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Growth dynamics in *Arabidopsis thaliana* root cambium

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Thesis

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Abstract

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<p>This work was done in a research group led by Ari Pekka Mähönen. The aim of this thesis was to find out if the Arabidopsis mutant lines under analysis had an altered phenotype compared to the Columbia control, meaning a larger or smaller vasculature width and secondary xylem vessel average. Gus- stained lines with T2 an T3 promoters were analysed to see where the stain would be situated in general and if the stained area was important in secondary root growth. The studied lines were either ordered from the stock centre or made in the laboratory using T-DNA insertion techniques.</p> <p>The mutant lines were chosen for this study based on previous experiments. In the Re-analysed lines there were 36 candidate mutant lines and in the other experiment 54 lines, which were ordered for this thesis. For the Gus-staining experiment there were several lines; RM9 1-16 which was a T2 selection and T3 selections RM13_16, RM13_13, RM13_8 with lines 1-4. RM13_5 had lines 1-5. These lines have been genetically modified by floral dipping, where the wanted gene was transferred to the plant via an Agrobacterium construct vector.</p> <p>The roots were grown in sterile conditions and processed to get histological crosscuts which were analysed using the microscope, an image analysis program and Microsoft Excel. The Gus- samples were only analysed visually. The results were compared to our wild type control (Columbia) to find out if the mutants had any significant changes in phenotype. For both the Re-analysed and the 54 lines four interesting mutant lines were found per experiment which might be eligible for further study. In the Re-analysed lines line II had the largest vasculature diameter average compared to Columbia at +33,98 µm. From the 54 lines the highest average was found in sample 6 at +36,6 µm.</p> <p>In the Gus-staining experiment the stain seemed to be situated in the phloem ball area for the T2 promoter samples and all around the vasculature are for the T3 samples. The T2 promoter genes might be linked to a specific area in the root, whereas the T3 seems to affect the whole vascular area and is more generalized. The results of this work might help narrow down the number of mutant lines to study in this line of research and confirm earlier research results.</p>	
Keywords	Arabidopsis thaliana, cambium, cytokinin, GUS, vasculature

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1 Introduction

I interviewed the research group leader Ari Pekka Mähönen for his opinion on why the research being conducted was important and what the possible benefits from it could be. He told me about the two main benefits of the research they were doing. First, research in this area is valuable because it provides more information about how biological mechanisms work. The activity of the cambium is currently not well known, so research in this area yields new information. The *Arabidopsis thaliana* plant is used as a model plant because it is easy to grow and study, and the results from the research can be applied to understanding tree growth as well. The second major yield from this research is in applying the information gained to bioengineering for example to find or create tree lines which have a faster wood formation. The nature of the wood created could also be affected, to create possible alternatives to oil based polymers. The wood creation process could be genetically altered to create different types of wood as an alternative to chemically modifying wood structure (Mähönen, Ari Pekka. 2017.)

This thesis is done as a part of a larger research project carried out in the Institute of Biotechnology in Helsinki. Plant biologist Tiina Blomster and PhD student Riikka Mäkilä will be assisting in the planning, execution and analysis of the research I will be doing.

2 The aim and of my thesis

My first project includes two separate lines of research which are done using the same methods and execution. I will be re-analysing 36 lines of *Arabidopsis thaliana* mutants previously analysed by another student and 54 lines ordered from the stock centre for this thesis. Both will be executed in the same way, by first growing the plants needed for the analysis and then processing their roots by methods based on the group's laboratory protocol. The results will be analysed by microscoping, image analysis and statistical calculations using Microsoft Excel. The mutant lines have been chosen by results from earlier research to possibly have an altered phenotype to the Columbia used as control. An altered phenotype, meaning here a larger or smaller secondary cambial growth in the root may indicate a change in the plant hormone cytokinin processes.

The second project is Gus-staining root samples and analysing the pictures taken of the roots. The Gus staining procedure coupled with the Gus gene attached to certain promoters creates a purple colour in the root where the genes are activated. I will see where if anywhere the root is coloured and deduce what colouring each T2 and T3 promoter causes in the area and what it might mean.

In conclusion, I have two hypotheses which I am testing:

1. Do the Salk lines have a phenotype that is remarkably different than that of the Columbia control and are there lines fit for further research
2. Which part of the root expresses the T2 and T3 promoters and is the expression meaningful for secondary cambial growth

The research will be done using quantitative methods and the lines, genes and results coded to protect the current research going on.

3 Importance of the research

3.1 The research group

The research group I will be working with is led by group leader Ari Pekka Mähönen and consists of several multinational researchers, including postdoctoral fellows, graduate students and undergraduate students, as well as research technicians. Several alumni have also worked with the group. The group has several publications to its name.

3.2 The aim and methods of the research group

The aim of the group is to study the mechanism by which the cambial stem cells regulate cell proliferation and differentiation, and how the obtained growth is then organized to achieve the correct vascular pattern. Studying this is important in order to develop mechanisms for enhancing wood proliferation in the future and thus increasing the biomass created (Plant Growth Dynamics web page.)

The group has taken four approaches to mapping the location and activity of the cambial stem cells and understanding their growth dynamics. First, they are searching for molecular markers which are specific for the different cell types in the cambium. Secondly, they use those molecular markers to monitor changes in cell identity or division activity during their cell ablation studies. The ablation studies will reveal the location and dynamics of the cambial stem cells. Third, they are using these and existing markers to record cell division and differentiation patterns during a span of time by using live imaging via microscope. The fourth approach is to use clonal analysis as a tool to map the cell lineage relationships inside the cambium, these different tools are used to study how the stem cell activity changes in various cambium mutants grown specifically for this purpose (Plant Growth Dynamics web page.)

Another line of the research is to screen for new cambial mutants by using a classical mutagenesis and candidate gene approach. Due to these different approaches the maintenance and activity of the cambium, as well as the stem cells therein are studied in detail in both wild type and mutant plants. The group uses novel techniques developed in the lab to achieve this. The plant used in the research is *Arabidopsis thaliana* (Plant Growth Dynamics web page.)

3.2.1 Climate change globally

The current warming of the planet is unprecedented and likely caused by humans.

The sea levels rose globally about 17 centimetres in the last century, which is nearly double the rate of the previous century. All three major global surface temperature reconstructions show that the Earth has warmed since 1880 and all of the then warmest years have occurred within the past 12 years and temperatures still continue to rise despite the 2000s solar output decline. The oceans have also warmed in response and the Greenland and Antarctic ice sheets have decreased in mass. All around the world glaciers are retreating, including the Alps, Himalayas, Andes, Rockies and Alaska and Africa. The number of extreme weather events has also risen and the acidity of surface ocean water has increased about 30 percent since the beginning of the Industrial Revolution. Rising levels of emitted carbon dioxide is the cause for this acidification. Satellite observations have also revealed that the amount of spring snow cover in the Northern Hemisphere has decreased over the past five decades and that the snow is melting earlier (Climate change: How do we know?)

3.2.2 Renewable energy use

Renewable energy use has the benefit of reduced greenhouse gas emissions, the diversification of energy supplies and reduced dependency on fossil fuel markets. The growth of these renewable energy sources may also stimulate employment in the EU through the creation of new jobs in the new 'green technologies' (Renewable energy statistics.)

The most important sources of renewable energies in the EU-28 2014 were solid biofuels and renewable waste. Hydropower was the second most important contributor, followed by wind energy. Solar energy use growth was rapid as was wind energy use. Geothermal energy use accounted for 3,2% of the total. There are currently low levels of tide, wave and ocean energy production (Renewable energy statistics.)

3.2.3 Biofuels

Biofuels are made from biomass or bio-waste and can be used for many purposes, but they are most commonly used to fuel transportation. Liquid fuels have the advantage of being easily pumped and handled. Biofuels are the best way of reducing the emission of greenhouse gases and provide energy security for fossil fuels which are limited in availability. Major producers and users of biogas are Asia, Europe and America. Biofuel can be theoretically produced from any carbon source but the most commonly used material are photosynthetic plants. A big problem is converting biomass energy into liquid fuel (What are biofuels?)

There are currently two methods to create biofuel. The first one is creating ethanol through the process of fermenting sugar crops or starch. The second is using normally grown plants to produce oil like jatropha and algae, which are heated to reduce their viscosity and used for diesel engines (What are biofuels?)

4 Knowledge base

4.1 Genetics

Understanding how Gregor Mendel's laws of heredity work is key when doing genetic studies and the base on which new knowledge is built. The basic underlying principles of heredity were discovered largely by Gregor Mendel, a European monk. His discoveries

of plant genetics also applied to people and other animals, because mechanisms of heredity are basically the same for all complex life forms. His research was done through selective cross breeding of common pea plants (*Pisum sativum*) over several generations (Mendel's genetics.)

He discovered that some traits are visible in offspring without the blending of parent characteristics. Pea flowers are purple or white and intermediate colours don't appear in the offspring of cross-pollinated plants. He found seven traits which are easily recognized and occur in two forms. The observation that these traits do not show up in intermediate forms in offspring was important, because the scientific consensus from before was that inherited traits blend from generation to generation (Mendel's genetics.)

Mendel's pick of common garden plants for his research is for the same reasons as many researchers choose to use *Arabidopsis Thaliana*; they can be grown easily in large quantities and their reproduction easily manipulated. (The *Arabidopsis* Information Resource). They have male and female reproductive organs and can either self-pollinate or be cross-pollinated with another plant so Mendel was able to selectively cross-pollinate purebred plants with selected traits over many generations to reach his conclusions about the nature of genetic inheritance (Mendel's genetics.)

He came to three important conclusions from his experimental results:

1. The inheritance of each trait is determined by "factors" that are passed on to descendants unchanged (now known as genes)
2. An individual inherits one such unite from each parent for each trait
3. The inherited trait may not show up in an individual but can still be passed on to the next generation

When starting the experiment his parent plants were homozygous for pea seed colour, which means they had two identical forms or alleles for this trait. The plants of the first f1 generation were all heterozygous, which means they had each inherited two different alleles, one from each parent plant. This is seen in the genetic makeup of the plant, the genotype. The phenotype is the plant's observable physical characteristics. When the f1 plants breed they have an equal chance of passing on both different alleles from the parents (Mendel's genetics.)

With the seven traits he observed he found one form dominant over the other, which means it masked the presence of the other allele. His observations can be summarized in two further principles:

1. The principle of segregation
2. The principle of independent assortment

The principle of segregation means that for any certain trait the pair of alleles of each parent separate and only one allele passes from each parent to an offspring. The inherited allele is based on chance and this segregation occurs during the sex cell formation or meiosis. The principle of independent assortment means that different allele pairs are passed off to offspring independently of each other, which results in possible new combinations of genes which neither parent has. The genes for independently associated traits are located in different chromosomes. Though these principles formed the basis for modern genetics there are some exceptions to these rules Mendel didn't have knowledge of. Some biologists refer to his principles as "laws" which also states the importance of his findings (Mendel's genetics.)

4.2 T-DNA tagging

Insertional mutagenesis is used to provide a more rapid way of cloning a mutated gene. Transposons and the T-DNA of *Agrobacterium tumefaciens* can be used as mutagens to create loss of function mutations in plants. The sequence of the inserted element is known and so the gene in which the insertion has occurred can be recovered using various cloning or PCR strategies. T-DNA use has advantages over transposons in that they do not transmit subsequent to insertion and they are chemically and physically stable for many generations. T-DNA disrupts the expression of the gene in which it is inserted and also acts as a marker for the identification of the mutation (Radharmony – Prasad – Srinivasan 2005.)

4.3 Dicotyledonous plants

Dicots have two cotyledons and the seed leaves are usually rounded and fat. The first leaves can be many different shapes. Most trees, shrubs, perennials and garden annuals are dicots and they are more common than monocots. In dicots, the cotyledons contain

the endosperm which supply the plant with food. Dicots have a long tap root which enables larger growth (Monocots and Dicots)

4.4 *Arabidopsis thaliana*

Wall cress is a small flowering plant, which is often used as a model organism in plant biology. It is from the mustard family (Brassicaceae). It has no commercial value and is considered a weed, though in biological study it has many uses. It has many advantages as a model organism. The plant has one of the smallest genomes in the plant kingdom; just 135×10^6 base pairs of DNA distributed in five chromosomes and almost all of this DNA encodes its 27407 genes. Transgenic plants are easy to make using the *Agrobacterium tumefaciens* as a vector for transmitting genes to the plant. The plant is under 30 cm high and can be easily grown in a small laboratory environment (Biology-pages.info.)

It develops rapidly, taking only five to six weeks from seed germination to the production of a new crop of seeds. *Arabidopsis* is a very prolific seed producer, making up to 10000 seeds per plant, thus making genetic studies easier. Mutations are also easily generated e.g. by irradiating the seeds or treating them with mutagenic chemicals. It is normally self-pollinated so recessive mutations become homozygous quickly and subsequently expressed. Other members of the plant's family cannot self-pollinate since they have an active system of self-incompatibility, whereas *Arabidopsis* has inactivating mutations in the genes SRK and SCR that cause this difference. *Arabidopsis* can be easily cross-pollinated for genetic mapping and to produce strains with multiple mutations (Biology-pages.info.)

Arabidopsis thaliana genome has been sequenced in the year 2000 and there are extensive genetic and physical maps of all 5 chromosomes. There are many mutant lines and genomic resources available from Stock Centres (The *Arabidopsis* Information Resource).

4.5 Basic plant anatomy

4.5.1 Root formation in *Arabidopsis*

After embryogenesis dicot plants have created two meristems, one to form the above-ground shoot systems and the other to form the roots. There is a great diversity in root systems in nature, but a simpler system has many advantages in the study of root development and other systems (Bowman 1993: 93.)

Arabidopsis roots have a small cell number and size, which make it an ideal system for the study of morphogenesis and root function examination. The root meristem is not surrounded by a developing primordia and axillary meristems like the shoot meristems, but is open and exposed. No removal of tissue is needed. The root's small size makes it quite transparent and subject to different experimental manipulations. Individual cells of the root can also be observed in real time and recorded. A meristem is the region capable of diversion and growth in plants (Bowman 1993: 93.)

The embryonic root meristem or radicle in the Brassicaceae is obtained from cells of the proximal suspensor cell and the adjacent cells of the lower part of the embryo proper. During germination, this meristem initiates the primary root growth. Lateral root primordia form from internal tissue layers some distance away from the primary root meristem. This new lateral meristem is formed in the pericycle to emerge on the surface after bursting through the surface layers of the endodermis, cortex and epidermis. Most roots on a plant are of lateral origin because there is only one primary root meristem (Bowman 1993: 93.)

4.5.2 Cell organization in the primary root

The distal part of the root is covered by a root cap, which is composed of two cell populations clonally unrelated to each other. The cells of the central cap are formed from initials originating from the hypophysis. The cells on the lateral root cap on the sides of the root are derived from the embryo proper cells and they also form epidermal tissues. The cells on the outer layer of the root cap secrete a wide range of molecules, including complex polysaccharides and glycoproteins. These secretions may lubricate the growing root as it grows through the soil. Cells of the lateral root cap are torn apart because of expansion of the underlying epidermal layer, which appears at the root surface only after the root cap tissue is destroyed. These emerging epidermal cells are in the process of elongation (Bowman 1993: 94.)

Root hairs grow from the base end of the cells about the stage when cell lengthening in the epidermis stops. The epidermal cells are arranged with separate files of hair- and nonhair cells, where the hair-forming cell files are usually interspersed with a couple of nonhair cells. The hair grows by a process called tip growth, which is characterized by the polar deposition of membrane and wall material at the tip of the growing hair (Bowman 1993: 94.)

4.5.3 Cellular organization of the root meristem

The *Arabidopsis* root meristem can be considered to be composed of three tiers of cells, giving rise to different sets of tissues. The lower level of cells forms the root cap and epidermis. The middle tier cells create cortical and endodermal files and the upper cells appear to give rise to stele tissue containing the pericycle and vasculature. The cell organization is considered to be “closed” which means the cells are restricted to preordained tissue blocks, like in many of the Brassicaceae and other angiosperm families. Distinctive features in the organization of the *Arabidopsis* root are its small size and quite invariant number of cells in the primary root (Bowman 1993: 94.)

4.6 The plant vascular system

The plant vascular system transports water, nutrients and photosynthates between plant organs and undergoes developmental adaptations, like wood formation. Wood formation involves specific proliferation of the vascular tissue and so the pattern of cell divisions is an important determinant of the cellular organisation in the tissue. Vascular tissue is formed during embryogenesis as an undifferentiated procambial tissue in the most inner domain of the plant embryo, surrounded by the epidermal and ground tissue layers (Mähönen – Bonke – Kauppinen – Riikonen –N. Benfey – Helariutta 2000.)

After the phloem and xylem strands in this area have differentiated, cell proliferation originates from the initial cells of the procambial tissue, near to the mitotically quiescent regions of the terminal meristems. Later on a lateral meristem, the cambium, is formed as undifferentiated cells start to divide in the procambial tissue between the phloem and xylem strands. The cell-division patterns in the vascular tissue is very diverse in different plants and it is species specific. Because of this, it is probable that cell proliferation in

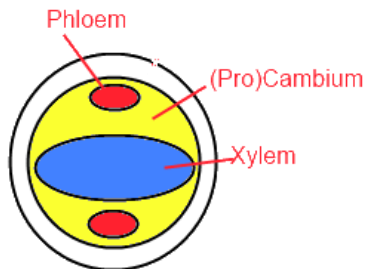
the vascular tissue is mainly genetically regulated (Mähönen – Bonke – Kauppinen – Riikonen –N. Benfey – Helariutta 2000.)

4.6.1 Vascular cambium development

Secondary phloem and xylem tissues are made through the vascular cambium, which is a cylindrical secondary meristem arising among primary plant tissues. Most dicotyledonous species go through secondary development, the Arabidopsis included. The plant is small and herbaceous in its nature but it has prominent secondary growth in many organs, including its root, hypocotyl and shoot. Due to these characteristics and vast genetic resources available to use in its study Arabidopsis is a versatile and accessible model organism for studying cambial development and wood formation (Nieminen–Blomster – Helariutta – Mähönen. 2015.)

Plant vasculature forms an organized mesh of interconnected cells throughout the whole plant, from the roots to the leaves. The vascular system in multicellular plants has two main functions; long distance transport and mechanical support. Xylem cells have thick secondary cell walls rich in lignin, cellulose and hemicellulose and they are mainly responsible for providing support for the plant. They also manage the bulk of the transportation of water, nutrients and minerals throughout the root system and shoots. Phloem handles the transportation of the autotrophic energy source, photoassimilates and signalling molecules, including plant hormones and peptides (Nieminen, Kaisa – Blomster, Tiina – Helariutta, Ykä – Mähönen, Ari Pekka. 2015.)

Plant growth arises from mitotic cell divisions in growth locations called meristems. The earliest primary meristems are of embryonic origin; these include the root apical meristem (RAM) and the shoot apical meristem (SAM). These contribute to root and shoot elongation, while also producing the primary plant body including the primary vasculature. The vascular anatomy in Arabidopsis differs between its roots and shoots. In the primary shoot, the vasculature is based in different collateral vascular bundles with the primary xylem towards the pith parenchyma cells. In the roots, the vascular tissue is organized in a bisymmetric pattern where primary xylem forms a central axis flanked by two poles of primary phloem. The procambial cells are surrounding them (Nieminen, Kaisa – Blomster, Tiina – Helariutta, Ykä – Mähönen, Ari Pekka. 2015.)



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At the beginning of secondary growth these procambial cells start to divide parallel to the plant axis/ surface which gives rise to secondary xylem (inwards), secondary phloem (outwards) and a secondary meristem called the vascular cambium, which forms a ring. The lateral or secondary growth in plants originates from the vascular cambium, and is a process which must be strictly regulated so that the plant vasculature is well formed (Nieminen– Blomster– Helariutta– Mähönen. 2015.)

Meristematic cells are small, cytoplasmic and undifferentiated; they divide by pushing away the outermost cells from the meristem, which subsequently cease division and initiate turgor-driven cell expansion and differentiation into specialized cell types. Cell proliferation and differentiation is crucial for meristem indeterminacy and they are both evidently under genetic control. There are many different features shared between the regulation of primary and secondary meristems and throughout plant life and a possibility that indeterminate growth may be sustained with the same hormones or genes/ functionally overlapping mechanisms (Nieminen– Blomster– Helariutta– Mähönen. 2015.)

Vascular meristems create cells which differentiate into xylem and phloem. The apical meristems in the shoot and root contain procambium, which is the primary vascular meristem; vascular tissue in the primary root and hypocotyl is derived from embryonic provascular tissue. Shoot vascular tissue originates from the shoot apical meristem. Species which undergo secondary growth develop a lateral vascular meristem called cambium, which develops mostly from procambium embedded between the differentiated xylem and phloem. In the shoot cambium arises from parenchyma and endodermis tissues. The complete ring of vascular cambium is formed early in the root/ hypocotyl area (Nieminen– Blomster– Helariutta– Mähönen. 2015.)

4.6.2 Plant hormones

Embryogenesis is a hormonally regulated event, where auxin plays a major role (Friml *ym.* 2003: 147-53). The roots and stems of dicotyledonous plants thicken by cambial cell proliferation, which is affected by environmental activities. The molecular mechanisms to regulate cambial activity are largely unknown. Cytokinins are important regulators of cambium development and the production of it in either the root or shoot is enough for normal development of both the root and shoot (Matsumoto-Kitano, M *ym.* 2008.)

When a quadruple *Arabidopsis thaliana* mutant *atipt1;3;5;7* had four genes encoding cytokinin biosynthetic isopentenyltransferases disrupted by T-DNA insertion, it was unable to form cambium and showed reduced thickening of the root and stem. The *atipt3* single mutant with slightly decreased levels of cytokinins showed decreased root thickening without other recognizable morphological changes. Exogenously supplied cytokinins to the quadruple mutant reactivated the cambium in a dose dependant manner (Matsumoto-Kitano, M *ym.* 2008.)

5 Methods

5.1 Sterilization

Seeds used in the laboratory are sterilized using chlorine gas or with a chlorine-ethanol combination. The sterilization is to ensure that all seeds used are without possible moulds or bacteria, which could contaminate the sterile growth environment and affect the plant growth, thus affecting the results of the research.

The chlorine gas sterilization is done in an exicator which is located in the acid laboratory to avoid poisonous chlorine gas being leaked. The seeds to be sterilized are placed in their tubes with the caps open into the exicator with 50ml of commercial Klorite in a 100ml beaker. The beaker size is large to contain any foam forming. Next a plastic pipet and pumpet will be used to put 1,5ml of 37% HCL into the glass beaker with Klorite. The chlorine gas formation starts immediately and the exicator hood must be closed quickly. The seeds are incubated for three hours in the gas, after which the gas will be let out and the seeds left to air for a while to make sure all gas is gone before taking them out. Lastly 0,1% agarose is added to the seeds and cold treatment applied. Gas sterilization

is optimal for sterilizing large quantities of seeds, because the exicator can fit many seed tubes at once.

The seeds can also be sterilized using liquid chlorite. Klorin with some Tween20 liquid is pipeted into the test tubes with the seeds and they are incubated for three minutes. After the incubation, the liquid is pipeted away and the seeds are washed once with 70% ethanol and three times with sterile water to remove any residue Klorin. Chlorine can damage the seeds and their germination if it is not removed well. The seeds are kept in a shaker during the washes to make sure all seeds are treated and fugged before removal of the wash water to make pouring the liquid easier. The seeds are lastly covered with 0,1 % agarose like in the previous method. This sterilization is better suited to smaller quantities of seeds.

5.2 Root collecting

The roots are collected from their growth plates using a scalpel and tweezers. The sample can be the whole root or just a part of it, cut usually roughly two centimetres below the hypocotyl area. Right under the hypocotyl is the oldest part of the root, where the secondary growth process has usually reached the furthest level. The leaves and the side roots are cut away with the scalpel to make analysing the samples easier. The cut samples are then moved to well-marked wells to await either GUS-staining or the normal fixation process.

5.3 GUS- staining

GUS refers to *Escherichia coli* β -glucuronidase gene which acts as a gene fusion marker when analysing gene expression in transformed plants. GUS expression can be measured with accuracy using fluorometric assays of small amounts of transformed plant tissue. Plants expressing GUS are normal, healthy and fertile and the fusion marker is very stable. Even after prolonged storage high levels of GUS activity can be measured from plant tissue (Jefferson – Kavanagh – Bevan 1987.)

First the samples are placed in 90% acetone on ice for 30 minutes for fixation, after which the acetone is washed away 2x with 0,5M NaPi with and incubation time of 10 minutes per wash. The pH used is 7,4-10. Next the GUS staining solution is added, with a 1,5mM ferri/ferri concentration if the Fi/Fo concentration is increased, it helps to transform the

soluble intermediate into un-diffusible blue colour faster, inhibiting signal diffusion but at the same time decreasing the GUS enzyme activity.

The stain is infiltrated into the sample using a vacuum for 5-30 min or 2 hours depending on the thickness of the sample. The vacuum must be gently released to avoid the liquid in the sample wells from splashing. The processed samples will now be incubated in 37 degrees centigrade and dark, until the desired staining efficiency is reached. The time usually varies from 20 minutes to overnight incubation. The samples should be checked hourly under microscope to avoid over staining the samples.

5.4 Fixation and tissue processing

The roots are fixed in a fixation solution containing 25% glutaraldehyde, 37% formaldehyde, some 1M sodium phosphate buffer (Nap) with a pH of 7,4 and water. The fixation liquid is toxic and waste containing it must be disposed to a specific waste collection container and work with fixed roots should be done in a laminar using gloves. Parafilm should be wrapped around the sample before putting it in a fridge preferably overnight but three hours is the minimum time.

After fixing the roots will be dehydrated using an ethanol gradient. The incubation times in each gradient can be 20 or 30 minutes, I have used both depending on my schedule. First the fixation solution must be removed from the samples before adding 10% ethanol (EtOH) for 20 minutes. The alcohol waste will be put into a separate waste collection bin until the gradient of 70% EtOH is added, to make sure no fixing solution is left. The gradients used next are 30%, 50%, 70%, 96% and 100% EtOH. The samples can be left in the 70% EtOH overnight and the 100% is used twice.

After the dehydration solution A will be added to the samples, beginning with a 1:1 solution A, where half of the volume is absolute EtOH. Solution A is made using 50ml of basic Resin and one sachet of Activation powder. No GUS samples should be incubated in the 1:1 solution A for 3 hours to several days, whereas GUS samples 1 hour.

5.5 Moulding roots

The roots have to be moulded into plastic to be later cut into microscope slides with the microtome. The first step is to place the roots into chambers made from thin plastic sheets, into which they are arranged into straight rows so that the hypocotyl areas of the

roots are at the same level with each other, so that we can get cuts from the same area of the root and the results from analysis are comparable.

The chambers are filled with 14:1 ratio of Solution A and Histo-resin hardener, which will harden into plastic when the chambers are covered with thin plastic sheets to induce anaerobic conditions. Filled chambers are left to harden overnight. Challenges include avoiding air bubbles in the chamber and getting the roots aligned so that the images taken later on will have straight root cross sections.

The hardened sheets of plastic with the roots are cut into 0.5 cm pieces (from the hypocotyl of the root) and stacked on top of each other using the same hardening mix. The area under inspection is placed along the edge of the petri dish the stack is made on. The stack order must be well documented, so that you will know which sample is which under microscope. The stack must be made unsymmetrical using empty sheets of plastic. After the stack has dried, it will be placed into a larger mould so that the cut, smooth edge of the stack is facing the bottom of the mould.

The moulds are to make big chunks of plastic to be cut with the microtome and are filled with the same 14:1 hardener mix and left to dry.

5.6 Sectioning, colouring and observation

Once the moulds have dried overnight to make solid plastic chunks, they will be removed from the moulds and glued into wooden blocks marked with the corresponding stack name and date. The hypocotyl area is facing the wood so that the cut area is facing the microtome blade.

Essentials needed for using the microtome are tweezers, sample blocks, microscope slides, slide holder box, a pencil and a brush. The blocks are first cut using a 10 μ m depth to get the excess plastic away until the sample is reached. Then it is cut about 60 times to even out the sample and to make sure all roots will be visible in the slides. The samples will be then cut with 5 μ m thickness, unless it is a Gus coloured sample where the same 10 μ m is used. Once the cuts seem like they have the whole block you will start cutting at set depths in sets of three.

First cuts are number 1, 2 and 3 then 11, 12 and 13 and finally 21, 22 and 23. The plastic cuts are thrown into a 50°C water bath and then picked up on the glass, which is placed on a 50 degree Celsius heat block. One block will be made to a total of three microscope slides, each containing a set of three from different depths. Only the best slide out of the three will be coloured and microscoped and the rest are in reserve. In the best slide only one of the three cuts are needed to take pictures.

The chosen slide is coloured using Ruthenium Red 0,05% in H₂O and Toluidine Blue 0.05% in 0.1M sodium phosphate buffer which has a pH of 7.4. The glass is first dipped into the Ruthenium Red for five seconds, rinsed twice with water and then dipped into the Toluidine Blue for four seconds, after which it is rinsed and put to dry. The blue colour is stronger than the red so it is used last and for a shorter time. The slide is now ready for microscoping.

6 Execution

I used the methods outlined in the "Methods" section in all the experiments. The methods explained are based on the laboratory protocols I have used while working on my Thesis. Straying from these is noted in the text. All seeds were sterilized using the gas sterilization method and seeds ordered from the stock centre NASC, except for the ones grown from previous plant seeds

6.1 Salk- lines

6.1.1 Re-analysed lines

In this experiment, I analysed 36 lines of Arabidopsis mutants previously analysed by another student. The mutants of interest were chosen by the researchers from earlier experiments.

The seeds for this experiment were grown from seeds collected from another student's plants. This shouldn't affect the results, because the seeds should be basically the same as those ordered from the stock centre. The seeds were sterilized and then plated on 1/2GM plates. Once old enough the roots were harvested from the samples and fixed until in solution A, where they were kept overnight in a fridge. The roots were placed in chambers with historesin and after they were set I made stacks 1, 2 and 3. Consequently

they were moulded into moulds for the microtome. After cutting the samples were coloured with red and blue colouring and microscoped.

Stack three had a confusing possible extra sample which we concluded was just an accidental root in an empty plastic piece, because there were so few roots. When adding empty plastic in the stack to make it unsymmetrical I must have taken a part with some residue roots by accident. After microscoping the roots were analysed with the ImageJ program and the root width and vessel number counted. I did the excel analyses and graphs to get the final results. This having been my first experiment from start to finish I was quite inefficient with the analysing, which made everything more difficult. Later with more knowledge everything was easier.

6.1.2 54 lines

The mutants screened in this work were chosen by results from previous experiments. These mutants have a mutation which might affect the cytokinin amount in the roots, which in turn affects root growth. The process was basically the same as for the 36 lines screened before.

The seeds were sterilized according to laboratory protocol and after cold treatment in the fridge plated on 1/2GM plates with the Columbia control. I used running numbers to replace the line names in order to make handling of the names easier and also so I can code the results later on. I missed number 16 by accident so that sample doesn't exist. The plants were grown for two weeks before harvesting and fixing. This growth time assures secondary growth in the xylem and phloem of the roots.

The roots were processed in the same manner as with the previous experiment, made into moulds which were cut using the microtome. The roots were coloured with red and blue colour according to protocol. The samples were microscoped and photographed before analysing root width and secondary xylem vessel numbers.

SALK- lines

6.1.3 Gus- staining experiment

For the Gus-staining procedure I had several lines; line RM9 1-16 which was a T2 selection and T3 selections RM13_16, RM13_13, RM13_8 with lines 1-4. RM13_5 had lines

1-5. These lines have been genetically modified by a procedure called floral dipping, where the wanted gene is transferred to the plant via an *Agrobacterium* construct vector.

The RM9 and RM13 are referring to two different promoters. The promoters might control the activation of certain genes of interest, which might affect the cytokinin amount in the roots, therefore affecting the secondary cambial growth either positively or negatively. Both results are interesting, because it suggests the genes affect the cytokinin regulation processes.

After Gus staining, active promoters with the attached Gus-gene cause a colour to be produced in the root tissue, creating the purple/bluish stain. From the area of the stain we can try and deduce what kind of genes the promoter might be controlling in the root and consequently the root growth processes. If a part of the root vasculature is coloured, it suggests that at least part of the hypothesis is correct because the promoters are active in the right area to probably affect root growth.

The original lines had already undergone a T1 selection, where the candidate lines were spread on an antibiotic Basta plate. From this plate, the surviving lines were chosen. The T2 lines have also been segregated again on an antibiotic plate to find lines with the correct number of insertions, which is one. One insertion is recognised by 25% of the plants being sensitive to the antibiotic and dying, while the other 75% are resistant. The T3 lines have survived the T3 selection, which finds homozygote lines, so they all have an identical pair of alleles for this specific trait. The lines which were survived these selections and moved to further processing were from the T2: RM9_4,8,9,10,12 and 15 (six lines). From the T3 the lines were: RM13_16-1, RM13_13-1, RM13_5-3, RM13_8-2 and 8-3 (5 lines).

The procedure for these lines was mostly the same as for the Salk-lines, except for a few differences. All the seeds were sterilized and then plated on Basta plates with four lines per plate. The T2 lines were plated with 40 seeds per line and the T3's with 20 seeds per line. The plants were grown in the Sanyo and removed after one week of growth. Now the procedure changes a bit; the roots are Gus-stained using the laboratory protocol before beginning the fixing. After the colour has been infiltrating the roots in the vacuum they were put into 37 degrees centigrade to colour for the time needed for the colour to become strong enough. I was checking the roots once an hour, avoiding light exposure to the roots which might affect the colouring. The T3 roots seemed to colour quite quickly compared to the others. They were a good blue/purple hue after three hours

whereas the T2 roots were basically uncoloured. The T2 roots were kept in the warm 37°C overnight and were sufficiently coloured in the morning.

After colouring the roots underwent the same procedure as the others. I accidentally added 0,1% agarose to the roots instead of 10% ethanol at first in the ethanol series, but I remedied it by washing the samples with 10% ethanol twice before continuing the procedure as my tutor instructed me to. After the roots were moulded they were microtomed with a difference of cutting the samples thicker than other root samples so the colour would show properly. The thickness was 10µm instead of the normal 5µm.

The microscope pictures were taken as with the other samples. The analysis however was different, because no width or vessel numbers were counted from the roots. Only the Gus-colouring was analysed if it was present.

6.2 Microscope analysis

The slides are analysed using a Leica microscope, usually at 20 or 40 times magnification depending on the root size and age. The slide is covered with a glass cover using MQ water. The sample orientation is deduced from the un-symmetry of the sample and pictures of each line of roots are taken and named accordingly. Knowing which sample you are analysing is crucial and sometimes quite difficult, since the borders between different samples can be unclear. Microscope analysis was very familiar to me from my studies but these samples were very different from the blood cells and bacteria I was used to.

6.3 Analysis using image programs

The program used was called ImageJ. I had no previous experience with this program, but once I had been taught the basics I was able to do the analysing quite efficiently. Before analysing the scale of 100 µm had to be set using a scale bar corresponding to the magnification used on the microscope. First I measured the width of each root by a segmented line, from the phloem to the xylem axis, to the phloem on the other side.

The phloem ball was quite easy to distinguish after some time of getting used to looking at the pictures. It was usually quite red compared to the rest of the root. The results were moved from the program to excel where they were arranged. It was important to keep

saving the results, because at one point I accidentally deleted about 50 measurements and had to do them all again. The second analysis was done counting the secondary xylem vessels in the roots and marking the result on the excel spread next to the width. The vessels counted had a certain blue colour to their walls which signified that they were mature.

6.4 Excel analysis

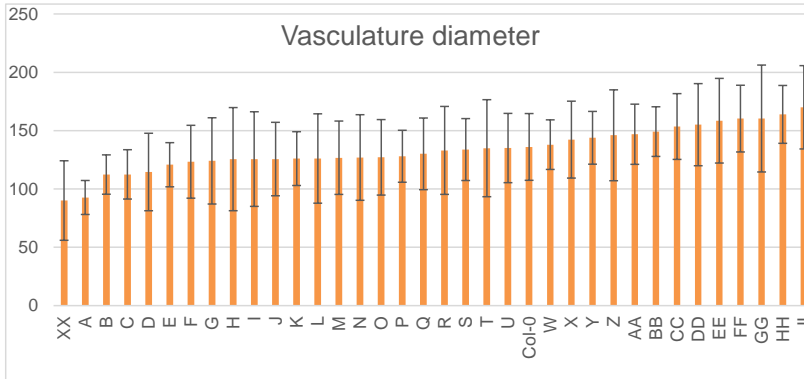
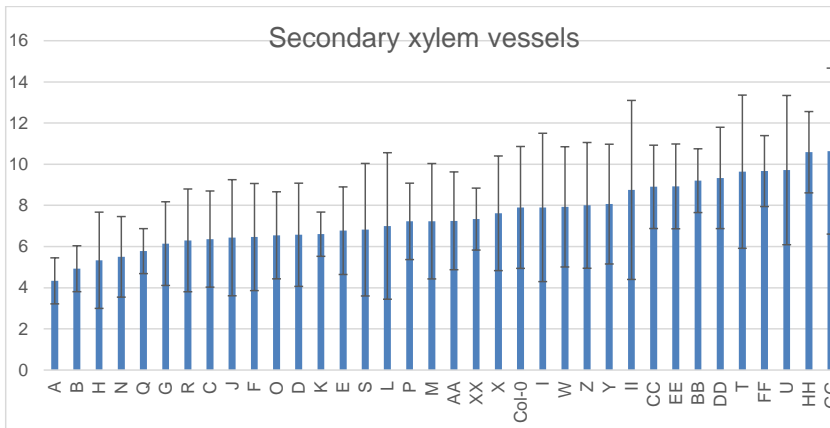
The data for the graphs and values was analysed on the Microsoft excel program. First all the measurements for each line were added to the table and then arranged. I calculated the averages and standard deviation for each line's results which I then used to form graphs for the xylem vessel number and root vasculature width averages. I arranged the graph values by order of size so the results are easier seen. The graphs were analysed by eye, which means I looked at which lines I thought had especially large or small values compared to the control. Statistical analysis of the results isn't a standard procedure in the research group so I didn't use that method.

7 Results

7.1 Re-analysed lines

A code number/letter has been used to replace the names of each line in the graphs shown because the results are part of ongoing unpublished results by the group.

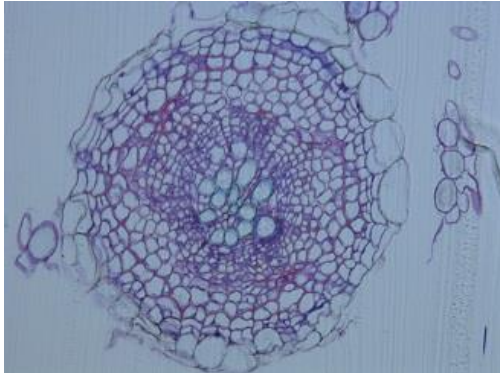
I analysed the graphs I had made from my excel analyses and chose some lines of interest and made a graph containing the vasculature diameter averages of each line arranged in order of size (graph 1) and a graph with the xylem vessel averages for each line (graph 2).

Graph 1: Vasculature diameter averages in μm 

Graph 2: Secondary xylem vessel averages

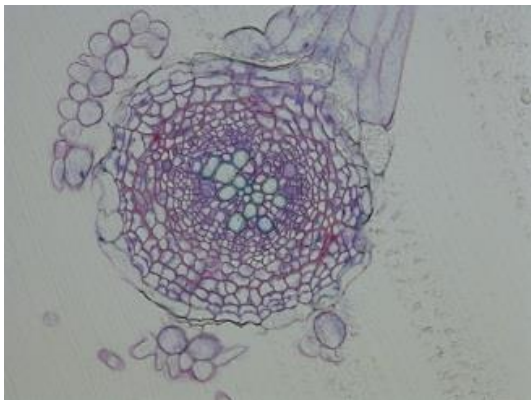
I chose two lines with the largest vasculature diameter and two smallest for further observation in these results. I chose a picture to represent each line, with the diameter and vessel number correlating as closely as possible with the average of that line.

The two largest root averages were in lines II and HH. Line II had a vasculature diameter average of $170 \mu\text{m}$ which is $33,98 \mu\text{m}$ larger than Columbia's $136,01 \mu\text{m}$. It had a vessel number average of $8,75$ which is $0,85$ vessels larger than Columbia's average of $7,9$ vessels (picture 1). This line however had only four pictures for the measurements so the results might not be reliable. Ten measurements would be better for counting the average.



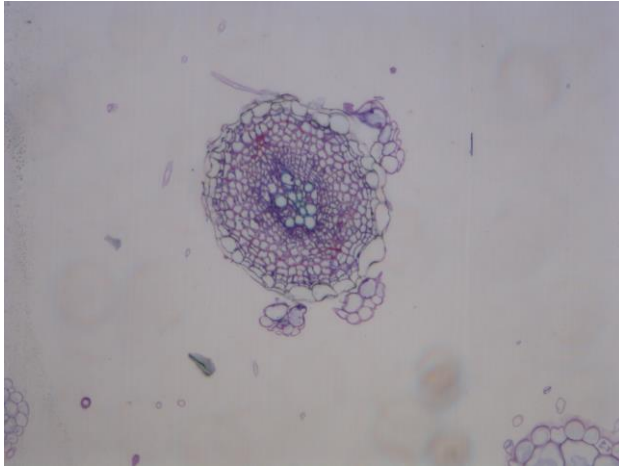
Picture 1: Line II root sample

Line HH had a vasculature diameter average of 163.92 μm , which is 27,92 μm larger than that of Columbia's. The vessel number average was 10,58 which is 2,68 vessels more than the control's (picture 2).

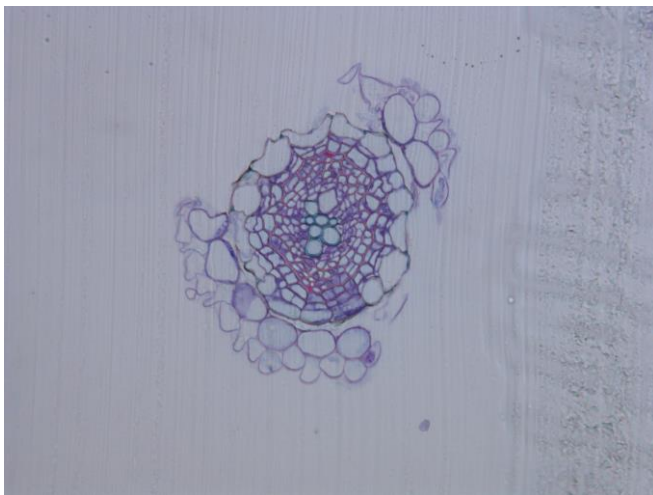


Picture 2: Line HH root sample

The lines with the smallest root and xylem vessel averages were lines XX and A. Line XX had a vasculature diameter average of -45,93 μm compared to Columbia and a vessel average of -3,6 compared to the control (picture 3). Line A had -43,31 μm vascular diameter and -3,6 xylem vessels compare to those of Columbia (picture 4).



Picture 3: Line XX root sample

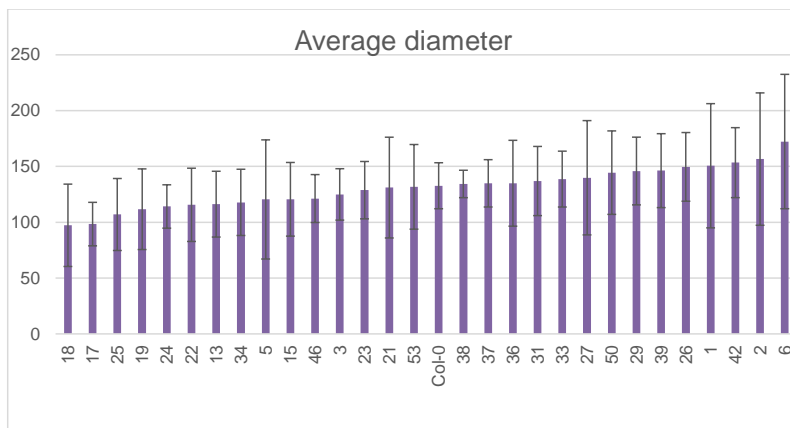


Picture 4: Line A root sample

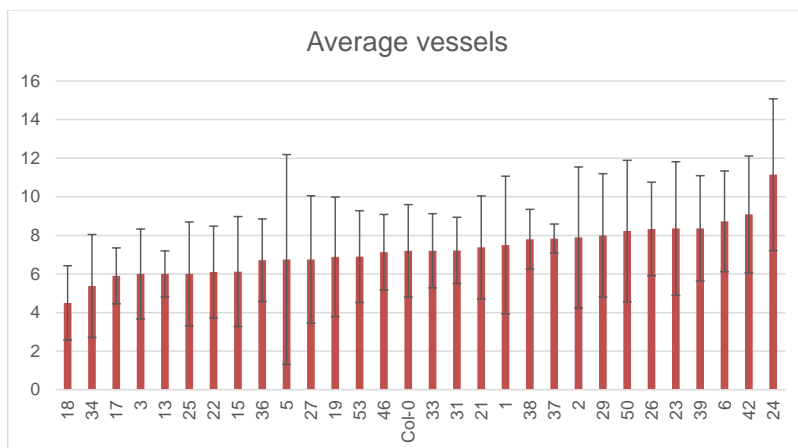
7.2 52 Salk lines

I analysed the same things with these lines as with the previous ones; vascular diameter averages and xylem vessel numbers. I made graphs from these values. I chose two lines with the highest and lowest of these values compared to the control to analyse further.

The two lines with the highest values were samples code named 6 and 2. Lines 18 and 17 had the smallest values in comparison to Col-0 (graphs 3 and 4).

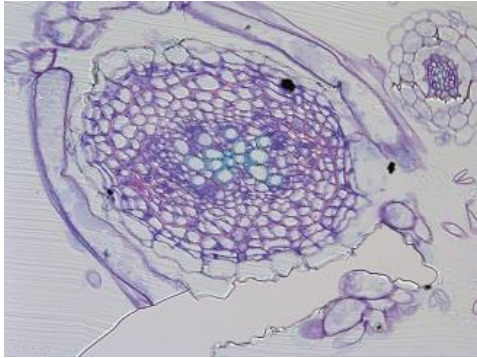


Graph 3: average vascular diameter in µm

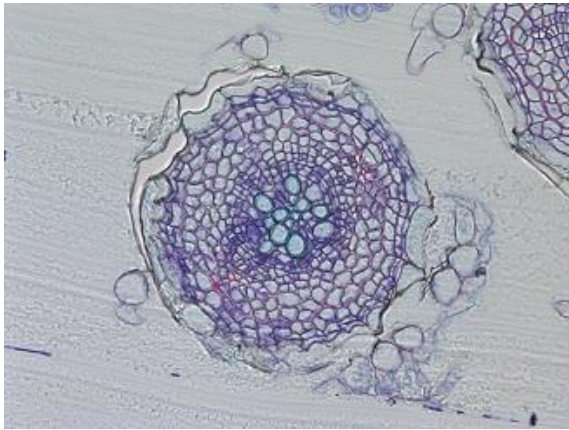


Graph 4: average vessels

Line 6 had a vascular diameter average of +36,6 µm compared to Columbia and 1,5 more vessels (picture 5). Line 2 had a diameter average of +23,87 µm in comparison to the control, but interestingly 1,3 vessels less on average than Columbia (picture 6).

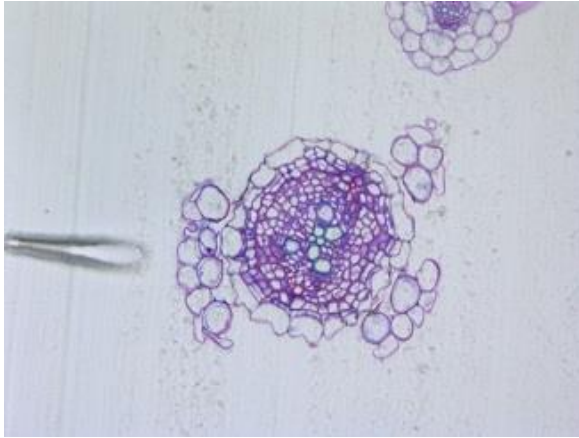


Picture 5: line 6 root sample

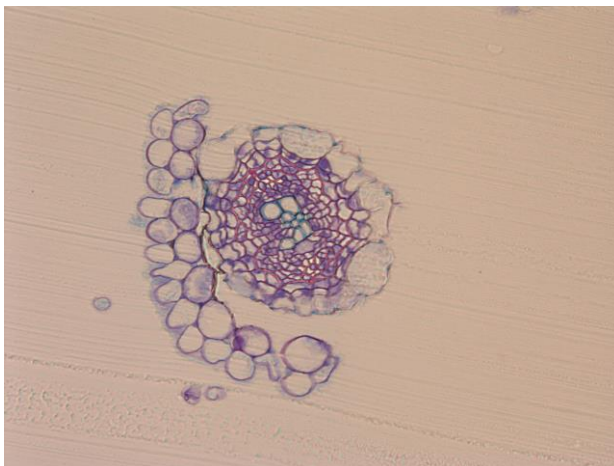


Picture 6: line 2 root sample

On the lines with smaller than the control averages line 18 had an average vascular diameter of $-35,43 \mu\text{m}$ and $-2,7$ vessels on average compared to Columbia (picture 7). Line 17 had an average root diameter of $-34,34 \mu\text{m}$ and xylem vessel number average of $-1,3$ compared to the control (picture 8).



Picture 7: line 18 root sample

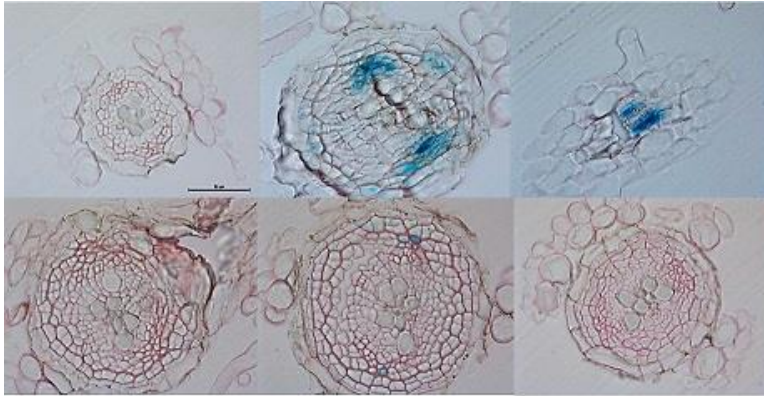


Picture 8: line 17 root sample

7.3 Gus- staining experiment

I analysed the results by first looking through each line of pictures and choosing one picture to represent each line. For example, if the line was not coloured at all I chose I root with no stain or if the stain was focused on a certain area in most pictures I chose one which most closely represented all the roots. I then made a collection of pictures for the T2 and T3 promoter selections.

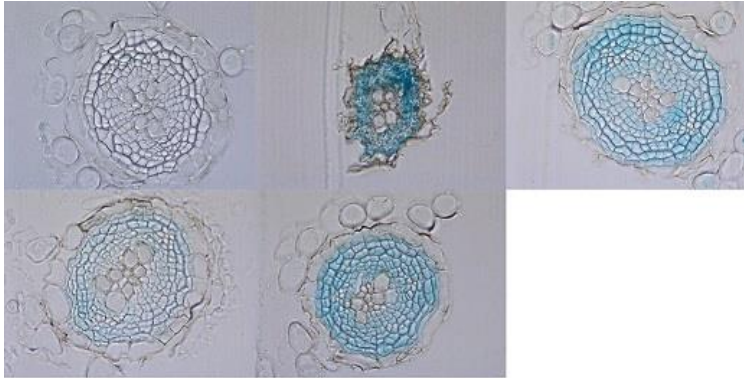
The T2 line staining seemed to be focused on the phloem balls of the vasculature in the root. Lines RM9_4,10 and 15 were not coloured (picture T2).



Picture T2: from the top going right: RM9_4, RM9_8, RM9_9 and the bottom going right: RM9_10, RM9_12 and RM9_15

The phloem is an essential part of the root vasculature, providing a pathway for the photosynthates to pass throughout the whole plant (Nieminen– Blomster– Helariutta– Mähönen. 2015). A gene in this area might be important for secondary cambial growth. The colouring also suggests a function of the gene which is quite specific in nature.

The T3 samples were mostly stained around the whole area of the root vasculature (picture T3). The line RM13_13-1 seemed disfigured for some reason, maybe due to drying out during the tissue processing; the stain seemed quite clear nonetheless. Line RM_16-1 was not coloured at all.



Picture T3: from upper left to right: RM13_16-1, RM13_13-1, RM13_5-3 and from bottom left to right: RM13_8-2 and RM13_8-3.

Staining of the whole vasculature area suggests that the gene controlled by the promoter is expressed all over the root. This expression covers a wide range of root functions, such as water and nutrient transportation in the xylem, photosynthate transfer in the phloem and cellular proliferation in the root (Nieminen– Blomster– Helariutta– Mähönen. 2015).

8 Reflection

8.1 Results

8.1.1 Re-analysed lines

For the re-analysed Salk lines, I found four interesting Arabidopsis mutant lines; two larger than average lines II and HH and two smaller than average, lines XX and A. Line II had a vascular diameter average of +33,98 μm 0,85 vessels more compared to Columbia. Line HH had a vasculature diameter average of +27,92 μm and 2,68 vessels more than the Columbia average. From the smaller lines, line XX had a vasculature diameter average of -45,93 μm and a vessel average of -3.6 compared to Columbia. Line A had -43,31 μm vascular diameter and -3,6 xylem vessels compared to the control.

8.1.2 54 lines

I chose interesting lines from the 54 lines analyzed in the same way as for the re-analyzed lines. The two lines with the highest values were samples code named 6 and 2. Lines 18 and 17 had the smallest values in comparison to Col-0. Line 6 had a vascular diameter average of +36,6 μm compared to Columbia and 1,5 more vessels, whereas line 2 had a diameter average of +23,87 μm in comparison to the control, but 1,3 vessels less on average than Columbia. Line 18 had an average vascular diameter of -35,43 μm and -2,7 vessels on average compared to Columbia. Line 17 had an average root diameter of -34,34 μm and xylem vessel number average of -1,3 compared to the control.

The secondary xylem vessel numbers seem to mostly correlate with the root vasculature size, but there are exceptions too. The line HH had a large amount of xylem vessels compared to Columbia, but line II vessel numbers weren't situated at the highest end of the graph. Maybe this result was affected by this line only having four pictures to count the averages from, so the results may not be very reliable. Line 2 was also interesting in terms of vessel numbers, because it had less vessels on average than the wild type though based on the root vasculature size one would assume the opposite.

As I chose these lines by analyzing the graphs visually, I can't be definite about where the line of meaningful difference goes in terms of having a larger or smaller vasculature width or vessel number average, but I think these lines might be of interest for further study. Both the larger and smaller than average lines seem to have some sort of difference in their root growth mechanism compared to the wild type and further study might reveal the reason behind that. The plant hormone cytokinin production or regulation is most likely involved, as that is an important part of what this research group is studying.

8.1.3 Gus- staining experiment

For the Gus- staining experiment I found that T2 and T3 promoter lines had different staining in their root vasculature area. T2 line staining seemed to be concentrated on the phloem ball area while in the T3 lines the whole vasculature was stained. It would seem the T2 promoter operates in a specific area whereas the T3 quite generally all around the root vasculature. Both are active in areas important for root growth and the stained lines might be valuable in further study. Some of the roots were not stained, which might be due to the staining being at a different part of the root than where the samples were

taken, but I am not sure of the reason. All roots were colored in the same way. The T3 roots were colored much quicker than the T2 which might be due to their homozygous genotype.

8.2 Credibility

All the research work was done according to lab protocol and if I made mistakes I corrected them as best I could with my tutor's help and recorded it in my lab book. We had controls for all the experiments and the Columbia results seemed to be as they should, situating themselves in the middle of the graphs. We also had multiple samples for each average, except for some exceptions. I think my results are reliable, though my inexperience in this work should be taken for account. I had very experienced lab personnel helping me throughout the whole process so any major mistakes would have been found and corrected.

8.3 Ethics

This research was done according to good research ethics, meaning it complies with the Nuremberg code. This research does not use any human or animal subjects so the main issues are related to the genetically modified plants. The laboratory operates according to Finnish laws for handling GMO material and all waste containing it is disposed of in the correct manner. The modified plants are usually modified to such an extent that they wouldn't do well even if they were exposed to the environment.

8.4 Professional improvement

During this thesis process I have learned vast amounts about subjects I had almost no previous knowledge. I was familiar to some of the laboratory techniques like pipetting, sterile working, doing the ethanol series and others which I had learned at school. I had plenty of experience with microscope analysis, though I had never analysed the kinds of samples as I did while working with the research group. Techniques involved in growing and sterilizing seeds, greenhouse working, collecting and fixing roots and many others were completely unknown to me. I feel that have vastly improved my laboratory skills in many areas from histology to microbiology and feel more confident doing laboratory work as a result.

I have learned that researching in general requires great patience and enthusiasm for the work, because small mistakes can easily ruin whole experiments and fixing them is hard work (see picture 8). Sometimes I had no idea how to deal with a difficult situation, but thanks to endless encouragement and patience from my tutors and the rest of the laboratory crew I could find solutions to problems and get the job done. The most valuable lessons I take away from this experience are never giving up when things seem hard and taking responsibility for my own work. I am grateful to have had the chance to see how valuable research work is done and to have learned so many new skills. I hope my work will have some value in their research.



Picture 8: Cleaning up after an overboiled 1/2GM growth medium is hard work, however you can always learn from your mistakes!

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Appendix 2

1 (1)