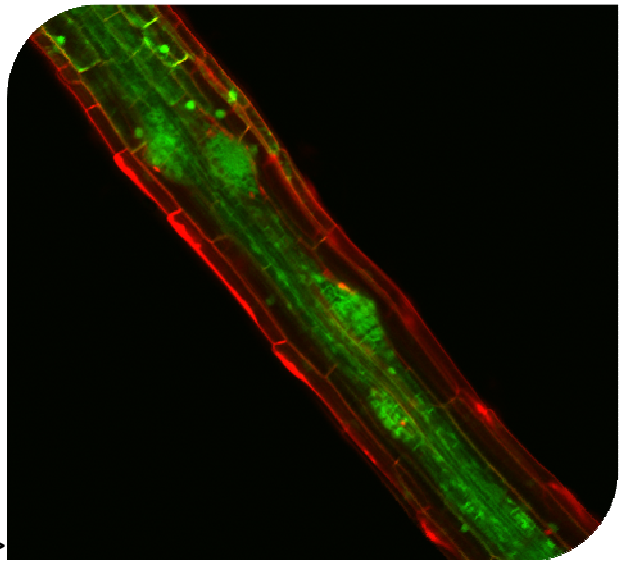
University of Antwerp,
Department of Biology,
laboratory of Plant Growth and
Development

Technological Educational
Institute of Crete, Department
of Biological Floriculture and
Greenhouse Crops

<<*Characterization of genes with potential involvement in the cell elongation of the root of Arabidopsis thaliana.*



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By Smyrnaki Rodanthi

Promoters

Prof. Dr. Kris Vissenberg

Prof. Dr. Konstantinos A. Loulakakis

Dr. Marios Nektarios Markakis

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1. Introduction

In biology, an organism is any living system such as humans, animals and plants. The main characteristic of the organisms is the ability to reproduce, develop and maintain life during their living time. All organisms are the result from a molecular unit of heredity called **genes**. Genes are stretches of DNA that, when expressed, transcribed into RNA will be translated to proteins. In genes all the necessary genetic information for the development and maintenance of an organism's cell is included and this information is passed to the future generations (Watson and Crick, 1953; Pearson et al., 2006).

1.1 DNA

Deoxyribonucleic acid (DNA) is a polynucleotide chain that carries the genetic information for the entire living organisms except RNA viruses. Along with RNA and proteins it is one of the three major macromolecules. The first DNA isolation was performed by a Swiss physician (1869) and according to James Watson and Francis Crick (1953), this polynucleotide chain is accepted as "model of the double helix", because of its structure (Watson and Crick, 1953). DNA consists of two long chains, which twist helically between them. These two chains are in opposite direction to each other, so they are anti-parallel. Those chains consist from four types of molecules called nucleotides or bases and they are; adenine (A), thymine (T), guanine (G) and cytosine (C), which are hold together by 2 or 3 hydrogen bonds depending on the nucleotide pairs that are opposite to each other (A=T and G Ξ C). The genetic information is coded in the sequence composed by the four nucleotides. This information encodes the sequence of the amino acids within proteins. The code is read by coping stretches of DNA into the related nucleic acid RNA in a process called transcription.

Nearly every gene contains the genetic information for a protein in a sequence of DNA and it can therefore influence the phenotype of an organism. Within a gene, the sequence of bases along a DNA strand determines a messenger RNA sequence, which then determines one or more protein sequences. The relationship between the nucleotide sequences of genes and the amino acid sequences of proteins is defined by the rules of translation. Each three bases of a coding region will translate to one amino

acid as a general rule. The RNA polymerase will copy the codons composed by three bases as mention before, into messenger RNA. This RNA copy is then decoded by a ribosome that reads the messenger RNA sequence and that, with the help of many other proteins and factors (see next paragraph), connects the correct amino acids to form one protein.

1.2 RNA

Ribonucleic acids (RNA) are made of a long single chain composed from nucleotides. Each nucleotide consists of a base, a ribose, sugar and a phosphate group. The sequence of nucleotides allows RNA to encode genetic information. All cellular organisms use messenger RNA (mRNA) to carry the genetic information that syntheses proteins. Some RNA molecules play an active role in cells by catalyzing biological reactions, controlling gene expression, or sensing and communicating responses to cellular signals (Barciszewski et al., 1999). One of these active processes is protein synthesis. This process uses transfer RNA (tRNA) molecules to deliver amino acids to the ribosome, where ribosomal RNA (rRNA) and enzymes link amino acids together to form proteins. The chemical structure of RNA is very similar to that of DNA, with three differences: (a) RNA contains the sugar ribose, while DNA contains a different sugar, deoxyribose (a type of ribose that lacks one oxygen atom), (b) RNA has the base uracil (U) while DNA contains thymine and lastly (c) RNA is single-stranded instead of DNA which is a double- strand (Crick, 1970).

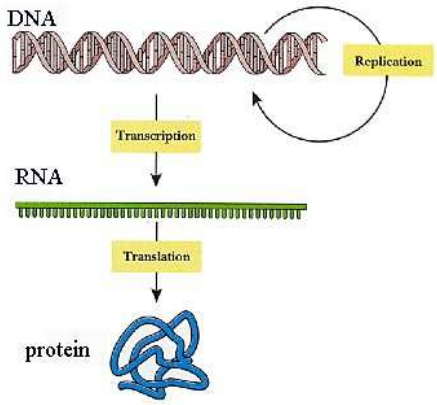


Figure 1: Central dogma of DNA-RNA-Protein.

1.3 Protein

Proteins are complex biological molecules, consisting from one or more polypeptides; a single linear polymer chain of amino acids bonded together by peptide bonds between the carboxyl and amino groups of neighboring amino acids. Proteins are essential for organisms because they participate in every procedure in the cells such as the maintenance of cell's shape, in cell signaling or responses and in the cell cycle. Also, many proteins are enzymes that catalyze biochemical reactions and are vital to

metabolism. In all organisms, there are two major steps building proteins; firstly, the gene must be transcribed form DNA to messenger RNA (mRNA) and secondly it must be translated from mRNA to protein, a process that is called protein synthesis. The end of each protein, which has a free carboxyl group, is known as the C-terminus (carboxy terminus), whereas the end with a free amino group is known as the N-terminus (amino terminus). Proteins are always biosynthesized starting from N-terminus and ending at the C-terminus (Alberts, 2002).

In order to understand the functions of the genes and proteins in organisms, the effect of knock out (K.O.) or overexpression of the genes on the phenotype can be studied. It will show in which process the gene is normally active. To know exactly where and when the genes are expressed, the promotor (that under normal conditions is controlling the expression of the gene of interest) is placed before a coding sequence for a reporter-gene, such as GUS or GFP. When these constructs are transformed into plants, this will lead to blue deposition and green fluorescence in the cells that normally express the gene.

1.4 Arabidopsis thaliana

Arabidopsis thaliana is a small flowering plant that belongs to the family of *Brassicaceae* and related to cabbage (*Brassica oleraceae*) and turnip (*Brassica rapa*). From the beginning of the 1900s (Weiling, 1991) *Arabidopsis* plants are of great interest since they are used in physiology studies as model organisms, but since 1945 they are widely used for studying and researching plant biology and genetics (Leutwileret, 1984). *Arabidopsis* has one of the smallest genomes among plants (5 chromosomes and 157 mega base pairs) so it is a great tool to understand molecular biology and plant development (Rensink and Buell, 2004). This model organism is inexpensive and convenient for cultivation due to its small size, usually growing 20–25 cm tall, so it does not need a lot of space in laboratories to grow. Usually, it completes its biological lifecycle in one year but under specific conditions and treatments it has the advantage to produce several thousand seeds in approximately six weeks (from germination to mature seed). *Arabidopsis* plays the role in plant biology that mice and *Drosophila* play in animal biology.

An extra benefit is that protocols exist to genetically alter or transform *Arabidopsis* plants. *Agrobacterium tumefaciens* is commonly used to transfer foreign DNA to the plant genome (Clough and Bent, 1998).

Figure 2: *Arabidopsis thaliana*.

1.5 The *Arabidopsis* root

The root is one of the most important plant organs that usually lay under the surface of the soil, however, a root can also grow above the ground (aerial) or above the water (aerating). Root has very important functions during the plant development. First of all, the root absorbs, stores and transports the water and the nutrient elements through the reams to other places of the plant. Secondly, it holds the plant body in the ground and lastly, it prevents erosion of the soil. On a transverse section, the *Arabidopsis* root consists from the vascular tissue (xylem and phloem) in the center of the root, surrounded by the endodermis, the pericycle, the cortex and finally the epidermis (Figure 3 A-B, Di Laurenzio et al., 1996). Normally, roots grow downward due to gravitropism instead of the stems that grow in the opposite direction (upwards) of the gravitational pull (Perrin et al., 2005).

Also, along the root surface different zones of development can be recognized. At the extreme tip there is the root cap which is not only responsible to protect the meristematic zone but also serves to guide the root through the soil according to the law of gravity (Figure 3C, Mariella, 2001). Specialized cells in the root cap sense the gravity vector and signal to other zones when growth is not along this vector. The zone of active cell division or the meristematic zone is the layer where the cells pass through the cell cycle. The meristem in *Arabidopsis* Col-0 is up to 200 μm away from

the root cap junction (RCJ) (Figure 3C, Verbelen et al., 2006). When cells exit the meristem, the cell elongation process is initiated. The elongation zone extends about 520 up to 850 μm away from the RCJ (Figure 3C, Dolan et al., 1994) and contains cells that elongate dramatically in a short time. When they stop elongating, they enter the zone of cell differentiation, where they take up their final functions and mature. The differentiation zone is characterized by very active tip growth of root hairs and extends from about 850 up to about 1500 μm away from the root cap junction (Figure 3C, Dolan et al., 1994). Root hairs are tubular projections from root epidermal cells that enormously increase the surface area of the root and they are the principal absorbing tissue of the plant. Water enters root hairs from soil by osmosis, while the mineral salts are absorbed by active transport. The water and mineral salts then pass through the cortex cells and enter xylem vessels from where they are transported throughout the plant via the transpiration stream.

Arabidopsis thaliana is a great model to understand the development of the root and the cell elongation mechanism also in higher plants. The cell elongation in the root is studied to explore the possibility to grow plants in different environmental conditions, for instance in drier countries. This would give us the chance to create transgenic plants that are adapted to, for example particular environmental conditions.

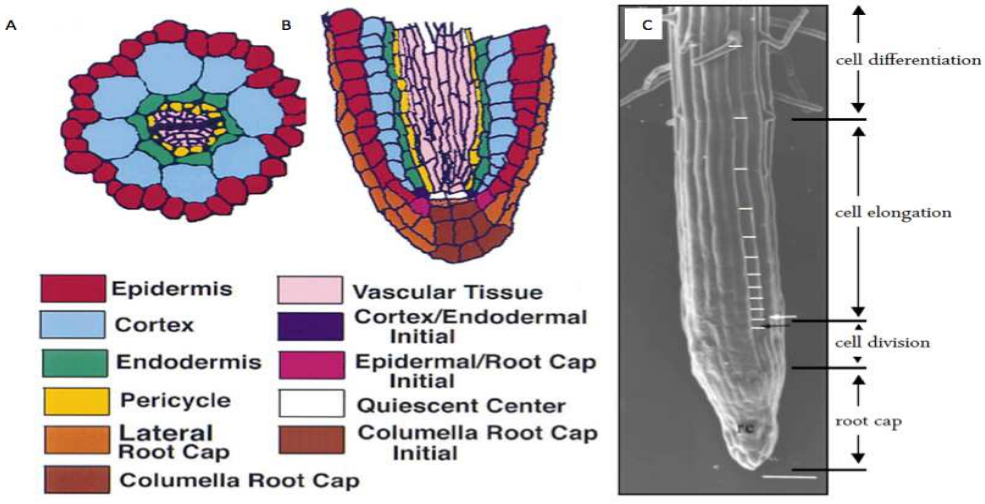


Figure 3: A) Cross section of the root tip B) Longitudinal section of the root (Di Laurenzio et al., 1996) C) Developmental zones along the root **(**Dolan et al., 1994).

1.6 Agrobacterium tumefaciens

Agrobacterium tumefaciens is the most known pathogenic bacterium, which belongs to the class of Alphaproteobacteria and to the family of *Rhizobiaceae* (Stanton, 2003). This microorganism is responsible for causing the formation of tumors in more than 140 species of dicotyledons flowering plants (Binns and Thomashow, 1988).

Contrary to all known phytopathogens, *A. tumefaciens* infects a wide range of plants by introducing a transferred fragment called T-DNA (transferred DNA) semi-randomly into their genome (Moore et al., 1977). The infection occurs through *Agrobacterium's* Ti plasmid. In experimental conditions, the Ti region is removed from the T-DNA and replaced with the gene of interest and a marker. The Ti plasmid then integrates a fragment of its DNA (T-DNA), into the plants chromosomal DNA (Stanton, 2003). The T-DNA contains genes encoding enzymes that produce two plant hormones (auxins and cytokinins) and one gene encoding the synthesis of certain amino acids known as "opines". The infected plant's cells are forced to synthesize opines, which are not catabolized by the plant, as a source of energy for *Agrobacterium*. Auxins and cytokinins cause the infected plant's cells to divide. T-DNA is transferred into the plant's cells and finally enters to the nucleus and integrates into the DNA (Moore et al., 1997). For the fact that, *Agrobacterium tumefaciens* has the ability to transfer DNA between itself and plants, it has become a very important and useful tool for laboratory studies, especially for plant transformation.

1.7 Plant transformation

In molecular biology, transformation in plants is the most common process to make transgenic plants by introducing foreign genes into plants genome with the use of the microorganism *Agrobacterium tumefaciens*.

Plant transformation in *Arabidopsis* is routine. There are a lot of protocols for introducing exogenous DNA into the plant chromosomes but the most simple and usual method is the mediated gene transfer by using *Agrobacterium* (Clough and Bent, 1998). The current protocol is known as "floral-dip" and involves simply dipping *Arabidopsis* flowers into a solution containing *Agrobacterium*, the DNA of interest, and a detergent, which will assist the *Agrobacterium* during the infections. With this method we don't need to culture tissues or to regenerate plants, however, sometimes it is possible that many plants are not transformable (Peters, 2010).

In order to transform plants with exogenous DNA sequences we worked with transformed *Agrobacterium* cells. Its T-DNA is now specifically modified and acts as a gene vector. Vectors are tools used in molecular biology for transferring foreign genetic information and the aim of this vector is to express the gene of interest in the transformed organism, in this case *Arabidopsis*.

In simple words, through this method we firstly isolate a sequence of DNA that codes for the fragment of interest, which we then insert into a plasmid. Afterwards this plasmid was transformed into *Agrobacterium tumefaciens*. A large amount of the bacterium containing this plasmid was then grown in order to transform the plants. Important is that the plants have flowers in order to be receptive, so that the new plants will get the DNA of interest. Seeds from these transformed plants can contain the plasmid. In order to select and identify the plants with the insertion, we use a feature from the plasmid, which contains a coding sequence for resistance to a specific antibiotic. In the case of pDONR207 the antibiotic is kanamycin and only the seeds that inherited the resistance gene (and therefore the seeds that also contain the DNA of interest) will grow under these conditions (Binns and Thomashow, 1988).

At this point, we have plants with a specific piece of DNA inserted. Usually, we will study the offspring (or offspring's offspring) of this plant in order to make sure that the plant is homozygous for the insertion (the cell has two identical copies of the gene of interest on both homologous chromosomes) (Clough and Bent, 1998). In the present thesis, we didn’t have the time to study the second generation of these plants.

1.8 Reporter gene

Reporter genes are chosen as reporters due to certain characteristics, namely that they allow visual identification or localisation. These types of genes (**selectable markers**) are used in laboratory studies in order to indicate the success of the procedure of introducing exogenous DNA into a plant cell (Koo et al., 2007).

Reporter genes have two functions. They are used to analyze the expression of a gene in the cell or organism population and also to analyze the activity of a specific promoter (in time and in space). The promoter is a region of DNA responsible for beginning the gene expression and for determining where and when the gene becomes expressed. The promoter of the gene of interest replaces the original promoter of the reporter gene. The new promoter connected to the reporter gene is introduced into the

plant and the expression of the gene under study is visualised by the reporter genes protein, GUS or GFP in this study (Jefferson, 1987).

Secondly, reporter genes can be used to localise proteins and even to find the subcellular localisation. For this, the coding sequence of the reporter gene, usually GFP is directly attached to this of the gene of interest. This makes that the two genes are now under the same promoter region and are transcribed into one mRNA strand. This mRNA is then translated into one protein with GFP fused to the N- or C-terminus.

The most common reporter genes in plant biology which are used by scientific researchers are:

1.8.1 GUS (β-glucuronidase)

The bacterium *Escherichia coli* encode the enzyme β-glucuronidase, which is often used as a reporter for gene activity. For this detection method one of the most common glucuronide substrates for GUS histochemical staining, 5-bromo-4chloro-3indolyl glucuronide (X-Gluc) is used. When the GUS enzyme incubates with the colorless X-Gluc substrate, it can transform it into a colored product, in this case it is a clear blue color, and in most of the cases it is even visible with the naked eye (Koo et al., 2007).

Higher plants or bacterial cells, which do not express the GUS enzyme, have very little endogenous activity, indicating that the blue color is the result form the activity of the promoter that is under study. The advantages of using GUS are its sensitivity, it is easy to perform the staining, it is highly visible, reliable and stable, and also GUS activity can be assayed at a wide pH range (Jefferson et al., 1986).

1.8.2 GFP (green fluorescent protein)

GFP is a protein that is first isolated from the jellyfish *Aequorea victoria*. This protein is composed of 238 amino acids. In genetics, the GFP gene is commonly used as a reporter of expression and can be introduced into organisms and fused in their genome through cell transformation. The discovery of GFP is related to a property of one part of the molecule, namely the chromophore. The chromophore is responsible for the green fluorescence when molecules are exposed to blue light. GFP has its excitation peak at approximately 400 nm and its emission peak at approximately 500 nm (Tsien, 1998).

This chromophore is formed spontaneously from a tri-peptide motif in the primary structure of GFP, so that its fluorescence is “automatically” turned on in every cell where it is expressed. In other words, the maturation of the tri-peptide-based chromophore in GFP only requires oxygen and does not depend on the presence of enzymes or other auxiliary factors. In order to visualize GFP, a blue light with wavelength 400nm is shined in its direction and a green fluorescent color is visible where the protein is located. In the lab, this protein is examined under a confocal microscope in which one can stimulate and capture fluorescence on digital images. Furthermore, when the gene for GFP is fused to the gene of a protein to be studied in an organism of interest, the expressed protein of interest retains its normal activity and, likewise, GFP retains its fluorescence, so that the location, movement and other activities of the studied protein can be followed by microscopic monitoring of the GFP fluorescence (Wang and Hazelrigg, 1994). GFP is generally non-toxic and can be expressed to high levels in different organisms with minor effects on their physiology.

1.9 Laser Scanning Confocal Microscopy (LSCM)

A laser scanning confocal microscope (LSCM) is a method for obtaining high-resolution optical images with depth selectivity. LSCM incorporates two principal ideas: Illumination point by point, of the sample and rejection of out of focus light, laser light (blue line) is directed by a dichromic mirror towards a pair of mirrors that scan the light in x and y. The light then passes through the microscope objective and excites the fluorescent sample. The fluorescent (light green) light from the sample passes back through the objective and is scanned by the same mirrors used to scan the sample. The light then passes through the dichromic mirror through a pinhole placed in the conjugate focal (hence the term confocal) plane of the sample. The pinhole thus rejects all out-of-focus light arriving from the sample. The light that emerges from the pinhole is finally measured by a detector such as a photomultiplier tube. At any particular instant

Figure 4: Laser scanning confocal microscope.

only one point of the sample is observed. A computer reconstructs the 2D image one pixel at a time. A 3D reconstruction of the sample can be performed by combining a series of such slices at different depths (Pawley, 2006).

1.10 Phenotypes

Phenotype is the visual characteristics of an organism such as its morphology. Phenotype results from the expression of an organism's genes which are influenced generally from environmental factors. In order to analyze phenotypes of *Arabidopsis thaliana*, we need plants with different gene activity. Three different genotypes (the inheritance code for each organism) of *Arabidopsis* were used in this phenotype experiment. The first is the wild type (**WT**) or control, which is the normal plant without any changes of the genome. The second genotype is characterized as 35s promoter (Koo et al., 2007). The **35s promoter** is a very strong constitutive promoter, causing high levels of gene expression, using it to over express genes that are of great interest for research. The last one genotype of *Arabidopsis thaliana* is the knock out (**K.O.**) lines of the gene. K.O. lines are plant lines created with the help of *Agrobacterium* and contain a random insertion of a T-DNA fragment. This results in the fact that the gene will not be translated into a protein anymore. By analysis of knock out mutants we can observe the effect of the absence of the gene of interest in the whole plant.

1.11 Plant hormones

Plant hormones or phytohormones are naturally produced products by the plants and always in very low concentrations which are able to alert cells metabolism. Hormones are regulated with the plant growth like the formation of the flowers and the time of flowering, the gene expression and the cell division. Each cell is capable to produce hormones. Hormones are crucial for the plant growth and in lack of them; plants would be a mass of undefined cells (Srivastava, 2002). So, phytohormone experiments are very interesting and many of them took place in our laboratory, in order to specify the reactions of the *Arabidopsis thaliana* root in the presence of different chemical substances, added to ½ MS medium plates.

In this thesis, six different hormone regulators were used in different concentrations: ACC, ABA, IAA, NAA, GA_3 and MJ_3.

Ethylene (with its precursor ACC) is one of the most popular hormones that is produced from all parts of higher plants including fruits, leaves, and roots and its production is also regulated by a variety of developmental and environmental factors, during certain stages of growth (Yang and Hoffman, 1984). Ethylene affects cell elongation, for example when a seedling hits an obstacle under the surface, ethylene production is increased, preventing cell elongation and causing the stem to move. Ethylene biosynthesis can be induced by endogenous and exogenous factors. Researchers have proved that ACC synthesis is increased with high levels of auxins, especially IAA, and cytokinins, on the other hand ABA can inhibit the synthesis of ACC (Rocklin, 2004).

Abscisic acid (ABA) is found in very high concentrations in newly abscised or freshly fallen leaves. In general, it acts as a chemical inhibitor. In plant species, without ABA, buds and seeds would start to grow during warm periods in winter and die when they are frozen again by changes in the temperature. In other plants, ABA levels decrease when gibberellins levels start to increase (Feurtado et al., 2004).

The most common auxins found in plants are acetic acid (IAA) and NAA. Auxins were the first class of growth regulators discovered. Leaf abscission is initiated by the growing apex of a plant ceasing to produce auxins, on the other hand it promotes lateral root development and growth. Auxins are toxic to plants in large concentrations so they are used as natural herbicides. IAA has many different effects on plant development. As all auxins IAA influences cell elongation and cell division with all subsequent results for plant growth and development (Simon and Petrášek, 2011).

Gibberellins (GA_3) includes a large range of chemicals which are produced naturally by plants and fungi. Gibberellins are important in seed germination, affecting enzyme production that mobilizes nutrients. Also, they promote cellular division, flowering etc. Absorption of water by the seeds causes production of GA_3 so it is very important during the germination and start of embryonic growth (Tsai et al., 1997).

1.12 Polymerase Chain Reaction (PCR)

Polymerase chain reaction is a scientific in vitro process, developed in 1983 (Bartlett and Stirling, 2003), used for amplifying a single or more copies of a piece of DNA, generating thousands or millions of copies of this particular DNA sequence. The method is based on thermal cycling; cycles of repeated heating and cooling of the

PCR sample for melting DNA and replicate enzymes of the DNA. Primers and DNA polymerase (such as Taq polymerase) are the keys to enable selective and repeated amplifications. Primers are pieces (10 to 30 nucleotides) of a single-stranded DNA, which make the starting point for DNA synthesis. Under these thermal cycling conditions the two strands of the double helix DNA are separated, in high temperatures (DNA melting). At lower temperatures, the DNA single-strands are used as a template for the synthesis by the DNA polymerase, which selectively amplifies the target DNA.

Figure 5: PCR cycler- Applied Biosystem.

Other applications of PCR are: DNA cloning, DNA sequencing, generating hybridization probes ect, where PCR enables analysis of DNA samples even from very small amounts of material.

1.13 Agarose Gel Electrophoresis

In order to check whether the PCR generated the DNA sequence of interest, an agarose gel electrophoresis is used. Agarose is a polysaccharide that forms a gel, and the electrophoresis is basically used to separate DNA molecules of different sizes. Using an electrical field the negatively charged nucleic acid molecules move through the pores of the gel towards the positive side. Molecules move with different speed through the gels' pores according to their size; larger DNA molecules move slower than smaller molecules because the smaller run more easily through the pores of the gel. The speed also depends on the length of the DNA fragment, the concentration of the electrophoresis buffer and the voltage that is applied to the gel. Afterwards, an electrophoresis tank is used to separate the molecules and finally the gel is incubated in an ethidium bromine-solution. Ethidium bromide (EtBr) is a fluorescent orange dye that intercalates between bases of nucleic acids and allows the detection of DNA fragments in gels. EtBr fluoresces under ultraviolet lamp when it has intercalated between the base pairs and finally the DNA bands are visible (Smisek and Hoagland, 1989).

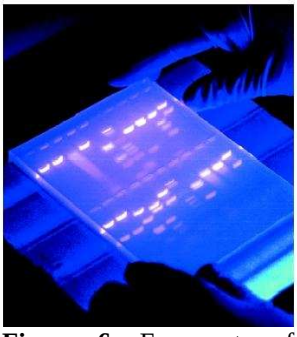


Figure 6: Fragments of DNA under ultraviolet light.

2. The aim of the study

The aim of this study is to investigate genes involved in the cell elongation process. After observations of Le et al. (2001) that ACC/ethylene can inhibit cell elongation in the root and a microarray study done in the laboratory by previous researchers to identify genes with an altered expression after ACC/ethylene addition, we selected genes with a potential involvement in the root elongation. Our focus here is to take over the remaining work from previous students and push it forward.

This includes the transformation of *Arabidopsis* with different constructs that were created before, selection of transformants and the analyses of some of them.

In a second set of experiments I looked for the phenotype in root of WT, KO and 35s-overexpression plants.

3. Material and Methods

3.1 Plant materials and growth conditions

Wild type seeds (Col-0, N1093) and a K.O. line for the genes ***At2g43590*** and ***At2g25150*** were obtained from the *Arabidopsis* stock centre (NASC, Loughborough, Leistershire, UK). All plants were grown in an environmentally controlled growth chamber (15 photons/cm/s; 16h light/8h dark; 24°C).

For soil cultivation we used Tref substrate soil.

For in vitro experiments sterilized seeds were placed on ½ strength MS plates containing 2.2g/l Murashige and Skoog (1965) salts including vitamins (Duchefa, the Netherlands), 1% (w/v) sucrose (Duchefa), adjusted to pH 5.7 [KOH] and solidified with plant agar 8g/l (Duchefa), for extraction of DNA/RNA or root measurement.

Seeds from transformed plants were harvested when they were mature and dry, and the sterilized seeds are placed on ½ MS agar plates supplied with 1 ml kanamycin per 1L to select transformants. Plates are placed in the growth chamber for 10-15 days. The wild type plants die on the medium whereas transformants survive and grow normally. These will be transferred to soil in order to grow and collect the second-generation seeds.

3.2 Seed sterilization

Seeds were surface-sterilised for 5 minutes in 6% commercial bleach followed by 3 rinses in EtOH. Plates were closed with one revolution of laboratory film (Parafilm, Pechiney Plastic Packaging, Menasha, WI, USA) and subsequently stratified for 2 days at 4°C in the dark. Plates were then placed in a growth chamber (15 photons/cm/s; 16h light/8h dark; 24°C) for the next days.

3.3 Plant cultivation

For in vitro cultivation of *Arabidopsis* we used:

- ½ Murashige & Skoog medium 1962 (MS)
- Sucrose : Merck
- Plant agar : Sigma-Aldger
- HCl and KOH
- Sterilized square plastic Petri dishes
- Sterilized toothpicks and filter paper

- Sterilized seeds

And we proceed as follow:

2, 2 g/l of half strength Murashige & Skoog-medium (1962) was used to grow plants on plates with 10 g/l sucrose. The pH of the medium was adjusted to 5, 7 with the use of the HCl or KOH solutions. After the pH is optimal, we put the solution in a Schott bottle adding 6 g/l of plant agar. Finally, the medium is autoclaved at 121°C for 20 minutes. Depending on the subsequent experiment after autoclaving, we add hormones or antibiotics and distribute the solution into sterilized square plastic Petri dishes. When the medium is solidified, sterilized seeds of *Arabidopsis* placed on the plates in a specific order with the help of sterilized toothpicks. Finally, the plates are closed with parafilm to prevent them from contaminations, covered with aluminum foil and placed in a 4°C fridge for 3 days. After 3 days, the seeds are placed in the growth cabinet under long-day conditions (16h light/8h dark and approximately 24°C).

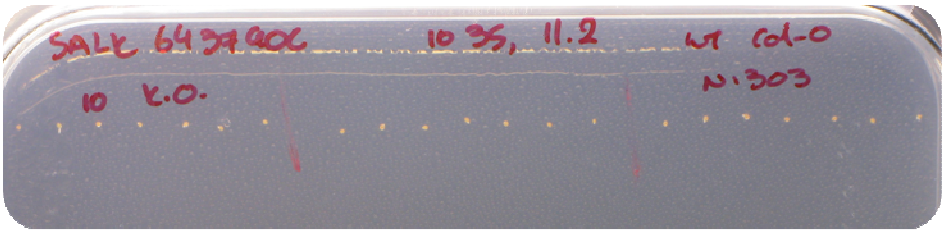


Figure 7: Sterilized seeds of three different lines (k.o., 35s, wt) of *Arabidopsis* placed on the plates in a specific order.

Alternative to solid medium is the liquid ½ MS medium with almost the same process for cultivating plants in solid ½ MS Medium with the exception of the addition of agar, which solidifies the solution, so that it remains liquid during the treatments. This liquid ½ MS medium was used for GFP or GUS experiments due to the fact that plant material grows faster in liquid media than in solid media. So we can easily check the promoter activity under a confocal microscope.

3.4 Plant transformation

For plant transformation we were following the protocol as shown below:

First of all, healthy *Arabidopsis* plants are growing until they are flowering, under long day conditions (16h light/8h dark/23°C). At the same time, *Agrobacterium tumefaciens* is growing in 5ml LB* pre-culture, with the appropriate antibiotics [(R-

G-K (Rifampicin 25 µg/ml, Gentamycin 50 µg/ml and Kanamycin 50 µg/ml)] for 24h at 28°C and 200rpm in order to select the plasmid. In each 250ml of LB medium we add 1ml of pre-culture and then we leave it to grown as before for 20-24h. This is enough for 6 trays with *Arabidopsis thaliana*.

All antibiotics are to insure that all the resistance genes are present and working in *Agrobacterium* and to insure that rearrangements or loss of markers is unlikely to occur. Also, only the bacteria that have successfully taken up the resistance gene become resistant and will grow under certain conditions considering that only the original vector does not have the resistance gene (Langridge et al., 1989).

The *Agrobacterium* cells were harvested by centrifugation at 8000rpm for 10 minutes in 4°C. Then the *Agrobacterium* solution was re-suspended in the transformation buffer (TB 50g/l sucrose + 0.74g/l $MgCl_2$). We should shake so well that the solution is homogenized.

Just before the dipping of the flowering part of the plant in the solution, we add 100µL per 500 ml TB silvvet-L 77, to a concentration of 0.05%. We dip gently, the *Arabidopsis* flowers in the *Agrobacterium* solution for 2.5-3 minutes. The dipped plants are then placed under long day conditions, for one week.

Important is to water regularly!!! After approximately 5-6 days the whole transformation procedure is repeated for a second time in order to increase the transformation events (Bechtold et al., 1993).

*LB (lysogeny broth) medium: materials: Triptone 10gr/L, yeast extract 5gr/L and NaOH 5gr/L. Mix and sterilize by autoclaving.

3.5 GUS staining

The most usual GUS histochemical staining is X-Gluc (5-bromo-4-chloro-3-indolyl glucuronide) and the product of this reaction is a blue color. In order to test our plants we follow the next protocol:

We transfer the plants from the liquid ½ MS, where they have been grown for 6 days, in 5ml GUS staining solution and incubate them overnight at 37°C. The plants are now rinsed three times with distilled water and fixed for 1 hour in a fixation solution (ethanol 100%: acetic acid 100% 3:1). Now, we dispose the solution, we incubate with NaOH (8M) for at least 1 hour. After this step, the plants washed again for 3 times with distilled water and analyzed under the confocal microscope.

GUS staining solution:

FerroCN 1mM, FerriCN 1mM, Potassium phosphate buffer 50%, Na_2EDTA 10mM, X-Gluc 2 mM.

3.6 Root measurements

The purpose of the root measurements is to find differences in growth between the genotypes of *Arabidopsis thaliana* such as: WT, overexpression lines (35s) and knock out (K.O.) lines. The length of the roots is measured and the numbers of the lateral roots are counted with the ImageJ program, which is freely available on internet.

Three plates for each experiment are produced and seeds added in specific order (35s-WT-K.O) usually 7-8 seeds per line. When the plates are ready, we close them with parafilm and cover them with aluminum foil and place them in the fridge (4°C). After 3 days the plates are placed in the growth chamber where a robot with a camera automatically takes pictures from the plates during approximately 3 weeks. Afterwards, the roots are measured with the computer program ImageJ.

Figure 8: The action bar of the ImageJ program.

3.7 Hormone experiments

Stock solutions:

- ACC 100µM
- ABA 1mM
- IAA 100mM
- NAA 10mM
- GA_3 50µM
- MJ_3 1mM
- Final concentration 5µM

For this kind of experiments, seedlings are growing as usual in normal ½ MS agar plates. On the 5th day of seedlings growth the experiment take place because now the

transferred plants are neither too small nor too big and with no lateral roots yet. Hormones are added in certain concentrations in autoclaved ½ MS and the solution is put in square sterilized Petri dishes. After the plates are solidified, by using a pincett, we transfer half of the plants from the normal ½ MS plate to the new hormone plate; the other halves are transferred to a new normal ½ MS plate in order to keep a control. The experiment has to be in the laminar flow in order to avoid contaminations in the new plates. After the transfer, the plates are closed with parafilm and placed in the photographic robot for at least 2 weeks in order to compare the growth of the roots of the different genotypes in each hormone.

3.8 PCR

The components that are essential for the basic PCR set up are:

1. The DNA sequence to be amplified
2. A reverse and a forward primer
3. The enzyme Taq polymerase
4. dNTPs - the building-blocks from which the DNA polymerase synthesizes a new DNA strand (adenine, guanine, cytosine and thymine).
5. Reaction buffer, providing a certain environment for optimum activity and stability of the DNA polymerase.
6. Nuclease-free water

The system that we use is Applied Biosystems Veriti 96 wells Thermal cycler. PCR consists of 20-40 cycles, means temperature changes, which each cycle usually consisted from 3 different temperature steps.

Firstly, the double stranded DNA is denaturated at 94°C. This is the step of the first cycle and consists of heating the reaction to 93-95°C for 5 minutes. Those high temperatures cause DNA melting which means that the hydrogen bonds between the complementary bases broke and then we have two separate single-stranded DNA strands, which they will become the templates for the primers and the DNA polymerase. Then follows the annealing step; the reaction temperature is lowered to 50-60°C for 20-40 seconds, allowing the annealing of the primers to the single-stranded DNA sample. After the primers are attached, the elongation step starts at 72°C (optimum temperature for using the enzyme Taq polymerase). In this phase the DNA polymerase will make a new DNA strand complementary to the first DNA

strand by adding dNTPs in the 5'-3' direction. By repeating these steps (usually 30 times), an exponential growth in amount of DNA is obtained. The final elongation occurs at 70-74°C for 5-10 minutes to ensure that the entire single DNA strands are extended. We hold the samples in 4°C till we will use them (Chien et al., 1976).

In order to check whether the PCR generated the DNA sequence of interest, an Agarose Gel Electrophoresis is used.

In order to make an agarose gel you should:

- Mix the agarose powder (3gr) with the electrophoresis buffer (50 X TAE buffer 200 ml) to the concentration of 1, 5%.
- Heat in the microwave oven for 2-3 minutes till the powder is completely melted.
- Place the gel in a gel rack (approximately 5ml), insert a comb at the one side of the gel and wait till it will be solidified (10-15 minutes).
- The electrophoresis tank is filled with 50X TAE buffer, ensure that the gel is completely covered.
- Remove the comb and now you can load the gel with 6µl of each DNA sample. Don't forget to put first a comparison- 10µl of DNA ladder, which contains DNA fragments of known size.

Start the electrophoresis tank with a voltage of 70V for approximately 40 minutes so that the separation of the molecules begins. After electrophoresis the gel is incubated in ethidium bromine (EtBr) -solution for 15 minutes. Due to the EtBr fluoresces under ultraviolet lamp the DNA bands can be visible.

3.9 RNA isolation

The RNA isolation is a complicated procedure, which demands specific steps in each protocol in order to be successful. For this thesis we used the following protocol:

We start with the homogenization of 50-100mg plant tissue in 0.5 ml TRI REAGENT. We centrifuge the samples for 5min at 7.000g.

Next follows the separation phase where we add and homogenate 0.2ml chloroform by mixing vigorously. Store the samples for 2-15min; centrifuge them for 15min, at 12.000g, in 4°C.

Next step is the RNA precipitation. We transfer the aqueous phase to a clean tube; we add 0.5ml isopropanol and mix. Store the samples for 5-10min, centrifuge them for 8min, at 12.000g, in 4-25oC.

Afterwards, we use 1ml 75% ethanol for washing RNA pellet. Centrifuge for 5min, at 7.500g, in 4oC.

Final step is the RNA solubilization. We add 0.5% SDS formazolR or water and we incubate at 50-60^{o}C for 10 min. Dry the RNA pellet for 5-10 min and dissolve by pippeting in 50-200μl of formazol.

The RNA samples are completed in approximately 1h and they are ready for concentration measurements. This is done using a NanoDrop spectrophotometer. NanoDrop has two functions: it measures the absorbance of small volume samples of *nucleic acids* and *proteins*. The NanoDrop is designed to measure from just 5ng/ul to 3.000 ng/ul samples. The sample is first pipetted directly onto the measurement surface. The surface tension then holds the sample between the two optical surfaces while the absorbance measurement is made. Then, the software automatically determines which path length to use for concentration calculations. Cleanup is performed by simply wiping both the upper and lower measurement surfaces with a dry laboratory wipe.

Figure 9: The NanoDrop spectrophotometer.

4. Results and Discussion

4.1 Plant transformation experiment

WT plants of *Arabidopsis thaliana* were transformed with selected *A. tumefaciens* colonies, using the "floral dip" method. After collecting seeds from the first generation transformed plants, we harvested the seeds and selected the transformed seeds on kanamycin plates (only seeds with the T-DNA will grow). In the figure 10-11 you can clearly see the transformants containing the construct for the genes *At2g43590* and *At2g25150*, marked with blue arrows. These plants are characterized by a clear green color instead of the other plants, which don't have resistance to the antibiotic, that are yellow or pale green. These will not survive. The seedlings that survived and produced leaves and roots were transferred to soil to grow and produce the next generation of seeds which can theoretically contain plants homozygous for the insertion.

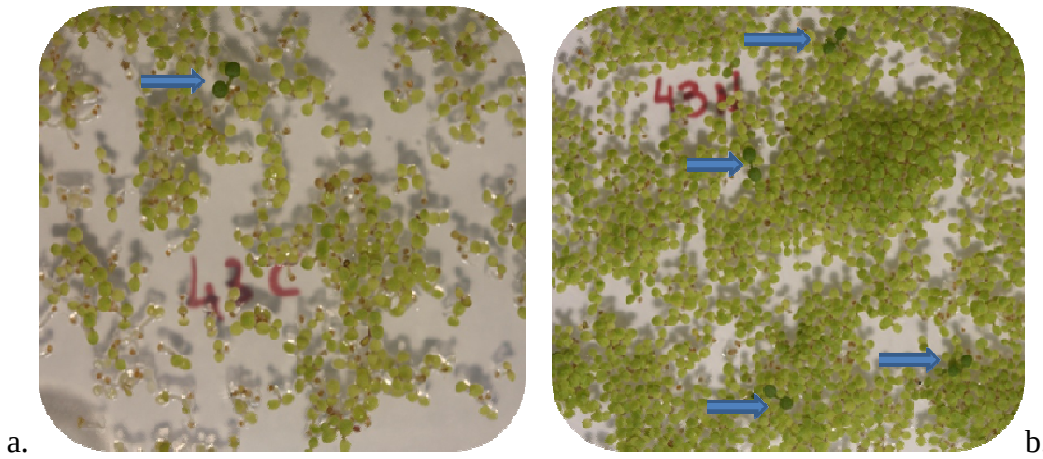


Figure 10: Mutant plants (blue arrows) on the ½ MS medium including kanamycin (a. 43C-terminus b. 43 N-terminus).

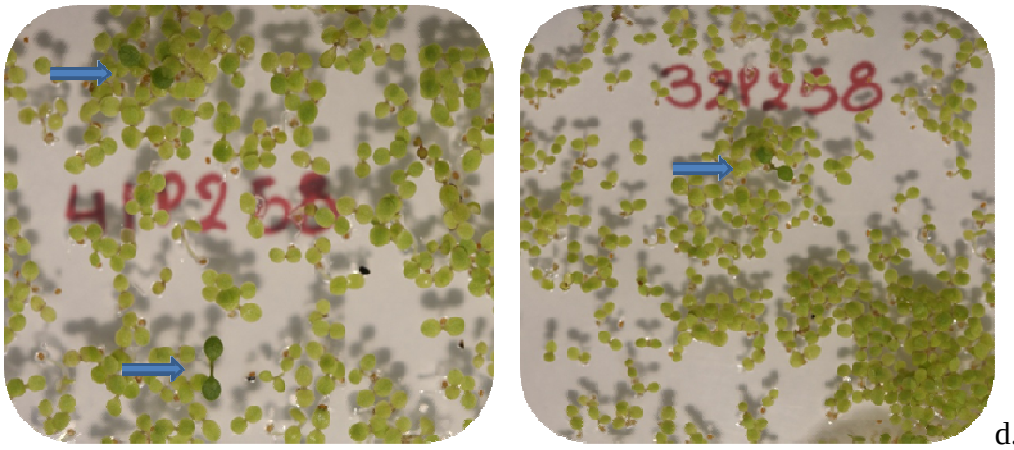
c.  d.

Figure 11: Mutant plants (blue arrows) on the ½ MS medium including kanamycin (c. 41P258 line d. 32P258 line).

4.2. GUS and GFP experiment

T1 generation seeds of transformed plants with promoter::GFP and promoter::GUS construct for the genes *At2g43590* and *At2g25150* were selected. The seedlings that grew in kanamycin plates for sure contained the promoter::GUS or promoter::GFP construct. These plants were checked under the light microscope after GUS staining or with a confocal microscope to visualize GFP presence. This was done in control condition and after different treatments.

The next pictures show GUS or GFP signals in the root of seedlings with the promoters::GUS or promoter::GFP construct. Blue and green mark the cells where the promoter is activated in control conditions or due to different conditions or treatments. Additional verification was performed with PCR in order to check if seedlings indeed include the constructs in cases that no GUS or GFP was present.

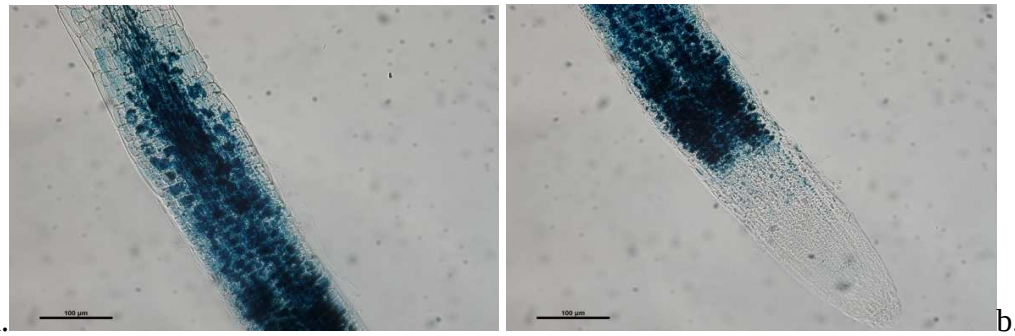
a.  b.

Figure 12: GUS signals in the root of *Arabidopsis* seedlings (At2g43590 gene).

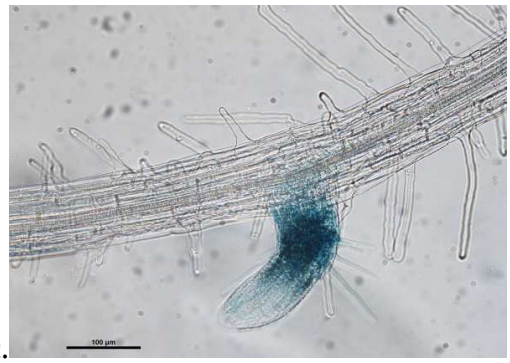
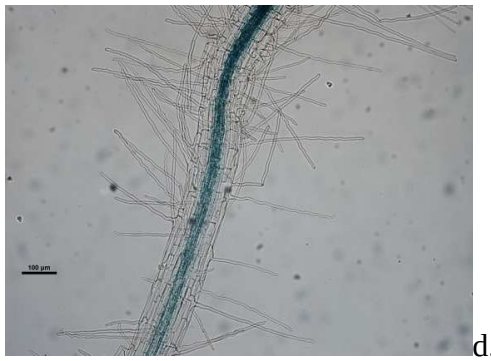
c.   d.

Figure 13: GUS signals in the root of *Arabidopsis* seedlings (At2g25150 gene).

Furthermore, GFP was localized in transformants that contained a protein-GFP fusion construct. As a result the protein contains an N- or C-terminal GFP addition, making the protein fluorescent under blue light. Some representative pictures are shown below.

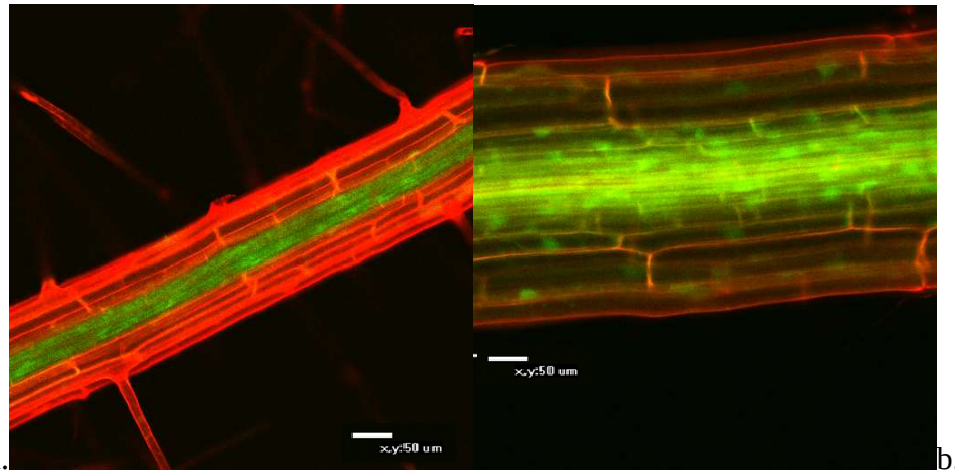
a.  b.

Figure 14: Pictures from confocal microscope with green GFP signal (1gfp n-terminus At2g43590).

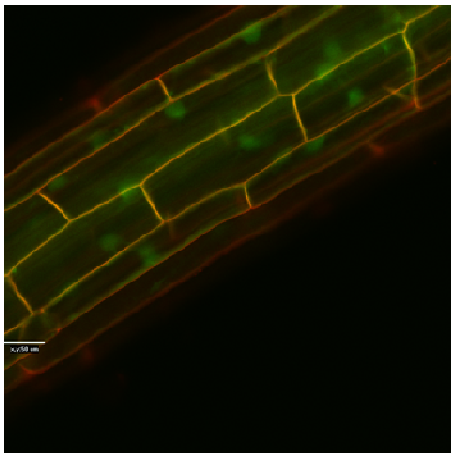
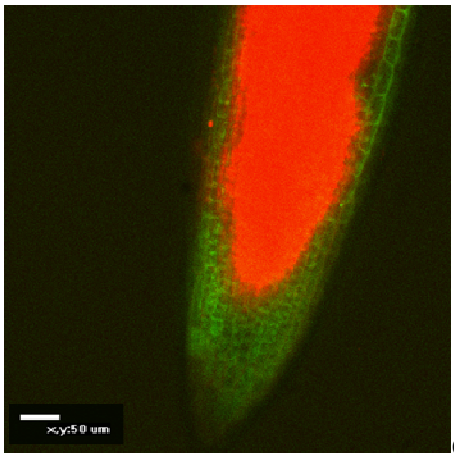
c.   d.

Figure 15: Pictures from confocal microscope with green GFP signal (1gfp c-terminus At2g43590).

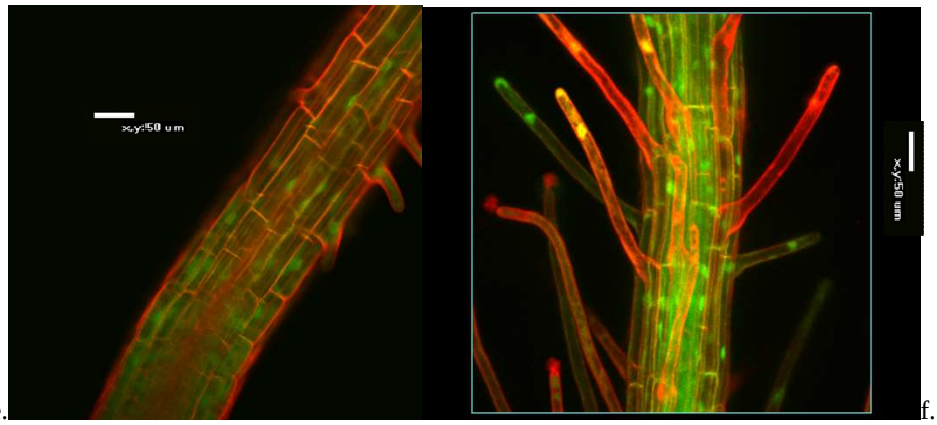
e.  f.

Figure 16: Pictures from confocal microscope with green GFP signal (1 gfp n-terminus At2g25150 gene) (f.: 3D version).

To see the effect of hormones on the expression patterns of the genes, promoter::GUS or GFP lines where treated with ACC and IAA which inhibits the elongation of the cells. On the pictures is clear that the elongation is affected, seen as shorter cells and a thicker root after 48hours of treatment. The GFP signal seems present in similar cells as under normal growth conditions, but the signal here seems stronger. This is not only seen in the primary root, but also in different stages of lateral root development.

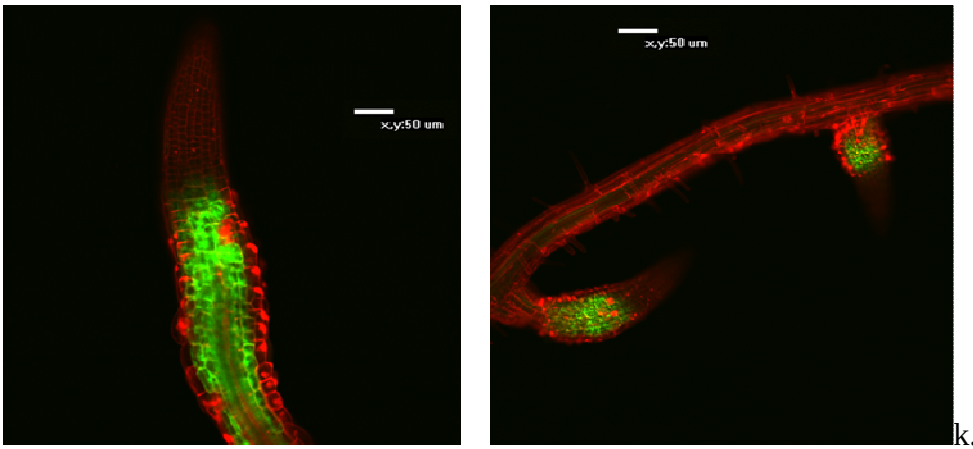
j.  k.

Figure 17: Pictures from confocal microscope with green GFP signal after 48h ACC treatment (1gfp).

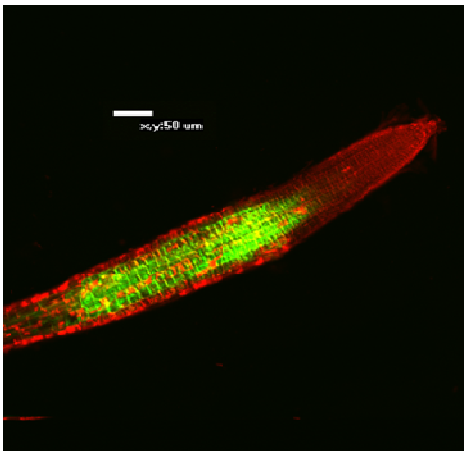
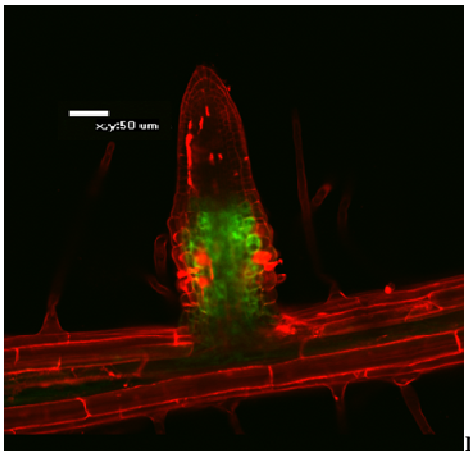
l.   m.

Figure 18: Pictures from confocal microscope with green GFP signal after 48 h IAA treatment (1gfp).

4.3 Hormones experiments

A growth phenotyping experiment was performed as described in material and methods on wild type plants and on plants with an altered gene expression level (knock-out and over-expression plants) of the genes *At2g43590* and *At2g25150*. On figures 20-25 the different reactions that are induced by different hormones on *Arabidopsis thaliana* root development, are compared to control plants. Different lines (35s-WT-KO) were placed in each agar plate including hormones (ACC, GA_3, IAA, MJ_3, NAA, ABA) with a final concentration of 5µM.

Experiment 1 At2g43590 gene

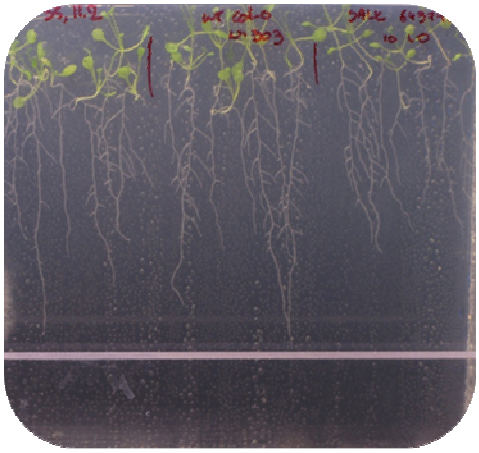
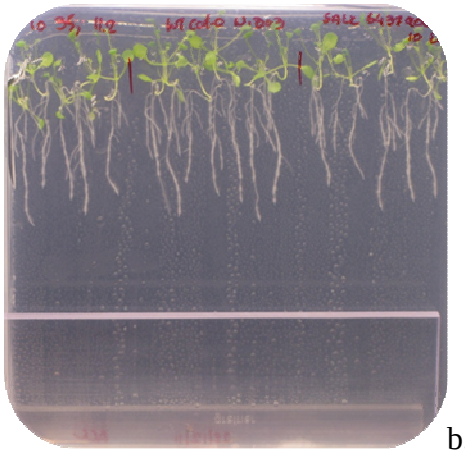
a.   b.

Figure 19: a. Lines of *Arabidopsis thaliana* growing in normal ½ MS medium b. Lines of *A. thaliana* growing in ACC agar plates.

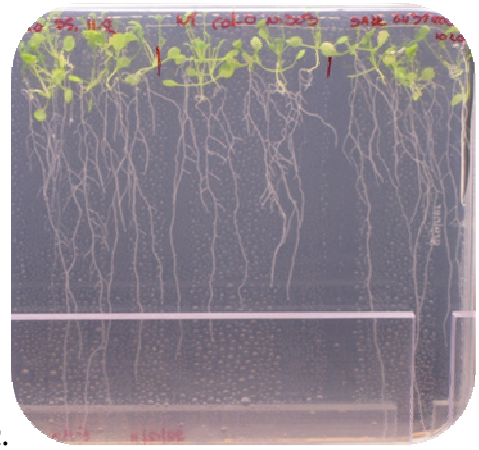
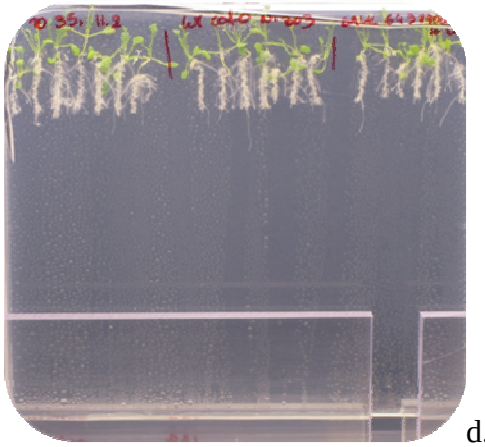
c.   d.

Figure 20: c. Lines of *Arabidopsis thaliana* growing in GA_3 agar plates. d. Lines of *A. thaliana* growing in IAA agar plates.

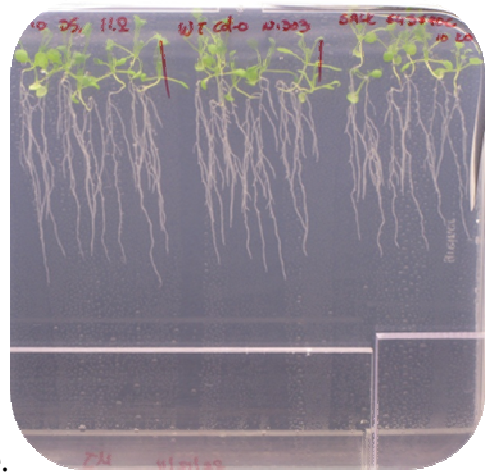
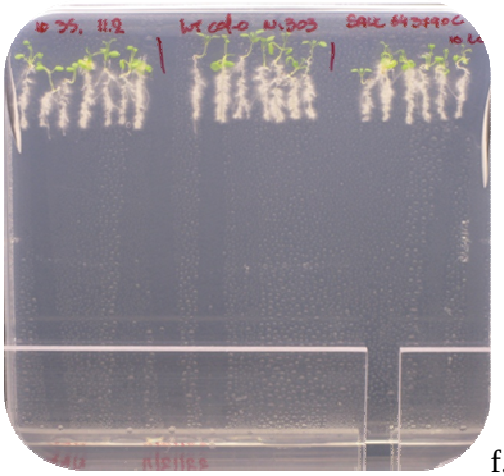
e.   f.

Figure 21: e. Lines of *Arabidopsis thaliana* growing in MJ_3 agar plates. f. Lines of *A. thaliana* growing in NAA agar plates.

Experiment 2 At2g25150 gene

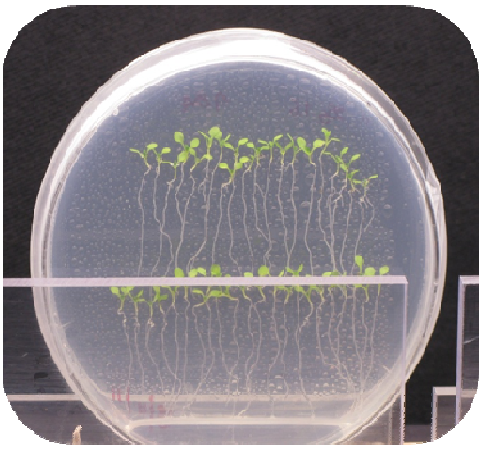
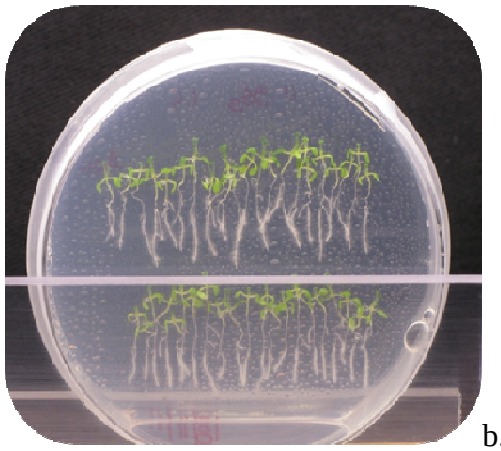
a.   b.

Figure 22: a. 11 35s 1.6 line of *Arabidopsis* growing under ABA treatment b. 11 35s 1.6 line of *Arabidopsis* growing under ACC treatment.

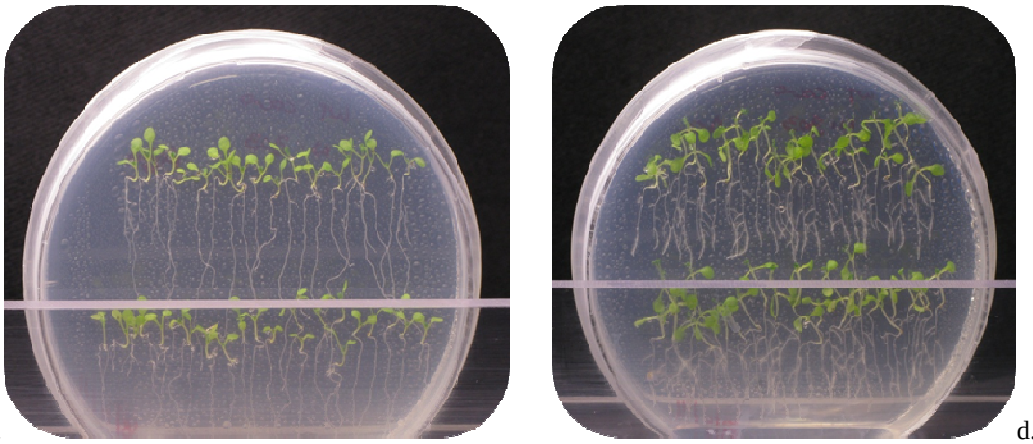
c.  d.

Figure 23: c. WT Col-o n1303 line of *Arabidopsis* growing under ABA treatment. d. WT Col-o n1303 line of *Arabidopsis* growing under ACC treatment.

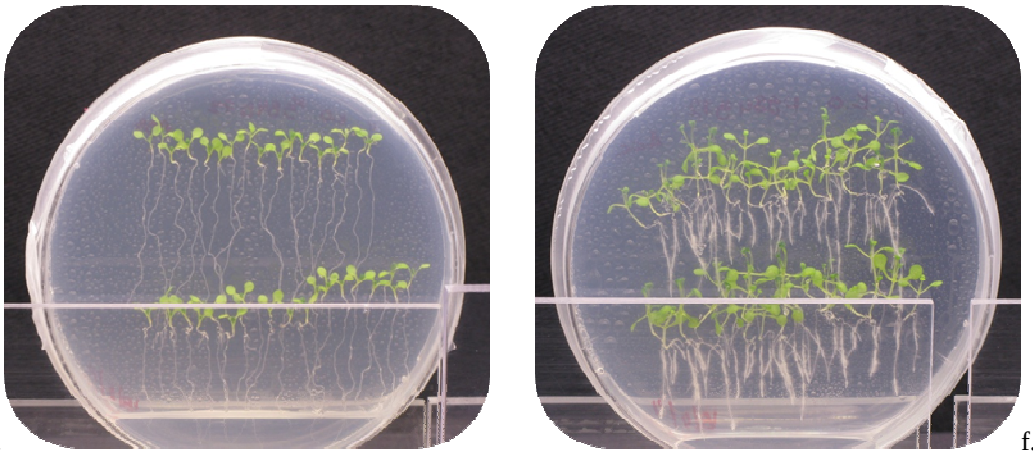
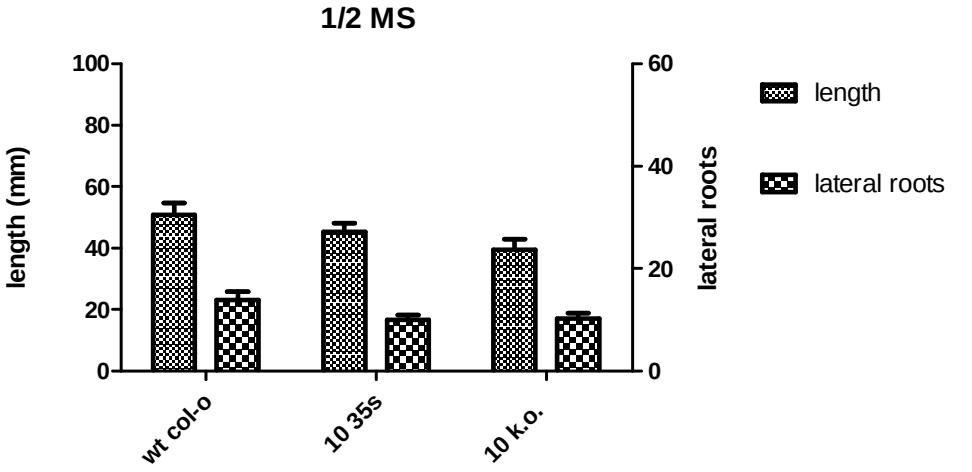
e.  f.

Figure 24: e. 11 ko 4584372 line of *Arabidopsis* growing under ABA treatment f. 11 ko 4584372 line of *Arabidopsis* growing under ACC treatment.

The major aim of the second set of experiments is to analyze the phenotype in the root of WT, KO and 35s-overexpression plants. By analysis of 35s and knock out transformants we can observe the effect of the overexpression or absence, of the gene of interest respectively, of the gene of interest in the whole plant and specific in the root.

Also, with a first observation of the above figures we can result to the fact that *Arabidopsis thaliana* root reacts in a different way in each hormone treatment, concerning the length and the number of the lateral roots. For instance, plants growing in ACC agar plates have shorter roots than plants growing in GA_3 agar plates, but with the first having more lateral roots than the second.

In the next figures is shown the numerical data of root length and number of lateral roots seen on plates in the previous two experiments.



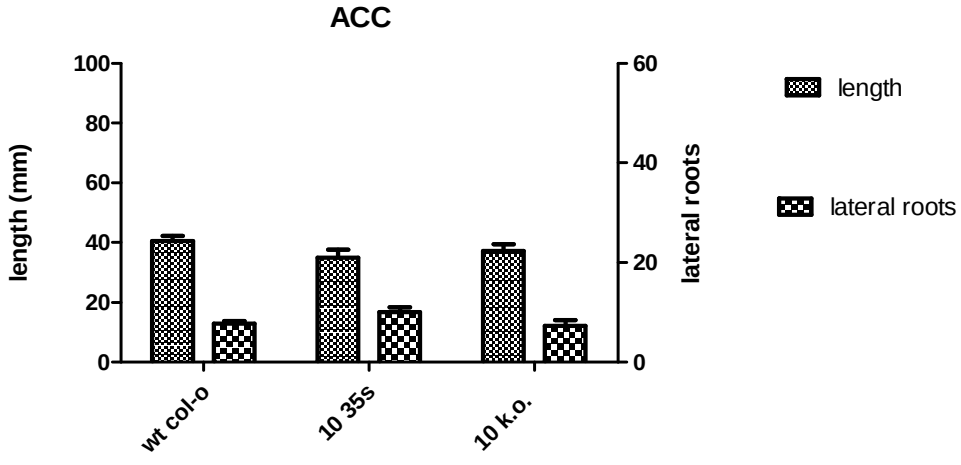


Figure 25&26: Analyzing the reactions of *Arabidopsis thaliana* root, in three different lines (10 35s 11.2 - WT Col-O N1303- Salk 643790C 10 k.o.), concerning the length and the number of the lateral roots as a wild type and under ACC treatment correspondingly.

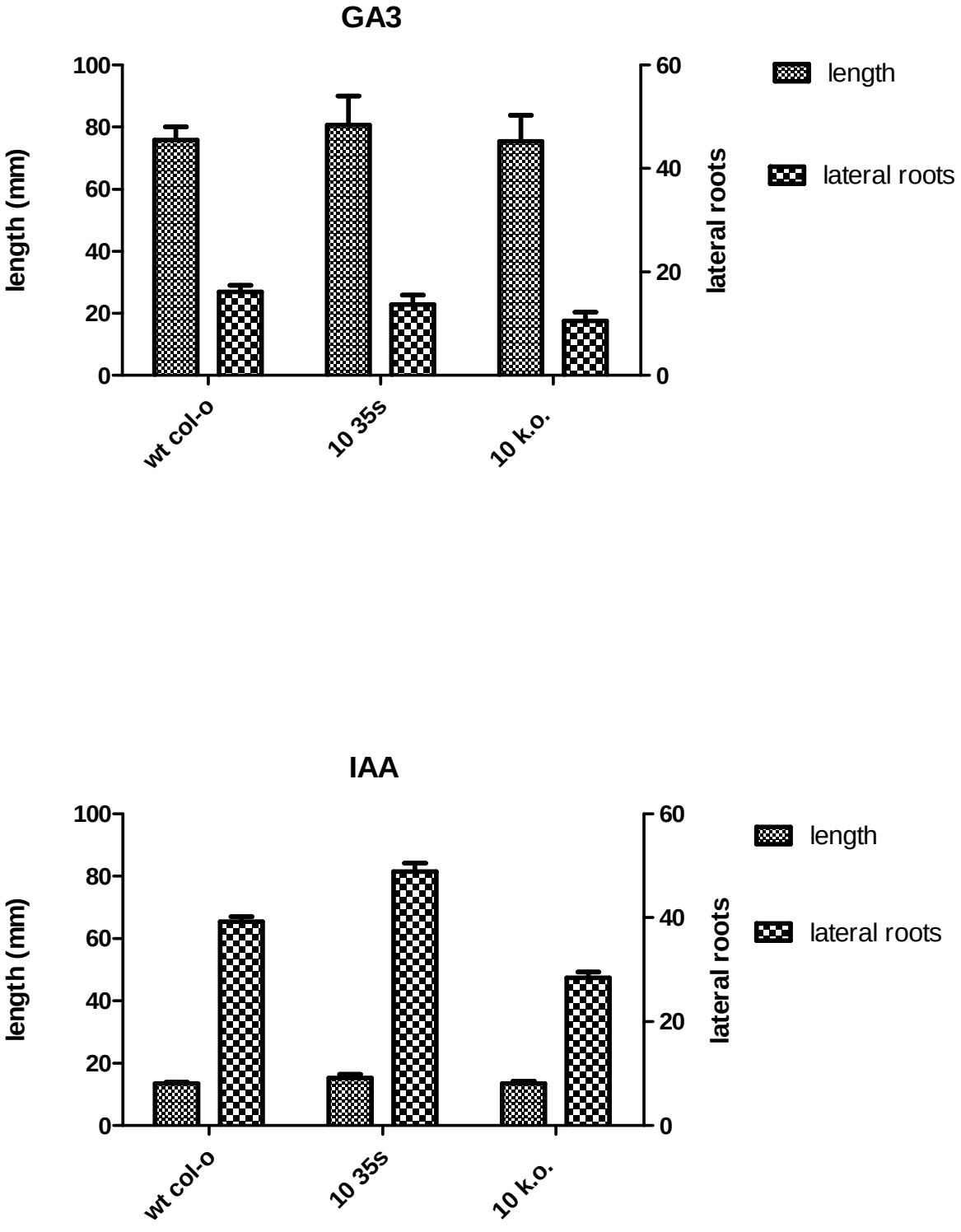


Figure 27&28: Analyzing the reactions of *Arabidopsis thaliana* root, in three different lines (10 35s 11.2 - WT Col-O N1303- Salk 643790C 10 k.o.), concerning the length and the number of the lateral roots under GA_3 and IAA treatment correspondingly.

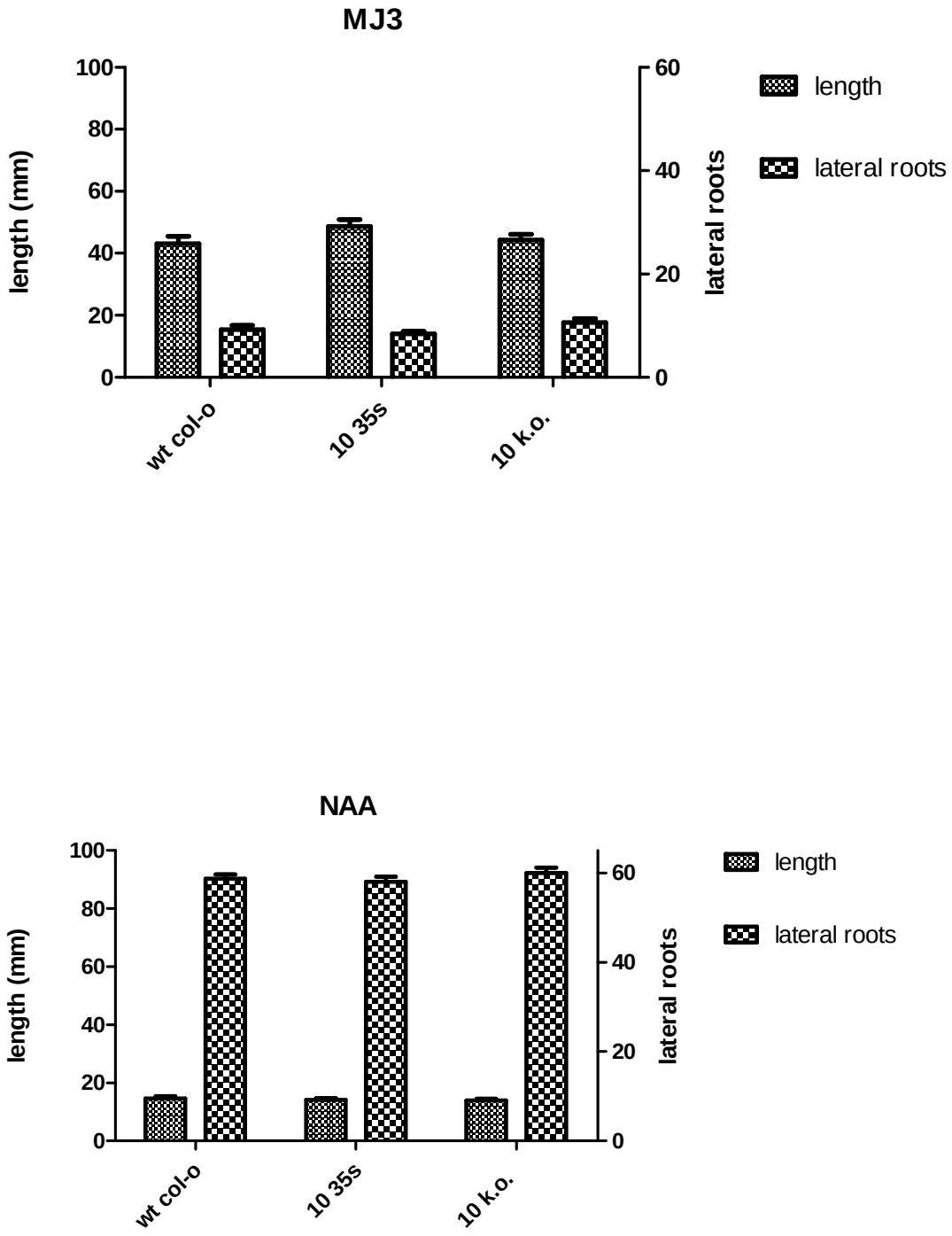
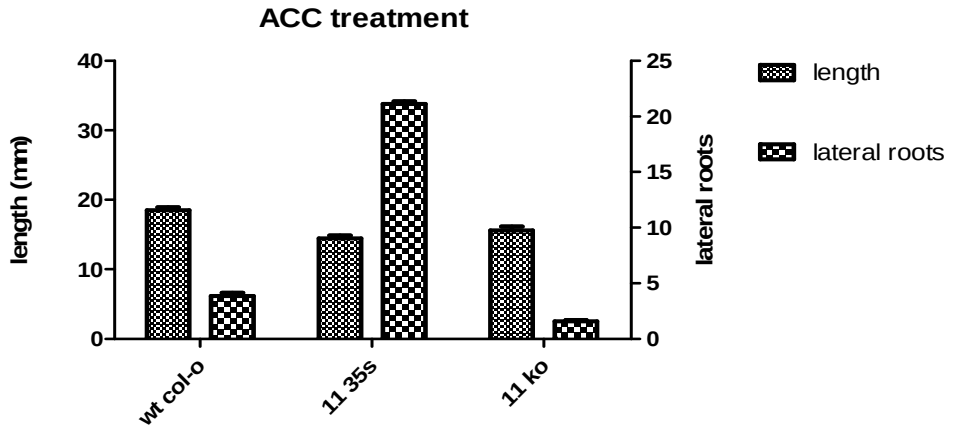


Figure 29&30: Analyzing the reactions of *Arabidopsis thaliana* root, in three different lines (10 35s 11.2 - WT Col-O N1303- Salk 643790C 10 k.o.), concerning the length and the number of the lateral roots under MJ_3 and NAA treatment correspondingly.



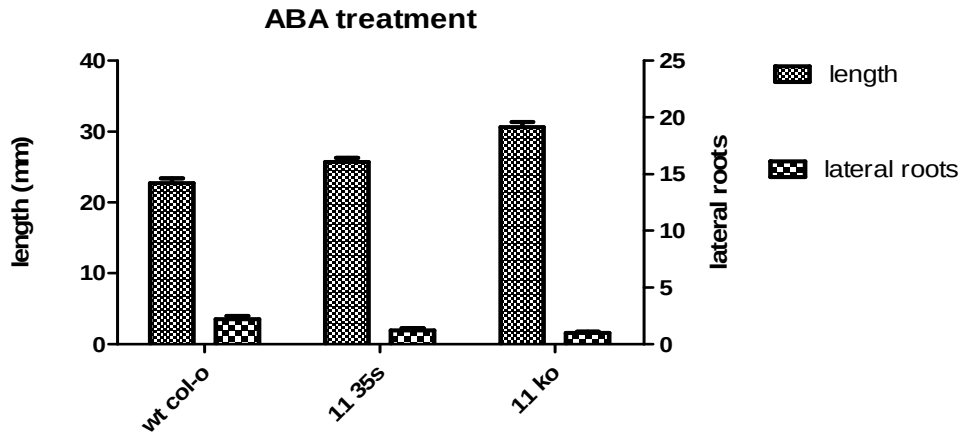


Figure 31&32: Analyzing the reactions of *Arabidopsis thaliana* root, in three different lines (11 35s 1.6 –WT Col-O N1303- 11 k.o. 4584872), concerning the length and the number of the lateral roots under ACC and ABA treatment correspondingly.

In statistics, a result is called statistically significant if it is unlikely to have occurred by chance alone, according to a pre-determined threshold probability, the significance level.

Based on the statistical analysis ($P < 0.05\%$) made related to the hormone experiment, we can result in a fact that the phenotype on wild type plants and on plants with an altered gene expression level (k.o. and over-expression plants) of the genes *At2g43590* and *At2g25150*, has some differences in each case.

Arabidopsis thaliana root development of the 35s- k.o. lines is always compared to control plants, placed in each agar plate including hormones.

Starting with the ½ MS plate for the gene At2g43590, we can see that there is not any significant difference between the three different lines of *Arabidopsis* concerning the length and the number of the lateral roots as well.

In ACC treatment the results as the same as seen before.

In GA_3 treatment the results are the same as seen before.

In MJ3 treatment the results are the same as seen before.

In NAA treatment the results are the same as seen before.

The IAA treatment shows that plants have the same length but with a significant difference in the number of the lateral root between all the lines with the overexpressed lines having the most laterals roots and the knock out the fewer.

On the other hand for the gene At2g25150 there are observed significant differences ($P < 0.05\%$) concerning the length between WT col-o vs. 11 35s and WT col-o vs. 11 ko but not between 11 35s vs. 11 ko in the ACC treatment with the WT line having the longer roots. The number of the lateral roots has significant differences between all the lines with the overexpressed lines having much more lateral roots than WT and the knock out much less.

In ABA treatment the length between WT col-o vs. 11 35s, WT col-o vs. 11 ko and 11 35s vs. 11 ko has significant differences with the knock out lines having the longer roots. The number of the lateral roots has significant differences between WT col-o vs. 11 35s, WT col-o vs. 11 ko and 11 35s vs. 11 ko with the knock out having the fewer.

5. Conclusion

WT plants of *Arabidopsis thaliana* were transformed with selected *A. tumefaciens* colonies successfully, containing the construct for the genes *At2g43590* and *At2g25150*. After collecting seeds from the first generation transformed plants our focus is to examine the next generation plants in order to check if plants are homozygous for the insertion. This is a work for the next researchers.

T1 generation seeds of transformed plants with promoter::GFP and promoter::GUS construct for the genes *At2g43590* and *At2g25150* were selected. The seedlings that grew in kanamycin plates for sure contained the promoter::GUS or promoter::GFP construct. These plants were checked under the light microscope after GUS staining or with a confocal microscope to visualize GFP presence. This was done in control condition and after different treatments. Additional verification was performed with PCR and we checked that seedlings indeed included the constructs.

A growth phenotyping experiment was performed on wild type plants and on plants with an altered gene expression level (knock-out and over-expression genes) of the genes *At2g43590* and *At2g25150*. *Arabidopsis thaliana* root development has different reactions that are induced by different hormones and compared always to control plants.

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