIAEX KIT

Qiaex l buffer

Qiaex ll buffer

PE buffer

- 10mM TRIS/HCl pH 8.50
- DNAse l (1 U)
- 5x RT Buffer (contains the restriction enzyme)

Contains 50 mM Tris/HCl pH 8.30,50 mM KCl, 4 mM $MgCl_2$, 10 mM DDT

- 100 µL oligo $d(T)_{18}$ (50 pmol)
- Peq GOLD M-MulV H Plus 200 U/ µL (peqlab) (200 U)

2.3 Plant material samples

Three different genotypes of plants one the control called wild type; the other two are containing the two methyltransferases nsun5-1 and Nsun5-2.

Control	University of Adelaide, Adelaide, South Australia 5005, Australia
nsun5-1	University of Adelaide, Adelaide, South Australia 5005, Australia
nsun5-2	University of Adelaide, Adelaide, South Australia 5005, Australia

2.4.1 RNA Isolation

The procedure of RNA isolation was performed as detailed below:

Cut around 300 mg of material from leaves of three genotypes: *Wild type*, *nsun5-1* and *nsun5-2* and store the material in 15 mL Falcon Tubes.

Grind the samples with a drill and place the samples in a Liquid Nitrogen bottle.

Add 3 mL of pre-heated RNA Extraction Buffer at 65°C and incubate the samples at 65°C for 5 minutes.

Add 3 mL solution of ice-cold chloroform, placed beforehand on ice, in the 15 mL Falcon tubes and vortex the samples for 10-15 seconds to assure a good mixing.

Incubate for 5 minutes on ice before centrifuge at 4230 rpm for 20 minutes in the 4°C chamber, removing the supernatant and transfer into a new 15 mL Falcon tube.

Add again 3 mL of ice-cold chloroform, vortex for 10-15 seconds and centrifuge again at 4230 rpm for 20 minutes in the 4°C chamber.

Transfer the supernatant to a 2 mL Eppendorf tube to wash the pellet.

Finally add ¼ of volume of an ice-cold 10 M Lithium chloride and incubate the samples at 4°C overnight in order to ensure a good RNA precipitation overnight (18 hours).

Centrifuge the samples at 12.000 rpm for 1 hour in the 4°C chamber and remove the supernatant carefully.

Add 2 ml of 75% Ethanol and centrifuge again at 12.000 rpm for 20 minutes to remove any possible salts in the sample.

Remove the ethanol and let the pellet dry for 30 minutes at room temperature or until the ethanol is completely evaporated.

Dissolve the pellet in 30 µL RNAse free water [double-distilled] and shake the samples at 4°C for 5 minutes at high velocity to ensure that the solution is properly mixed.

Store at -80°C.

2.4.2 Nano drop RNA quantification

The Nano drop micro volume sample retention system [Thermo Scientific Nano drop Products] combines fibre optic technology and natural surface tension properties to capture and retain low amount of sample [Desjardins.P and Cocklin.D, 2010]. Just pipette 1µL is needed to obtain the concentration of the desired sample.

2.4.3 RNA electrophoresis gel

RNA handling has to be as clean as possible so that all the equipment have to be washed with 96% ethanol and RNAse free water, furthermore, the RNA electrophoresis apparatus has also to be cleaned before performing any assay.

For RNA samples 1, 2 % containing agarose gel is used. Agarose acts as a molecular sieve: DNA migrates through the gel during electrophoresis and is separated according to size. Small fragments migrate faster than larger ones and are thus, further down on the gel. Increased agarose content aids in the resolving quality of the gel. More agarose means that DNA is separated according to size more specifically so that is easier to see the difference in size between the RNA fragments that could have a similar size.

2 µL of Ethidium Bromide is added in the agarose gel after heating it up and dissolved completely to detect nucleic acids as double stranded DNA or single single-stranded RNA. When exposed to UV light, it will fluoresce with an orange color.

Following formula is used to calculate the amount of agar powder and X1 TAE buffer are needed:

$$8\ cm * 8\ cm * 0{,}70\ cm = 44{,}8\ cm3$$
$$\approx 45\ \text{mL}$$

$$\frac{45\ mL * 1{,}2}{100} = 0{,}54\ g\ of\ agarose\ powder$$

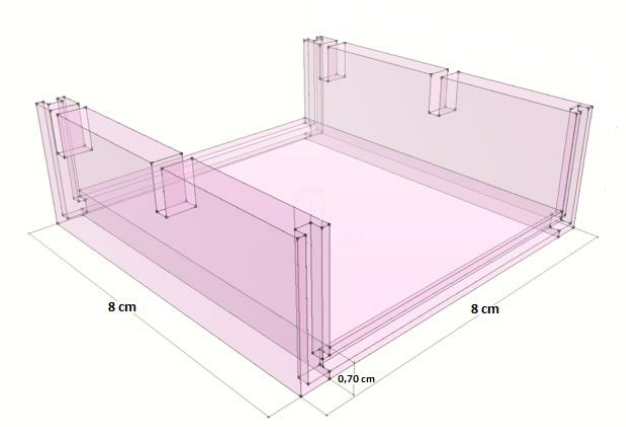


Figure 5: ***Drawing shows an electrophoresis tray and its dimension for only RNA apparatus***

2.5.1 Genomic DNA preparation

The procedure of genomic DNA preparation was performed as detailed below:

Half of a leaf is taken from each genotype plant and put it into an Eppendorf 1, 5 mL tube.

Grind the fresh tissue with liquid Nitrogen until a powder is created.

Add 200 µL of 2x CTAB buffer and vortex it.

Add 200 µL of chloroform, vortex and centrifuge at 13200 rpm for 2 minutes.

Transfer the supernatant into a fresh tube, in this step you should be very careful cause the interface contains CTAB buffer and Polysaccharides.

Add 500 µL of 96-100 % ethanol and place it at -20°C for 20 minutes.

Centrifuge the samples for 15 minutes at 13.200 rpm and rinse the pellet with 70% ethanol.

After removing the last drop of ethanol, let the pellet dry at room temperature for 10 minutes or until becomes off-white.

Resuspend the pellet in 40 µL of autoclaved high quality water and store it at 4°C.

2.5.2 Polymerase Chain Reaction

All the primers are provided with a concentration of 100 µM at -20°C and a concentration of 20 µM is needed so that the dilution is done as it follows:

$$10\ \mu L\ 100\ mM\ Primer + 40\ \mu L\ Water \rightarrow 50\ \mu L\ 20\ \mu M\ solution$$

Preparation of the mix

I take out the genomic DNA samples of the 4°C fridge, centrifuge for 10 seconds at 13.000 rpm and keep on ice.

Table 1: ***Materials needed for a preparation of 20 samples mix example.***

Reagent	Volume x n samples	volume needed
10 x buffer	1 µL * 20 samples	20 µL
2 mM dNTP	1 µL * 20 samples	20 µL
Primer F	0,25 µL * 20 samples	5µL
Primer R	0,25 µL * 20 samples	5µL
dH20	6,5 µL * 20 samples	130 µL
taq DNA polymerase	0,08 µL * 20 samples	1,60 µL

Pipette 1 µL of DNA sample and 9 µL of the mix into a 200 µL PCR tube.

Add one drop of mineral oil sigma to minimize evaporation in the PCR tubes during the changes of the temperature in the lid

Use the Eppendorf master cycler gradient machine and select program SSLP-55/40 cycles (Table 2):

Table 2: ***Steps from the PCR SSLP55-40 cycles program at 55*****°C.**

Cycle step	Temperature [ºC]	time [s]
Denaturation	95	180
Hybridization	55	15
Elongation (Polymerization)	72	30
Denaturation	95	15
Cooling	22	∞

2.5.3 DNA electrophoresis gel

Agarose gel for DNA samples

Following the same procedure as explained (Chapter 2.4.3) but this time total amount of Ethidium Bromide is 5 µL and the volume of X1 TAE buffer it is calculated as:

$$12\ cm * 14\ cm * 0{,}70\ cm = 117{,}6\ cm3 \approx 120\ \text{mL}$$

A 1, 2 % Agarose concentration is used so that the total amount of the required agarose powder can be calculated as:

$$\frac{120mL * 1{,}2}{100} = 1.44\ g\ of\ agarose\ powder$$

For 2% Agarose gel content:

$$\frac{120mL * 2}{100} = 2.40\ g\ of\ agarose\ powder$$

For 3% Agarose gel content:

$$\frac{120mL * 3}{100} = 3.60\ g\ of\ agarose\ powder$$

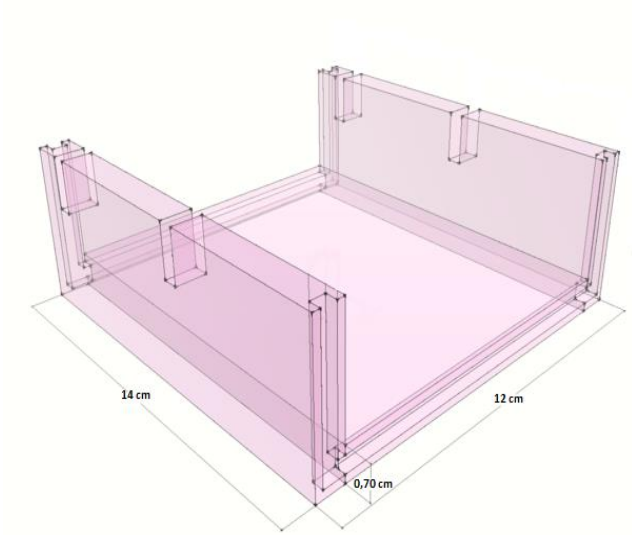


Figure 6: ***Drawing shows an electrophoresis tray and its dimension for only DNA apparatus.***

2.6 Complementary DNA (cDNA) synthesis

Complementary DNA is a double-stranded DNA synthetized from a single strand RNA sample catalyzed by the enzyme reverse transcriptase.

DNAse treatment

To remove the traces of genomic DNA prior the cDNA synthesis, 4 µL of total RNA in water is treated with 1µL of DNase l (RNAse free, room temperature), 3,7 µL sterile water and 0,3µL $MgCl_2$.

Incubate the solution for 30 minutes at 37°C and promptly stopped incubating during 10 minutes at 75°C.

For the reverse transcription

Add 3 µL 5x RT Buffer (provided with the reverse transcriptase enzyme), 1, 5 µL 10 mM dNTP, 0, 5 µL 100µM oligo d $(T)_{18}$ and 1µL peqGOLD M-MuLV H Plus,200 U/µL (peqlab®) to the previous solution.

Mix properly and incubate for 60 min at 37°C, 5 minutes at 75°C and store the samples at -20°C.

2.7 New primers design

To guarantee that the gene sequence in which I expect that my mutant *nsun5-1* is going to be expressed; a design of new primers that follow four simple rules is needed.

- First, the gene sequence should, at least, contain 9 or 10 G+C.
- Avoid having four identical nucleotides in a row.
- The fusion temperature should be around 60°C and can be calculate as it follows:

$$Tm = (G + C) * 4 + (T + A) * 2$$

- The resulting cDNA fragment size should be around 200 bp.

2.8.1 Heavy metal plates preparation

Prior to the root measurement, seedlings have to be planted in each plate to test how they grow in different medium following the next protocol:

Collect around 210 seedlings with the help of zooming googles and laboratory pincers. Prepare 50 mL of high quality water with 0.60 g low melting agarose and autoclave at 121°C for one hour; also 100mL double distilled water is necessary before start.

From now on, work in the laminar flow cabinet is necessary to guarantee the cleanest atmosphere possible.

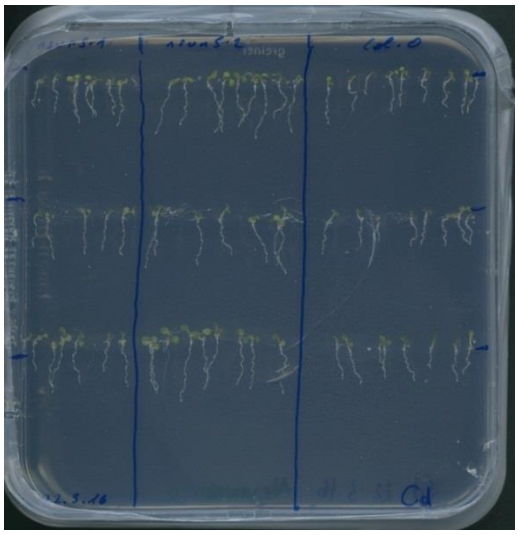
Using sterile 20 mL pipette to fill up 210 seeds of Col, *nsun5-1* and *nsun5-2* in 2 mL tubes. With a solution of 5% Na-Hypochlorite and 5 or 6 drops of Polysorbate 20 (also known as tween 20) to get a proper washing of the seedlings.

Keep the seedlings in the detergent solution for three minutes and every 15 seconds vortex them removing the supernatant at the end of the time. Washing the samples twice with autoclaved high quality water is necessary to get rid of all the possible vestige detergent liquid still present in the seedlings.

Put low melting agarose gel bottle in the microwave for 30 seconds to assure a liquid state from the original solid state and fill up the 2 ml tube samples to the top.

A pattern is needed to organize the distribution of my seedlings. Choose the top of the plate for Col, the middle for the mutant *nsun5-1* and the bottom for *nsun5-2* (Picture 2). Plant 9 seedlings per column of each genotype of plant having around 30 seeds per line and follow the pattern on every heavy metal medium. With paper film Parafilm seal the borders of the plate to avoid the elimination of the humidity inside the plate and any possible exogenous contamination during the growth of the seedlings in the 4 °C chamber.

Keeping the samples for two or three days in the chamber is needed to normalize the quantity of water of all seedlings , after, put the samples in the growth chamber at room temperature (22-24°C and light conditions 75 mM/m^2/s) during twelve days.



Picture 2: ***An example of seedling plate in a medium with Cadmium on its 5th day.***

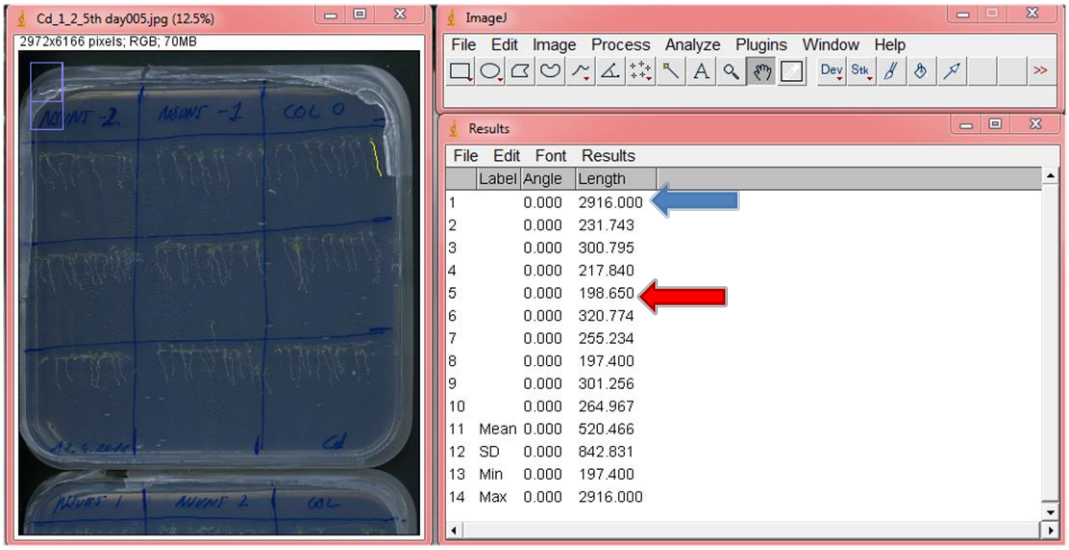
2.8.2 Seedling measurement

The procedure of seedling measurement starts taking pictures of each plate during the 3rd, 4th, 5th and 12th day. Harvest and weight the samples before the liquid nitrogen grind prior the RNA isolation and finally label everything properly and store the samples at -80°C.

The importance of cultivate the seedlings is relevant regarding two aspects. First, to obtain material to work with and secondly, to find out if there is any difference when my seedlings grow on different heavy metals medium. In order to do this, measure the length of the roots of the seedlings is necessary using the program Image J on the picture that has been previously taken with a photograph machine in the laboratory.

The length of the plate is twelve centimeters, then a comparison of the measures obtained using Image J with the plate and using the program Excel to draw a graphic showing the results. This assay is been performed three times to minimize the error of any possible problem in the medium preparation or any abnormal growth that could show unreal results.

The blue arrow shows the measured value of the plate and the red arrow shows one of the measured values of the root.



Picture 3: ***Example of the measurement of the root length in cadmium medium in the 5th day of growth using the program Image J.***

With the following formula you can easily measure the root length of each seed:

$$\frac{Measured\ \ value\ root\ seed}{Measured\ valued\ plate}\ x\ 12\ cm = \ X\ cm\ \ root\ length$$

2.9.1 Agarose gel extraction

The procedure of agarose gel extraction was performed as detailed below:

Weight one Eppendorf tube at the beginning and wrote down the value.

Use UV light and a little scalpel to cut down the zone of gel of interest and put the sample in the previous weighted Eppendorf tube and write down the value:

$$After\ adding\ the\ sample\ weight - Before\ adding\ the\ sample\ weight = Sample\ weight$$

Following the manual that the kit brings says having 3 % of agarose content in the gel fill a 2 mL tube 6 times to the initial volume with buffer QX1 and vortex vigorously:

$$Sample\ weight * 6\ times = X\ mL\ buffer\ QX1$$

Add 10 µL of cold QIAEX ll buffer and incubate the solution at 50°C for 10 minutes vortexing every minute to be sure the buffer is in suspension.

Centrifuge for 30 seconds at 13.000 rpm and remove the supernatant.

Add 500 µL buffer QX1, vortex and centrifuge for 30 seconds at 13.000 rpm and remove the supernatant.

Wash the pellet two times adding 500 µL buffer PE, vortex and centrifuge for 30 seconds at 13.000 rpm and remove the supernatant.

Finally, let the pellet dry at room temperature for 20 minutes or until becomes off-white.

To elute the cDNA, add 20 µL of 10 mM Tris/Hcl pH 8, 50, vortex and incubate the solution at room temperature for 5 minutes.

Centrifuge for 30 seconds at 13.000 rpm and pipet carefully the supernatant into a new clean tube (first elution).

A second elution is made repeating the last two steps.

Note: A second elution step increase the yield by approximately 10–15%

2.9.2 Standard curve

Using the following formula is easy to calculate the initial number of molecules to start building my standard curve for my real time qPCR:

$$\frac{X\ ng}{1\ \mu L} * \frac{1\ g}{10^9\ ng} = X * 10^{-10} g/\mu L$$

$$\left[\frac{\frac{X * 10^{-10} g}{\mu L}}{Fragment\ size * 660}\right] * 6{,}022 * 10^{23} = X * 10^9\ \frac{molecules}{\mu L}$$

Knowing the starter number of molecules that the sample contains the standard curve can be built as it follows:

$1\ \mu L\ sample + 99\ \mu L\ sterile\ water \rightarrow 100\ \mu L\ of\ 1 * 10^7\ molecules\ dilution$

From 100 μL 10^7
$\rightarrow 10\ \mu L\ sample + 90\ \mu L\ sterile\ water = 100\ \mu L\ of\ 1 * 10^6 molecules\ dilution$

From 100 μL 10^6
$\rightarrow 10\ \mu L\ sample + 90\ \mu L\ sterile\ water = 100\ \mu L\ of\ 1 * 10^5 molecules\ dilution$

From 100 μL 10^5
$\rightarrow 10\ \mu L\ sample + 90\ \mu L\ sterile\ water = 100\ \mu L\ of\ 1 * 10^4 molecules\ dilution$

From 100 μL 10^4
$\rightarrow 10\ \mu L\ sample + 90\ \mu L\ sterile\ water = 100\ \mu L\ of\ 1 * 10^3 molecules\ dilution$

From 100 μL 10^3
$\rightarrow 10\ \mu L\ sample + 90\ \mu L\ sterile\ water = 100\ \mu L\ of\ 1 * 10^2 molecules\ dilution$

+ Water control containing 0 molecules

Performing this assay allows you to build your standard curve for the real time qPCR in order to compare the gen of interest, *nsun5* and three different housekeeping genes as *tub9*, *ubq5* and *pp₂a*.

2.10.1 First run real time qPCR

In this assay, all three genotypes from RNA isolation with leaves were tested Col 2, *nsun5-1.2* and *nsun5-2.2* to find there were and how much different was the expression between mutants and wild type.

Firstly a 10 times dilution taking 2 µL sample and 18 µL of water were done to test if RNA could be inhibitory.

QIAGEN's real time PCR cycler has 72 spaces to use so that a prior planning has to be done.

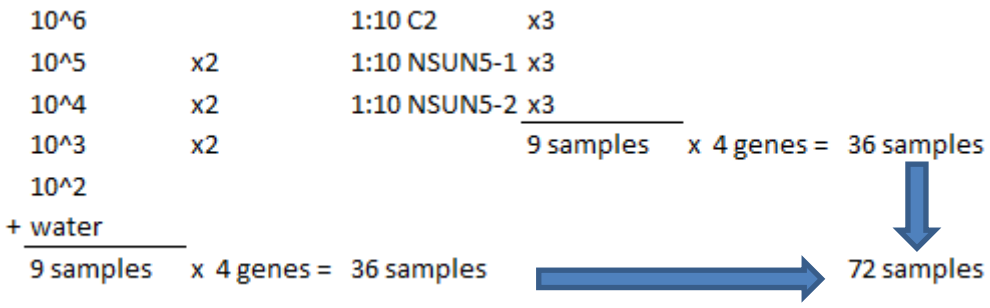


Figure 7: ***Planning of the first real time PCR with 9 different samples and 9 different dilutions using four genes [gen of interest nsun5-1 and three housekeeping genes tub9, ubq5 and pp2a]***

Preparation of the real time qPCR mix for 20 samples was needed with 5x hot five Eva green mix, forward and reverse primers and distilled water:

5x HOT FIRE EVA GREEN MIX	$\rightarrow 2{,}8\ \mu L\ x\ 20\ samples = 56\ \mu L$
PRIMER F	$\rightarrow 0{,}25\ \mu L\ x\ 20\ samples = 5\ \mu L$
PRIMER R	$\rightarrow 0{,}25\ \mu L\ x\ 20\ samples = 5\ \mu L$
dH2O	$\rightarrow 9{,}70\ \mu L\ x\ 20\ samples = 194\ \mu L$

Total volume: 260 µL

With a total volume of 260 µL and 20 different samples 13 µL per 200 µL PCR tube in addition of 1 µL of cDNA sample is needed.

2.10.2 Second run real time qPCR

In this assay, just control Col and control with Nickel as medium from seeds were tested to find if there was any difference between them and also if a dilution was needed and how much the expression changes if so to follow another protocol in the next real time PCR assays.

Ten different dilutions were used and twelve samples but they did not fill up all the spaces in the QIAGEN's real time PCR cycler so that six spaces were filled with empty 200 µL PCR tubes to complete all the remaining empty spaces in the rotor.

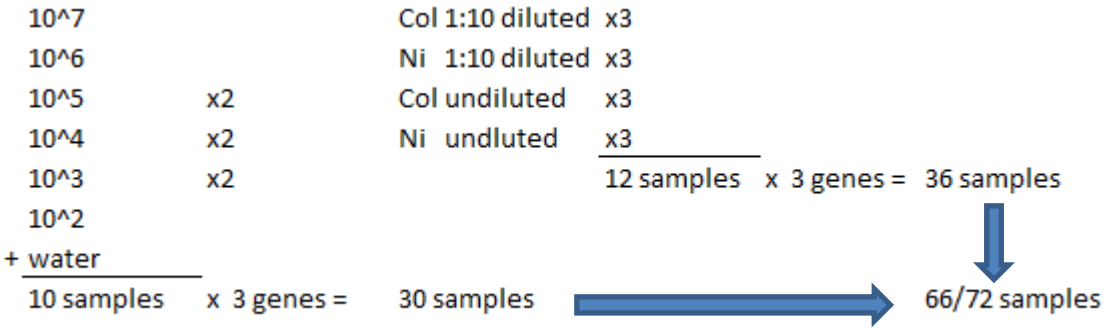


Figure 8: ***Planning of the first real time PCR with 12 different samples and 10 different dilutions using three genes [gen of interest nsun5-1 and two housekeeping genes tub9 and ubq5]***

Preparation of the real time qPCR mix for 23 samples is needed with 5x hot five Eva green mix, forward and reverse primers and distilled water:

5x HOT FIRE EVA GREEN MIX	$\rightarrow 2{,}8\ \mu L\ x\ 23\ samples = 64{,}4\ \mu L$
PRIMER F	$\rightarrow 0{,}25\ \mu L\ x\ 23\ samples = 5{,}75\ \mu L$
PRIMER R	$\rightarrow 0{,}25\ \mu L\ x\ 23\ samples = 5{,}75\ \mu L$
dH2O	$\rightarrow 9{,}70\ \mu L\ x\ 23\ samples = 223{,}10\ \mu L$

Total volume: 299 µL

With a total volume of 299 µL and 23 different samples 13 µL per 200 µL PCR tube in addition of 1 µL of cDNA sample is needed.

2.10.3 Third run real time qPCR and new dilutions

In this assay, before start, a new standard curves for all three housekeeping genes are built to test if I they were good enough because in the first and second real time qPCR all standard curves of all three housekeeping genes were given by the supervisor. First of all, the samples from the second real time qPCR were put all together in a 2 mL tube (table 3).

Table 3: ***showing the different total volumes of all three housekeeping genes [tub9, ubq5 and pp2a]***

TUB9 [ALL DILUTIONS + ALL SAMPLES] = 13 µL * 22 samples = 286 µL
UBQ5 [ALL DILUTIONS + ALL SAMPLES] = 13 µL * 22 samples = 286 µL
PP2A [ALL DILUTIONS + ALL SAMPLES] = 13 µL * 18 samples = 234 µL

All dilution were loaded in a gel in order to make a new quantification using quantity one program to build all three new housekeeping genes curves. Once all the curves were correctly done, start the real time qPCR, this time with 9 different dilutions were used and three samples of each Col, *nsun5-1* and *nsun5-2* from leaves. They did not fill up all the spaces in the QIAGEN's real time PCR cycler so that eighteen spaces were filled with empty 200 µL PCR tubes to complete all the remaining empty spaces in the rotor.

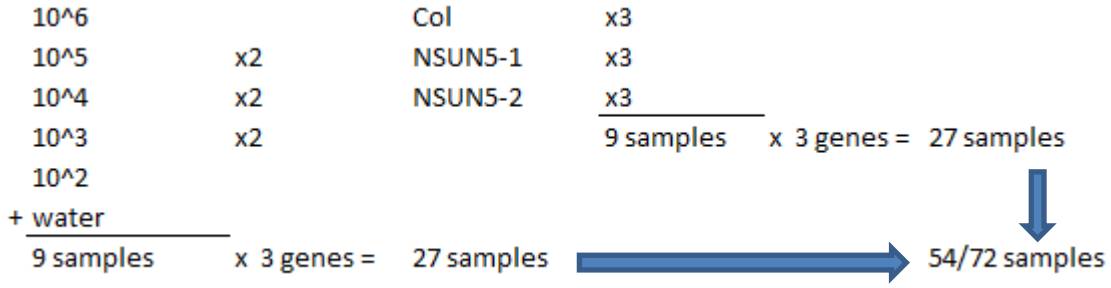


Figure 9: ***Planning of the first real time PCR with 9 different samples and 9 different dilutions using three genes [gen of interest nsun5-1 and two housekeeping genes tub9 and ubq5]***

Preparation of the real time qPCR mix for 20 samples was needed with 5x hot five Eva green mix, forward and reverse primers and distilled water.

5x HOT FIRE EVA GREEN MIX	$\rightarrow 2{,}8\ \mu L\ x\ 20\ samples = 56\ \mu L$
PRIMER F	$\rightarrow 0{,}25\ \mu L\ x\ 20\ samples = 5\ \mu L$
PRIMER R	$\rightarrow 0{,}25\ \mu L\ x\ 20\ samples = 5\ \mu L$
dH2O	$\rightarrow 9{,}70\ \mu L\ x\ 20\ samples = 194\ \mu L$

Total volume: 260 µL

2.10.4 Real time qPCR: fourth run

In this assay, just control Col and control with Nickel as medium from seeds were tested to find if there was any difference between them with the new standard curves that were built in the past third real time qPCR.

Ten different dilutions were used and also ten samples but they don't fill up all the spaces in the QIAGEN's real time PCR cycler so that twelve spaces are filled with empty 200 µL PCR tubes to complete all the remaining empty spaces in the rotor.

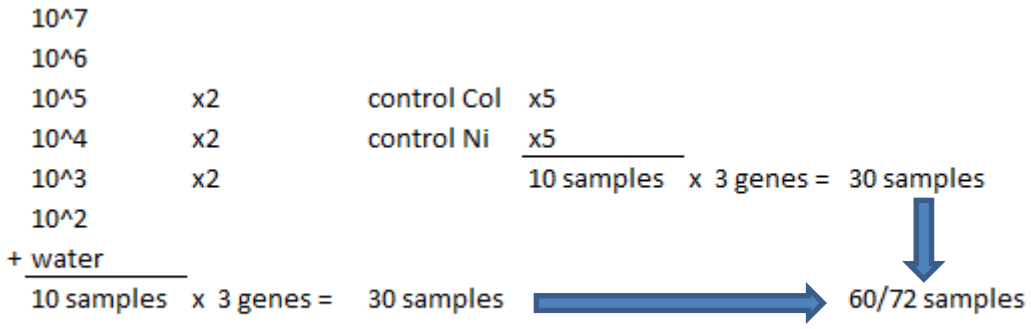


Figure 10: ***Planning of the first real time PCR with 10 different samples and 10 different dilutions using three genes [gen of interest nsun5-1 and two housekeeping genes tub9 and ubq5]***

Preparation of the real time qPCR mix for 22 samples is needed with 5x hot five Eva green mix, forward and reverse primers and distilled water:

5x HOT FIRE EVA GREEN MIX → 2,8 µL x 22 $samples$ = 61,6 µL

PRIMER F → 0,25 µL x 22 $samples$ = 5,5 µL

PRIMER R → 0,25 µL x 22 $samples$ = 5,5 µL

dH2O → 9,70 µL x 22 $samples$ = 213,4 µL

Total volume: 286 µL

With a total volume of 286 µL and having 22 different samples I need 13 µL per 200 µL PCR tube in addition of 1 µL of cDNA sample.

Chapter 3: Results

3.1 RNA Isolation and Nano drop quantification

To test if the RNA isolations worked properly they were loaded in RNA electrophoresis gel showing the 20s rRNA fragment size around 1100 bp and 16s rRNA fragment size around 850 bp (Figure 11). The blue arrow shows the 1000 bp band size from the marker.

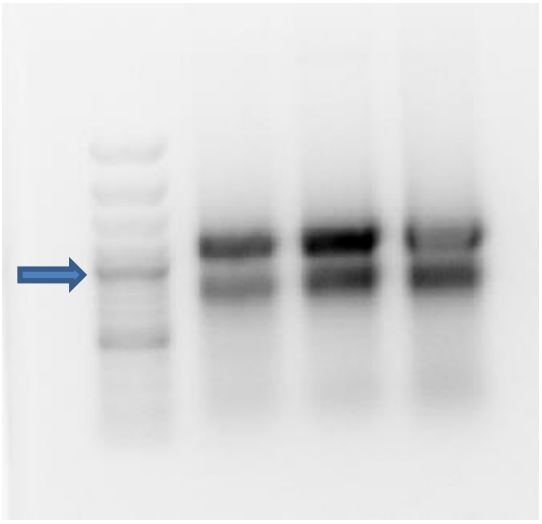


Figure 11: ***From left to right: 100 bp marker, Col, nsun5-1 and nsun5-2. [70V, 76 mA, 45 min, 1, 2% Agarose gel]***

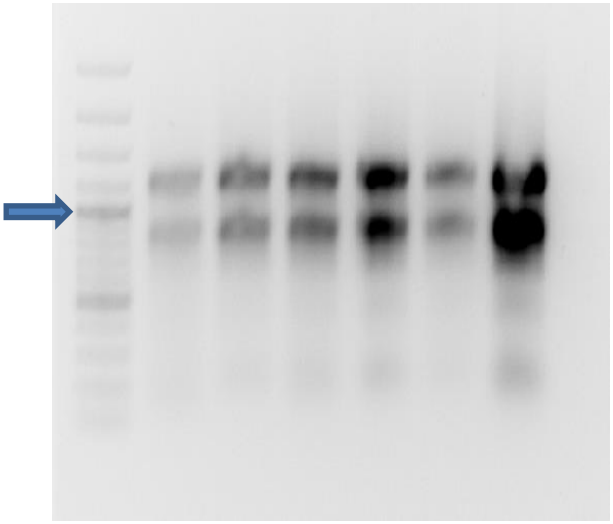


Figure 12: ***From left to right: 100 bp marker, 2 samples Col, 2 samples nsun5-1 and 2 samples nsun5-2. [70V, 76 mA, 60 min, 1, 2% Agarose gel]***

Table 4: ***Results regarding RNA isolation from leaves as starter material.***

#	Sample ID	User name	Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type	Factor
1	control0	h94000_nanodrop	04.03.2016 15:20:19	667.1	ng/µl	16.678	7.415	2.25	2.16	RNA	40
2	nsun5-1	h94000_nanodrop	04.03.2016 15:21:47	757	ng/µl	18.924	8.381	2.26	2.11	RNA	40
3	nsun5-2	h94000_nanodrop	04.03.2016 15:22:37	882.8	ng/µl	22.071	9.842	2.24	2.17	RNA	40

3.2.1 Genomic DNA (gDNA): TUB9

To find out if the DNA samples were good or the genomic DNA protocol was properly performed, DNA samples were tested and a few ones from another student that it weres already proved that they worked previously and proceed to test the primer TUB9. The results are shown in the figure 13, with an expected genomic DNA fragment size of 470 bp.

The blue arrow shows the 500 bp band size from the marker.

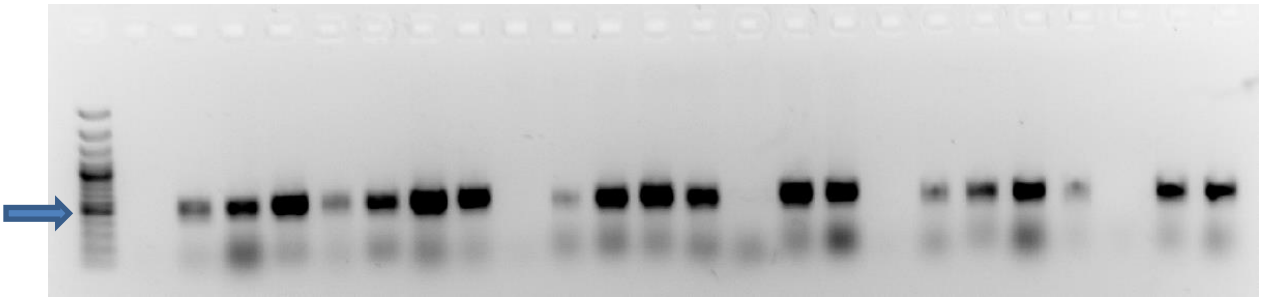


Figure 13: ***from left to right: Marker 100 bp, 1-8 DNA samples [nsun5-2] 30 µM $MgCl_2$, 1-8 DNA samples 30 µM $MgCl_2$ from Marie's and 1-8 DNA samples [nsun5-2] 25 µM $MgCl_2$ [80V, 92 mA, 45 min, 1,2 % Agarose gel, 470 bp expected fragment size, Primers TUB9_F/R].***

3.2.2 Genotyping of t-DNA Insertion

In total, eight different mixes were tested in order to find if my mutants are truly expressed in the gene sequence.

The first mix tested was one with SALK_4337 forward and reverse primers that should be expressed in the mutant NSUN5-1 sequence.

The second mix is provided with salk_204104 forward and reverse primers that should be expressed only in the mutant NSUN5-2 sequence.

Third and fourth mixes are with the tDNA border primer SALK_LB1C and SALK_43337_R / SALK_204014_R respectively.

Fifth and sixth mixes are with the tDNA border primer SIGNAL_LBB1 and SALK_43337_R / SALK_204014_R.

Seventh and eighth mixes are with the tDNA border primer SALK_LBA1 and SALK_43337_R / SALK_204014_R.

- The first mix tested was with SALK_4337 forward and reverse primers that should be expressed in the mutant *nsun5-1* sequence. The blue arrow shows the 500 bp band size from the marker.

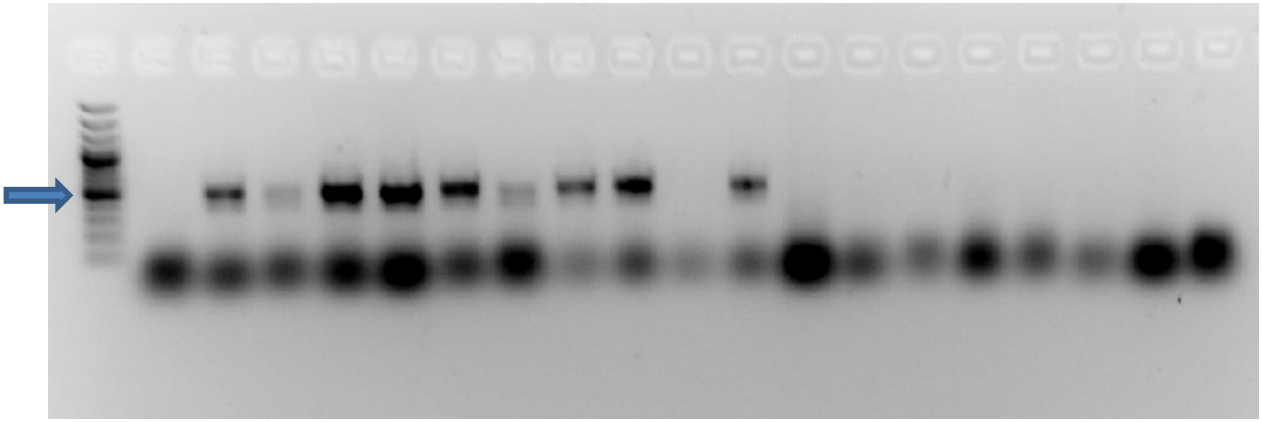


Figure 14: ***from left to right: Marker 100 bp, 1 blanket, Control 1 and 2,1-8 DNA samples nsun5-1, 1-8 DNA samples nsun5-2 [80V, 65 mA, 45 min, 1,2% Agarose gel, 469 bp expected length, Primers SALK_4377 R/F]***

Both control samples and all *nsun5-1* samples except the seventh show a band around 469 bp.

- The second mix is provided with salk_204104 forward and reverse primers that should be expressed only in the mutant *nsun5-2* sequence. The blue arrow shows the 500 bp band size from the marker.

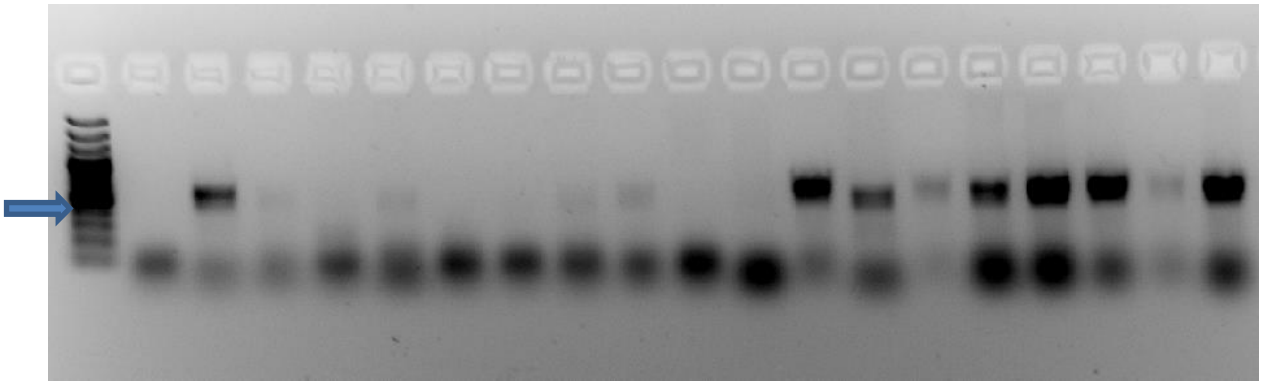


Figure 15: ***from left to right: Marker 100 bp, 1 blanket, Control 1 and 2,1-8 DNA samples nsun5-1, 1-8 DNA samples nsun5-2 [[80V, 65 mA, 45 min, 1,2% Agarose gel,553 bp expected length, Primers SALK_204104 R/F]***

Control 1 sample and all nsun5-2 samples show a band around 553 bp.

- Third mix is with the tDNA border primer SALK_LB1C and SALK_4377_R. and unfortunately it didn't work to show any expected result.

- Fourth mix is with the tDNA border primer SALK_LB1C and SALK_204104_R .The blue arrow shows the 200 bp band size from the marker.

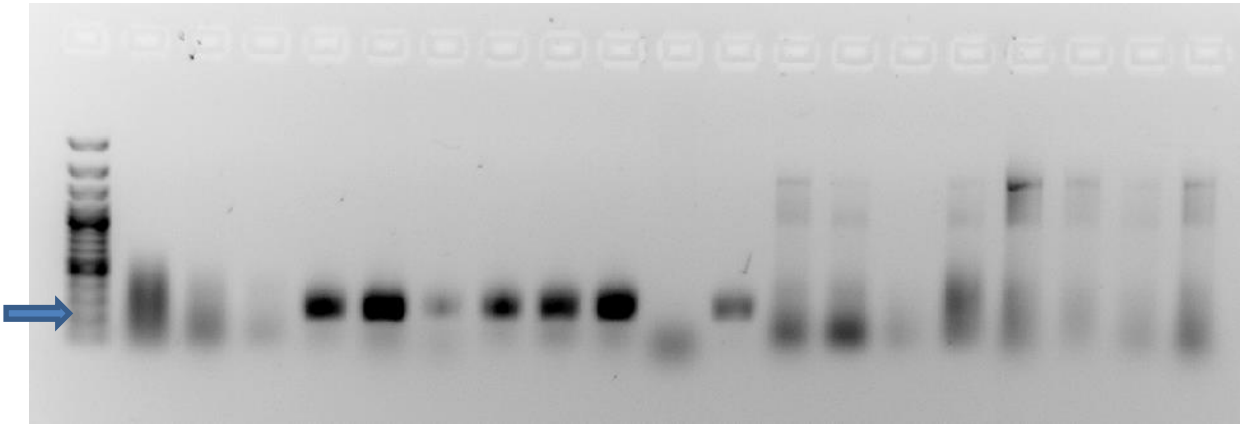


Figure 16: from left to right: Marker 100 bp, 1 blanket, Control 1 and 2,1-8 DNA samples *nsun5-1,* 1-8 DNA samples *nsun5-2* [80V, 55 mA, 45 min, 1,2% Agarose gel, Primers SIGNAL_LB1C+SALK_204104_R]

Samples one, two, four, five and six from *nsun5-1* showed a strong band .Samples from *nsun5-2* looked diluted.

- Fifth mix is with the tDNA border primer SALK_LBB1 and SALK_4377_R .The blue arrow shows the 500 bp band size from the marker.

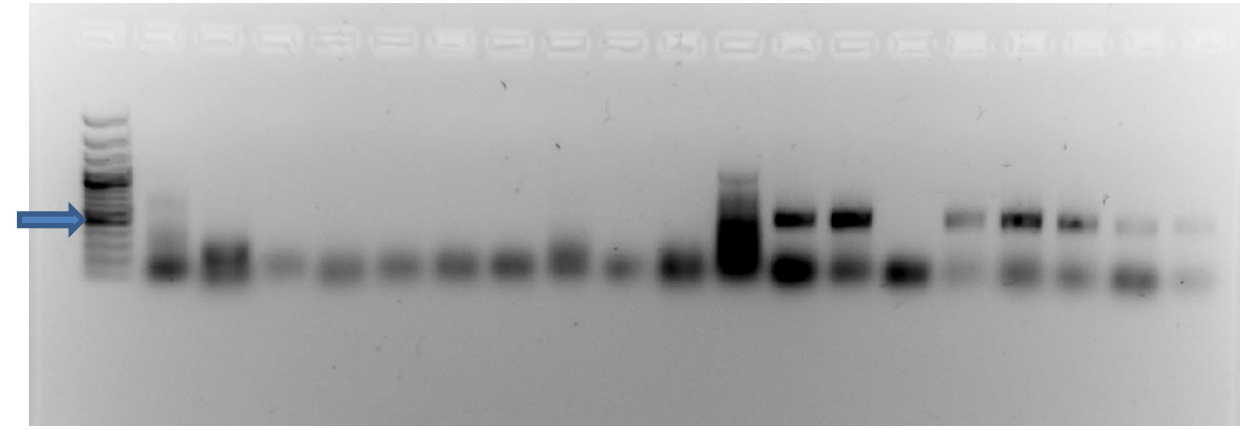


Figure 17: from left to right: Marker 100 bp, 1 blanket, Control 1 and 2,1-8 DNA samples *nsun5-1,* 1-8 DNA samples *nsun5-2* [80V, 55 mA, 45 min, 1,2% Agarose gel, Primers SIGNAL_LBB1+SALK_4377_R]

Samples one, two, four, five and six from *nsun5-2* showed a strong band.

- Sixth mix is with the tDNA border primer SALK_LBB1 and SALK_204014_R. The blue arrow shows the 200 bp band size from the marker.

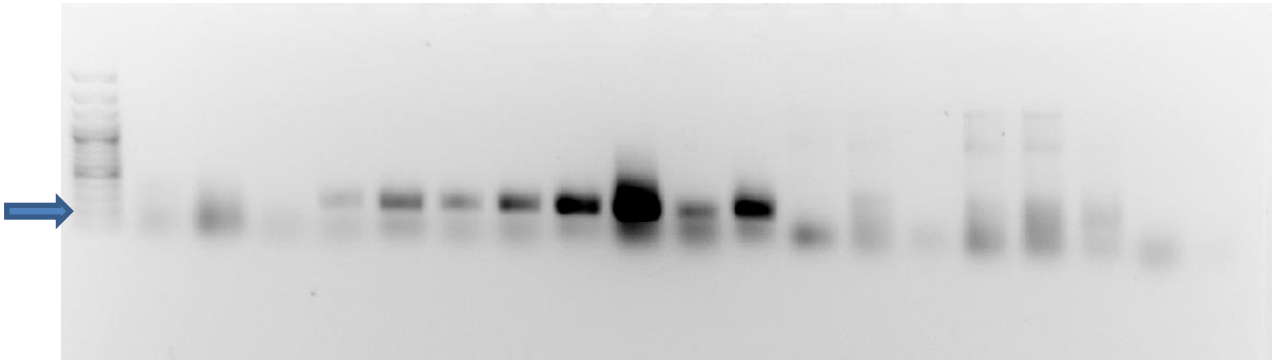


Figure 18: from left to right: Marker 100 bp, 1 blanket, Control 1 and 2,1-8 DNA samples *nsun5-1*, 1-8 DNA samples *nsun5-2* 80V, 55 mA, 45 min, 1,2% Agarose gel [Primers SIGNAL_LBB1+SALK_204104 R]

Samples two, four, five, six, seven and eight of *nsun5-1* samples show a strong band.

- Seventh and eighth mixes are with the tDNA border primer SALK_LBA1 and SALK_43337_R / SALK_204014_R. Unfortunately, border primer LBA1 only shows results in NSUN5-2 for the seventh mix. The blue arrow shows the 700 bp band size from the marker.

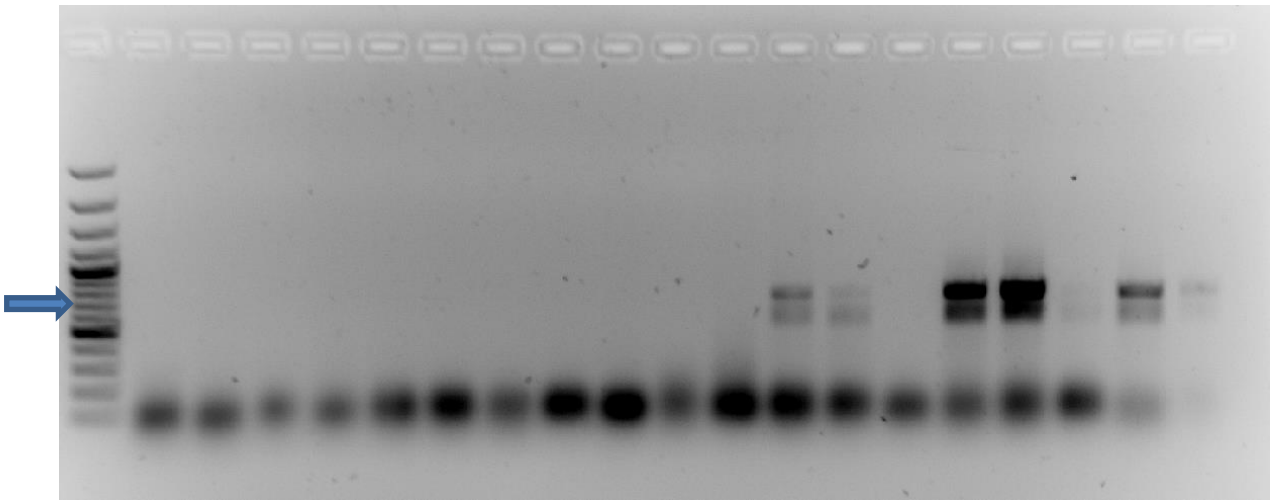


Figure 19: from left to right: Marker 100 bp, 1 blanket, Control 1 and 2,1-8 DNA samples *nsun5-1*, 1-8 DNA samples *nsun5-2* [80V, 64 mA, 70 min, 1,2% Agarose gel, [Primers SIGNAL_LBA1+SALK_4377 R]

Just samples four and five from *nsun5-2* show a strong band.

All the results are summarized in the table:

Table 5: ***Showing the results of the different mixes proving that my plants are homozygous for the mutants [+ positive result, strong band; ? positive result, weak band; - negative result, no band showed].***

Samples	SALK_4377 F/R	SALK_204104 F/R	SALK_LB1C SALK_4377_R	SALK_LB1C SALK 204104_R	SALK_LBB1 SALK_4377_R	SALK_LBB1 SALK_204104_R	SALK_LBA1 SALK_4377_R	SALK_LBA1 SALK_204104_R
NSUN5-1 1	+	-	-	+	-	?	-	?
NSUN5-1 2	+	-	-	+	-	+	-	?
NSUN5-1 3	+	-	-	?	-	?	-	?
NSUN5-1 4	?	-	-	+	-	+	-	-
NSUN5-1 5	+	-	-	+	-	+	-	-
NSUN5-1 6	+	-	-	+	-	+	-	-
NSUN5-1 7	+	-	-	-	-	+	-	-
NSUN5-1 8	+	-	-	+	-	+	-	-
NSUN5-2 1	-	+	-	-	+	-	?	-
NSUN5-2 2	-	+	-	-	+	-	?	-
NSUN5-2 3	-	?	-	-	-	-	-	-
NSUN5-2 4	-	+	-	-	?	-	+	-
NSUN5-2 5	-	+	-	-	+	-	+	-
NSUN5-2 6	-	+	-	-	+	-	-	-
NSUN5-2 7	-	?	-	-	?	-	+	-
NSUN5-2 8	-	+	-	-	?	-	-	-
col 1	+	+	-	-	-	-	-	-
col 2	?	-	-	-	-	-	-	-

3.3 Complementary DNA (cDNA)

To test if the cDNA protocol worked properly and figure out the expression of the mutants within the gene sequence two different mixes were tested. The first was prepared with the SALK_204104 forward and reverse primers and the second one with 5g26180 forward and reverse primers. The blue arrow shows the 500 bp band size from the marker.

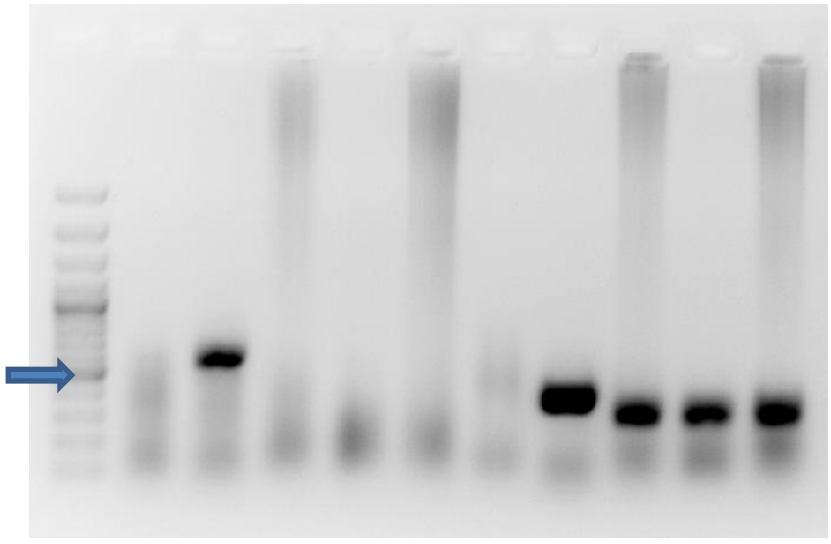


Figure 20: from left to right: Marker 100 bp, 1 blanket, Col genomic DNA, col, *nsun5-1* and *nsun5-2* [genomic DNA 553 bp expected length, cDNA 206 bp expected length Primers SALK_204104 F/R], 1 blanket, Col genomic DNA, col,*nsun5-1* and *nsun5-2* [80V, 54 mA, 60 min, 1,2% Agarose gel, genomic DNA 344 bp expected length, cDNA 264 bp expected length [Primers 5g26180 F/R]

Following, the primers from an alternative splicing for *nsun5-1* mutant were tested. Unfortunately, the first results were not as good as expected (figure not shown) not showing precise results around 155 bp, therefore an optimization with different concentrations of $MgCl_2$ as 25 and 30 mM or hybridization temperatures of 45 and 50 degree Celsius and increasing to 3 % of agarose content in the gel was performed. The blue arrows show the 100 bp band size from the marker.

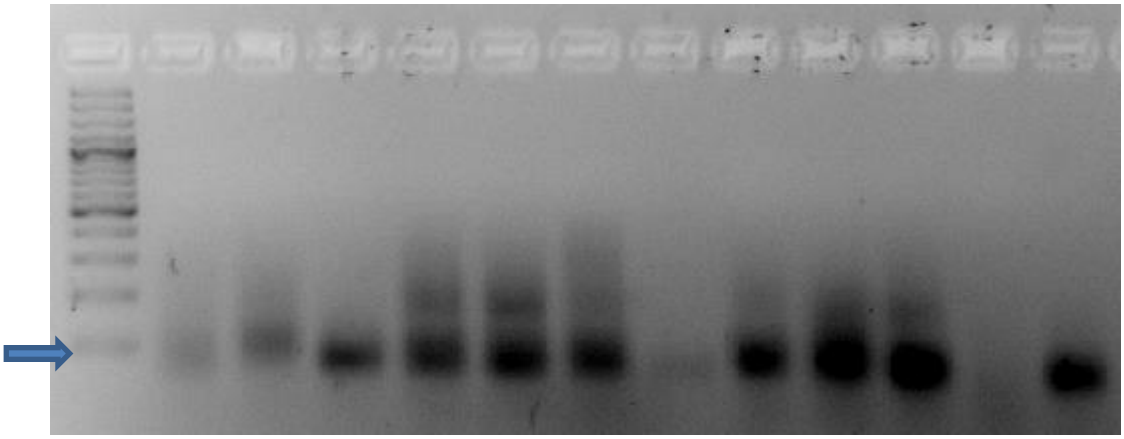


Figure 21: from left to right: Marker 100 bp, 1 blanket, Col 1 and 2 ,*nsun5-1* 1 and 2 and *nsun5-2*, 1 and 2 with a 30 mM $MgCl_2$ concentration , 1 blanket, Col 1 and 2 ,*nsun5-1* 1 and 2 and *nsun5-2*, 1 and 2 with a 25 mM $MgCl_2$ concentration [80 V, 44 mA, 75 min, 3% Agarose gel, genomic DNA 155 bp expected length, Primers 25_S rRNA_ F/R] at 45º Celsius.

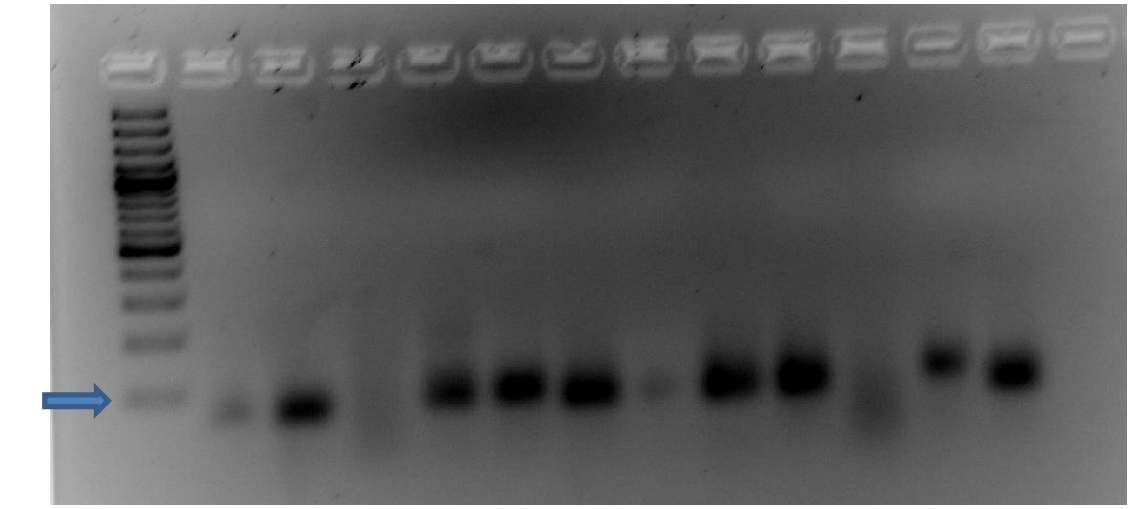


Figure 22: from left to right: Marker 100 bp, 1 blanket, Col 1 and 2 ,*nsun5-1* 1 and 2 *and nsun5-2*, 1 and 2 with a 30 mM $MgCl_2$ concentration , 1 blanket, Col 1 and 2 ,*nsun5-1* 1 and 2 and *nsun5-2*, 1 and 2 with a 25 mM $MgCl_2$ concentration [80 V, 65 mA, 90 min, 3% Agarose gel, genomic DNA 155 bp expected length, Primers 25_S rRNA_ F/R] at 50º Celsius.

3.4.1 New primers design

In order to know if mutant allele *nsun5-1* was expressed within the gene sequence, following the rules for a primer design, pair of new primers were designed with an expected fragment size of 447 bp in the genomic DNA and 172 bp in the cDNA.

Table 6: ***Design of a new pair of primers to test the expression of the mutant nsun5-1 within the gene sequence***

		Fragment size		
Primer used	Gene sequence	Genomic DNA	Complementary DNA	Tm [ºc]
5g26180_F2	GTCATTTGAAACCACGGTATG	447 bp	172 bp	60
5g26180_R2	CGTCCATTCGCAACCAAACG			62

To find out if the primers have worked properly and their expression was the expected, a test in genomic DNA samples was performed; the results (figure 23) show an overall positive strong band with an expected fragment size of 447 bp. The blue arrow shows the 500 bp band size from the marker.

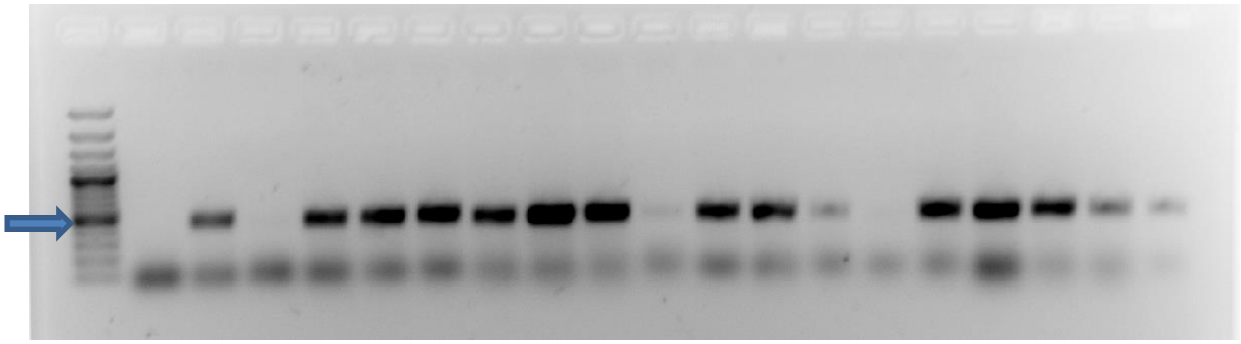


Figure 23: ***from left to right: Marker 100 bp, 1 blanket, Control 1 and 2,1-8 genomic DNA samples nsun5-1, 1-8 genomic DNA samples nsun5-2 [80 V, 55 mA, 60 min,2 % Agarose gel, Primers 5g26180_F2/R2]***

The next step was to test the new primers on cDNA samples with an expected fragment size of 172 bp. The resolution of the gel (figure 24) was not good enough to assure if there was just one or more bands of sample therefore an optimization of the primer was done in order to figure out the best parameters that fits for this specific primers. The blue arrow shows the 500 bp band size from the marker.

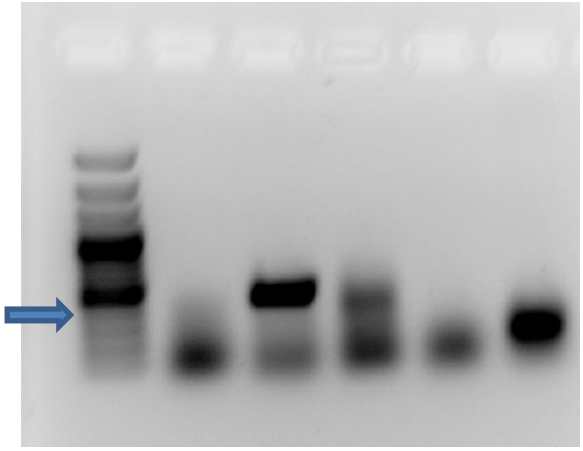


Figure 24: ***from left to right: Marker 100 bp, 1 blanket, Col genomic DNA, Col, nsun5-1 and nsun5-2 cDNA samples [80V, 55 mA, 60 min,1,2 % Agarose Gel, 30mM $MgCl_2$ Primers 5g26180_F2/R2] at 50 ªC Celsius***

3.4.2 New primers optimization

The optimization of the primers was done testing different temperatures and $MgCl_2$ concentrations to find out what option was the best to work with in the future. First test shows a concentration of 25 and 30 mM at 55°C. The blue arrows show the 200 bp band size from the marker.

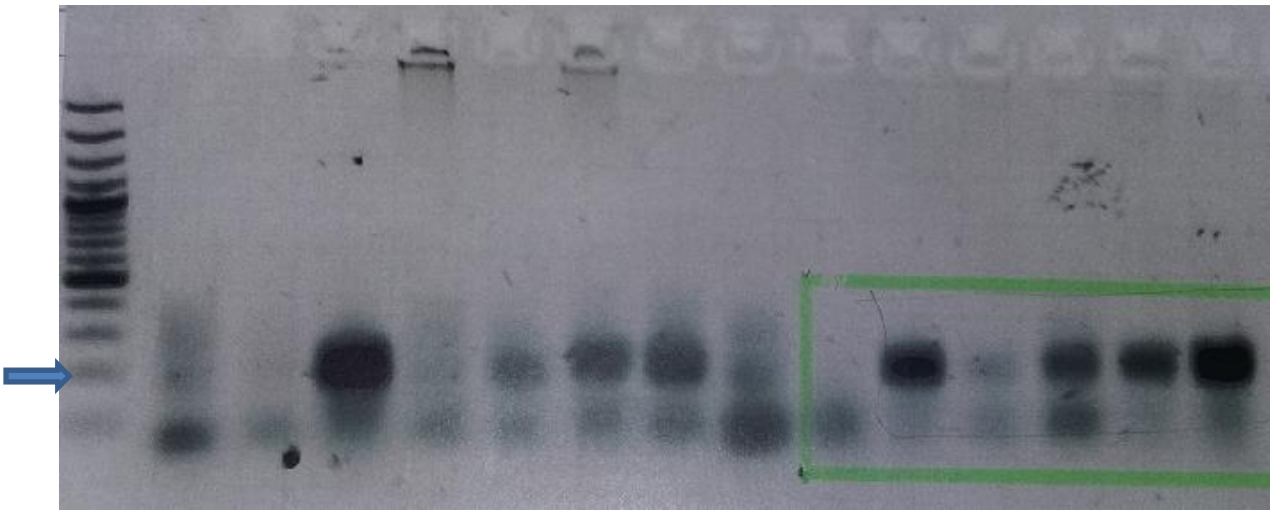


Figure 25: ***from left to right: Marker 100 bp, 1 blanket, Col 1, 2 samples nsun5-1 and 2 samples nsun5-2 with 30mM $MgCl_2$, 1 blanket, Col 1, 2 samples nsun5-1 and 2 samples nsun5-2 with 25mM $MgCl_2$ [80 V, 55 mA,75 min, 2% Agarose gel, genomic DNA 172 bp expected length, Primers 5g26180_F2/R2] at 55º Celsius.***

Second test showed a concentration of 25 and 30 mM at 60°C. The blue arrow shows the 200 bp band size from the marker.

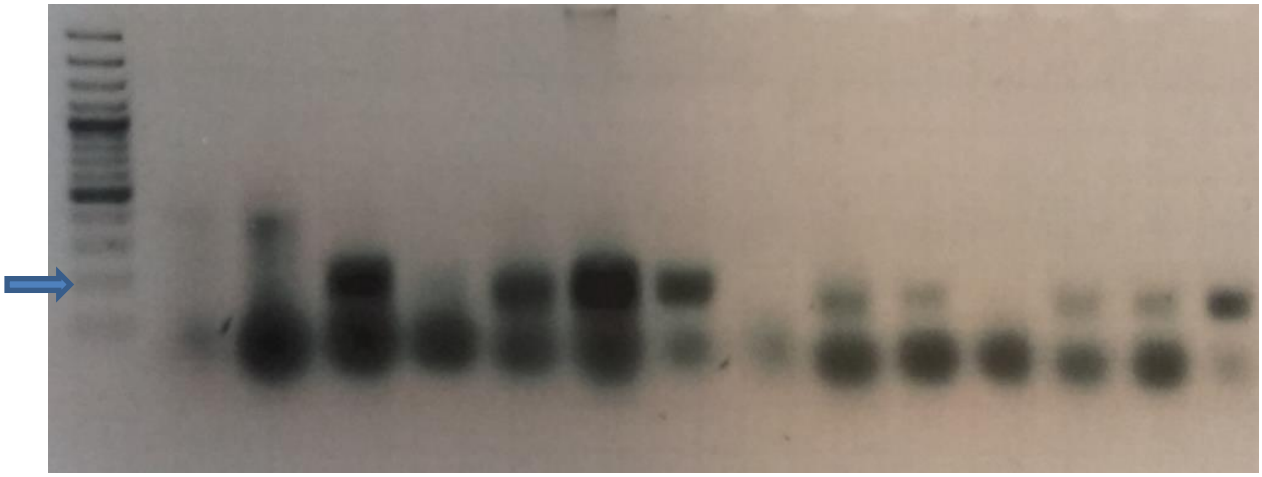


Figure 26: ***from left to right: Marker 100 bp, 1 blanket, Col 1, 2 samples nsun5-1 and 2 samples nsun5-2 with 30mM $MgCl_2$, 1 blanket, Col 1, 2 samples nsun5-1 and 2 samples nsun5-2 with 25mM $MgCl_2$ [[80 V, 55 mA,75 min, 2% Agarose gel, genomic DNA 172 bp expected length Primers 5g26180_F2/R2] at 60º Celsius.***

Regarding the optimization, the test with 25 mM $MgCl_2$ concentration and 55°C showed the best results among the other temperatures and concentration (figure 25).

3.4.3 Phusion® Master mix and Dream taq

Since the beginning of the project, homemade taq DNA polymerase was used because it worked greatly with short size DNA fragments. In the following tests, primers that include mostly the whole gent were tested and because of that, different taq's were needed to be able to work with larger DNA fragments as the expected one [genomic DNA 2.246 bp, cDNA 961 bp].

In this assay the primers SALK_204104_F and 5g26180 _R and two different taq polymerase were tested: Phusion® Master Mix which already includes the 10 x taq buffer, 2 mM dNTP and 25 mM $MgCl_2$ in the solution and also the DREAM taq. The results showed that the Phusion® Master Mix worked contrary to DREAM taq polymerase. The blue arrow shows the 1000 bp band size from the marker.

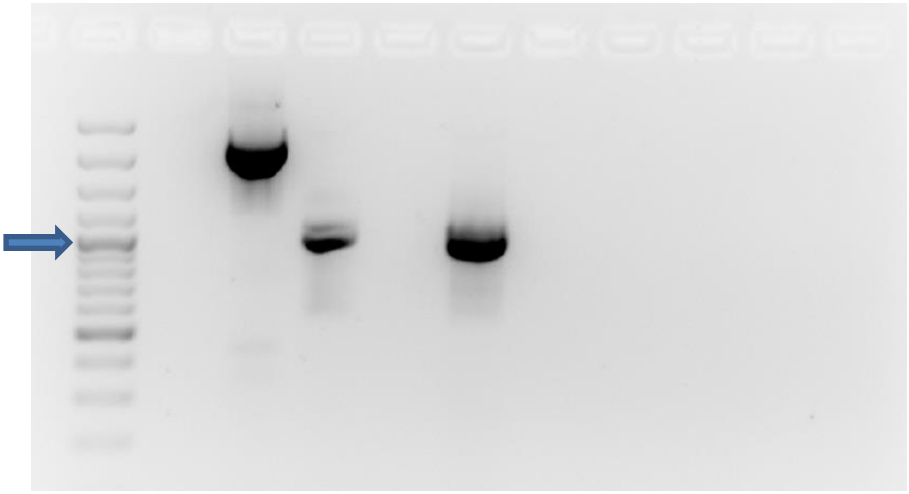


Figure 27: ***from left to right: Marker 100 bp, 1 blanket, Control genomic DNA, Col, nsun5-1 and nsun5-2 cDNA samples from Phusion® Master mix, 1 blanket, Control genomic DNA, Col, nsun5-1 and nsun5-2 cDNA samples from DREAM taq [80 V, 78 mA,75 min,2 % Agarose gel, genomic DNA 2246 bp fragment size,961 bp cDNA fragment size, Primers SALK_204104_F+ 5g26180_R] at 55º Celsius.***

The resulting gel shows that Phusion mastermix worked properly and prove that both mutants are expressed in the line gene sequence (figure 27).

3.5.1 First root growth assay

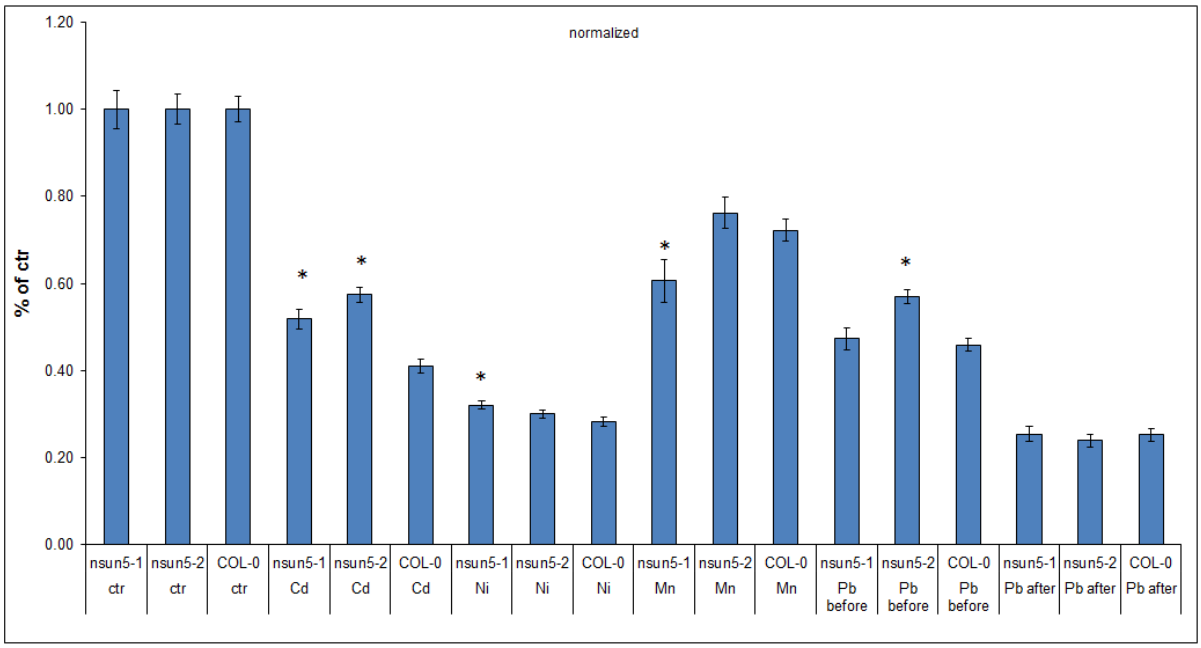


Figure 28: ***Showing the results from the first seedling measurement from the 5th growth day with six different heavy metal mediums.***

Table 7: ***First attempt seedling measurement highlighting that nickel, lead before, manganese and cadmium could be interesting mediums to work with in the future.***

		mean	SD	SE	p-values
ctr	nsun5-1	1	0.20538651	0.04378855	1
ctr	nsun5-2	1	0.14544626	0.0352759	1
ctr	COL-0	1	0.1269035	0.02991144	-
Cd	nsun5-1	0.51791738	0.1031933	0.02307472	0.000283827
Cd	nsun5-2	0.57474259	0.08703027	0.01740605	7.66279E-09
Cd	COL-0	0.41088825	0.07854726	0.01570945	-
Ni	nsun5-1	0.32056263	0.04258964	0.00888055	0.010401772
Ni	nsun5-2	0.29996239	0.04664427	0.00994459	0.258916269
Ni	COL-0	0.28285725	0.04111058	0.01061471	-
Mn	nsun5-1	0.60557854	0.20531947	0.04839426	0.030302073
Mn	nsun5-2	0.7617976	0.15324128	0.03515596	0.363384302
Mn	COL-0	0.72230668	0.12524821	0.02611606	-
Pb before	nsun5-1	0.47283079	0.10820009	0.02550301	0.625795007
Pb before	nsun5-2	0.56898821	0.08467866	0.01660685	1.50534E-05
Pb before	COL-0	0.45887366	0.06307084	0.01410307	-
Pb after	nsun5-1	0.25410452	0.07689571	0.01812449	0.925998331
Pb after	nsun5-2	0.23959028	0.07454169	0.01434555	0.534767287
Pb after	COL-0	0.25202316	0.06840709	0.01368142	-

In this first assay was shown that both alleles were hypersensitive to cadmium medium: on nickel and manganese, *nsun5-1* allele was hypersensitive and tolerant for *nsun5-2*. Regarding led medium assay, the *nsun5-2* allele was high tolerant except in the assay putting the heavy metal before autoclave.

3.5.2 Second root growth assay

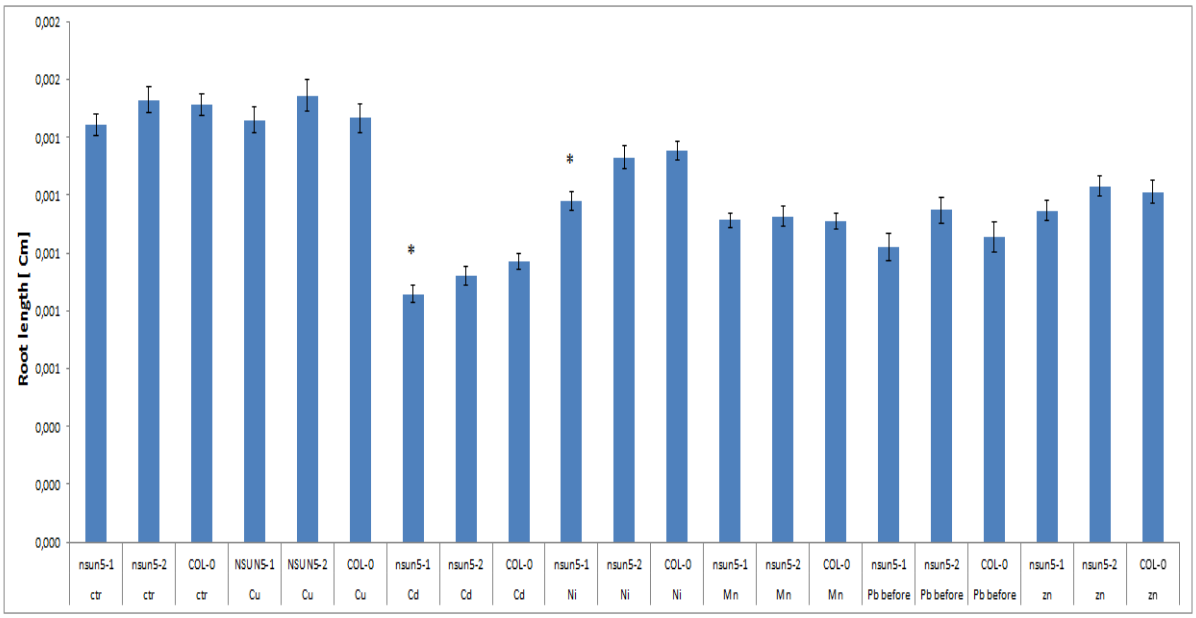


Figure 29: ***Showing the results from the second seedling measurement from the 5th growth day with six different heavy metal mediums.***

Table 8: ***Second attempt seedling measurement highlighting that Nickel and Cadmium could be interesting mediums to work with in the future.***

mean (cm)		col 1	col 2	col 3	col 1-3	p values
ctr	nsun5-1	1.50169896	1.47717917	1.35890833	1.442448	0.19676521
ctr	nsun5-2	1.65756019	1.57364537	1.35376991	1.52832515	0.79252666
ctr	COL-0	1.5008787	1.60356111	1.43388796	1.51277593	-
Cu	nsun5-1	1.4753505	1.45177522	1.45224256	1.45919092	0.92491938
Cu	nsun5-2	1.57830788	1.61040778	1.42419226	1.54405746	0.28627617
Cu	COL-0	1.41303026	1.5064121	1.45959126	1.46550883	-
Cd	nsun5-1	0.9127762	0.7966983	0.86018171	0.8562617	0.00873097
Cd	nsun5-2	0.93764117	0.98707554	0.83920916	0.92130862	0.25579597
Cd	COL-0	0.96381539	1.01835883	0.92760293	0.96992572	-
Ni	nsun5-1	1.2037784	1.15790042	1.17818887	1.1799559	0.00032266
Ni	nsun5-2	1.29107153	1.44389154	1.25727863	1.33074723	0.65149158
Ni	COL-0	1.40434128	1.35447779	1.30387564	1.35423157	-
Mn	nsun5-1	1.14655405	1.15683662	1.03693239	1.11216744	0.94421301
Mn	nsun5-2	1.17482723	1.11139067	1.08930516	1.1257045	0.7217704
Mn	COL-0	1.19906937	1.10421127	1.03486197	1.10939285	-
Pb before	nsun5-1	1.01893629	1.18722161	0.8584663	1.0215414	0.65089501
Pb before	nsun5-2	1.26756279	1.24395239	0.91546797	1.14733712	0.19230414
Pb before	COL-0	1.17865512	1.14830402	0.84531256	1.05392851	-
zn	nsun5-1	1.17434419	1.21210293	1.03753683	1.14531995	0.22365178
zn	nsun5-2	1.27465911	1.26896601	1.15046789	1.23136434	0.68399377
zn	COL-0	1.32479887	1.25058121	1.00890146	1.20962924	-

From now on I decided to not use led added after the autoclave and also copper and zinc were added as new mediums. In this second assay is shown that *nsun5-1* was hypersensitive for cadmium and nickel. *Nsun5-1* had a high tolerance to copper, manganese and led before autoclave while *nsun5-2* allele had it to nickel, manganese and zinc.

3.5.2 Third root growth assay

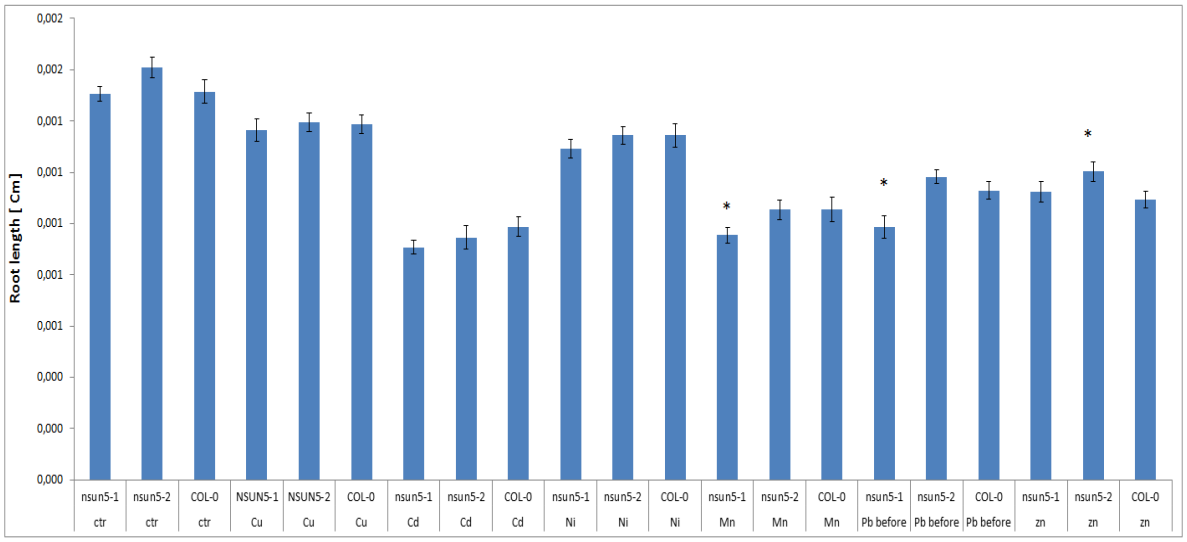


Figure 30: ***Showing the results from the third seedling measurement from the 5th growth day with six different heavy metal mediums.***

Table 9: ***Third attempt seedling measurement highlighting that Nickel, Lead and Zinc could be interesting mediums to work with in the future.***

mean (cm)		col 1	col 2	col 3	col 1-3	p values
ctr	nsun5-1	1.47462915	1.56966049	1.47204511	1.50544492	0.8635953
ctr	nsun5-2	1.65535708	1.68171652	1.49474596	1.61060652	0.12354829
ctr	COL-0	1.44918851	1.55099199	1.55245994	1.51481248	-
Cu	NSUN5-1	1.50800836	1.24195682	1.33759889	1.36451515	0.68090456
Cu	NSUN5-2	1.39405246	1.45084587	1.34182916	1.39557583	0.86910592
Cu	COL-0	1.44145543	1.44548979	1.27473305	1.38722609	-
Cd	nsun5-1	0.92501681	0.96328992	0.84011578	0.90753134	0.10554913
Cd	nsun5-2	0.96221148	1.04286712	0.77236555	0.9462123	0.492137
Cd	COL-0	1.05108824	1.04556536	0.8533104	0.98832175	-
Ni	nsun5-1	1.37553069	1.31674127	1.17585393	1.2937415	0.38683014
Ni	nsun5-2	1.32874363	1.36309018	1.33976157	1.34386512	0.99427677
Ni	COL-0	1.310483	1.33926346	1.37181303	1.34427443	-
Mn	nsun5-1	0.99670986	0.96902254	0.89845775	0.95473005	0.06907154
Mn	nsun5-2	1.11779437	1.05568498	0.98802488	1.05383474	0.9699036
Mn	COL-0	1.20716318	1.03323427	0.8858831	1.05612696	-
Pb before	nsun5-1	0.88980729	1.05419886	1.01824905	0.9874184	0.01477136
Pb before	nsun5-2	1.16341619	1.21257623	1.16784748	1.1817966	0.23783076
Pb before	COL-0	1.17033191	1.21082173	1.01499716	1.1290206	-
zn	nsun5-1	1.27454476	1.20461619	0.89636095	1.12517397	0.54018263
zn	nsun5-2	1.31458071	1.26392286	1.02315161	1.20308657	0.0296195
zn	COL-0	1.10161143	1.13300381	1.0466819	1.09376571	-

In the last assay showed that the allele *nsun5-1* was hypersensitive to manganese and led and had a high tolerance to copper and zinc. Concerning *nsun5-2* showed a high sensitivity to zinc medium and a high tolerance to copper, nickel and manganese.

3.6.1 First run real time qPCR

Since the mutant alleles were proved to be expressed in the gene sequence of the plants but how much is this expression and the difference between each mutant allele and the control plant was still unknown, four different real times quantitative PCR in order to know the % of expression and its interpretation.

First, quantification was performed in a gel to be able to build the standard curve to compare the housekeeping genes *tub9, ubq5* and *pp2a with nsun5.*

To do this, a repetition of agarose electrophoresis gel was done with designed primers 5g26180_F2/R2 at 55°C and 25 mM of $MgCl_2$ concentration because it was the experiment with better results in the optimization assay as it is shown in figure 31. The blue arrows show the 200 bp band size from the marker.

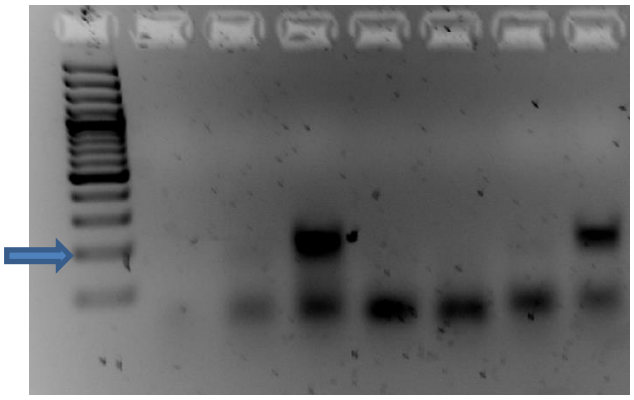


Figure 31: ***from left to right: Marker 100 bp, 1 blanket, Col 1 and 2, 2 samples NSUN5-1 and 2 samples NSUN5-2 with 25 mM with 25mM*** $MgCl_2$ ***[80 V, 55 mA, 50 min, 1,2 % Agarose gel, cDNA 172 bp expected fragment size, Primers5g26180_F2/R2] at 55º Celsius.***

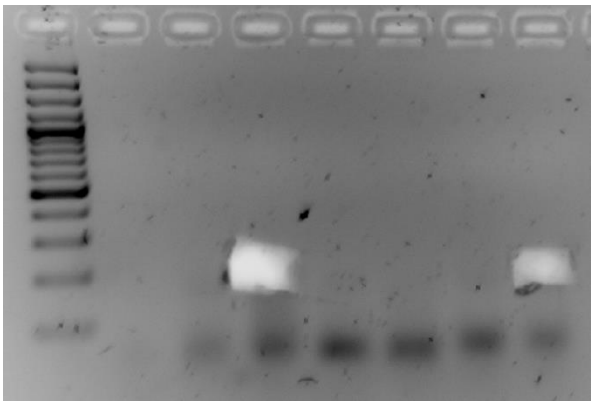


Figure 32: ***Picture X after cutting the gel prior to use it for real time PCR***

The recovery rate of the agarose gel extraction is usually around 70 %:

$$100\ ng\ sample \rightarrow 70\ \%\ recovery\ rate$$

$$70\ ng\ sample\ in\ 20\ \mu L \rightarrow 7\ ng\ sample\ in\ 2\ \mu L$$

Therefore 2 µL for the first elution and 3 µL for the elution 2.

The blue arrows show the 200 bp band size from the marker.

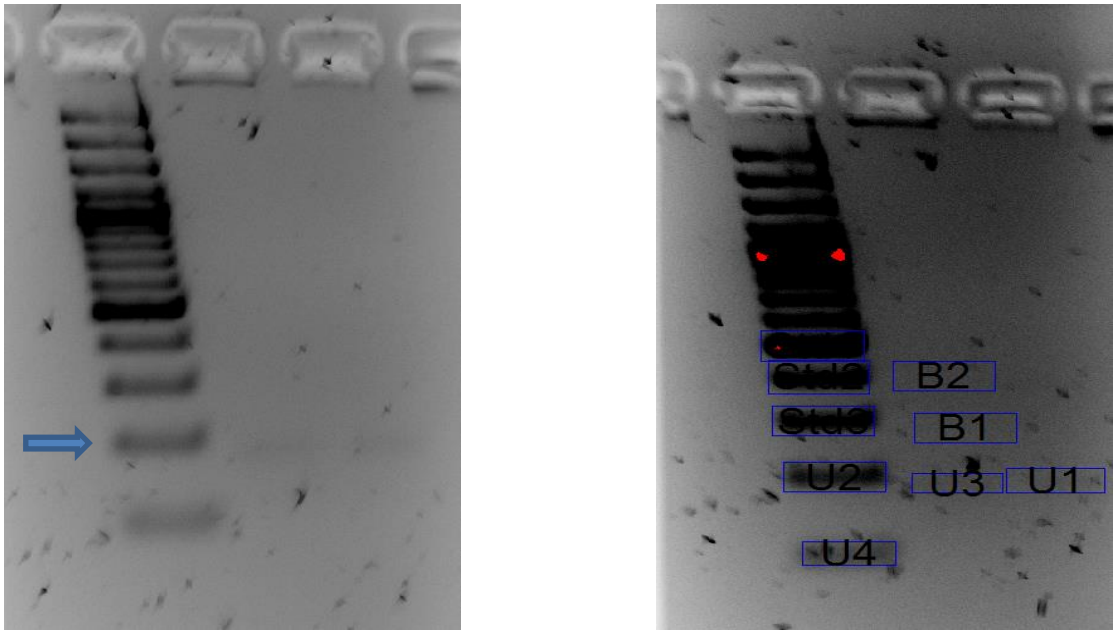


Figure 33: ***Showing elution 1 and 2 loaded in the gel (left) and the quantification method using the program quantity one (right), [80 V, 64 mA, 90 min, 1,2 % Agarose gel]***

Index	Name	Volume INT*mm2	Adj. Vol. INT*mm2	% Adj. Vol.	Concentration
1	Std1	80199.77837	29607.56809	62.72	40.00000000000
2	Std2	67673.12706	10258.25013	21.73	15.00000000000
3	Std3	57929.25604	7337.045765	15.54	12.00000000000
4	U1	37632.58393	-2701.88159	N/A	N/A
5	U2	54576.83569	3984.625416	N/A	7.413120861363
6	U3	28419.56296	-834.005431	N/A	1.294222077482
7	U4	35668.90372	-660.608978	N/A	1.514408138054
8	B1	49898.71520	-693.495072	N/A	N/A
9	B2	51285.70535	693.4950723	N/A	N/A

Table 10*: Results shows a concentration of 1, 29 ng/ 2 µL for elution 1 and 0 for elution 2 therefore I work now on just with the first elution.*

Because the 2 µL loaded sample was showing 1.294 ng/µL therefore a concentration of 0.66 ng/µL is the starter concentration of elution 1.

Using the following formula was easy to calculate the initial number of molecules to start building the standard curve for the first real time qPCR:

$$\frac{0{,}66\ ng}{1\ \mu L} * \frac{1\ g}{10^9\ ng} = 6{,}6 * 10^{-10}\, g/\mu L$$

$$\left[\frac{\frac{6{,}6 * 10^{-10} g}{\mu L}}{172\ bp * 660}\right] * 6{,}022 * 10^{23} = 3{,}5011 * 10^9\ \frac{molecules}{\mu L}$$

Knowing the starter number of molecules that the sample contains the standard curve can be built as it is detailed below:

$1\ \mu L\ sample + 99\ \mu L\ sterile\ water \rightarrow 100\ \mu L\ of\ 1 * 10^7\ molecules\ dilution$

From 100 μL 10^7
$\rightarrow 10\ \mu L\ sample + 90\ \mu L\ sterile\ water = 100\ \mu L\ of\ 1 * 10^6 molecules\ dilution$

From 100 μL 10^6
$\rightarrow 10\ \mu L\ sample + 90\ \mu L\ sterile\ water = 100\ \mu L\ of\ 1 * 10^5 molecules\ dilution$

From 100 μL 10^5
$\rightarrow 10\ \mu L\ sample + 90\ \mu L\ sterile\ water = 100\ \mu L\ of\ 1 * 10^4 molecules\ dilution$

From 100 μL 10^4
$\rightarrow 10\ \mu L\ sample + 90\ \mu L\ sterile\ water = 100\ \mu L\ of\ 1 * 10^3 molecules\ dilution$

From 100 μL 10^3
$\rightarrow 10\ \mu L\ sample + 90\ \mu L\ sterile\ water = 100\ \mu L\ of\ 1 * 10^2 molecules\ dilution$

+ Water control containing 0 molecules

For the first real time PCR just the samples *Columbia 2*, *nsun5-1* number 2 and *nsun5-2* number 2 were used because were the ones who showed better results in the electrophoresis gel (picture not shown). I received the dilutions from 10^6 to 10^2 for three housekeeping genes, *tub9*, *ubq5* and *pp2a from the supervisor, MS Hauser.*

Table 11: ***Number of copies from the first real time qPCR from every gene, showing a really low expression from Nsun5 gene comparing it with the three housekeeping genes.***

NSUN5	Ct	Given Conc (copies/ul)	Calc Conc (copies/ul)	Mean
nsun 10^6	12.5	1,000,000	1,049,560	
nsun 10^5	15.79	100,000	99,356	
nsun 10^4	19.16	10,000	8,845	
nsun 10^3	22.11	1,000	1,063	
nsun 10^3	22.18	1,000	1,010	
nsun 10^2	25.38	100	101	
H2O	26.96	Unknown	33	-
C2	30.49	Unknown	3	9
C2	28.15	Unknown	14	
C2	28.65	Unknown	10	
nsun5-1.2	30.74	Unknown	2	3.5
nsun5-1.2	29.71	Unknown	5	
nsun5-1.2		Unknown		
nsun5-2.2	31.62	Unknown	1	1
nsun5-2.2		Unknown		
nsun5-2.2	33.44	Unknown		

TUB9	Ct	Given Conc (copies/ul)	Calc Conc (copies/ul)	Mean
TUB9 10^6	19.94	1,000,000	894,927	
TUB9 10^5	23.83	100,000	147,383	
TUB9 10^5	25.03	100,000	84,718	
TUB9 10^4	29.88	10,000	8,925	
TUB9 10^4	29.63	10,000	10,027	
H2O		Unknown	0	-
C2	22.64	Unknown	256,401	255640
C2	22.37	Unknown	290,817	
C2	22.97	Unknown	219,703	
nsun5-1.2	28.78	Unknown	14,912	23025
nsun5-1.2	27.77	Unknown	23,815	
nsun5-1.2	27.24	Unknown	30,348	
nsun5-2.2	28.32	Unknown	18,468	16870
nsun5-2.2	28.62	Unknown	16,052	
nsun5-2.2	28.61	Unknown	16,090	

UBQ5	Ct	Given Conc (copies/ul)	Calc Conc (copies/ul)	Mean
UBQ5 10^6	20.02	1,000,000	1,115,576	
UBQ5 10^5	23.89	100,000	86,098	
UBQ5 10^4	27.19	10,000	9,717	
UBQ5 10^3	30.6	1,000	1,018	
UBQ5 10^3	30.55	1,000	1,053	
H2O		Unknown	0	-
C2	23.53	Unknown	109,550	79661
C2	29.14	Unknown	2,677	
C2	23.31	Unknown	126,757	
nsun5-1.2		Unknown	0	0
nsun5-1.2		Unknown	0	
nsun5-1.2		Unknown	0	
nsun5-2.2		Unknown	0	0
nsun5-2.2		Unknown	0	
nsun5-2.2		Unknown	0	

PP2A	Ct	Given Conc (copies/ul)	Calc Conc (copies/ul)	Mean
PP2A 10^6	27.12	1,000,000	696,296	
PP2A 10^5	30.28	100,000	133,420	
PP2A 10^5	30	100,000	154,594	
PP2A 10^4	35.65	10,000	8,092	
PP2A 10^4	35.53	10,000	8,604	
H2O		Unknown	0	-
C2	30.16	Unknown	142,290	97555
C2	30.65	Unknown	109,999	
C2	32.57	Unknown	40,377	
nsun5-1.2		Unknown	0	0
nsun5-1.2		Unknown	0	
nsun5-1.2		Unknown	0	
nsun5-2.2		Unknown	0	0
nsun5-2.2		Unknown	0	
nsun5-2.2		Unknown	0	

Table 12: ***Comparison between the gene Nsun5 and the other three housekeeping and its ratio between the mutants and the Columbia control.***

	NSUN5 / TUB9	RELATION TO COL 2	NSUN5/UBQ5	RELATION TO COL 2	NSUN5/PP2A	RELATION TO COL 2
C2	3.52057E-05	-	0.000112978	-	9.22553E-05	-
nsun5-1.2	0.000152009	431.77	-	-	-	-
nsun5-2.2	5.92768E-05	168.37	-	-	-	-

3.6.2 Second run real time qPCR

Since different RNA isolations have been performed during this thesis, different samples were tested to figure out the expression of the mutants under the stress of different heavy metals that have shown a special treatment the root length measurement. One of these heavy metals of interest is Nickel, but this time few samples were diluted to 1:10 and the rest are kept undiluted to figure out if there is any difference.

Table 13: Number of copies from the second real time qPCR from every gene.

NSUN5	Ct	Given Conc (copies/ul)	Calc Conc (copies/ul)	Mean
nsun 10^7	7	10,000,000	10,827,445	
nsun 10^6	10.42	1,000,000	1,040,452	
nsun 10^5	14.08	100,000	84,414	
nsun 10^4	17.36	10,000	8,923	
nsun 10^3	20.42	1,000	1,093	
nsun 10^3	20.42	1,000	1,095	
nsun 10^2	23.94	100	98	
H20		Unknown		-
COL diluted	25.17	Unknown	42	23.67
COL diluted	25.72	Unknown	29	
COL diluted		Unknown	0	
Ni diluted	27.18	Unknown	11	14.5
Ni diluted	26.42	Unknown	18	
Ni diluted		Unknown	0	
Col	21.38	Unknown	569	422.33
Col	22.3	Unknown	301	
Col	21.9	Unknown	397	
Ni	23.31	Unknown	152	141
Ni	23.46	Unknown	136	
Ni	24.80	Unknown	135	

TUB9	Ct	Given Conc (copies/ul)	Calc Conc (copies/ul)	Mean
tub9 10^7	16.1	10,000,000	21,550,552	
tub9 10^6	19.39	1,000,000	1,975,368	
tub9 10^5	24.6	100,000	45,141	
tub9 10^5	27.46	100,000	5,672	
tub9 10^4	25.03	10,000	33,075	
tub9 10^4	25.28	10,000	27,738	
H20		Unknown		-
COL diluted	28.31	Unknown	3,079	32,746
COL diluted	25.07	Unknown	32,172	
COL diluted	24.14	Unknown	62,988	
Ni diluted	24.99	Unknown	34,154	31,528
Ni diluted	24.67	Unknown	43,091	
Ni diluted	25.92	Unknown	17,339	
Col	19.96	Unknown	1,314,480	610,571
Col	21.42	Unknown	454,246	
Col	24.14	Unknown	62,988	
Ni	20.34	Unknown	993,435	1,891,830
Ni	19.78	Unknown	1,492,083	
Ni	18.73	Unknown	3,189,972	

UBQ5	Ct	Given Conc (copies/ul)	Calc Conc (copies/ul)	Mean
UBQ5 10^7	14.63	10,000,000	13,535,057	
UBQ5 10^5	23.87	100,000	77,061	
UBQ5 10^5	24.04	100,000	70,083	
UBQ5 10^4	28.51	10,000	5,764	
UBQ5 10^4	26.5	10,000	17,723	
UBQ5 10^3	31.12	1,000	1,339	
H20		Unknown		-
Col diluted	26.97	Unknown	13,605	109,665
Col diluted	22.73	Unknown	146,272	
Col diluted	22.47	Unknown	169,118	
Ni diluted	24.11	Unknown	67,423	67,423
Ni diluted	24.29	Unknown	60,980	
Ni diluted	25.44	Unknown	32,171	
Col		Unknown	0	876
Col	31.88	Unknown	876	
Col		Unknown	0	
Ni	20.5	Unknown	509,341	517,798
Ni	25.36	Unknown	33,533	
Ni	19.27	Unknown	1,010,520	

Table 14: Comparison between the gene Nsun5 and other two housekeeping and its ratio between the mutants and the control. The reduction grows when the sample is diluted in both scenarios.

	NSUN5 / TUB9	RELATION TO COL DIL/UNDIL	NSUN5/UBQ5	RELATION TO COL
COL diluted	0.000722727	-	0.000215809	-
Ni diluted	0.000459909	63.64	0.00021506	99.65
Col	0.000691702	-	0.482115677	-
Ni	7.4531E-05	10.78	0.000272307	0.06

3.6.3 Third run real time qPCR

Checking last two real time qPCR, especially for the housekeeping genes I decided to remake the standard curves for all three. In order to do that they have to be loaded again and be quantify using the program quantity one.

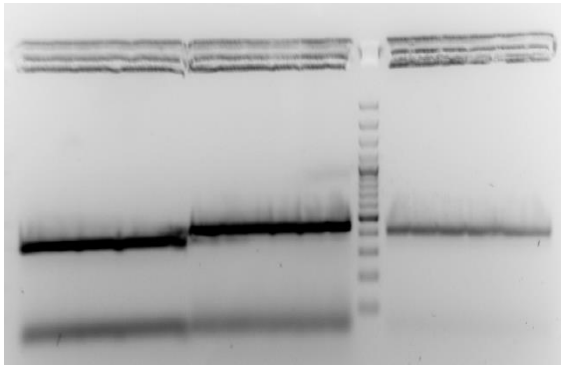


Figure 34: ***From left to right: TUB9, UBQ5, 100 bp marker and PP2A loaded in the gel before the quantification, [70 V, 85 mA, 90 min, 1,2 % Agarose gel, cDNA TUB9 360 bp, cDNA UBQ5 426 bp, cDNA PP2A 401 bp]***

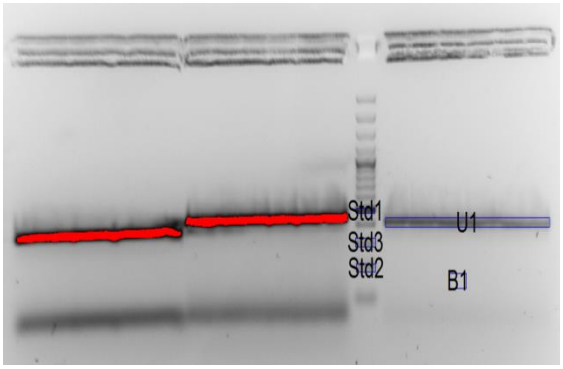


Figure 35: ***Same picture as the shown in the left but after the quantification***

Table 15*: Results shows a concentration of 662.47 ng/ 20 µL for elution 1 and 0 for elution 2 therefore I work now on just with the first elution.*

Index	Name	Volume INT*mm2	Adj. Vol. INT*mm2	% Adj. Vol.	Concentration
1	Std1	9216.190961	6215.147466	42.25	40.00000000000
2	Std2	8869.890925	3725.244934	25.32	15.00000000000
3	Std3	10838.19113	4769.414285	32.42	15.00000000000
4	U1	117852.9123	65834.82505	100.00	662.4675652022
5	B1	8174.270853	0.000000000	N/A	N/A

Unfortunately, UV overexposure just allowed to quantify the primer *pp2a* therefore it was needed to load all the samples again in a gel to quantify them with a better picture exposure: The recovery rate of the agarose gel extraction is usually around 70 %:

$$662.47\ \ ng\ sample \rightarrow 70\ \%\ recovery\ rate$$

$$463.4\ ng\ sample\ in\ 20\ \mu L \rightarrow 46.3\ ng\ sample\ in\ 2\ \mu L$$

Assuming the quantification by eye as 3 times more of the other two genes [*tub* and *ubq5*] was not assumable so that all genes were loaded again in a gel using 5 µL per each elution and using again the program quantity one. The blue arrows show the 500 bp band size from the marker.

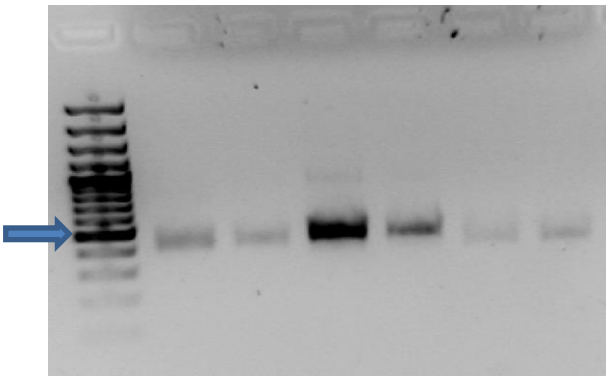


Figure 36: ***from left to right: 100 bp marker, elution 1 and 2 Pp2a, elution and 2 UBQ5, elution 1 and 2 TUB9[70 V, 50 mA, 45 min, 1,2 % Agarose gel, cDNA TUB9 360 bp, cDNA UBQ5 426 bp, cDNA PP2A 401 bp fragment size expected]***

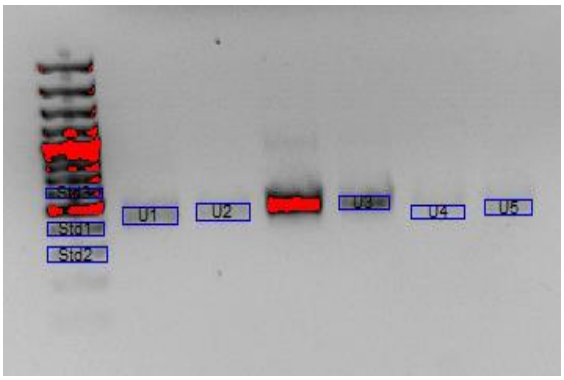


Figure 37: ***Same picture as the shown in the left but after the quantification***

Table 16*: Results shows a concentration [ng/µL] of all elutions from each housekeeping gene. The marked with the blue arrow are the one I took to go further to build the new standard curves.*

Index	Name	Volume INT*mm2	Adj. Vol. INT*mm2	% Adj. Vol.	Concentration	
1	Std1	5876.939786	5876.939786	26.43	7.400000000000	
2	Std2	7173.665638	7173.665638	32.27	12.00000000000	
3	Std3	9182.536158	9182.536158	41.30	15.00000000000	
4	U1	8736.301332	8736.301332	21.79	14.42181656364	←
5	U2	7472.331014	7472.331014	18.63	11.60332187128	
6	U3	10359.22620	10359.22620	25.83	18.04073471381	←
7	U4	6670.057763	6670.057763	16.63	9.814353515742	
8	U5	6862.928864	6862.928864	17.11	10.24443179158	←

From the results using quantity one program, the quantification showed valid values of the initial concentration to start to work with:

PP$_2$A: Elution 1

$14{,}42\ ng : 5\ \mu L\ \ loaded\ sample = 2{,}884\ ng/\mu L$

$2{,}884 \frac{ng}{\mu L} = 2{,}884\ x\ 10^9 \frac{g}{\mu L}$

$$\frac{2{,}884\ x10^9 \frac{ng}{\mu L}}{1401\ bp * 660}\ x\ 6{,}022\ x\ 10^{23} = 6{,}56\ x10^9 \frac{molecules}{\mu L}$$

Taking 1 µL sample + 55.6 µL H_2O I get 56.6 µL of 1x 10^8 molecules / µL

$10\ \mu L\ sample + 90\ \mu L\ RNAse\ free\ water \rightarrow 100\ \mu L\ of\ 1\ x\ 10^7$ and so on until 10^2 to build the standard curve.

UBQ5: Elution 2

$18{,}04\ ng : 5\ \mu L\ \ loaded\ sample = 3{,}608\ ng/\mu L$

$3{,}608 \frac{ng}{\mu L} = 3{,}608\ x\ 10^9 \frac{g}{\mu L}$

$$\frac{3{,}608\ x10^9 \frac{ng}{\mu L}}{426\ bp * 660}\ x\ 6{,}022\ x\ 10^{23} = 7{,}72\ x10^9 \frac{molecules}{\mu L}$$

Taking 1 µL sample + 76,2 µL H_2O I get 77,2 µL of 1x 10^8 molecules / µL

$10\ \mu L\ sample + 90\ \mu L\ RNAse\ free\ water \rightarrow 100\ \mu L\ of\ 1\ x\ 10^7$ and so on until 10^2 to build the standard curve.

TUB9: Elution 2

$10{,}24\ ng : 5\ \mu L\ \ loaded\ sample = 2{,}048\ ng/\mu L$

$2{,}048 \frac{ng}{\mu L} = 3{,}608\ x\ 10^9 \frac{g}{\mu L}$

$$\frac{2{,}048\ x10^9 \frac{ng}{\mu L}}{360\ bp * 660}\ x\ 6{,}022\ x\ 10^{23} = 5{,}190\ x10^9 \frac{molecules}{\mu L}$$

Taking 1 µL sample + 50, 90 µL H_2O I get 51.90 µL of 1x 10^8 molecules / µL

$10\ \mu L\ sample + 90\ \mu L\ RNAse\ free\ water \rightarrow 100\ \mu L\ of\ 1\ x\ 10^7$ and so on until 10^2 to build the standard curve.

Table 17: ***Number of copies from the third real time qPCR with Nsun5 and tub9 .***

NSUN5	Ct	Given Conc (copies/ul)	Calc Conc (copies/ul)	Mean
nsun 10^6	10.31	1,000,000	1,267,785	
nsun 10^5	13.93	100,000	108,960	
nsun 10^5	14.05	100,000	100,642	
nsun 10^4	18.13	10,000	6,377	
nsun 10^4	18.05	10,000	6,695	
nsun 10^3	20.47	1,000	1,308	
nsun 10^3	20.55	1,000	1,231	
nsun 10^2	24.2	100	105	
H2O			-	-
c2	25.74		37	52
c2	24.86		67	
c2			0	
nsun5-1			0	0
nsun5-1			0	
nsun5-1			0	
nsun5-2	20.83		1,023	919
nsun5-2	21.17		815	
nsun5-2			0	

TUB9	Ct	Given Conc (copies/ul)	Calc Conc (copies/ul)	Mean
TUB9 10^6	13.02	1,000,000	1,005,883	
TUB9 10^5	16.3	100,000	106,461	
TUB9 10^4	19.91	10,000	8,972	
TUB9 10^3	23.11	1,000	1,006	
TUB9 10^2	26.43	100	103	
H2O			-	-
c2			1,162	1,547
c2	22.9		2,181	
c2	21.98		1,297	
nsun5-1	22.74		0	0
nsun5-1			0	
nsun5-1			0	
nsun5-2			35,047	22,836
nsun5-2	17.92		33,425	
nsun5-2	17.99		34.586	

Table 18: ***Comparison between the gene Nsun5 with tub9 and its relation between the mutants and the Columbia control.***

	NSUN5 / TUB9	RELATION TO COL 2
c2	0.03362069	-
nsun5-1	0	0.00
nsun5-2	0.040244306	119.70

3.6.4 Fourth run real time qPCR

Since the UBQ5 dilutions did not worked in the 3rd run of real time qPCR, a repetition of the dilutions was needed and this time just with undiluted control Columbia and sample from control Nickel to finally test if the new housekeeping gene curves worked properly:

Table 19: ***Number of copies from the fourth real time qPCR from Nsun5, tub9 and ubq5 genes.***

NSUN5	Ct	Given Conc (copies/ul)	Calc Conc (copies/ul)	Mean
nsun 10^7	7.97	10,000,000	7,592,789	
nsun 10^6	10.76	1,000,000	1,097,639	
nsun 10^5	13.43	100,000	172,557	
nsun 10^5	14.66	100,000	73,369	
nsun 10^4	17.92	10,000	7,627	
nsun 10^4	17.13	10,000	13,264	
nsun 10^3	20.54	1,000	1,240	
nsun 10^3	20.77	1,000	1,060	
nsun 10^2	24.66	100	71	
H2O				-
col				26
col				
col				
col				
col	26.09		26	
Ni				2,543
Ni				
Ni				
Ni				
Ni	19.51		2,543	

TUB9	Ct	Given Conc (copies/ul)	Calc Conc (copies/ul)	Mean
TUB9 10^7	14.66	10,000,000	12,214,585	
TUB9 10^6	19.63	1,000,000	752,102	
TUB9 10^5	22.71	100,000	133,939	
TUB9 10^5	23.45	100,000	88,628	
TUB9 10^4	27.86	10,000	7,494	
TUB9 10^3	31.09	1,000	1,224	
H2O				-
col				0
col				
col				
col				
col				
Ni				16,043
Ni	26.5		16,043	
Ni				
Ni				
Ni				

UBQ5	Ct	Given Conc (copies/ul)	Calc Conc (copies/ul)	Mean
UBQ5 10^7	18.67	10,000,000	11,130,282	
UBQ5 10^6	22.69	1,000,000	1,043,343	
UBQ5 10^5	27.33	100,000	67,892	
UBQ5 10^4	30.55	10,000	10,245	
UBQ5 10^4	30.26	10,000	12,150	
UBQ5 10^3	34.47	1,000	1,019	
H2O				-
Col				25,825
Col	27.88		49,158	
Col				
Col				
Col	32.95		2,492	
Ni				98.5
Ni	38.95		73	
Ni				
Ni				
Ni	38.04		124	

Table 20: Comparison between the gene Nsun5 and other two housekeeping and its ratio between the mutants and the control.

	NSUN5 / TUB9	RELATION TO COL	NSUN5/UBQ5	RELATION TO COL
Col	0	-	0.001006776	-
Ni	0.1585115	-	25.81725888	2564349

Chapter 4: Discussion

4.1 Genotyping the t-DNA insertion

4.1.1 RNA isolation

The low yield of several RNA isolations performed was the main drawback in order to reach the original goal of performing bisulfite conversion and C2278 methylation analyses. The first thing I realise when I started the experiments was how unstable and delicate RNA could be if you compare it with genomic DNA in terms of temperature storage, handling and time work.

The results that the RNA isolation showed were really good, showing two clear band fragments around 1100 bp for 20S RNA and 850 bp for 16S RNA. Although the previous results were from leaves and around 300 mg was used (table 4), if seedlings are used as starter material, results were relatively lower in comparison (table 18). Several isolations have been performed taking care of the temperature of storage, speeding up the velocity of my work and protocoling the time of every step in the assay. Even with that, similar results were obtained.

Table 21: ***Results from RNA isolation from seed showing a lower results in comparison with leaves (Table 4).***

#	Sample ID	User name	Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type	Factor
1	control_col_2	h94000_nanodrop	23.05.2016 12:48:27	231.5	ng/µl	5.788	2.667	2.17	1.77	RNA	40
2	control_nsun5-1_2	h94000_nanodrop	23.05.2016 12:49:51	198.8	ng/µl	4.97	2.273	2.19	1.88	RNA	40
3	control_nsun5-2_2	h94000_nanodrop	23.05.2016 12:50:28	130.4	ng/µl	3.261	1.468	2.22	1.53	RNA	40
4	Ni_col_2	h94000_nanodrop	23.05.2016 12:51:12	155.4	ng/µl	3.886	2.805	1.39	1.07	RNA	40
5	Ni_nsun5-1_2	h94000_nanodrop	23.05.2016 12:52:14	68.9	ng/µl	1.723	0.776	2.22	1.33	RNA	40
6	Ni_nsun5-2_2	h94000_nanodrop	23.05.2016 12:53:08	329.4	ng/µl	8.236	3.784	2.18	2	RNA	40

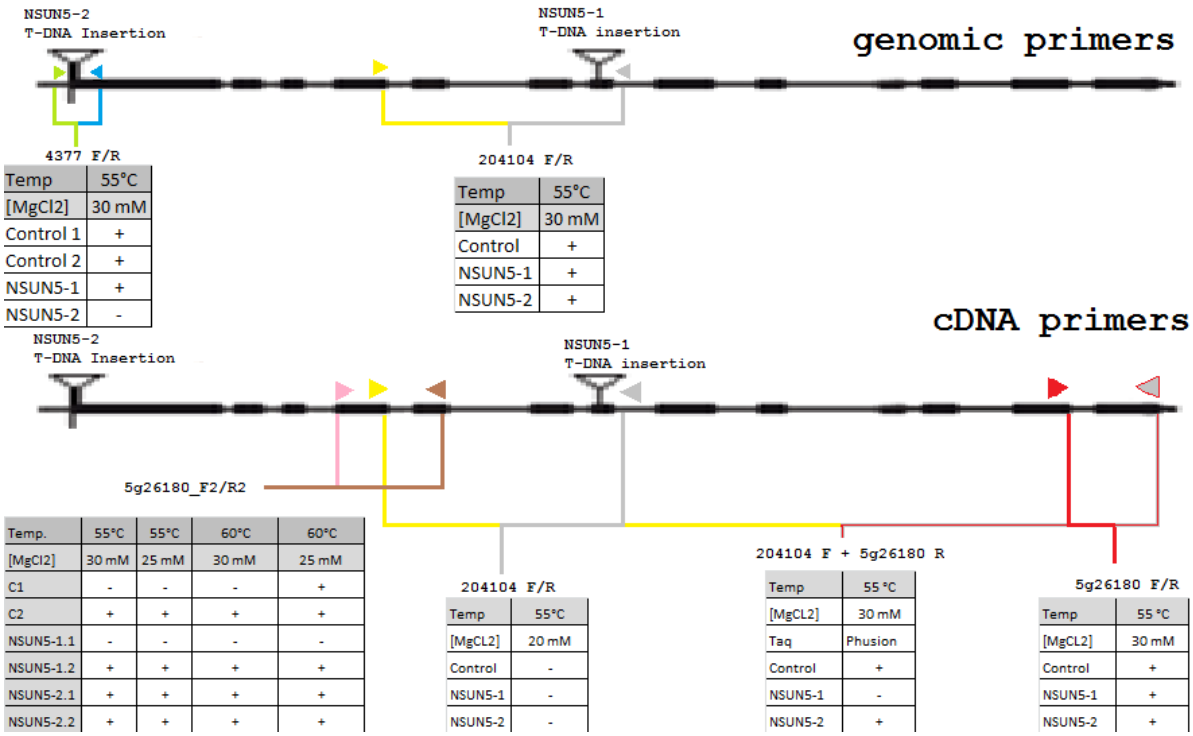
One of the possible solutions could have been working with a higher starter amount of sample because just around 300 mg from leaves were used and as it is shown in table 19, an average of 80 mg were harvested from all three genotypes.

Table 22: ***Example of harvesting material from seeds in two different medium getting results around 70-90 mg each***

[mg]	Control	Nickel
Col 1	77	86.9
Col 2	79.6	64.6
Col 3	-	-
NSUN5-1.1	78.8	106.2
NSUN5-1.2	93	84.5
NSUN5-1.3	53	73
NSUN5-2.1	79.7	103.5
NSUN5-2.2	76.3	56
NSUN5-2.3	78.7	85.5

4.1.2 Complementary DNA and its optimization

Concerning the test with the primers 5g26180, as it is shown in the figure 20; it was demonstrated that the mutant allele *nsun5-1* was expressed in the gene sequence. Testing the primers Salk_204104 went as expected because the reverse primer was located in an intron, the noncoding section of the RNA transcript. Testing the primers 25_s rRNA and its optimization regarding different temperature and magnesium chloride concentration did not show a really improvement assay because they were primers designed specifically for the bisulfite treatment as an undigested product after the hinfI restriction enzyme elimination.



Temp	55°C
[MgCl2]	30 mM
Control 1	+
Control 2	+
NSUN5-1	+
NSUN5-2	-

Temp	55°C
[MgCl2]	30 mM
Control	+
NSUN5-1	+
NSUN5-2	+

Temp.	55°C	55°C	60°C	60°C
[MgCl2]	30 mM	25 mM	30 mM	25 mM
C1	-	-	-	+
C2	+	+	+	+
NSUN5-1.1	-	-	-	-
NSUN5-1.2	+	+	+	+
NSUN5-2.1	+	+	+	+
NSUN5-2.2	+	+	+	+

Temp	55°C
[MgCl2]	20 mM
Control	-
NSUN5-1	-
NSUN5-2	-

Temp	55 °C
[MgCL2]	30 mM
Taq	Phusion
Control	+
NSUN5-1	-
NSUN5-2	+

Temp	55 °C
[MgCL2]	30 mM
Control	+
NSUN5-1	+
NSUN5-2	+

Figure 38: ***Summarize the entire primers test performed during the genotyping of both t-DNA insertion.***

4.2 Seedling measurement

Find out if there was any difference between seedlings cultivated in a medium containing heavy metal and the medium control. In order to do that, a security line of 5% difference was imposed and every sample within this range would become an interesting heavy metal medium to work further with.

The first assay proved that both alleles were hypersensitive to cadmium medium: on nickel and manganese, *nsun5-1* allele was hypersensitive and tolerant for *nsun5-2*. Second assay *nsun5-1* was hypersensitive for cadmium and nickel. *Nsun5-1* had a high tolerance to copper, manganese and led before autoclave while *nsun5-2* allele had it to nickel, manganese and zinc. For the last assay the allele *nsun5-1* was hypersensitive to manganese and led and had a high tolerance to copper and zinc. Concerning *nsun5-2* showed a high sensitivity to zinc medium and a high tolerance to copper, nickel and manganese.

The addition of heavy metals before or after autoclaving prior its use to plant the seedlings could affect their future growth. First and third root growth assay demonstrated that just led added before the autoclaving showed a truly difference in the allele *nsun5-1*. Adding the heavy metal medium before autoclaving could affect the composition of the medium regarding the pH effect that had to be adjusted beforehand between 6 and 6.4 pH and it is really difficult to check it afterwards. In spite of everything, just led was tested but other heavy metals could be also interesting to test to see how much can the seedling growth be affected.

I chose Nickel over the other possibilities because when I was working in the seedling measurement at the beginning just nickel showed as an interesting heavy metal medium but performing a normalization of the results in the measurement I realize that other heavy metals could be also interesting to test. Nickel is considered as essential element in plant growth but due to its low requirement in plants, it can be also found as a contaminant in soils.

Cadmium and led could be also a good choices in future research because they are considered as non-essential element for plants and also it is known to be also toxic for human beings.

Regarding copper, manganese or zinc, they are considered as micronutrients which means the plants need them to perform their physiological plant functions properly but as a remind, a contamination is considered where it is unwanted, or in a concentration or form that causes a detrimental environmental effect to the plants so they could also be tested as potential contaminants in a growth medium.

4.3 Real time qPCR

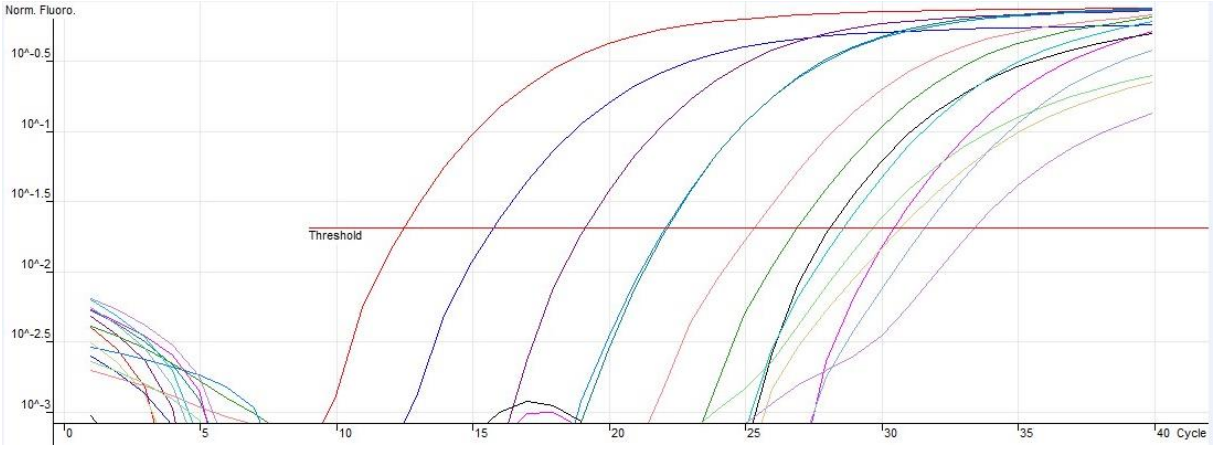


Figure 39: ***Real time q PCR standard curve for Nsun5 in the first run.***

Summarizing up the results, the expression reduction from *nsun5-1* to Columbia-0 was 431.7 % and 168.4 % reduction from *nsun5-2* comparing it with *tub9* in the first run.

The second run, the reduction comparing if the sample was diluted or undiluted 10 times with columbia-0 and nickel was 63.6 % and 10.70 % respectively comparing it with *tub9*. If the comparison was with *ubq5*, the reduction of methylation was 99.7 % and 0.06 %.

In the third run, the reduction from *nsun5-1* was not measured because none of my samples showed a single number of copies. Nevertheless, *nsun5-2* showed a reduction of the expression of 119.70 % if compared with *tub9* and it was not possible to compare it with *ubq5* because the standard curve did not work as expected.

Fourth run showed an unlikely result that is the reason I could not include it within the discussion. The dispersed results leaded me to think several explanations:

Firstly, in all four runs, the expression of both mutant *nsun5* was lower expressed if compared with any other housekeeping genes which maintain the number of copies higher and stable. Secondly, building the gene curves I realized that the results obtained were difficult to compare because the dilutions were dispersed in every gene. For this reason, I decided to maintain the same threshold every run to normalize the results that would allow me to compare them. Setting a threshold of 0.0250 in the normalized fluorescence showed that the reduction of expression from *nsun5-1* to Columbia-0 is lowered to 391.6 % and 98.93 % from *nsun5-2,* comparing it with *tub9* in the first run. In the second run, the reduction with columnia-0 and nickel is 29.86 % and 28.75 % in *tub9* and 89.7 % and 0.15 % with *ubq5*. In the third run, *nsun5-2* shows a reduction of the methylation of 205.1 %. Fourth run again shows an unlike results out for the expected range of possible expectations.

Even though the numbers showed dispersed results within every real time PCR, what can truly be concluded is that the expression of our gene under heavy metal stress with Nickel is highly influenced.

Chapter 5: Conclusion

Since the beginning of the thesis the RNA isolation it is been the biggest drawback because of the low yield resulting was essentially different if the starter material were leaves or seedlings. Around 4 times reduction was shown, when the isolation was performed with seedling around 200-300 ng/µL and from leaves 700-800 ng /µL.

Concerning the genotyping of T-DNA insertions, both mutants were expressed in the line gene sequence of AT5g26180 gene being the plants homozygous for each mutant, fact that allowed me to work further with the project.

Comparing each mutant with Columbia-0 and setting an interval of confidence of 95 %, any sample within it would immediately become a sample of interest. Being within this range means that sensitivity towards the medium and root growth is proved. First root growth assay showed that both alleles in cadmium, *nsun5-1* in nickel or manganese and *nsun5-2* with led-before had high sensibility towards the medium that contained them. The second root growth assay showed a hypersensitive behavior when *nsun5-1* was in cadmium or nickel and the third root growth assay when *nsun5-1* was on manganese or led and *nsun5-2* on zinc.

Only nickel it's been tested on a real time qPCR showing an average of expression reduction of 431.7 % in *nsun5-1* and 119.7-168.3 % of reduction in *nsun5-2* comparing it with Columbia-0. If the sample is diluted with nickel as medium the reduction is in a range of 63.6-99-6% and the undiluted is up to 0.06-10.70 %. After checking the results and setting a fix threshold 0.0250 in the normalized fluorescence the reduction of gene expression in *nsun5-1* is updated around 391.5 % and *nsun5-2* is 98.9-205.1 %. If the sample is diluted with nickel as medium the reduction is in a range of 29.8-89.64 % and the undiluted is up to 0.15-28.75 %

The use of mediums containing heavy metals and trace elements as cadmium, manganese, led and zinc as stressors to modify the gene expression in comparison with a control medium is still unknown and need further research. Different stresses can be also tested such as UV-B treatment, temperature changes, drowning samples or also under different vitamin reduction or absence. The effect of heavy metals solutions on the medium that are poured in is still unknown and furthermore if this solution is introduced before or after autoclaving and how this metal affects the Hoagland solution contained in the medium could be a really interesting field of future research.

Originally, this thesis was divided in two main parts; first analyze *NSUN5* expression in wildtype and two mutant alleles under different heavy metal and trace elements stresses and second to perform a bisulfit conversion and C2278 methylation analyses; however, due to the low yield of RNA isolation I could not reach one of the initials goal. Further studies are needed for a better understanding of the effects of both mutants alleles and the gene for their implication on the translation and gene expression.

Acknowledgments

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List of references

- Arabidopsis thaliana (thale cress). (n.d.). Retrieved June 6, 2016, from http://www.kew.org/science-conservation/plants-fungi/arabidopsis-thaliana-thale-cress

- Azpiroz-Leehan R., Feldmann K.A. (1997). T-DNA insertion mutagenesis in Arabidopsis: Going back and forth. Trends Genet. 13, pp. 152–156.

- Burgess, A. L., David, R., & Searle, I. R. (2015). Conservation of tRNA and rRNA 5-methylcytosine in the kingdom Plantae. BMC Plant Biol BMC Plant Biology.

- Complementary DNA. (n.d). In wikipedia. Retrieved May 29, 2016, from https://en.wikipedia.org/wiki/Complementary_DNA

- Feldmann, K. A., & Goff, S. A. (2014). The First Plant Genome Sequence—Arabidopsis thaliana. Genomes of Herbaceous Land Plants Advances in Botanical Research, 91-117.

- Gigova A, Duggimpudi S, Pollex T, Schaefer M, Kos M. (2014). A cluster of methylations in the domain IV of 25S rRNA is required for ribosome stability. RNA.2014; October 20: pp. 1632–1644.

- Hua J., Meyerowitz E.M.(1998). Ethylene responses are negatively regulated by a receptor gene family in Arabidopsis thaliana. Cell 94, pp. 261–271.

- Krysan, P. J., Young, J. C., & Sussman, M. R. (1999). T-DNA as an Insertional Mutagen in Arabidopsis. The Plant Cell December 1999 vol. 11 no. 12. pp. 2283-2290

- Krysan P.J., Young J.C., Tax F., Sussman M.R.(1996). Identification of transferred DNA insertions within Arabidopsis genes involved in signal transduction and ion transport. Proc. Natl. Acad. Sci. USA 93, pp. 8145–8150.

- Meinke, D. W. (1998). Arabidopsis thaliana: A Model Plant for Genome Analysis.Science, 282(5389), pp. 662-682.

- QIAEX® II Handbook. (n.d.). Retrieved June 5, 2016, from https://webcache.googleusercontent.com/search?q=cache:v_IJOMFLdxkJ:https://www.qiagen.com/resources/download.aspx?id=13d33145-9f64-426a-a43b-394211d8cf2b&lang=en

- Quires JE et al.(2012) .Widespread occurrence of 5-methylcytosine in human coding and non-coding, Nucl. Acids Res. (2012) 40 (11): pp. 5023-5033.

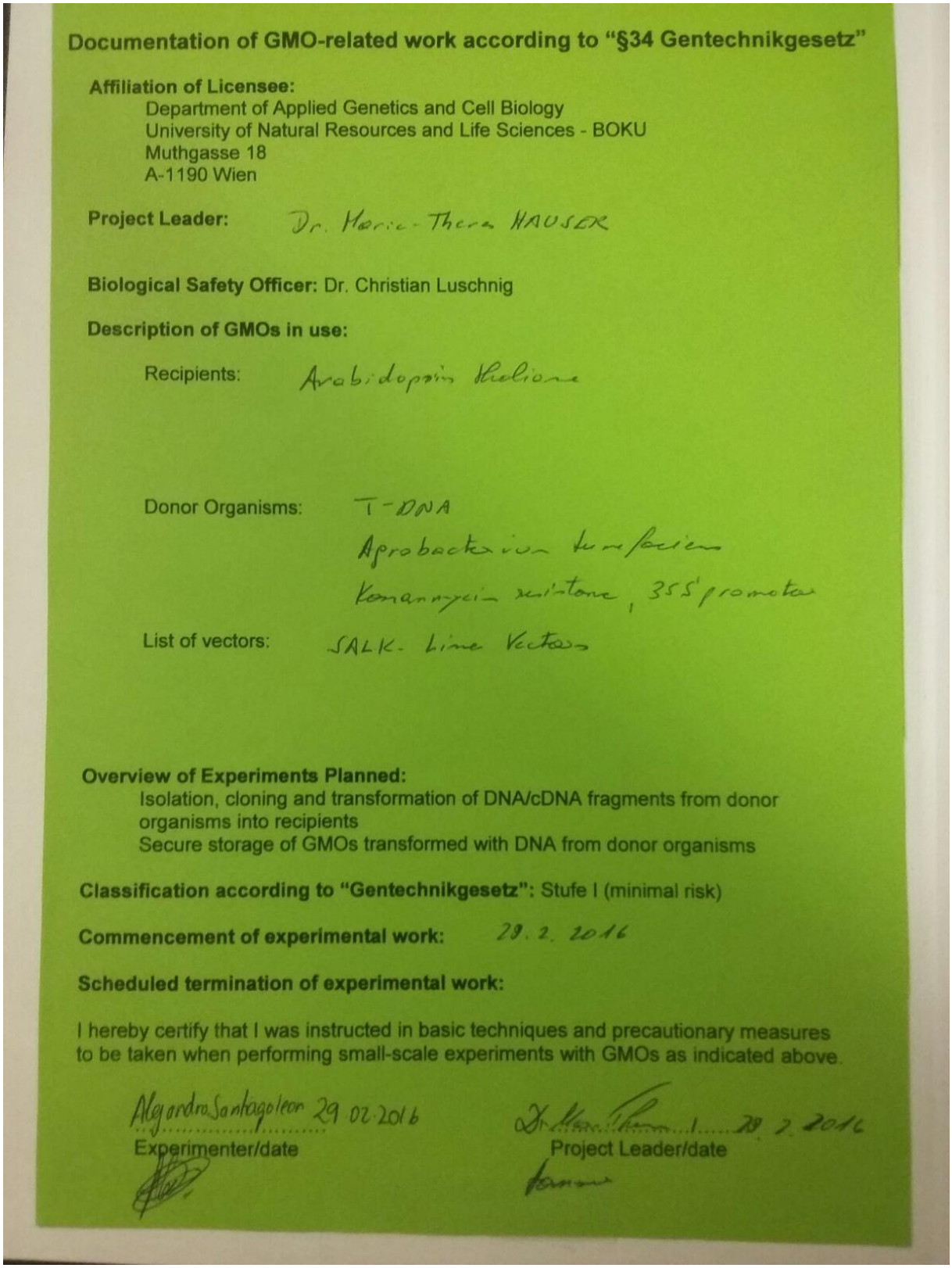
- TAIR - Genome Assembly. (2010, April 5). Retrieved June 6, 2016, from http://www.arabidopsis.org/portals/genAnnotation/gene_structural_annotation/agicomplete.jsp

- Shaefer M, Pollex T, Hanna K, Lyko F. RNA cytosine methylation analysis by bisulfite sequencing. Nucleic Acids Res. pp. 2009-2037,

- TAIR - About Arabidopsis. (n.d.). Retrieved June 6, 2016, from https://www.arabidopsis.org/portals/education/aboutarabidopsis.jsp

- Transfer DNA. (n.d.). Retrieved June 5, 2016, from https://en.wikipedia.org/wiki/Transfer_DNA

Annex



Documentation of GMO-related work according to "§34 Gentechnikgesetz"

Affiliation of Licensee:
Department of Applied Genetics and Cell Biology
University of Natural Resources and Life Sciences - BOKU
Muthgasse 18
A-1190 Wien

Project Leader: Dr. Marie-Thera HAUSER

Biological Safety Officer: Dr. Christian Luschnig

Description of GMOs in use:

Recipients: Arabidopsis thaliana

Donor Organisms: T-DNA
Agrobacterium tumefaciens
Kanamycin resistance, 35S promotor

List of vectors: SALK. Line Vectors

Overview of Experiments Planned:
Isolation, cloning and transformation of DNA/cDNA fragments from donor organisms into recipients
Secure storage of GMOs transformed with DNA from donor organisms

Classification according to "Gentechnikgesetz": Stufe I (minimal risk)

Commencement of experimental work: 29. 2. 2016

Scheduled termination of experimental work:

I hereby certify that I was instructed in basic techniques and precautionary measures to be taken when performing small-scale experiments with GMOs as indicated above.

Alejandro Santiagoleon 29.02.2016
Experimenter/date

Dr. Marie-Thera ... 29. 2. 2016
Project Leader/date

Table 23: *Root growth assay, first attempt.*

	DAY 5	Normalized																			
	CONTROL	CONTROL	CONTROL	Cu	Cu	Cu	Cd	Cd	Cd	Ni	Ni	Ni	Mn	Mn	Mn	Pb BEFORE	Pb BEFORE	Pb BEFORE	Pb AFTER	Pb AFTER	Pb AFTER
	NSUN5-1	NSUN5-2	COL-0	NSUN5-1	NSUN5-2	COL-0	NSUN5-1	NSUN5-2	COL-0	NSUN5-1	NSUN5-2	COL-0	NSUN5-1	NSUN5-2	COL-0	NSUN5-1	NSUN5-2	COL-0	NSUN5-1	NSUN5-2	COL-0
seed 1	1.235	1.184	1.165				0.566	0.556	0.463	0.370	0.261	0.283	0.395	0.716	0.791	0.584	0.571	0.407	0.278	0.307	0.095
seed 2	1.285	1.010	0.348				0.471	0.462	0.421	0.301	0.318	0.203	0.432	0.714	0.830	0.507	0.440	0.482	0.225	0.343	0.253
seed 3	1.269	1.024	1.084				0.403	0.667	0.414	0.293	0.297	0.265	0.631	0.631	0.798	0.534	0.671	0.508	0.288	0.291	0.277
seed 4	0.992	1.036	1.049				0.496	0.492	0.358	0.307	0.305	0.288	0.361	0.638	0.740	0.540	0.413	0.481	0.258	0.277	0.240
seed 5	1.106	1.049	1.056				0.395	0.436	0.356	0.316	0.253	0.262	0.647	0.826	0.795	0.612	0.522	0.456	0.261	0.240	0.329
seed 6	1.033	1.194					0.608	0.635	0.467	0.296	0.268		0.885		0.818	0.321	0.640	0.437	0.215	0.215	0.178
seed 7	1.030						0.506	0.615	0.202	0.336	0.319		0.662		0.817	0.380	0.581	0.465	0.300	0.228	0.405
seed 8							0.371	0.508	0.375								0.612	0.474		0.235	0.350
seed 9								0.551	0.490								0.524			0.231	0.297
seed 1	0.997	1.081	1.020				0.520	0.556	0.342	0.334	0.357	0.182	0.272	0.578	0.920	0.552	0.557	0.448	0.208	0.166	0.187
seed 2	0.414	1.181	1.146				0.456	0.582	0.455	0.372	0.342	0.311	0.346	0.810	0.769	0.483	0.540	0.392	0.258	0.251	0.185
seed 3	1.215	0.836	1.032				0.513	0.478	0.462	0.291	0.353	0.316	0.249	0.638	0.638	0.338	0.395	0.479	0.501	0.426	0.194
seed 4	1.002	0.964	1.141				0.526	0.516	0.483	0.369	0.285	0.294	0.833	0.641	0.737	0.305	0.594	0.466	0.256	0.158	0.230
seed 5	0.590	1.166	1.232				0.452	0.532	0.393	0.194	0.329	0.312	0.794	0.874	0.721	0.517	0.653	0.494		0.183	0.354
seed 6	1.081	1.076	0.818					0.436	0.259	0.353	0.392		0.512	0.806	0.750	0.287	0.504			0.334	0.329
seed 7	0.860		1.018					0.666	0.444	0.355	0.330			0.845	0.581		0.583			0.114	0.227
seed 8	0.901		0.820						0.469	0.384					0.810		0.522			0.324	0.268
seed 9																	0.643			0.338	
seed 1	0.947	0.852	0.883				0.600	0.618	0.433	0.328	0.356	0.290	0.741	0.710	0.887	0.528	0.549	0.455	0.158	0.225	0.288
seed 2	1.063	0.825	0.934				0.453	0.570	0.432	0.329	0.276	0.306	0.491	1.281	0.538	0.606	0.542	0.282	0.122	0.159	0.230
seed 3	1.035	0.822	0.855				0.575	0.522	0.293	0.314	0.273	0.337	0.729	0.689	0.559	0.349	0.642	0.377	0.227	0.205	0.236
seed 4	1.153	0.981	0.832				0.816	0.699	0.495	0.273	0.326	0.310	0.648	0.858	0.586	0.578	0.765	0.431	0.229	0.240	0.233
seed 5	1.006	0.722	0.967				0.633	0.536	0.349	0.283	0.222	0.271	0.614	0.762	0.541	0.426	0.664	0.553	0.262	0.246	0.285
seed 6	0.875						0.409	0.711	0.450	0.285	0.248			0.585	0.749		0.563	0.541	0.302	0.206	0.184
seed 7	0.893						0.584	0.805	0.532	0.326	0.213			0.751	0.426		0.478	0.543	0.225	0.154	0.202
seed 8								0.563	0.437	0.363	0.279				0.753		0.626			0.117	0.246
seed 9								0.539												0.251	

Table 24: *Root growth assay, second attempt.*

	DAY 5																				
	CONTROL	CONTROL	CONTROL	Cu	Cu	Cu	Cd	Cd	Cd	Ni	Ni	Ni	Mn	Mn	Mn	Pb BEFORE	Pb BEFORE	Pb BEFORE	Zn	Zn	Zn
column 1	NSUN5-1	NSUN5-2	COL-0	NSUN5-1	NSUN5-2	COL-0	NSUN5-1	NSUN5-2	COL-0	NSUN5-1	NSUN5-2	COL-0	NSUN5-1	NSUN5-2	COL-0	NSUN5-1	NSUN5-2	COL-0	NSUN5-1	NSUN5-2	COL-0
seed 1	1.619	1.745	1.182	1.875	1.357	0.697	0.796	0.809	0.854	1.137	1.156	1.418	1.215	1.393	0.966	1.079	0.886	1.158	1.438	1.071	1.140
seed 2	1.328	1.722	1.445	1.687	1.735	1.439	0.937	0.819	1.199	1.223	1.449	1.076	0.871	0.961	1.331	1.072	1.222	1.158	1.407	1.416	1.797
seed 3	1.547	1.595	1.666	1.517	1.613	1.515	0.907	0.937	0.803	1.033	1.239	1.513	1.070	1.194	1.334	0.975	1.254	1.220	1.149	1.376	1.204
seed 4	1.431	1.456	1.531	1.346	1.795	1.599	1.025	1.021	1.136	1.267	1.409	1.340	1.141	0.931	1.312	0.915	1.385	1.348	1.082	1.526	1.269
seed 5	1.364	1.696	1.611	1.513	1.493	1.583	0.579	0.775	0.781	1.085	1.631	1.319	1.245	1.525	1.230	0.901	1.483	0.881	1.131	1.244	1.396
seed 6	1.351	1.431	1.521	1.157	1.589	1.646	1.142	0.981	0.988	1.336	0.883	1.523	1.313	0.842	1.108	0.907	1.428	1.480	1.303	1.289	1.225
seed 7	1.877	1.601	1.476	1.220	1.456		0.883	1.234	0.798	1.358	1.148	1.510	1.200	1.197	1.129	1.116	1.361	0.886	0.987	1.117	1.368
seed 8	1.437	2.041	1.380	1.487	1.871		1.040	0.958	1.130	1.157	1.379	1.603	1.118	1.315	1.181	1.030	1.116	1.222	1.026	1.283	1.276
seed 9		1.633	1.695		1.297		0.906	0.845	0.985	1.238	1.266	1.338		1.215		1.176	1.273	1.254	1.046	1.148	1.208
column 2																					
seed 1	1.532	1.778	1.303	1.014	1.608	1.526	0.750	1.033	0.855	0.783	1.037	1.364	0.936	1.102	0.955	1.123	1.260	1.045	1.179	1.211	1.263
seed 2	1.558	1.852	1.536	1.557	1.601	1.293	0.757	1.074	0.905	1.390	1.680	1.254	1.044	1.260	1.128	1.226	1.279	1.198	0.997	1.303	1.183
seed 3	1.545	1.737	1.545	1.121	1.504	1.516	1.028	0.589	0.995	1.286	1.273	1.292	1.166	0.968	1.073	1.407	1.457	1.377	1.422	1.495	1.254
seed 4	1.623	1.649	1.556	1.663	1.685	1.477	0.712	0.972	1.011	1.165	1.484	1.531	1.116	1.125	1.413	1.187	1.377	0.785	1.053	0.938	1.385
seed 5	1.344	1.266	1.735	1.256	1.599	1.463	0.646	0.936	0.934	1.360	1.447	1.207	1.067	1.050	0.916	1.217	1.026	1.200	1.141	1.483	1.015
seed 6	1.592	1.304	1.431	1.972	1.754	1.847	0.818	1.184	1.002	1.031	1.373	1.453	1.146	0.811	1.039	1.218	1.463	1.195	1.385	1.383	1.183
seed 7	1.277	1.758	1.803	1.546	1.533	1.454	0.813	0.874	1.053	0.994	1.513	0.965	1.329	1.141	1.100	0.975	1.169	1.142	1.195	1.423	1.420
seed 8	1.346	1.255	1.800	1.531	1.599	1.746	0.616	0.995	1.046	1.164	1.644	1.514	1.262	1.434	1.253	1.240	0.320	1.245	1.324	1.396	1.221
seed 9		1.564	1.663	1.345		1.230	1.029	1.161	1.365	1.243	1.545	1.610	1.284		1.001	1.085			1.214	0.788	1.332
column 3																					
seed 1	1.526	1.377	1.467	1.537	1.442	1.612	0.661	0.692	0.852	1.366	1.168	1.426	0.986	1.182	1.007	0.590	0.853	1.152	1.194	1.282	1.053
seed 2	1.430	1.364	1.315	1.247	1.708	1.506	0.871	0.709	0.953	1.230	1.399	1.447	1.200	1.068	0.909	1.172	0.745	0.455	0.683	1.142	0.799
seed 3	1.328	1.404	1.780	1.635	1.591	1.057	0.997	1.041	0.724	1.138	0.969	1.002	0.952	1.175	1.006	0.933	1.105	0.472	0.896	1.134	0.990
seed 4	0.894	0.953	1.466	1.643	1.664	1.623	0.804	0.809	0.730	1.102	1.048	1.387	1.077	1.043	1.177	1.012	0.879	0.531	0.951	1.210	1.048
seed 5	1.338	1.451	1.427	1.299	1.575	1.618	0.964	0.912	0.951	1.243	1.290	1.176	0.820	0.997	1.084	0.925	0.742	1.245	1.162	1.206	0.893
seed 6	1.087	1.667	0.910	1.485	1.533	1.319	0.744	0.943	1.120	1.112	1.305	1.276	1.095	1.346	1.072	1.222	1.023	0.957	1.057	0.850	1.137
seed 7	1.584	1.365	1.423	1.176	0.456	1.652	0.960	0.891	0.981	0.761	1.406	1.306	0.930	0.991	1.215	0.922	1.024	0.874	1.123	1.107	1.137
seed 8	1.499	1.349	1.611	1.377		1.414		0.643	0.895	1.433	1.163	1.432	1.274	1.029	0.811	0.100	0.347	1.062	1.227	1.263	
seed 9	1.544	1.254	1.506	1.545		1.335		0.913	1.142	1.217	1.568	1.283	0.938	0.972	1.033	0.843		0.861		1.160	

Table 25: *Root growth assay, third attempt.*

	DAY 3																				
	CONTROL	CONTROL	CONTROL	Cu	Cu	Cu	Cd	Cd	Cd	Ni	Ni	Ni	Mn	Mn	Mn	Pb BEFORE	Pb BEFORE	Pb BEFORE	Zn	Zn	Zn
column 1	NSUN5-1	NSUN5-2	COL-0	NSUN5-1	NSUN5-2	COL-0	NSUN5-1	NSUN5-2	COL-0	NSUN5-1	NSUN5-2	COL-0	NSUN5-1	NSUN5-2	COL-0	NSUN5-1	NSUN5-2	COL-0	NSUN5-1	NSUN5-2	COL-0
seed 1	1.519	1.733	1.260	1.590	1.239	1.155	0.987	0.885	0.728	1.646	1.458	1.027	1.058	1.020	1.143	0.552	1.287	0.934	1.237	1.356	0.767
seed 2	1.561	1.539	1.453	1.782	1.111	1.369	0.896	1.063	1.134	1.437	1.420	1.484	1.229	1.239	1.237	1.103	1.270	1.203	1.225	1.292	0.846
seed 3	1.192	1.546	1.359	1.647	1.094	1.418	0.934	1.167	0.934	1.494	1.718	1.092	0.547	1.113	1.182	1.198	1.103	1.166	1.221	1.357	1.177
seed 4	1.551	1.610	1.478	1.481	1.602	1.618	0.839	1.137	1.163	1.371	1.243	1.718	1.070	1.116	1.183	0.667	1.043	1.232	1.384	1.208	1.236
seed 5	1.570	1.534	1.584	1.599	1.412	1.577	0.885	0.579	1.161	1.323	1.278	1.163	1.031	1.360	1.559	1.188	1.014	1.161	1.317	0.967	1.257
seed 6	1.228	1.571	1.702	1.458	1.463	1.459	0.949	0.942	1.204	1.330	1.381	1.379	1.008	1.007	1.054	0.870	1.321	1.177	1.238	1.537	1.297
seed 7	1.568	1.667	1.804	1.260	1.536	1.473			1.185	1.237	1.124		1.057	1.034	1.093	1.021	1.030	1.287	1.407	1.463	1.113
seed 8	1.747	2.004	1.152	1.505	1.538	1.214			0.941	1.362	1.068		0.903	1.111		0.835	1.197	1.270	1.316	1.337	1.177
seed 9	1.336	1.635	1.252	1.251	1.487	1.630			1.003	1.173	1.270		1.061	1.058		0.574	1.140	1.103	1.127		0.385
column 2																					
seed 1	1.782	1.095	2.086	0.943	1.421	1.230	1.046	1.219	1.269	1.378	1.053	1.302	1.136	1.166	0.755	0.638	0.938	1.024	1.142	1.255	1.122
seed 2	1.521	1.843	1.443	1.122	1.379	1.312	1.041	0.956	1.306	1.162	1.436	1.046	1.065	0.821	1.037	0.885	1.258	1.055	1.216	1.119	0.973
seed 3	1.597	1.630	1.431	1.449	1.365	1.393	0.993	1.283	0.954	0.976	1.439	1.427	0.937	0.657	0.679	1.363	1.400	1.075	1.301	1.208	1.172
seed 4	1.361	1.606	1.447	1.483	1.503	1.532	0.895	0.943	0.896	1.357	1.438	1.397	1.015	1.184	1.210	1.080	1.271	1.341	1.456	1.244	1.193
seed 5	1.622	1.693	1.446	1.127	1.233	1.386	0.876	1.182	0.833	1.401	1.451	1.381	0.904	0.966	0.864	1.153	1.005	1.252	1.245	1.422	1.201
seed 6	1.598	1.844	1.579	1.357	1.679	1.543	0.754	0.851	1.197	1.300	1.106	1.297	1.041	1.154	1.021	0.965	1.126	1.388	1.243	1.263	1.129
seed 7	1.506	1.772	1.382	0.969	1.466	1.585	0.922	0.921	0.883	1.626	1.535	1.302	1.012	1.142	1.154	1.090	1.439	1.184	1.130	1.191	1.129
seed 8	1.516	1.783	1.591	1.358	1.466	1.459	1.020	0.988	0.924	1.381	1.536	1.413	0.839	1.150	1.219	1.041	1.168	1.368	1.077	1.277	1.026
seed 9	1.625	1.797		1.370	1.545	1.570	1.122		1.146	1.263	1.213	1.488	0.772	1.259	1.261	1.273	1.248		1.033	1.392	1.251
column 3																					
seed 1	1.323	1.554	2.089	1.333	1.370	0.938	0.886	0.544	1.124	1.266	1.095	1.571	0.939	1.134	0.527	1.196	1.224	1.119	0.894	1.165	1.418
seed 2	1.317	1.467	1.116	1.110	1.366	1.181	0.914	0.828	0.500	1.216	1.577	1.507	0.841	0.638	0.963	1.240	1.197	0.536	0.755	0.997	1.010
seed 3	1.557	1.464	1.525	1.373	1.017	1.062	0.604	0.745	1.009	1.247	1.432	1.713	1.047	0.735	1.019	1.077	0.918	0.727	1.084	1.116	1.147
seed 4	1.368	1.440	1.545	1.663	1.624	1.337	0.940	0.846	0.815	1.096	1.200	1.166	0.967	1.214	0.972	0.650	1.179	1.159	1.096	0.980	1.193
seed 5	1.315	1.441	1.575	1.237	1.352	1.509	0.746	0.898	0.944	1.332	1.392	0.725	0.511	1.001	0.948	1.143	1.032	1.188	0.639	1.209	1.043
seed 6	1.673	1.045	1.493	1.269	1.232	1.362	1.102		0.646	1.439	1.045	1.329	0.973	1.259		1.062	1.178	1.196	0.954	0.720	1.016
seed 7	1.517	1.600	1.510	1.312	1.566	1.222	0.643		0.722	0.971	1.407	1.416	0.875	0.592		1.046	1.204	1.170	0.754	1.042	0.636
seed 8	1.709	1.783	1.567		1.001	1.268	0.733		1.067	0.840	1.431	1.485	0.973	1.214		0.956	1.411	1.071	0.798	0.956	0.951
seed 9	1.469	1.659			1.547	1.595	0.992				1.473	1.434	0.960	1.046		0.794		0.908	1.033		0.939