Universitat de Girona

Facultat de Ciències

Memòria del Treball Final de Grau

Títol del treball:

Molecular study of ORPHEUS, a transcription factor potentially involved in (cell) expansion of Arabidopsis thaliana hypocotyls

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Abstract

Nowadays society faces a worrying energy sources shortage due to the growth of the world population and the rapid exhaustion of fossil fuel reserves. Renewable energy as plant biomass plays a central role to face the future, thus the increase in plant mass is an important target. Consequently, cell expansion mechanism, greatly contributing to biomass, is a current topic in scientific studies. Because of the suitable characteristics as a model plant, *Arabidopsis thaliana* was chosen in order to perform the present study. An interesting *Arabidopsis* mutant, *apollo*, was isolated at Professor Vissenberg's lab. Light-grown *apollo* shows a 4 times hypocotyl length increase over WT plants due to increased cell elongation. In this work we introduce ORPHEUS, a gene affected by the T-DNA insertion in *apollo*. Its study can provide useful knowledge about the mechanism/regulation of cell expansion.

Abstract

De hedendaagse wereld maakt zich op voor een zorgelijke energieschaarste door de groei van de wereldwijde bevolkingsaantallen en de snelle verdwijning van fossiele brandstofvoorraden. Hernieuwbare energie, zoals biomassa van planten, speelt een central rol in de toekomst. Om de biomassa van een enkele plant te vergroten kunnen mechanismen zoals celexansie bestudeerd worden. Vanwege zijn geschikte karakter als modelplant is *Arabidops thaliana* gekozen om deze moleculaire studie uit te voeren. Een interessante *Arabidopsis* mutant is *apollo*, die geïsoleerd is in het labaratorium van professor Vissenberg. *Apollo*, gegroeid in het licht, toont een 4 maal langer hypocotyl dan een wild type van deze plant, dankzij toegenomen celexpasie. In deze studie introduceren wij ORPHEUS, een gen dat beïnvloed wordt door de T-DNA insertie in *apoll*o. Het bestuderen van ORPHEUS kan nieuwe informative geven over het mechanisme en de regulatie van celexpansie.

Extracte

Avui dia la societat ha de fer front a una escassetat de fonts d'energia degut al creixement de la població al món i a l'esgotament imminent dels combustibles fòssils. Com a energia renovable, la biomassa vegetal, juga un paper central de cara al futur. Per augmentar la quantia de biomassa per planta, mecanismes com l'expansió cel·lular són força estudiats. La planta *Arabidopsis thaliana*, per les seves innegables característiques com planta model, és utilitzada en aquest treball per realitzar el present estudi molecular. Un mutant d'interès, *apollo,* va ser aïllat en el laboratori del Professor Doctor Vissenberg. A causa de mecanismes d'elongació cel·lular, les plantes *apollo* desenvolupen el seu hipocòtil 4 vegades més que les silvestres. En aquest treball presentem ORPHEUS: un gen afectat per la inserció del T-ADN en *apollo*. El seu estudi pot proporcionar coneixements útils sobre el mecanisme / regulació de l'expansió cel·lular.

Extracto

Hoy en día la sociedad se enfrenta a una preocupante escasez de fuentes de energía debido al crecimiento de la población a nivel mundial y a la disminución imparable de las reservas de combustibles fósiles. La biomasa vegetal como una energía renovable juega un papel central de cara al futuro. Para aumentar la masa de la planta, y así mejorar la cuantía de biomasa por planta, mecanismos como la expansión celular son estudiados. *Arabidopsis thaliana*, por sus innegables características como planta modelo, es utilizada en este trabajo para realizar el presente estudio molecular. Un mutante de interés, *apollo,* fue aislado en el laboratorio del Profesor Doctor Vissenberg. Debido a mecanismos de elongación celular, las plantas *apollo* desarrollan su hipocotíleo 4 veces más que las silvestres. En este trabajo presentamos ORPHEUS: un gen afectado por la inserción de T-ADN en las plantas *apollo*. Su estudio puede proporcionar conocimientos útiles sobre el mecanismo / regulación de la expansión celular.

Content

1. Introduction

It is expected that the world population will triple between 1950 (2.5 billion) and 2020 (7.5 billion). Current estimates indicate that we need to increase food production by 70% in the next 40 years. Recent reports form the FAO (Food and Agriculture Organization of the United Nations) expose that the number of people in the world who are chronically hungry crossed the one billion mark in 2009 (FAO, 2015). Thus, food and energy production are the main challenges the world should deal with. Renewable sources as plant biomass can potentially provide liquid fuels, chemicals and materials that generate less carbon emissions than petroleum (some carbon emitted by the fuel is the carbon absorbed for the plant). Therefore plant growth (specifically cell expansion, to increase biomass) studies may be increased to provide applied knowledge to boost renewable energy and food fields.

Arabidopsis thaliana is a small flowering plant member of the mustard (Brassicaceae) family and is widely used as a model organism in plant biology. Although its agronomic importance is limited it offers important advantages for basic research in genetics and molecular biology (TAIR). Its importance lies not only in its small size, short generation time (approximately six weeks) or the fact that you can obtain thousands of seeds obtained from each plant (Haughn and Kunst., 2010), but also in the small size of its genome with five chromosomes (Leutwiler et al., 1984) that is ideal for genetic mapping and has facilitated the complete sequencing (Arabidopsis Genome Initiative, 2000). In addition its transformation with *Agrobacterium tumefaciens* is simple and efficient which is an advantage not only in itself, but also has allowed the creation of an important collection of T-DNA insertion mutants (Birch, 1997; Krysan et al., 1999).

Plant growth is defined as an irreversible increase in mass and size. In non-mature plant organs, increasing size is mainly due to cellular processes: proliferation and expansion. In the first phase, proliferation, the cytoplasm increases in mass until a certain value when the cell is divided mitotically. In this process, the average cell size remains constant, because the cellular growth is coordinated with cell division. However, in the expansion phase, the organ grows

because the cells become larger. This expansion process occurs because the turgor pressure extends the cell wall thanks to the uptake of water that comes through semipermeable membranes by osmosis. In parallel, two main slow phenomenons occur: wall loosening and cell wall tightening. Some agents have been identified as the responsible of cellulose microfibril movements in the cell wall: hydrolases, transglycosylases, expansins and ·OH radicals (Roberts, 2007).

Cell expansion is crucial for plant growth and morphogenesis, therefore it is regulated by important internal and external factors, such as phytohormones and light exposition. In the *Arabidopsis* seedling, this process is decisive for cotyledon expansion and leaf development and it is the main responsible for the postembryonic growth in etiolated hypocotyls (with almost no contribution from cell division). That is the reason why the *Arabidopsis* hypocotyl is widely used as a model for physiological studies of the mechanism of cell elongation and its control. (Azpiroz et al., 1998; Gendreau et al., 1997). Cell expansion in the dark-grown hypocotyl occurs in two phases: at first, all cells have the same slow elongation rate but, after 48 hours, it has been observed that a growth the acceleration starts from the base and it goes towards the apex over time (Refrégier et al., 2004; Gendreau et al., 1997).

As is shown in the picture underneath (Fig. 1), the transversal section of the hypocotyl consists of concentric layers. The external layer is the epidermis that is in contact with the cortical cells. Within the inner cortex there is the endodermis, the pericycle and, located at the centre, there is the stele containing the vascular tissue (Derbyshire et al., 2007; Boron and Vissenberg, 2015).

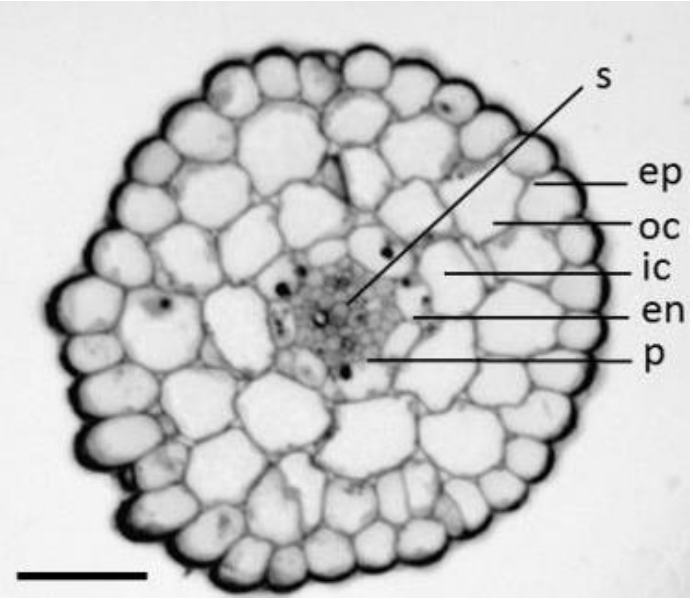

Figure 1. Etiolated hypocotyl Black and white bright-field picture of a toluidine blue-stained cross-section through an etiolated *Arabidopsis thaliana* hypocotyl, with reference to cell types (s=stele, ep= epidermis, oc= outer cortex, ic= inner cortex, en = endodermis, p = pericycle). Scale bar is 50 µm (Boron and Vissenberg, 2015)

The lab of Professor Vissenberg has recently isolated a mutant *apollo* that shows abnormal hypocotyl elongation (Fig. 2). The insertion position of the T-DNA was located in between two anti-parallel genes, one coding for a transcription factor called ORPHEUS (Fig. 3) and a second one important for seed development, LINUS. The expression of both genes was disturbed because of the T-DNA insertion (PhD Dr. Agnieszka Boron).

Figure 2. *Apollo* phenotype *Apollo* mutant grown in the light with non-apollo (=WT) seedlings.

As the transcription factor ORPHEUS can influence the expression of many downstream genes, it is worth studying it further, which is the objective of this thesis.

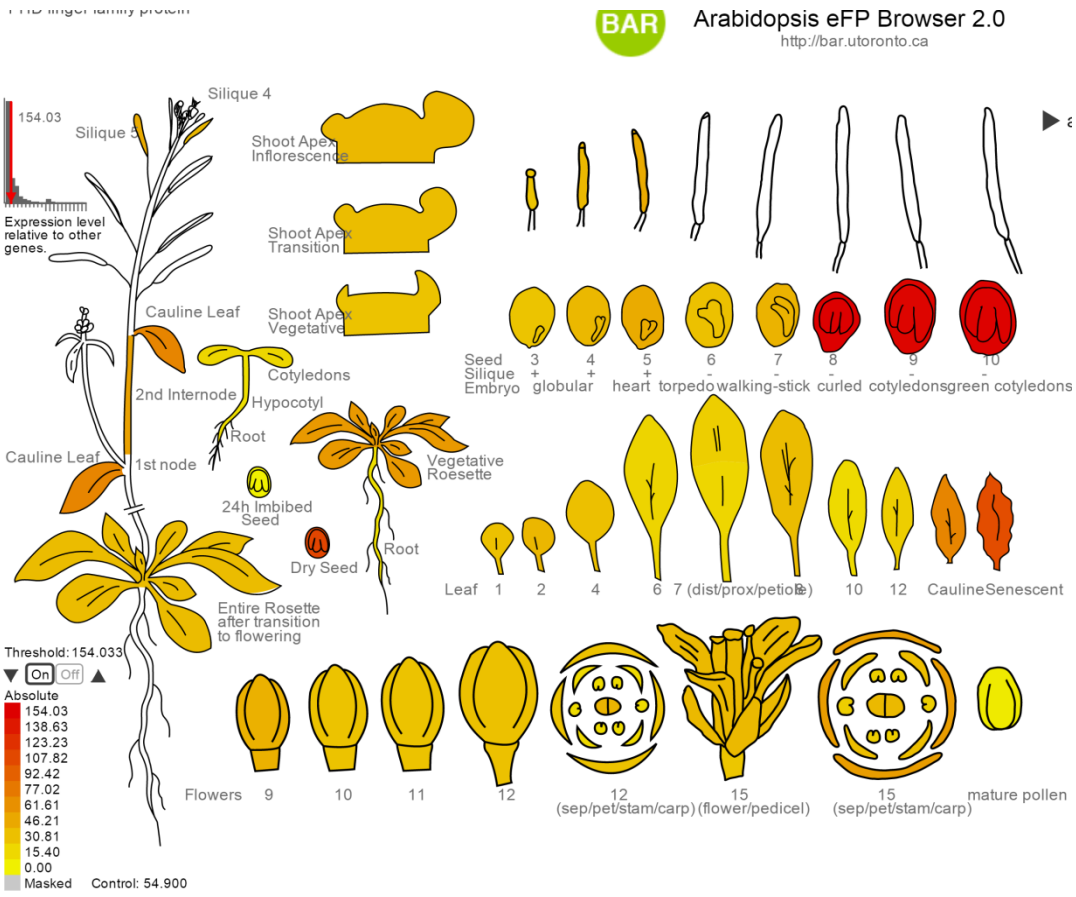

Figure 3. Expression pattern of ORPHEUS in *Arabidopsis* visualized with eFP Browser. It shows expression in most tissues and organs except roots and old siliques. The highlight expression seems to be in the more final stages of embryo formation and dry seeds.

2. Objectives

The goal of this project presented is to increase the knowledge on the regulation of plant cell elongation, as it plays a key-role in the primary production of biomass. The investigation of cell elongation was focused on *Arabidopsis thaliana* hypocotyls, as their growth occurs only through cell elongation.

As is mentioned above, the *apollo* mutant had a striking hypocotyl phenotype in the light and it thus forms a very useful object to study cell elongation and its control. The genes affected by the T-DNA insertion in *apollo* are poorly studied, and it is therefore useful to know the role of these genes in the regulation of cellular elongation. In order to do so, the aim is to phenotype hypocotyl growth in *apollo* and to create, as far as it's possible during the project time, transgenic lines of one of the identified genes in *apollo* coding for a transcription factor called ORPHEUS, allowing for example the precise localisation in time and space of gene expressions (ProORPHEUS::GFP-GUS), the measurement of the effect of overexpression on hypocotyl growth (CaMV35Sprom::ORPHEUS), and the subcellular localization of the final protein (CaMV35Sprom::GFP::ORPHEUS, CaMV35Sprom::ORPHEUS::GFP). As this procedure takes longer than the time I could spend in the lab, I could analyse other transgenic lines that were already available in the lab with the techniques that I should have used with my own lines (confocal microscopy to detect GFP and GUS-staining followed by bright-field microscopy for the promoter::GUS lines). By doing so, I would have done the whole set of procedures/experiments.

In summary the goals of the present work have been:

- To analyse the phenotypic effect of the T-DNA insertion of the hypocotyls length in *apollo* mutants
- To create transgenic lines of ORPHEUS promoter fused to reporter genes GUS and GFP
- To identify knock-out lines from ORPHEUS and LINUS

- To determine protein cell localization of At1g70990 in the roots and hypocotyls by confocal microscopy
- To check the localization of MAIA in the seedling by GUS staining.

3. Materials and methods

3.1 Plant material and growth conditions

Wild type *Arabidopsis thaliana* seeds (Col-0) were obtained from the European Arabidopsis Stock Centre (NASC, UK). For surface sterilization seeds were placed for 5 minutes in 6% commercial bleach diluted in 100% EtOH followed by two rinses in 100% EtOH. For most experiments, plants were grown on half-strength Murashige ans Skoog solidified medium (Duchefa, The Netherlands). All experiments were carried out in growth rooms (1.5E+15 photons cm-2 s-1, 16h light/8 h dark, 22ºC). Seeds were stratified at 4ºC for 3 days before transfer to the growth rooms.

For selection of tranformants 50 µg/mL kanamycin was added to the ½ MS medium after autoclaving.

For measurements of dark-grown hypocotyls, seeds were sown on solid ES medium (Estelle and Sommerville, 1987) and stratified for 3 days at 4º C. The synchronous germination was induced by exposure to fluorescent white light (150 mmol m–2 s –1 True Light; Philips, Eindhoven, The Netherlands) for 4 hours at 21 ºC. The transfer to light is referred to as time zero for all the experiments. Darkness was obtained by wrapping the Petri dishes in four layers of aluminium foil. Covered plates were afterwards placed vertically at 21ºC in an environmentally controlled growth cabinet.

Seedlings for GUS staining and GFP fluorescence detection in light grown conditions were grown in Gilroy medium (Wymer et al., 1997). However, the seedlings for GFP detection grown in dark conditions were sowed in ES medium.

3.2 Molecular cloning

All constructions were generated using the Gateway system (Life Technologies, http://www.lifetechnologies.com/).

Table 1. Oligonucleotides used to amplify the inserts of interest. The fractions of the sequences distinguished in red belong to attB recombination sites for the Gateway system.

Primer description	Sequence	Alias
ORPHEUS Promoter region	For 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTGAAGAGAAGGCGTTGGCAAT-3'	LP.PRO.Orph
	Rev 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCCCATATATCCTCACCCCAACA-3'	RP.PRO.Orph
ORPHEUS	For 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGCCTTTCACGTAGCTTGTC-'3	LP.GEN.Orph
	Rev 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAGCTCCTTGACCTCTTTTGC-'3	RP.GEN.Orph
	Rev (no STOP codon)5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCGCTCCTTGACCTCTTTTGCTT- '3	RP.NOSTOP.Orph

For the promoter:: reporter gene analysis a 1556 bp sequence upstream of the start codon of ORPHEUS was PCR amplified from Col-0 genomic DNA using Platinum high fidelity DNA polymerase (Life Tehnologies). The sequence of the gene (3782bp) and the gene without the STOP codon (3779bp) were also amplified by high fidelity PCR. The primers in the table above (Table 1) were used for the mentioned polymerase reactions. All the nucleotides were obtained from Eurogentech (Seraing, Belgium).

The PCR product was subcloned into pDONR 201 vectors (Invitrogen) by BP reaction and chemically competent *E.coli* cells (Invitrogen) were transformed with the product. To verify the transformants a colony PCR was done and subsequently DNA sequencing was done to check the nucleotides at VIB (Flemish Institute of Biotechnology, Antwerp) thanks to M13 flanking regions present in the vector.

Clones with the promoter found to have a correct DNA sequence were subsequently recombined by LR reaction with the destination vector pGWB3 and pGWB4, containing β-glucuronidase and GFP reporters respectively (Nakagawa et al., 2007). The resulting clones were checked by colony PCR and then used to electrophorate *Agrobacterium tumefaciens* (ElectroMAX™ LBA4404 Cells, Invitrogen).

Due to several problems to get an amplicon from the previous colony PCR, some additional polymerase reactions were done. Kanamycin, GFP and AttL recombination sites primers were

used. Moreover, to confirm the presence of the inserts two pairs of different specific primers for the promoter regions were ordered (Table 2).

Table 2. Additional primers used for checking the cloning process.

	Sequence	Alias	Annealing temperature, amplifX (ºC)	Amplified fragment size (nt)
ORPHEUS Promoter region	For 5'-GGCTGGTT CCAATAATAATTGGC-3'	2.LP.Pro.08020	52	827
	Rev 5'-GAGTAGCCA AGTCTTCCTTTACG-3'	2.RP.Pro.08020		
	For 5'-AGTTTGGAGC CCAAATGTATC-'3	2.LP.2.Pro.08020	51	956
	Rev 5'-CCCATATATC CTCACCCCAACA-'3	2.RP.2. Pro.08020		
AttL recombined sides	For 5'-TCGCGTTAAC GCTAGCATGGATCTC-'3	AttL 1	50	1816
	Rev 5'-GTAACATCAG AGATTTTGAGACAC-'3	AttL 2		
GFP	For 5'-CACATGAAG CAGCACGACT-'3	LP.sGFP	58.3	381
	Rev 5'-TGCTCAGG TAGTGGTTGTCG-'3	RP.sGFP		
Kanamycin	For 5'-TCATTTCGA ACCCCAGAGTC-3'	NptII.LP	50	~1000
	Rev 5'-GCGTTCAA AAGTCGCCTAAAG-'3	NptII.RP		

3.3 Plant Transformation

After checking the clones the transformation into *Arabidopsis thaliana* was carried by floral dip as described in Clough & Bent in 1998 using a transformation buffer containing 5% sucrose, MgCl2·6H2O (4mM) and 0.02% (v/v) Silwet L-77 (polyalkylenoxide modified heptomethyltrisiloxane) (Fig. 4).

Figure 4. This picture shows the transformation performed by floral dip in *Agrobacterium* solution.

3.4 Phenotypic analysis

Pictures of Petri plates containing etiolated *apollo* and WT seedlings were taken at day 8 using a digital camera (Canon, 50D). Then the hypocotyls were measured with the software ImageJ (available at http://rsbweb.nih.gov/ij/). Three sets of replicates with 10 *Apollo* and WT seeds on each were analysed.

3.5 Identification of knock-out lines

The knock-out (KO) lines (Table 3) were ordered from the Nottingham Arabidopsis Stock Centre (NASC). These mutant lines have a T-DNA insertion in the promoter region of the antiparallel genes affected in *apollo*. The seedlings were grown on half-strenght Murishige and Skoog medium (Duchefa, The Netherlands) supplemented with kanamycin (50 µg/mL) and their leaves were collected for DNA extraction. The presence of the T-DNA insertion and zygosity was verified by PCR using a T-DNA border LBb1.3 primer (5'-ATTTTGCCGATTTCGGAAC-3') and a pair of specifics primers for each SALK line (Table 3).

Table 3. Identity of the mutants and their primers for the identification of homozygous SALK lines.

SALK alias number	NASC alias number	LEFT Primer	RIGHT Primer	Annealing temperature (amplifx)	Amplified fragment size (nt)	
SALK_1	N65	CAAAGACGACAAAATTCCCAC	CGTGAGTGCGTAGAGAGAACC	53	1119	SALK lines from LINUS
SALK_2	N53	TGACACCAGATTCAAACCTCC	AACGTTTCGGGGAGATTTATG	52	1164	SALK lines from ORPHEUS
SALK_3	N66	TAGACAAGTGTTTTGCTCGGG	CATCCCATATATCCTCACCCC	51	1195	
SALK_4	N60	ACGTGTTGTGTAGGGTCCTTG	GGTGAGCTCTGTGAGTTTTGG	52	1169	

3.6 GUS-Staining

GUS activity staining was performed according to a modified protocol of Jefferson *et al*. (1987). Pictures were taken using a macroscope (Nikon AZ-100) equipped with a digital camera (Nikon DS-Ri1).

3.7 Confocal microscopy

Seedlings containing the construct Promoter$_{At1g07990}$::GFP from the PGO laboratory were sown on Gilroy medium. The fluorescence was checked at days 3, 5, 7 and 12 with a Nikon D-Eclipse C1 confocal microscope. Counter staining was performed by dipping the seedlings in propidium iodide (0, 1 mg/ml).

4. Results

4.1 Cloning and identification of ORPHEUS

High fidelity PCR was performed (Fig. 5) in order to isolate the promoter (1566bp), the gene (3782bp) and the gene without stop codon (3779bp) of ORPHEUS from an A. *thaliana* DNA sample (Col-0). As shown in figure 3 the fragments were also amplified by standard PCR (GoTaq®), to clearly visualize the amplicons.

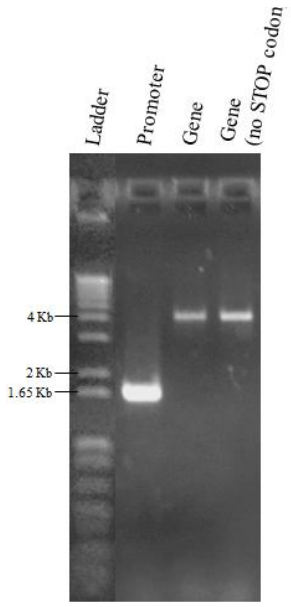

Figure 5. A green dye stained agarose gel showing DNA fragments produced by PCR amplification of ORPHEUS promoter and gene. The first lane contains a 1Kb plus DNA ladder (Invitrogen).

Once the BP reaction was completed, the pDONR201 was transformed into *E.coli* Bacteria were grown up, followed by a colony PCR (Fig. 6).

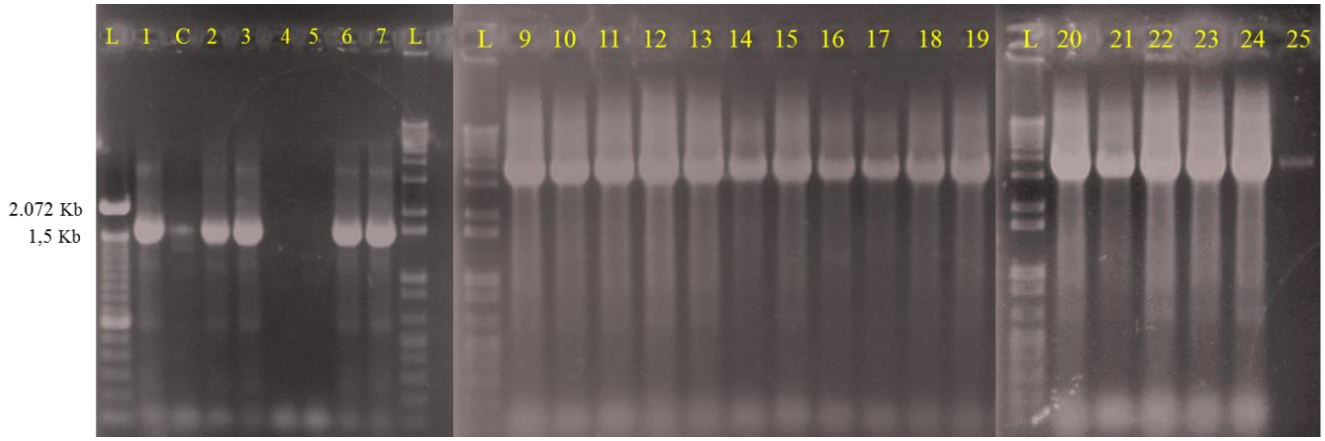

Figure 6. This stained agarose gel presents DNA fragments from transformed colonies. Colonies from 1 to 7 correspond to ORPHEUS promoter, the whole gene is present in colonies 9 to 24 and the gene without STOP codon is present in the last lane (25).

Correct clones were chosen and then were grown to extract the plasmids, which were sent to the VIB Sequencing Facility (University of Antwerp, Belgium) for sequencing. The results were aligned with BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the chromatogram results were read with Chromas Lite (http://technelysium.com.au/?page_id=13). This was done to identify those colonies that contained no basepair errors.

Due to lack of time and the vastness of the gene the complete sequencing of the gene and the gene without the STOP codon was not carried out. Even though, the vector containing the promoter was subcloned by LR reaction into two different types of pGWB vectors. *E.coli* competent cells were transformed with pGWB3 and pGWB4 vectors containing the promoter region. Colony PCR was performed to check which carry the insert (Fig. 7).

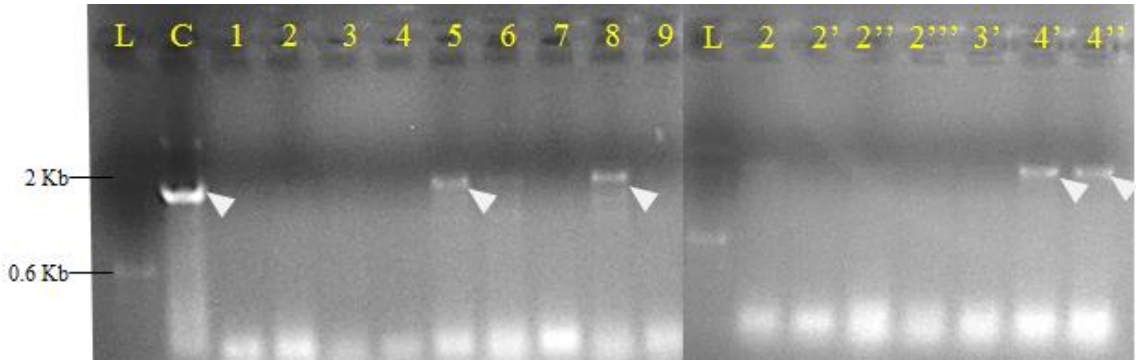

Figure 7. Stained agarose gel (with Midori Green dye, Nippon Genetics) showing amplicons from the promoter (1566bp) contained in pGWB3 (nº 4', 4'') and pGWB4 (nº5, 8) vectors. The numbers correspond to different *E.coli* colonies and the control is the sample from the vector pDONR 201 with the insert that was used for the LR reaction.

Plasmid DNA was extracted from the colonies that present a clear amplicon for the promoter. Those samples were measured by Nanodrop spectrophotometer (Table 4) to determine the suitable concentration for *Agrobacterium* transformation (no more than 100 ng/µl).

Table 4. Concentration measurements with Nanodrop of plasmid DNA extracted from *E. coli* colonies transformed with pGWB vectors with an insert.

DNA from colony number	Concentration (ng/µl)	260/280 quality ratio	260/230 quality ratio
4'	556.1	1.85	2.10
4''	593.0	1.90	2.12
5	580.2	1.85	211
8	447.3	1.86	2.05

After the transformation of electrocompetent Agrobacterium bacteria the colonies (Fig. 8) were analysed by colony PCR. In the gel any amplicon was visualized. Thence it was checked the plasmids extracts from the *E.coli* cultures again (Fig. 9).

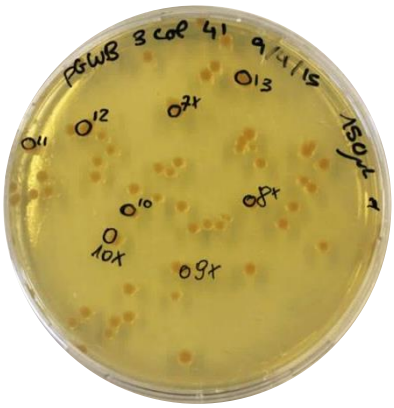

Figure 8. This picture shows a petri dish with LB agar medium containing spread *Agrobacterium tumefaciens* bacteria transformed with pGWB3 vector with ORPHEUS promoter as an insert.

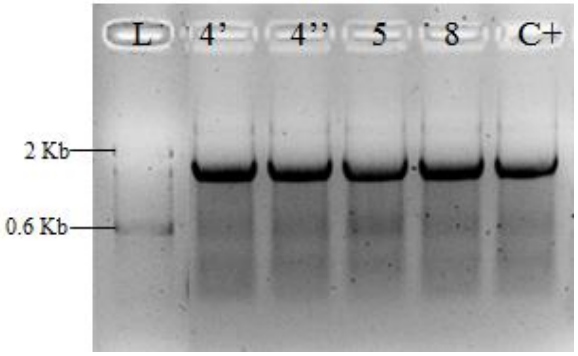

Figure 9. In this agarose gel there are amplicons correspond to the promoter region amplified with RP.PRO.Orph and LP.PRO.Orph. The control contains the product of BP reaction.

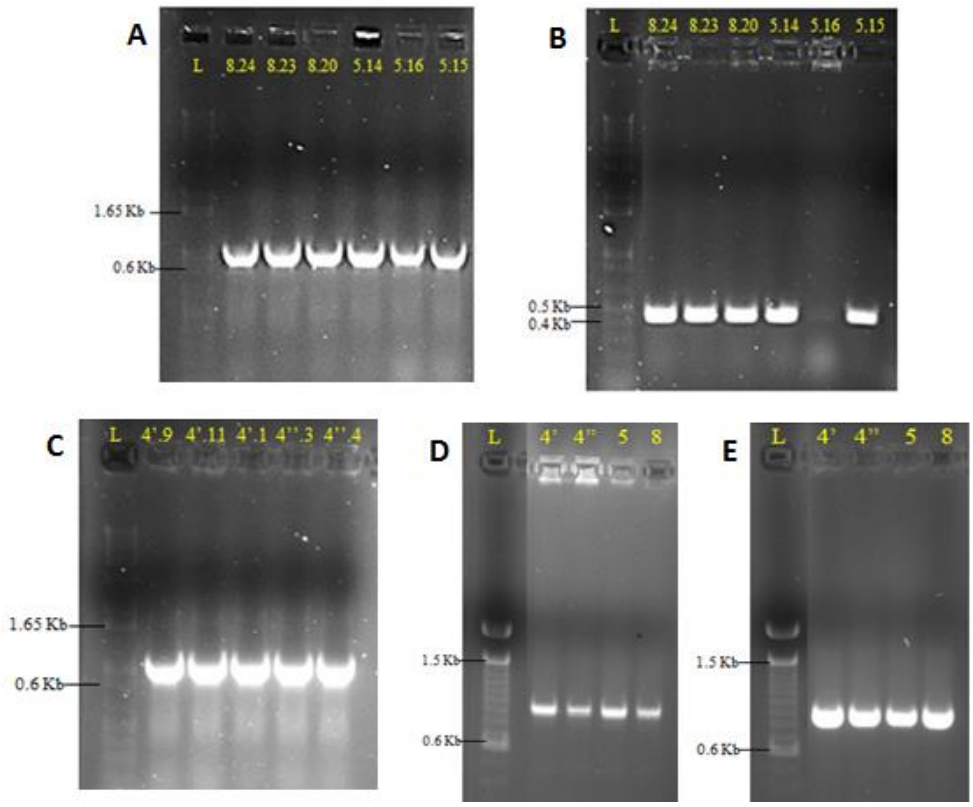

Figure 10. Agarose gels of *Agrobacterium* colony PCRs for amplification of: **A)** Kanamycin region of pGWB4 with NptII.LP and NptII.RP primers **B)**GFP gene from pGWB4 using LP.sGFP and RP.sGFP primers **C)** Kanamycin region of pGWB3 vector with the primers NptII.LP and NptII.RP **D)** ORPHEUS promoter region using 2.LP.Pro.08020 and 2.RP.Pro.08020 primers **E)** promoter region applying 2.LP.Pro.**2**.08020 and 2.RP.Pro.**2**.08020 primers

More PCRs were performed in order to make sure the correct insert is present in *Agrobaterium* (Fig 10).

Once confirmed that the contructs were correct the agrotransformation of WT *Arabidopsis thaliana* plants (T0) was performed to obtain the parental line that carry the T1 seeds (Fig. 11). It is not necessary to create more filial lines because the promoter is expressed altough the plant is heterozygous for the insertion.

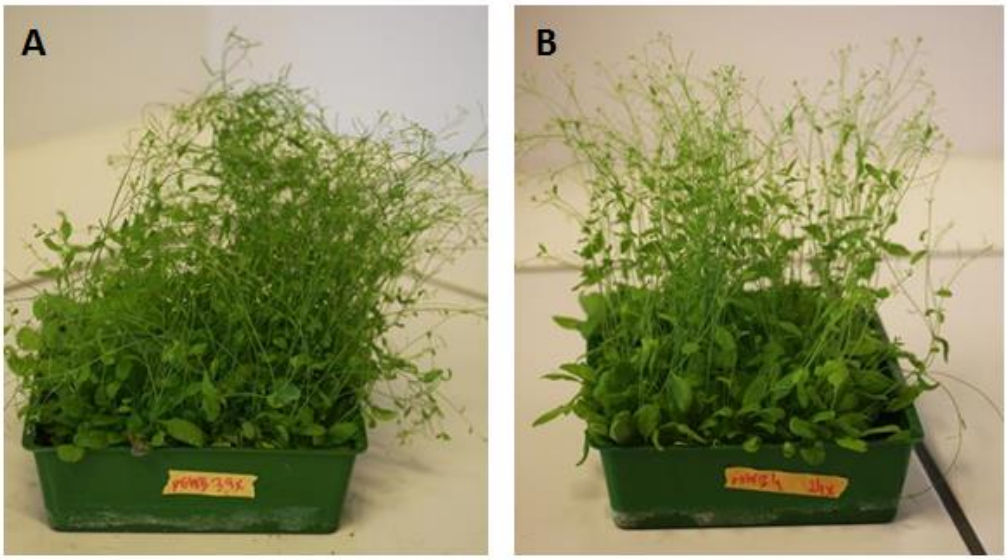

Fig 11. ***Arabidopsis thaliana*** **plants after agrotransformation. A)** Transformed with pGWB3 constructs (containing prom::GUS) **B)** Transformed with pGWB4 construct (containing prom::GFP)

The seeds (T1) were collected and were sowen on medium containing kanamycin. Several transformants for the pGWB4 construct were obtained (Fig. 12), although the ratio was low.

Fig 12. T1 germinating plant after being transformed with the pGWB4 vector containing as a insert the promoter region from ORPHEUS

4.2 Phenotypic analysis of apollo

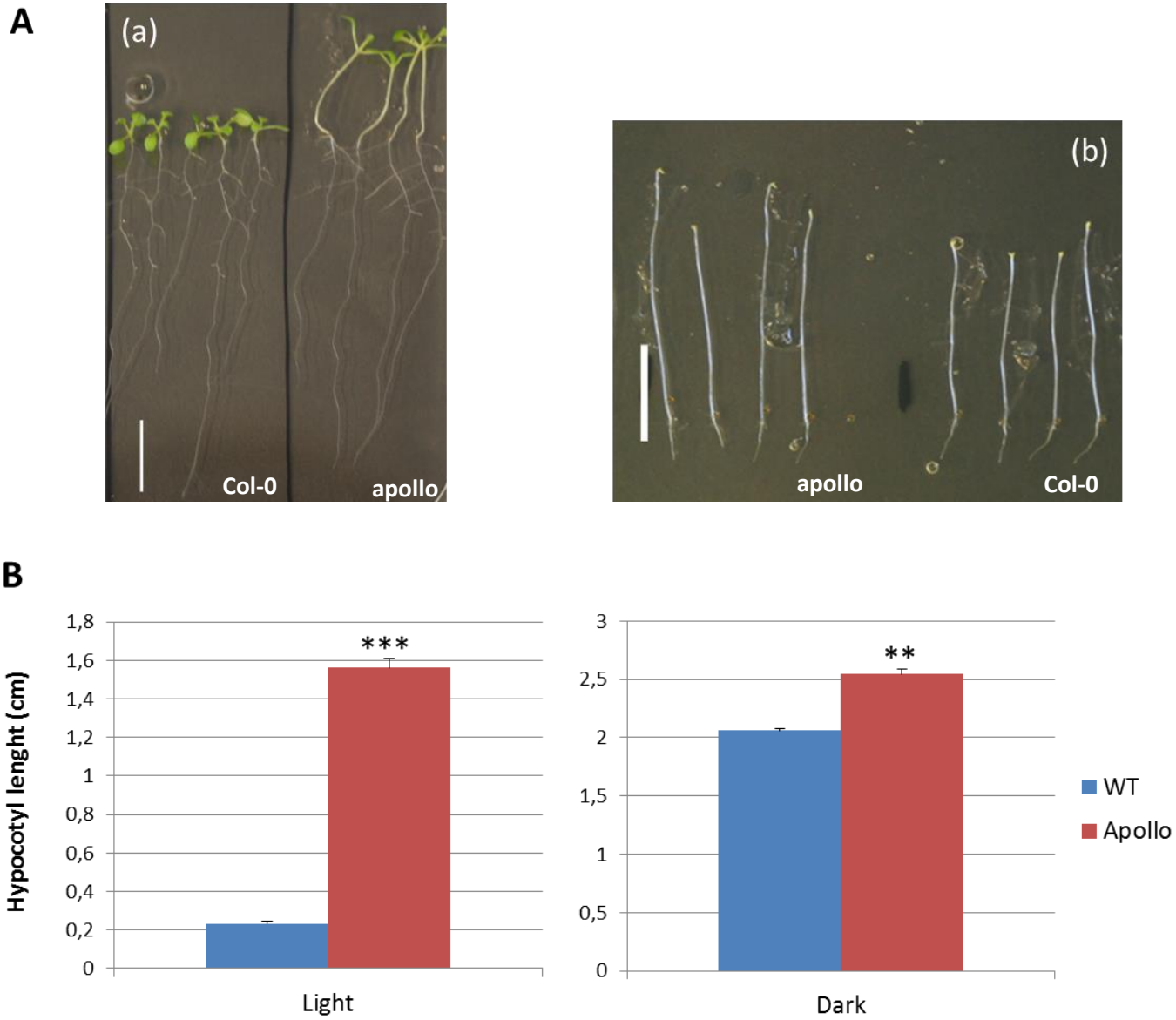

Figure 13. Light and dark-grown hypocotyl length in wild type and apollo.
A) Wild-type (Col-0) and apollo *Arabidopsis thaliana* seedlings were grown in the light (a) on ½ MS medium and in the dark (b) on ES medium for 8 days. Scale bar is 1 cm **B)** The length of the hypocotyl was measured at day 8. Three biological replicates were measured. Asterisks indicate significant differences (Student's t test, two tailed, $P < 0.001$).

In order to determine the phenotypic effect of *apollo* on hypocotyls a lenght study has been performed on etiolated and non-etiolated hypocotyls (Fig. 13). The T-DNA insertion in the promoter region of LINUS and ORPHEUS genes generates an abnormal hypocotyl elongation, which is more significant on non-etiolated hypocotyls.

4.3 knock-out lines from LINUS and ORPHEUS

PCR results from genotyping (Fig. 14) shows that not all the SALK lines plant analysed contain the T-DNA insertion. To identify the homozygote lines for the T-DNA three different primers have been used (Section 3.5). If the line is homozygote for the T-DNA insertion the lane show amplicon when the T-DNA primer is used and it doesn't show amplicon when the WT endogen primers are applied as it occurs in the picture A, lanes 1 and 2 (Fig. 14). Lines N53 and N65 are homozygote for the T-DNA. However, lines N60 and N66 are not knock-out lines.

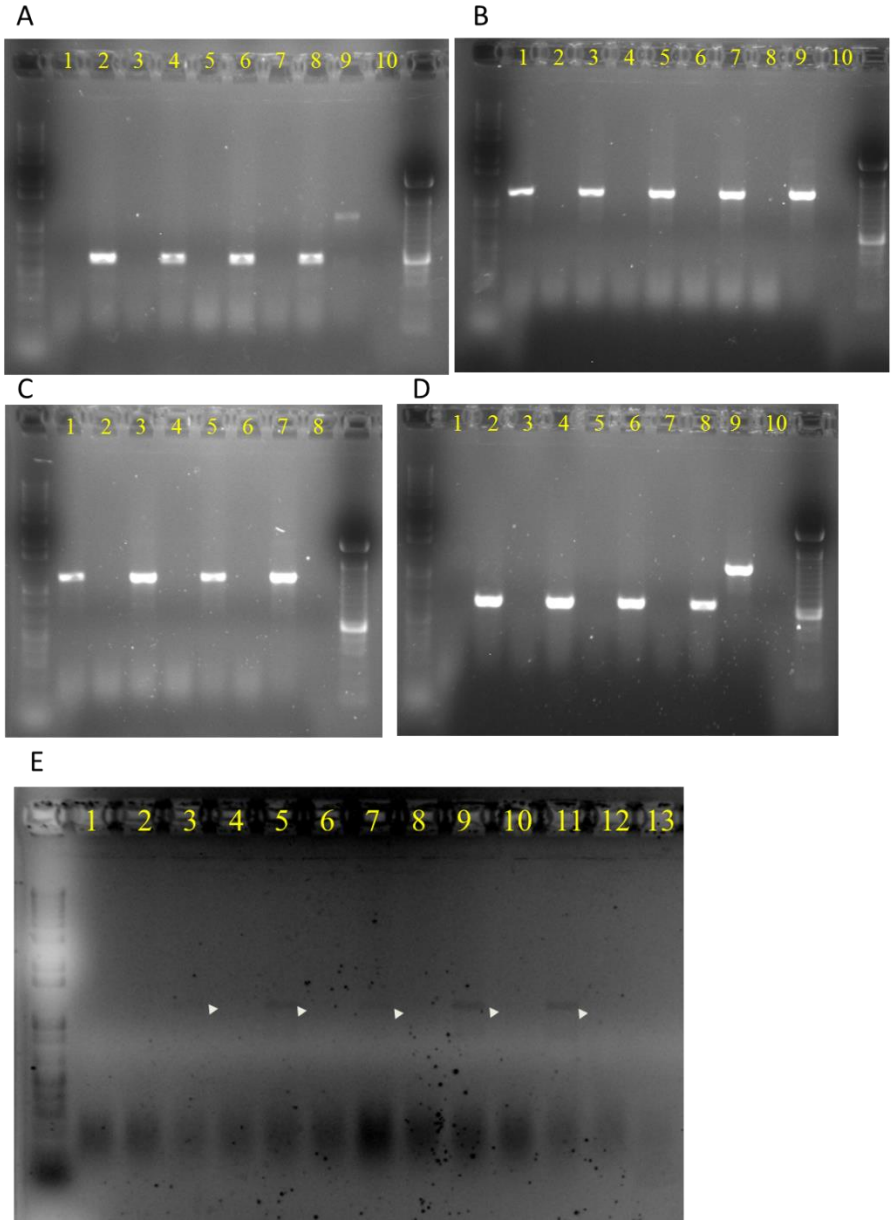

Figure 14. Agarose gels from the genotyping study of the SALK lines plants from LINUS (N65) and ORPHEUS (N53, N60, N65, N66).
Picture nomenclature:
Line – plant number or negative control (C*) -- primers used: left (L), right (R) or T-DNA primer (T)**

**primers indicated in the section 3.5

A)
1. N53-1-LR
2. N53-1-RT
3. N53-2-LR
4. N53-2-RT
5. N53-3-LR
6. N53-3-RT
7. N53-4-LR
8. N53-4-RT

B)
1. N60-1-LR
2. N60-1-RT
3. N60-2-LR
4. N60-2-RT
5. N60-3-LR
6. N60-3-RT
7. N60-4-LR
8. N60-4-RT
9. N60-WT-LR
10. N60-C*-LR

C)
1. N60-5-LR
2. N60-5-RT
3. N60-6-LR
4. N60-6-RT
5. N60-7-LR
6. N60-7-RT
7. N60-WT-LR
8. N60-C*-LR

D)
1. N65-1-LR
2. N65-1-RT
3. N65-2-LR
4. N65-2-RT
5. N65-3-LR
6. N65-3-RT
7. N65-4-LR
8. N65-4-RT
9. N65-WT-LR
10. N65-C*-LR

E)
1. N66-1-LR 2. N66-1-RT
3. N66-2-LR 4. N66-2-RT
5. N66-3-LR 6. N66-3-RT
7. N66-4-LR 8. N66-4-RT
9. N66-5-LR 10. N66-5-RT
11.N66-6- LR 12.N66-6-RT
13.N66-WT-LR

4.4 Identification of promoterAt1g70990::GFP expression

Green Fluorescent Protein (GFP) has became a powerful tool for visualising structures and processes in living cells and organisms since its clonation (Dimitry et al., 2010). The lab of Professor Vissenberg has generated transgenic Arabidopsis plants containing At1g70990 prom:: GFP contruct. Here the GFP is present where the gene is normally expressed, enabling the localization were At1g70990 is expressed.

At1g70990 codifies for a proline-rich family protein. Its expression shows differences depending on light grown conditions. In dark-grown hypocotyls the expression is present in all types of cells. However, the pattern in the root is different; GFP is visible in the vascular tissue, pericycle and, with less intensity, in the cortex (Fig. 16). Hypocotyls developed in light show expression in the epidermis and the cortex only. Apparently, in the roots the fluorescence is visible in the vascular tissue, the pericycle and the cortex. (Fig. 15).

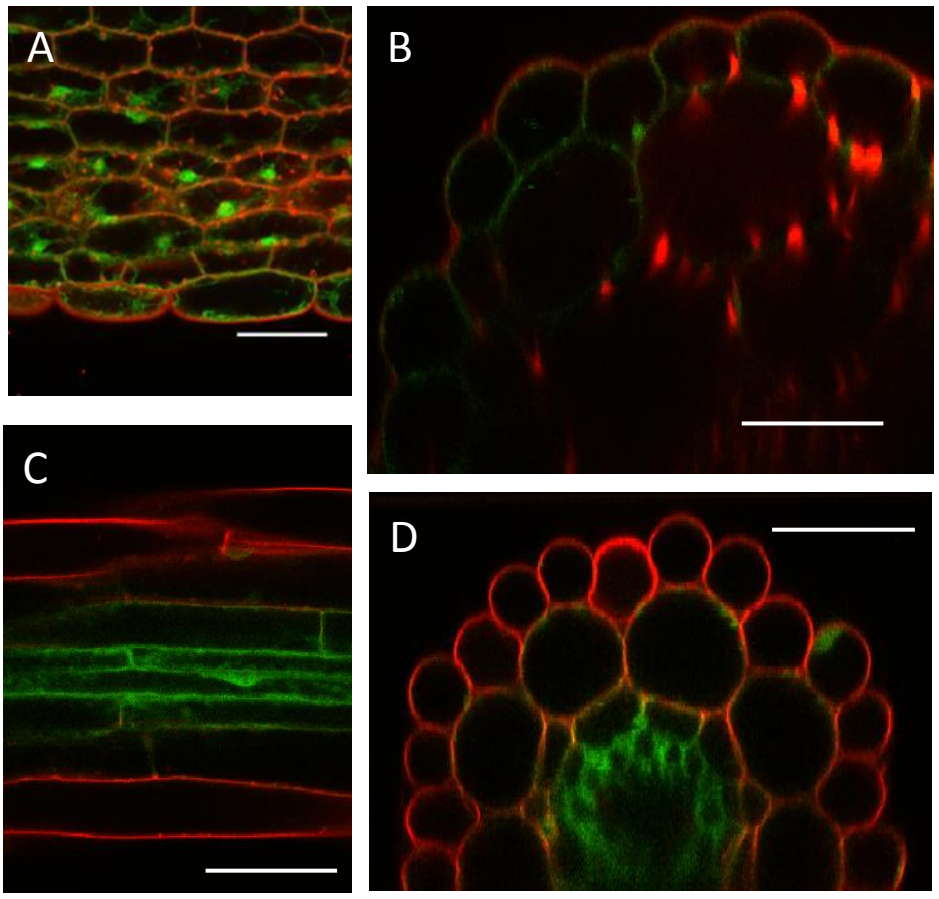

Figure 15. Confocal images of light-grown Arabidopsis seedlings expressing At1g07790prom::GFP.

A) Hypocotyl longitudinal section of 3-days-old seedling. This picture shows the epidermal layer **B)** Cross-section of 7-days-old hypocotyl. **C)** Longitudinal section of the root of a 7-days-old seedling. **D)** 7-days-old cross-section of the root. Cell walls are stained with propidium iodide. All scale bars: 50µm

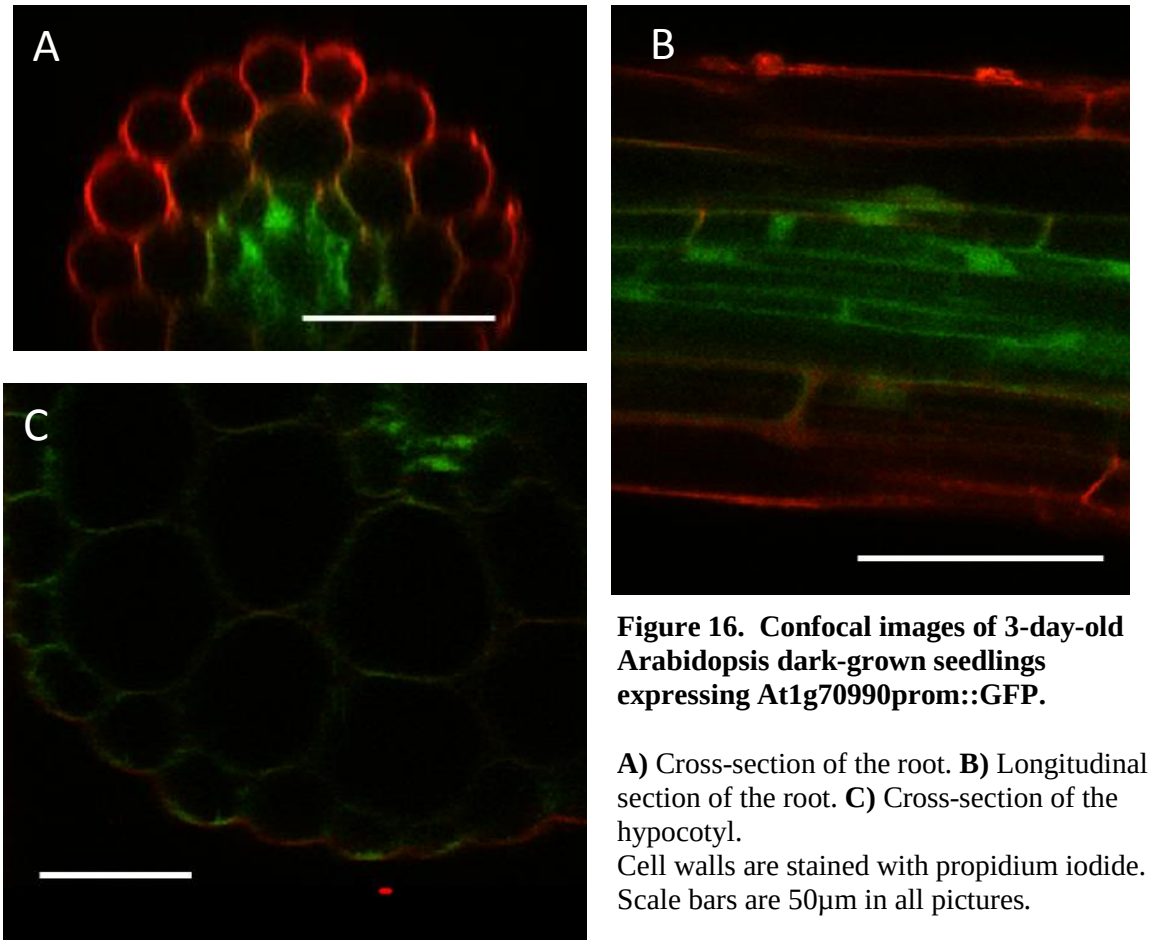

Figure 16. Confocal images of 3-day-old Arabidopsis dark-grown seedlings expressing At1g70990prom::GFP.

A) Cross-section of the root. **B)** Longitudinal section of the root. **C)** Cross-section of the hypocotyl.
Cell walls are stained with propidium iodide. Scale bars are 50µm in all pictures.

4.5 MAIA expression analysis on seedling

Characterized by PhD Dr. Daria Balcerowicz, MAIA is a serine/threonine kinase, more specifically a malectin/receptor-like protein kinase. Transgenic plants bearing MAIA promoter-reporter gene GUS were created at Professor Vissenberg´s lab with the purpose to examine the tissue-specific expression pattern of this gene of interest. The histochemical GUS staining confirms again that MAIA is expressed in the root (Fig. 17), and more precisely only in the trichoblasts cells.

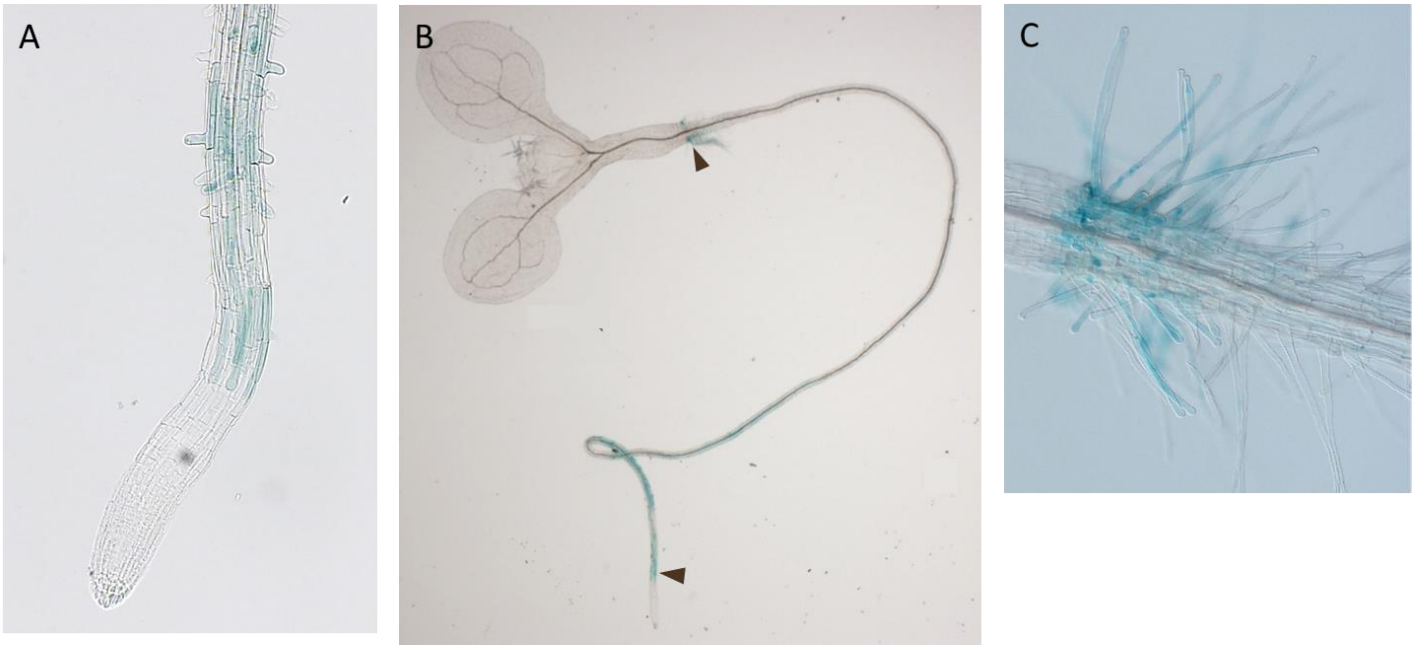

Figure 17. Histological localization of MAIA promoter::GUS expression.
6 days grown seedlings were bathed on β-gluconorosidase substract. **(B)** The product indicates that MAIA is only expressed in early root hair cell files. **(C)** as shown on the upper and the bottom **(A)** part of the root.

6. Discussion

Nowadays traditional energy sources (as fossil resources) don't correspond to the world's energy demand since they are running out. Alternative renewable sources should be the focus of world concern. Biomass consists in plant material generated thanks to photosynthesis, which, in general terms, involves CO_2, water and sun light energy. Lots of energy types come from biomass: electricity, fuels for vehicles or heat for homes are some common examples (Ciubota-Rosie et al., 2008).

Photosynthesis is a clean energy source which means that the potential of plants to extract renewable energy is high. Increasing plant biomass to get energy or improving biomass conversion into biofuels has been a recent central topic in scientific knowledge. Genetic modifications of cell wall composition (Abramson et al., 2013; Fornalé et al., 2012) and research of efficient lignocellulose digestion enzymes (King et al., 2010) are some examples of that.

Knowledge about cell expansion mechanism and the control of it can provide new ways to generate plant mass and consequently, more clean biofuels. For instance, there are genes that limit the cell expansion, creating mutants for those genes causes an increase in cell elongation that means an increase of biomass. The mutant of E2Ff , an atypical member of the E2F family of transcription factors, is a clear example of this phenomenon. *E2ff-1* mutants have longer hypocotyls than those of the wild type, whereas overexpression reduces hypocotyl lenghts due to changes in cell expansion. AtE2Ff has several direct targets that are involved in the biosynthesis of cell wall, so this transcription factor may limit cell expansion through the inhibition of the cell wall biosynthesis (Ramirez-Parra et al., 2004).

In previously works of PhD Dr. Agnieszka Boron, and also experimentally checked in this work (Fig. 13), the *apollo* mutant seems to avoid the effects of de-etiolation in light-condition presenting an abnormal hypocotyl length. Consequently, the two genes affected *ORPHEUS* and *LINUS* become genes of interest. Furthermore there is not much information available about them.

In etiolated hypocotyls from WT *Arabidopsis* it has been demonstrated that elongation is due to wall depositions of cell wall components and, consequently, changes in wall thickness (Derbyshire et al., 2007; Refrégier et al., 2004). Moreover in the non-published work of Dr. Boron a FT-IR analysis has been performed in order to determine if there are cell wall component differences. The results show that no changes are present in the *apollo* mutant. Despite that, FT-IR analysis results are very hard to read and can be easily misunderstood (Roberts, 2007) To make sure no component differences occur on mutants another cell wall analysis should be considered, as the one proposed by Pettolino et al., 2012.

Current unpublished work from PhD Dr. Balcerowicz characterizes the role of MAIA in plant development. *Maia* mutants show an abnormal phenotype in pollen tubes and root hair and also during their growth. In the present report the expression in the seedling is shown at the trichoblasts cells, the ones that start the root hair development.

Considering the expression pattern present at Arabidopsis eFP Browser for the gene At1g70990, that show high expression in the root, it was expected to found expression there. However, expression in dark conditions hasn't been performed before, showing a considerable change on hypocotyls.

7. Conclusion

Although a plant molecular study takes lot of time, in this work the first steps for their study have been successfully accomplished. I have successfully created constructs for the promoter::GFP/GUS analysis and have selected some Arabidopsis transformants where the GFP and GUS can be detected in the next step. Because of time constraint, I have done a similar analysis on lines that were available in the lab, making me familiar with the techniques. In addition, I have phenotyped hypocotyl length of wild-type and *apollo* mutants grown in the dark and the light, confirming their striking hypocotyl phenotype, especially in the light where they become 4 times longer than expected.

In conclusion the points achieved in this work are:

- The creation of the constructions promORPHEUS::GFP and promORPHEUS::GUS
- Obtaining kanamycin resistant plants transformed with pGWB4 vector (containing the promORPHEUS::GFP)
- The identification of knock-out lines to compare phenotype with ORPHEUS and LINUS
- The differences between the *apollo* hypocotyls length compared to the wild type.
- The localization of At1g70990 promotor expression in the roots and hypocotyls cell types by fluorescence
- The identification of MAIA expression in the seedling by GUS staining

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