Patron: Her Majesty The Queen

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Professor Maurice Moloney

Mr Colin MacEwan – Head of BBRO
British Beet Research Organisation
The Research Station
Great North Road
Thornhaugh
Peterborough
PE8 6HJ

2 November 2012

Dear Mr MacEwan,

Placement Studentship project 12/11:
Understanding the genetic determinants of dry matter composition.

Please find attached the Final Report for the above mentioned project in the form of an Executive Summary and a detailed scientific report.

The scientific report was prepared by the student, Matthew Stevenson, to document the aims, approaches and outcomes of his 12 month placement project based with Dr Belinda Townsend at Rothamsted Research – Broom's Barn. The report forms part of the requirements of an Industrial Placement by the University of Reading, at which Matthew is now commencing his final year of a Bachelor of Science. It represents the application of a variety of plant science skills from field work, to molecular biology and microscopy.

The project progressed very well. Matthew has contributed greatly to the resources and knowledge that is now available to progress our understanding of the biology of the sugar beet crop. This was both directly as evidenced by the outcomes of his project, and indirectly by enabling his supervisor to focus on other work in trying to secure the future of strategic sugar beet research in the UK. He received excellent training opportunities to contribute to maintaining the knowledge base for agricultural scientists of the future with interests in sugar beet production.

Matthew also received formal training in the following topics:
1. Reference Management using Endnote (run by Rothamsted Research)
2. Introductory Microscopy (run by Rothamsted Research)
3. Media training – John Forrest Memorial training course (run by Green Shoots Productions)

Matthew also attended the following meetings and events:
1. CEREALS 2012 – Arable agricultural trade and communication event, Peterborough, June 2012.
2. Rothamsted Research Day – October 2011
3. British Beet Research Organisation Winter Meeting – Closing the Gap conference, Peterborough, January 2012.

The laboratory work and results that Matthew produced during his placement project will underpin future publications and outcomes targeted at improving the sustainability of the sugar beet industry in the UK. The BBRO will be acknowledged and notified of the release of any communications, knowledge, or products to arise from their investment in this project.

I would like to express my gratitude to the BBRO for supporting this project. I am now formally based at the Harpenden site of Rothamsted Research to continue my work on sugar beet if the funding opportunities present themselves. Broom's Barn will still undertake near-market research into sugar beet and I will be visiting and liaising with colleagues and industry there at every opportunity. Although this represents a significant change to the nature of work on sugar beet biotechnology, I remain committed to research that will positively impact the future of the UK sugar industry.

Please feel free to contact me if you have any further queries about this project or any future projects.

Yours sincerely,

Dr Belinda Townsend
Project Leader

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Project 12/11 Final Report

Understanding the genetic determinants of dry matter composition

Project Leader: Dr Belinda Townsend
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Ph: 01284 812200 email: belinda.townsend@rothamsted.ac.uk

Project Duration: 30 August 2011 – 29 September 2012

Staff Input: 1 year 100% Undergraduate student time Matthew Stevenson
1 year 10% Supervision by Dr B. Townsend

Costs expended: Student Salary £13,000: BBRO contributed £6,500 and Felix Cobbold Agricultural Trust co-funded with £6,500.
No institutional costs, directly incurred costs, services, travel, consumables, university fees, or supervision time were cost into this studentship.

Executive Summary:

Overall Aim: To understand the key genetic factors influencing the composition and development of the cell wall in sugar beet roots. The cell walls make up the majority of non-sucrose dry matter that is left in the pulp after sugar extraction, and the size of cells as well as the thickness of the cell wall is thought to be related to the capacity to store high concentrations of sucrose. Therefore, by understanding how carbon is partitioned between sucrose storage and the structural components of the cell wall, we hope to discover genetic targets to improve sucrose yields and/or the value of the pulp.

Objectives and progress:

1. Harvesting and processing of a sugar beet root developmental time course grown under field conditions (a commercial variety).

Completed in full. The 2011/12 season was harvested regularly to plan, and both 2011/12 and 2010/11 season material was processed by drying and grinding for storage. Both seasons were also extracted for nucleic acids (RNA) that contain a catalogue of all the genes expressed in the plant tissue at the time of harvest, and also all the metabolites present. Data on dry matter content, fresh weight, and root diameter were also collected.

2. Growth, harvesting and processing of a sugar beet root developmental time course focussing on very early seedling development.

Completed. It was initially planned to conduct this experiment in hydroponic culture to enable a very clean system where lateral roots could be harvested independently of the main storage root. A lot of time was lost trying to overcome contamination problems that plagued the system and was likely due to the water supply. Ultimately, it was decided to revert to a soil/grit mix for growth under controlled conditions. This successfully produced a 6 week time course of root material for gene expression and microscopic analysis.

3. Consult the scientific literature for information on key cell wall components likely to impact composition and the genes known to be involved in their biosynthesis. Identify sugar beet gene

equivalents and determine the expression pattern of these genes during root development and in various tissue types, from the 2010/11 field time course.

Completed in full. Five key genes were identified, based mostly on studies in model plants such as *Arabidopsis*. These included genes involved in xyloglucan synthesis (which is present in low levels compared to other dicot species), genes involved in xylan and pectin synthesis (with pectin content being comparatively very high in sugar beet roots), and also cellulose biosynthesis (which is the main six-carbon sugar source contributing to a majority of plant biomass). All of these genes are classified as glycosyltransferases because of their activity in utilising sugar molecules. A prior deep-sequencing project conducted by B. Townsend in 2009/10 provided a very useful catalogue of genes expressed in expanding storage roots. This 'root transcriptome' provided the sequence information for sugar beet that was needed to identify the gene sequence information, and information on the level of expression within the growing root. Gene expression analysis showed variation during development for one gene involved in xyloglucan synthesis (RGP3). The same gene showed some increases in expression during young seedling development, as did QUA1 (pectin/xylan synthesis). The gene responsible for the high arabinose content of pectin is ARAD1, and it had strikingly higher gene expression level in leaves than other plant parts.

4. Develop assays for basic analysis of cell wall composition in roots using antibodies and tissue staining. Analyse the changes in cell wall composition in developing roots.

Partially completed. Given the time lost to contaminated hydroponics, there was insufficient time to progress with the antibody compositional assays. The focus was on developing the microscopic analysis of early root development in seedlings, when the vascular rings are being laid down to determine the ultimate potential for sucrose storage and root expansion. Significant testing was required to obtain quality embedded sugar beet roots for sectioning and viewing under the microscope. A fluorescent stain that highlighted lignified walls of the vasculature was chosen to render the rings clearly visible.

5. Analysing recombinant inbred lines with a 'floppy stem' phenotype that may indicate cell wall perturbation

Partially completed. Upon growth of the plants, it was unclear if the phenotype was genuinely a result of cell wall issues or hormonal defects, therefore this objective became less of a priority. There were also no suitable control lines available for parallel growth so the potential for analysis was limited. None the less plants were grown, details recorded and key tissues sampled for future analysis of cell wall composition and DNA sequencing if required.

6. Write up the experimental approach, methods and results in a detailed placement report to be submitted to the University of Reading for examination, and as a detailed final report for funding agencies.

Completed in full. A report was prepared and submitted by the final day of the placement and is attached to this summary.

Outcomes:

1. There is now a complete set of three replicated field plots sampling the developmental time course of sugar beet root material (and some aerial parts) from seedling through to flowering. There is also a young seedling developmental time course from one experiment. These are available for comparisons of the plant response to different environmental conditions and stages of development, using genetic or biochemical testing methods. Preliminary analyses have already been conducted on one season and so they can now be extended to produce robust results for identifying breeding targets.

2. Cell wall-associated gene expression patterns have provided markers for changes in cell wall biosynthesis that can serve as targets for manipulation or characterisation of natural variation with a view to selecting for sugar beet lines with and ideal composition or to find a link between different compositions and increased sucrose content.

3. Technical knowledge and materials are now available to quickly investigate the cell-specific and spatial expression of gene sequences within the sugar beet storage root, and link them to a basic understanding of cell types and cell wall composition of those cells.
4. Preliminary data and resources generated will now serve to strengthen future grant applications to attract public investment in sugar beet research.
5. Investment in quality training and career development of a science student to provide the skills for future research into agriculture and sugar beet in particular.

Potential for Intellectual Property Exploitation:

The identification of gene sequence information and expression data is not sufficient to acquire rights over their use by others. To progress to that stage we require functional characterisation of these genes as part of subsequent projects, such as by generating transgenic sugar beet for experimental use (just one example). However, this project represents a first step towards demonstrating a potential role or application of these genes that could then warrant a patent application for their exploitation and subsequent licensing to interested parties such as sugar beet breeding companies or agrochemical companies.

Further proposals for research:

This 12 month placement studentship generated the plant materials, preliminary root development understanding, and preliminary gene expression data that will underpin a future PhD studentship proposal. The four year PhD studentship is in collaboration with the University of Leeds, and will look specifically at the role of pectin in sugar beet cell walls in cell expansion, sucrose accumulation, and the applications of pulp to add value to the crop. This is a BBSRC Industrial CASE studentship, where the student is required to spend at least 3 months working with industry, for which Tony Sidwell at British Sugar Wissington factory has agreed to be the Industrial Supervisor. This represents an excellent opportunity to keep fundamental scientific research focussed on industry needs. This studentship requires at least £5,000 per annum support from industry (over 4 years) for which a forthcoming full proposal will be submitted to the BBRO to commence in April 2013 financial year (the student would not start until October 2013). The BBSRC decision to fund this project should be known by December 2012.

By generating data as part of this project, we are now more likely to succeed in attracting further sources of public funding such as Technology Strategy Board (TSB – for which the collaboration with British Sugar plc is very important) and BBSRC or EU funding opportunities. We anticipate submitting applications to extend the sugar beet genetic improvement program in the spring of 2013.

Communication of the results:

The preliminary data generated as part of this project will go towards in-depth analyses that are intended for publication in peer-reviewed scientific journals in 2013, and will also be described in lay terms in the British Sugar Beet Review. The BBRO will be acknowledged in all communications regarding this project.

The student attended CEREALS 2012 and the BBRO Winter Meeting in 2012 where he was available to communicate his work during informal discussions.

Results from the work have been included on poster presentations made to scientific staff at Rothamsted Research Day 2012, and the student formally presented the data and conclusions to staff at Broom's Barn. The placement report has been submitted to the University of Reading for examination.

Developing Methods to Assess Sugar Beet Pulp Composition

Matthew Stevenson
University of Reading

Industrial Placement with Rothamsted Research – Broom's Barn

August 2011 – August 2012

Funded by -

Felix Thornley Cobbold Agricultural Trust

British Beet Research Organisation

Developing Methods to Assess Sugar Beet Pulp Composition.

Introduction

Rothamsted Research is the longest running agricultural research station in the world, providing cutting-edge science and innovation for nearly 170 years. The institute's mission is to deliver the knowledge and new practices to increase crop productivity and quality and to develop environmentally sustainable solutions for food and energy production. My placement is at Broom's Barn, Department of Applied Crop Sciences. Broom's Barn is the UK's national centre for sugar beet research, located 20 miles east of Cambridge. Research spans a range of crops and scientific disciplines and includes liaison/extension work with growers and the industry.

SUGAR BEET

Beta vulgaris is a member of the Chenopodiaceae family, in the order Caryophyllales. It is a highly variable species with four main agriculturally significant groups, leaf beets, fodder beets, garden beets (such as beetroot) and sugar beet.

Sugar beet is a biennial plant. The first year's growth is characterised by vegetative growth and sucrose accumulation and the second by reproductive growth. It is sown in spring and harvested before the winter of the first year. In order for the plants to flower in the second year vernalisation is required. This can also occur if the seedlings are subjected to a late frost soon after establishment. Vernalisation is the process by which plants are triggered to flower in response to a period of cold weather. Once temperature and day length increase in the spring the plant grows an elongated stem from which the reproductive organs develop. At this stage the storage root becomes a source of energy rather than a sink for storage.

Most of the world food comes from around one hundred and fifty plant species, but sugar (the common name for sucrose) comes from just two crops, sugar cane and sugar beet (Draycott, 2006) . Sugar cane is grown in tropical regions. Cane sugar has been produced in large quantities for centuries and continues to dominate the world supply of sugar. By contrast sugar beet is a relatively new crop appearing in temperate regions in the nineteenth century and only becoming wide spread in the twentieth century. Sugar beet is now grown in over fifty countries and accounts for around a quarter of world sugar production (Draycott, 2006).

The cell wall

Plant cell walls develop in two stages. Primary cell walls are synthesised by growing cells and are relatively thin and flexible accommodating cell expansion. Primary cell walls also generate turgor pressure and mediate cell adhesion. Once cells have stopped expanding specific cell types then synthesise secondary cell walls between the primary wall and the plasma membrane. These are thicker than primary walls and resist compression forces. Many secondary cell walls are further strengthened by lignin, a complex polymer of phenolic residues. Secondary cell walls are particularly important in cell types involved in transporting water and providing mechanical strength to the plant (Lee et al., 2011). Cell walls do not have a fixed composition; they are continually being modified as the plant grows and develops and in response to the environments and interactions between plant and symbionts and pathogens.

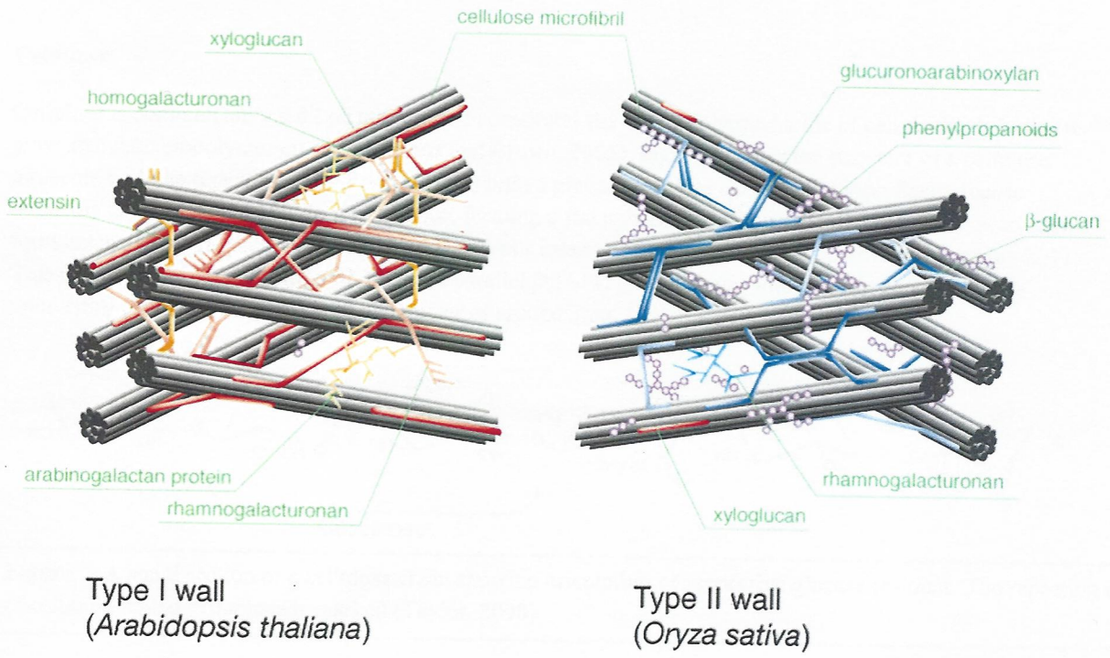


Figure 1. A schematic structural model of type I and II type wall as represented by Arabidopsis and rice cell walls respectively (Yokoyama and Nishitani, 2004)

The primary cell walls of plants are classified into two major groups, Type I and Type II primary cell walls, with respect to, wall architecture, the chemical structure of components and their biosynthetic processes (Carpita, 1996) shown in figure 1. Primary cell walls consist mainly of polysaccharides but also contain structural proteins, glycoproteins and many different enzymes (Liepman et al., 2007). Dicotyledonous plants and non-commelinoid monocotyledonous plants synthesise type I walls, which contain roughly equal amounts of cellulose and cross-linking xyloglucans (hemicellulose) (Yong et al., 2005), with minor amounts of arabinoxylans, glucomannans and galacto-glucomannans. The cellulose-xyloglucan framework is embedded in a matrix of pectic polysaccharides which is comprised primarily of homogalacturonans (HGA), rhamnogalacturonan I (RGI) and rhamnogalacturonan II (RGII) (Yokoyama and Nishitani, 2004). The pectin matrix controls several physiological properties such as microfibril spacing, charge density and wall porosity (Willats et al., 2001). Type II cell walls, found in commelinoid monocotyledonous plants which include cereals like rice, wheat and barley, contain the same cellulose microfibrils as type I walls but less xyloglucan. The predominant glucans that cross link cellulose microfibrils are glucuronoarabinoxylan and β1,3:β1,4 mixed glucans (Kato et al., 1982). Type II walls contain less pectin than type I walls but contain higher amounts of phenylpropanoids (Yokoyama and Nishitani, 2004) which form extensive interconnecting networks. An abundance of cell wall bound ferulic acid is a distinctive feature of type I cell walls in sugar beet and other members of the order Caryophyllales pectic polymers are feruloylated (Ralet et al., 1994; Renard et al., 1999). Sugar beet has unusual cell walls for a dicotyledon. Most dicotyledons have type I cell walls but sugar beet doesn't really fit into type I or type II. Unlike most dicotyledons (type I) sugar beet cell walls are almost devoid of xyloglucans and other hemicelluloses (Renard and Thibault, 1993; Renard and Jarvis, 1999) and therefore they play a negligible role in the cell wall architecture. Renard and Jarvis (1999) proposed that other molecules in the cell wall must be performing a similar function to xyloglucans. Zykwinska et al (2007) showed in sugar beet that neutral sugars on the side chains of RG1form non-covalent interactions with cellulose microfibrils thus linking pectin and cellulose together.

Cellulose

Cellulose is common among all plants where it constitutes the major polysaccharide of cell walls. It is also the most abundant biopolymer on earth (Saxena and Brown, 2005). Figure 2 shows the structure of a cellulose molecule with the repeating unit of two β(1→4) linked glucose residues called cellobiose. Each glucose molecule is orientated at 180^0 to it neighbour, forming a flat unbranched chain. Parallel β(1→4) glucan chains form extensive hydrogen bonding and Van Der Walls interactions with each other (Endler and Persson, 2011). This results in the crystallisation of thirty six parallel β(1→4) glucan chains into the functional cellulose microfibril (Somerville, 2006) with a diameter of around 3nm.

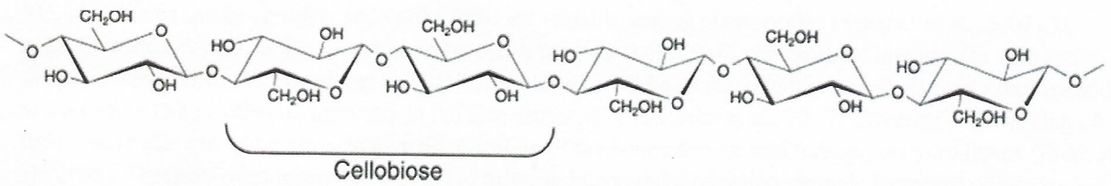


Figure 2. A small section of a cellulose chain showing orientation of successive glucose residues. The repeating unit of cellulose called cellobiose is marked (Taylor, 2008)

Pectin

Pectins are a group of acidic polysaccharides which can be divided into four main groups, HGA, RGI, RGII and xylogalacturonan (XGA). There is much species variation in the ratio of the different groups but in most angiosperms the most abundant pectic polysaccharide is HGA which makes up around 65% of pectin, RGI 20% to 35% and RGII and XGA less than 10 % each (Harholt et al., 2010). The different pectic polysaccharides are not separate molecules but covalently linked domains as shown in Figure 3 a representation of the four different domains.

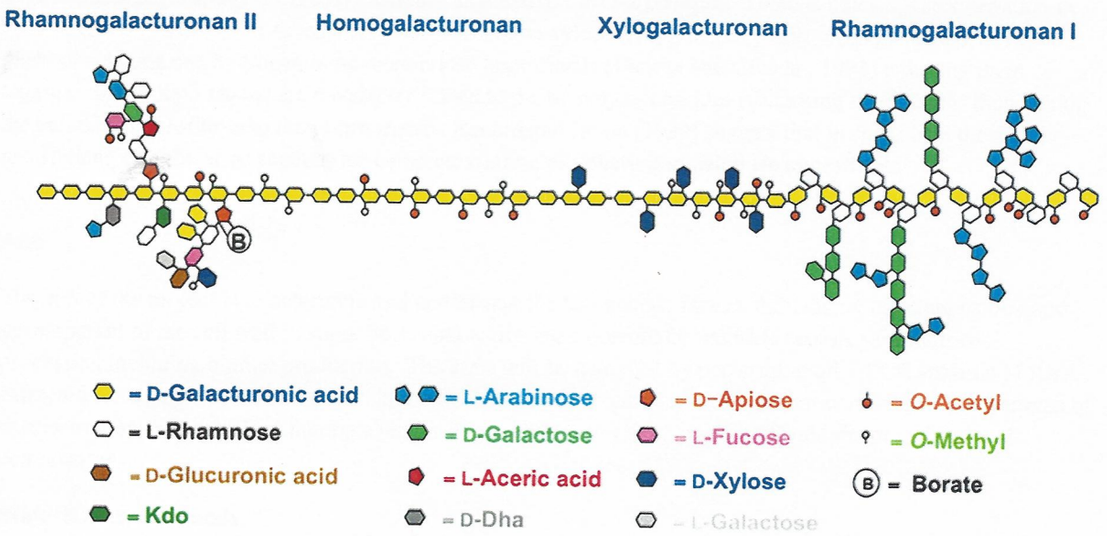


Figure 3. A schematic representation of the pectic polysaccharides RGII, HGA, XGA and RGI (Harholt et al., 2010)

The HGA domain is an unbranched polymer of α-1,4-linked galacturonic acid residues. Both RGII and XGA have the same α-1,4-linked galacturonic acid backbone as HGA but have side chains. In XGA xylose residues are attached to the O-3 position of some galacturonic acid residues. Additional xylose molecules can be attached to the first xylose (Zandleven et al., 2006). RGII is the most complex pectic polysaccharide with clusters of complex side chains attached to the galacturonic acid backbone at either the O-2 or O-3 position. Despite its complexity RGII has a highly conserved structure among vascular plants (Matsunaga et al., 2004). RGI is a branched polymer which differs from the other pectic polysaccharides in that instead of a galacturonic acid backbone; it has a repeating galacturonic acid-Rhamnose disaccharide unit as the backbone. The rhamnose residues often have galactan, arabinan and/or arabinogalactan side chains attached.

RGI side chains can be complex and unlike RGII are variable among plant species (Scheller et al., 2007). In sugar beet the galactan, arabinan and/or arabinogalactan side chains form non-covalent interactions with cellulose microfibrils (Zykwinska et al., 2007). Ferulic acid can be attached to the O-6 position of galactose and to the O-2 or O-5 position of arabinose in the side chains of RG1 (Ralet et al., 2005). Covalent cross linking of RG1 side chains can occur via oxidative dimerization of the bound ferulic acid forming dehydrodimers (Ralet et al., 2005). These dehydrodimers are thought to increase intercellular adhesion, provide increased protection against digestion by microorganisms and decrease wall extensibility (Waldron et al., 1997).

Hemicelluloses

Hemicelluloses are a very heterogeneous group of cell wall polysaccharides comprising of the remaining polysaccharides which are not pectin or cellulose. Scheller and Ulvskov (2010) suggest hemicelluloses be used to define glucans with a β-(1→4)- linked backbone of glucose, mannose or xylose. Xyloglucan (XyG) is the major hemicellulose in dicots accounting for 20-25% of the primary cell wall's dry weight (Popper and Fry, 2008). Members of the order Caryophyllales such as sugar beet and spinach have low levels of xyloglucan in their cell wall(Zykwinska et al., 2006). The hemicelluloses also contain xylans, mannans and glucomanans. Xyloglucans have the same linear β(1→4) glucan backbone as cellulose with the addition of xylosyl units added to the O-6 position of the glucosyl units of the chain in a regular pattern (Eckardt, 2008). Some xylose residues have additional galactosyl or fucosyl residues attached to the O-2 position. There is much species variation in the additional galactosyl or fucosyl residues attached to xylose (Hayashi and Kaida, 2011). The β(1→4) glucan backbone can hydrogen bond to cellulose microfibrils (Carpita and Gibeaut, 1993) tethering them together. Some XyG chains are covalently linked to pectic polysaccharides (Cumming et al., 2005) thus linking the cellulose microfibrils to the pectin matrix. Renard and Jarvis (1999) suggest that in sugar beet there is insufficient xyloglucan to account for complete coating or tethering of cellulose microfibrils.

Aim

The aim of the project is to determine and understand the key genetic factors influencing the composition and development of the cell wall in sugar beet roots which are a potentially valuable resource for industrial processing including biofuel production. The aims will be achieved by performing qRT-PCR analysis of RNA extracted form sugar beet roots grown in the field and under controlled environment conditions. The patterns of expression may give us clues linking changes in gene activity with changes in development and cell wall composition.

Materials and Methods

Chemicals and reagents

For RNA extractions, all chemicals used were molecular biology grade, phenol, chloroform, isoamyl alcohol, lithium chloride, ethanol, isopropanol, tris-HCl, sodium acetate, sodium chloride, sodium EDTA, SDS, PVP-40 and β-mercaptoethanol. All water used was Hyclone Hypure™ molecular biology grade water.

All kit-based protocols were carried out in accordance with the manufacturer recommendations. Kits used were; Turbo DNA free™ (Ambion), Affinity script™ multiple temperature cDNA synthesis kit (Stratagene) and Brilliant II SYBR® green qPCR low ROX master mix (Stratagene)

Harvesting Field Plant Material

The sugar beet variety "Sophia" (KWS SAAT Gmbt) was grown from commercially pelleted seed in keeping with local agricultural practice, planted at a similar time as commercial beet along with the same applications of fertiliser, herbicides and pesticides. Harvesting was done approximately every four weeks from emergence to flowering, which covers about 16 mounths. Twelve representative plants were harvested from the inner strips of the plot and split into two sets of six biological replicates. Each individual beet was then washed and all lateral roots removed. The crown was then removed and discarded. Six roots were then individually sliced into small pieces using an industrial grater (Hallde Hobart VPU350 with ¾ 4.5 grating disc) and a handful was immediately frozen in liquid nitrogen for subsequent isolation of total RNA. After removal of the crown the second set of six roots were weighed and the average diameter across the top of the root recorded before grating and freezing in liquid for future compositional analysis. Also, a handful of the second set was taken and dried in an oven for five nights at 85°C to measure dry weight. The first set of six was ground into a fine powder in liquid nitrogen and stored at -80°C. The second set was freeze dried for five nights in the dark before being ground into a fine powder and stored in the dark with silica.

At the start of the project two seasons (2009/10 and 2010/11) of material had already been harvested. During my project I harvested most of the 2011/12 material.

Harvesting plants grown in Controlled Environment (CE) room

Raw seed of the sugar beet variety "Sophia" (KWS SAAT Gmbt) was steeped in Thiram (300ppm) overnight. The seed was then transferred to boxes containing wetted Schleicher and Schuell pleated strips, two seeds placed in each pleat. The seeds were then incubated for 48 hours at 22°c. 5 inch pots were filled with a 50:50 mix of compost and Terra Green (OIL-DRI LTD Wisbech) and 2 germinated seeds planted in each pot at a depth of approximately 1 inch. The plants were then grown for 6 weeks after emergence (wae) at 22°C with a 16 hour photoperiod. Emergence (day 0) was defined as the point when all seedlings were visible and the cotyledons had unrolled.

The plants were removed from the pots taking care not to break the roots and the soil washed off in water. All of the lateral roots were removed and the main root was cut 1cm below the leaf base and the bottom root section collected. Roots were then pooled into two replicates for each of the three analyses (RNA extraction, microscopic analysis and dry matter analysis). Different numbers of plants were pooled at each time point in order to collect a sufficient quantity of sample. The numbers of plants polled into each rep are shown in table 1.

Replicate	Number of plants pooled for RNA extraction	Number of plants pooled for dry matter analysis	Number of plants used for microscopic analysis
1wae 1	20	20	4
1wae 2	20	20	4
2wae 1	20	16	4
2wae 2	20	16	4
3wae 1	14	14	4
3wae 2	14	14	4
4wae 1	8	8	3
4wae 2	8	8	3
5wae 1	8	6	3
5wae 2	8	6	3
6wae 1	6	6	3
6wae 2	5	6	3

Table 1. total numbers of plants pooled in to each replicate used for RNA extraction, Dry matter analysis and microscopic analysis

At each time point two reps were fresh frozen in liquid nitrogen to be used for RNA extractions and another two reps had the root diameter measured just below the cut and then the fresh and dry weight was measured.

From the final 2 reps sections were taken between 1cm-1.5cm below the leaf base (top section) and 2.5cm-3cm below the leaf base (bottom section) and fixed in 4% paraformaldehyde and PEM buffer (50mM Pipes, 5mM EGTA and 5mM MgSO4) overnight in a mild vacuum at 8oC. The sections were then dehydrated in the following ethanol series 10%, 20%, 30%, 40%, 50%, 60% for 80 minutes each then left in 70% ethanol overnight at 4°C. The following day they were further dehydrated in 80%, 90% and 100% for 2 x 80 minutes each and a final 15 minute vacuum in fresh 100% ethanol and left overnight at 4oC. The dehydrated sections were then embedded in Technovit® 7100 (Kulzer). First they were pre-infiltrated with the Technovit liquid with 1g of hardener 1 per 100ml of liquid (solution A) and ethanol in a ratio of 1:2 (2hours) 1:1 (2hours) and 2:1 (2hour). Sections were then infiltrated with 100% solution A with a vacuum for 30 minutes then left overnight in the fridge. Finally the sections were embedded in the solution A and hardener 2 in a ratio of 15:1 and left to polymerise at room temperature. 20µm sections were taken using a Leica RM2035 rotary microtome and mounted on poly-lysine slides. A 1:40 dilution from a 0.1% stock solution of Acriflavin (3,6-diamino-10-methylacridinium chloride) was used to stain the sections for 2 minutes and mounted under a coverslip in glycerol. The sections were viewed at 5 x magnification under Leica SP1 Confocal Laser Scanning Microscope.

RNA Extraction

RNA was extracted from the 2010/11 and 2011/12 seasons from frozen ground tissue using a method adapted from (Damaj et al., 2009), in which a high salt and detergent concentration extraction buffer called TENS (10mM Tris-HCl pH 7.5, 1mM NaEDTA pH8.0, 1% SDS, 2% PVP-40, 0.1M NaCl and 7% 2-Mercaptoethanol) and a phenol-chloroform-isoamyl alcohol mixture (1:0.8:0.2) are used. A lithium chloride precipitation step and the use of a DNAse enzyme "Turbo DNA-free®" ensured DNA contamination was kept to a minimum. The extracted RNA was quantified using a Thermo Scientific NanoDrop 2000C and stored at -80°C.

cDNA Synthesis

A cDNA synthesis reaction was carried out without the reverse transcriptase enzyme (RT) in order to check that the RNA wasn't contaminated with any genomic DNA. 2µg of total RNA are added to water to a final volume of 12.7µl and 1µl of oligo dT is added and incubated at 65°C for 5min. Then 2µl of 10x affinity script buffer and 2µl of 100mM DTT is added along with 0.8µl of dNTP mix (25mM each of dATP, dCTP, dGTP and dTTP) and 1.5µl of water. It is then placed in a PCR machine on the following programme: 5min at 25°C, 45min at 50°C, and 5min at 95°C then held at 8°C. 1µl of cDNA was used in a subsequent qRT-PCR reaction was carried out using GAPDH primers. Samples with high levels of DNA contamination (ct<34) were retreated with the Turbo DNA free enzyme and then requantified and retested. Once all samples were free of DNA contamination a cDNA synthesis reaction was carried out using the same reaction mixture as the –RT reaction except the 1.5µl of water was replaced with 1µl of the reverse transcriptase enzyme and 0.5µl of RNAse Out. The cDNA was then diluted 10 fold and stored at -20°C.

qRT-PCR Reaction

All qRT-PCR reactions were carried out in a 96 well plate format using a MX3000p QPCR system from Stratagene. The 15µl reaction volume contained 300nM of both the forward and reverse primer, 1x Brilliant II SYBR® green master mix and 1µl of cDNA. Every plate had sample cDNA, a NTC (No Template Control) and a Reference well all in triplicate. The reference well always contained cDNA from the same source and allows direct comparison between plates because expression levels are calculated relative to the reference wells.

The following thermal profile was used: DNA polymerase activation for 10 min at 90°C, then 40 amplification cycles consisting of a denaturing step for 30s at 95°C, an annealing step for 60s at 60°C and an extension step for 60s at 72°C. All of the data was automatically normalised to the ROX internal Dye in the SYBR green master mix. The expression levels of the gene of interest were calculated relative to the reference well.

The relative expression of GAPDH, EF1α and EF2 (using the primers in table 2.) was used to produce normalisation factors using geNorm v3.5 (Vandesompele et al., 2002) which uses a geometric averaging method. These normalisation factors were then applied to each sample to generate normalised relative expression levels (relative expression/normalisation factor). The normalisation factor was applied in order to account for variation between cDNA samples. A one way AVOVA analysis was carried out for each gene but interpretation of the results will have to be done by my supervisor at a later date.

Gene	Forward Primer Sequence	Tm (°C)	Reverse Primer Sequence	Tm (°C)	Primer Efficiency
GAPDH	GCTTTGAACGACCACTTCGC	67.0	ACGCCGAGAGCAACTTGAAC	66.6	107.1%
EF1α	GATTCCCACCAAGCCTATGG	65.3	GATGACACCAACAGCGACAG	64.5	105.7%
EF2	CCCCTCTACAACATCAAGGC	63.4	CCAGAGGGTCAGAAGGCATC	66.1	107.3%

Table 2. Primer information for the primers used to calculate normalisation factors

Primers were designed for the candidate gene sequences using the Primer3 (Rozen and Skaletsky, 2000) primer design program in Geneious (Table 3).

Gene of Interest	Forward Primer Sequence	Tm (°C)	Reverse Primer Sequence	Tm (°C)	Primer Efficiency
Reversibly Glycosylated Polypeptide 3	TGATGCTGTTCTGACCATCC	63.8	AGTACATAGCAGGGCCAATG	62.5	96.6%
Quasimodo 1	ACATTGTGGAAACTGGGGAC	63.6	GTTACGGATCTCATCCCAGC	63.4	96.3%
Arabinosyltransferase 1	CCAGCAGGAGATGAAGAAGG	63.8	AACCAGGGGAACCTTCAAAC	63.9	97.0%
Galacturonosyltransferase 1	TTGAAAAGGCAGCAGTTGTG	63.9	TCACTCAAGTTGCATCTCCG	64.1	92.2%
Cellulose Synthase 1	TCTGGGTGATTGCTCACTTG	63.9	CCAGAGCAAGGAGAAGATGG	63.8	89.5%

Table 3. Primer information for the primers used in the qRT-PCR analysis

Results and Discussion

The sugar beet variety Sophia was grown was grown for 60 weeks after emergence in the field in keeping with local agricultural practices. There are six developmentally important stages in sugar beet growth. There is an initial period of root patterning from emergence to around 6 to 7 weeks after emergence. There is then a period of rapid growth, expansion and sugar accumulation until the beet reaches maturity around 30 weeks after emergence. This is when commercially grown beet would be harvested. During the winter there is a period of vernalisation lasting until around 42 weeks after emergence. There is then there is a period of spring growth before the plants begin to bolt. By 52 weeks after emergence in the 2011/12 season plants had an average bolt height of 18cm and by 55 weeks after emergence bolts had reached an average height of 80cm. by the final harvest at 60 weeks after emergence the plants were flowering and beginning to produce seed. A representative sample of roots was harvested and processed at regular intervals throughout development. Leaf tissue was also harvest at 4 and 18 weeks after emergence, the plants harvested at 55 weeks after emergence also had leaf, floral leaf, floral bud and stem tissue collected. Sophia seeds were also grown in pots in a CE room for 6 weeks after emergence. A representative sample of roots was harvested and processed at weekly intervals to assess the development of young seedlings. The representative samples were then used to perform dry matter analysis or RNA extraction for use in qRT-PCR reactions.

Dry matter analysis

The dry matter is comprised primarily of cell walls and sucrose. Figure 4 shows the dry matter data for the field material. There is a rapid increase in dry matter between 10 and 14 wae when the root will be rapidly growing and synthesising new cell walls and accumulating sucrose which will both add to the dry weight. The amount of dry matter continues to increase until 22 wae when it appears to plateau, this is also when the root is reaching maturity and so has expansion is slowing down. The percentage of dry matter then drops after 38wae this could be caused by the transition of the root from a sucrose sink to a source of sucrose used in the production of the reproductive tissues. Figure 5 shows the data for the seedlings grown in the CE room. There is a gradual increase in the percentage dry matter from around 5% at 1 week after emergence to almost 16% at 6 weeks after emergence this value is similar to the early field harvest points (14%) shown in figure 4.

qRT-PCR gene expression analysis

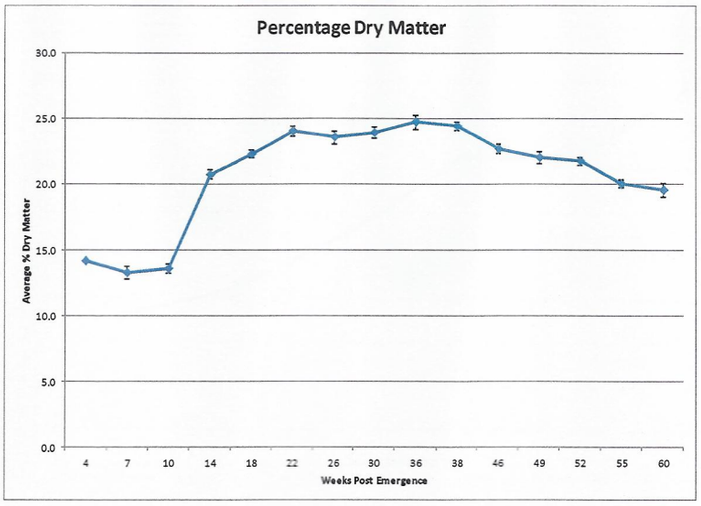
The gene expression analysis was carried out on RNA extracted from material harvested during the 2010/11 season. Before the expression on the genes of interest could be analysed normalisation factors had to be calculated for the cDNA. The relative expression of three housekeeping genes (GAPDH, EF1α and EF2) was used the calculate normalisation factors. These were then applied to the relative expression data for the genes of interest to give normalised relative expression levels.

A literature search was carried out to identify important genes involved in primary cell wall synthesis. Five key genes were selected for qRT-PCR analysis, Cellulose Synthase 1 (CESA1) GT Family 2, Reversibly Glycosylated Polypeptide 3 (RGP3) GT Family 75, Galacturonosyltransferase 1 (GAUT1) GT Family 8, Quasimodo 1 (QUA1) also known as GAUT8 GT Family 8 and Arabinan Deficient 1 (ARAD1) GT Family 47. A transcriptome cataloguing all genes expressed in young expanding sugar beet storage roots (B. Townsend unpublished) provided candidate genes and sequences. A comparative bioinformatics approach was used to identify Contigs with homology to glycosyltransferase families in the Carbohydrate Active Enzymes database (http://www.cazy.org/(Cantarel et al., 2009)). A protein blastx was carried out in the computer programme Geneious (Kearse et al., 2012) to identify protein sequence homology to the model plant species Arabidopsis thaliana. Sequences with high sequence homology suggest that the genes are homologues. Reservations have to be made about the role of these genes until functional genomics has proven their function in sugar beet.

RGP3 (GT 75)

Bvcontig09324 was found to have 94% protein sequence similarity with Arabidopsis thaliana RGP 3 (At3g08900). The reversibly glycosylated proteins (RGPs) are a group of soluble proteins found in association with the Golgi that can reversibly bind UDP-glucose, UDP-xylose and UDP-galactose (Zhao and Liu, 2002). It has been suggested that RGPs are involved in Xyloglucan synthesis because the steady state glycosylation of RGPs with UDP-glucose, UDP-xylose and UDP-galactose is in the same ratio as the typical sugar composition of xyloglucan (Dhugga et al., 1991; Dhugga et al., 1997; Delgado et al., 1998).

We chose to analyse RGP3 expression as a marker of xyloglucan synthesis because it was the most highly expressed RGP in our sugar beet transcriptome. The expression of RGP3 in the field follows a pattern of increasing expression as the root is expanding and accumulating sugar with a dramatic drop in expression during the winter when there is little growth and increasing again as spring growth begins (figure 6a). RGP3 was expressed in similar levels in all of the tissues analysed (Figure 6b). There appears to be a similar pattern of expression in the leaf with highest expression when the root is expanding and accumulating sugar (Figure 6b). These findings seem to correlate to findings in Arabidopsis (Delgado et al., 1998), rice (Gupta et al., 2000) and cotton (Zhao and Liu, 2002), which show high levels of expression during periods of non-cellulosic polysaccharide synthesis and active cell growth. The expression pattern in the young seedlings (Figure 6c) shows an increase in expression between the first and second week post emergence.



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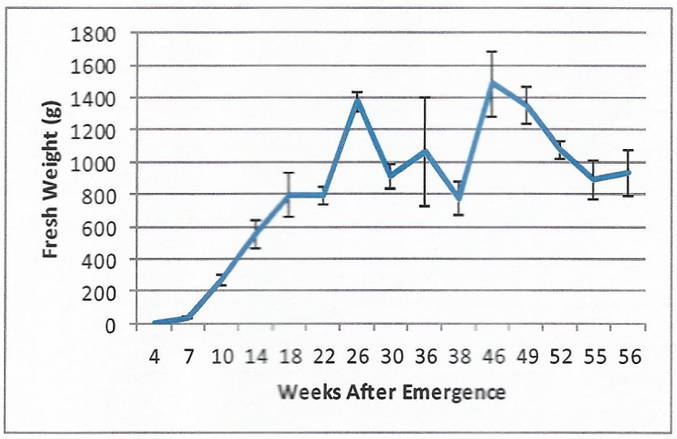

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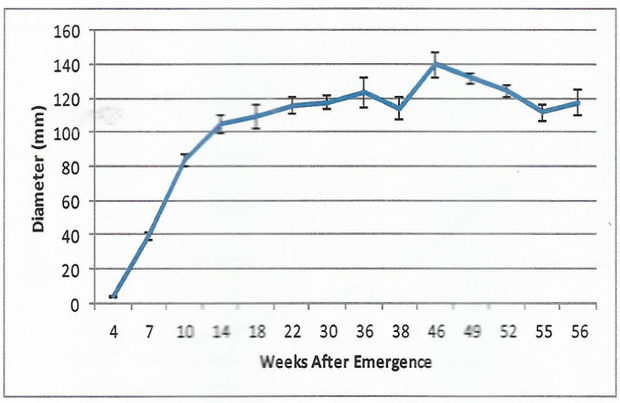
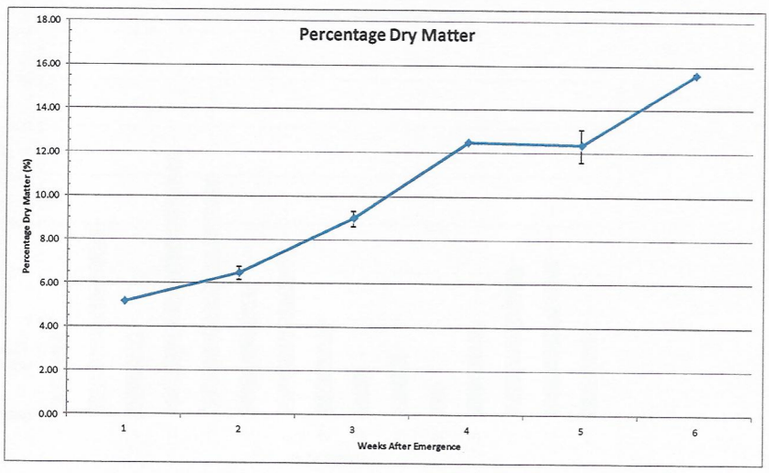


c.

Figure 4. Sugar beet roots storage during development in the field in 2011/12. Error bars indicate the standard error of the mean for 6 biological replicates.

- **a.** Dry matter composition
- **b.** Fresh weight
- **c.** Root diameter



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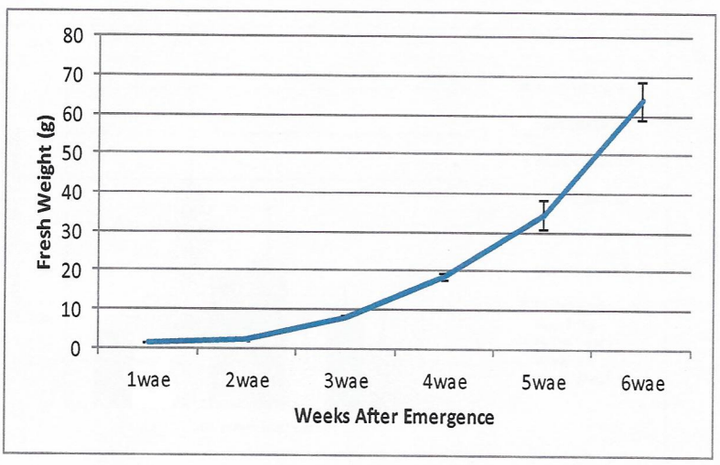

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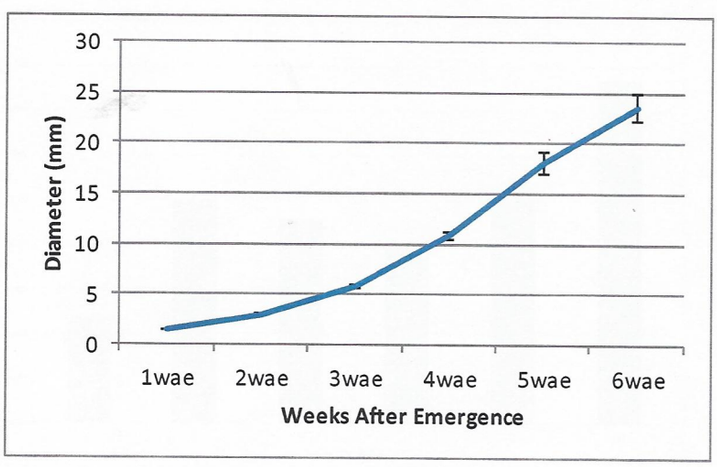
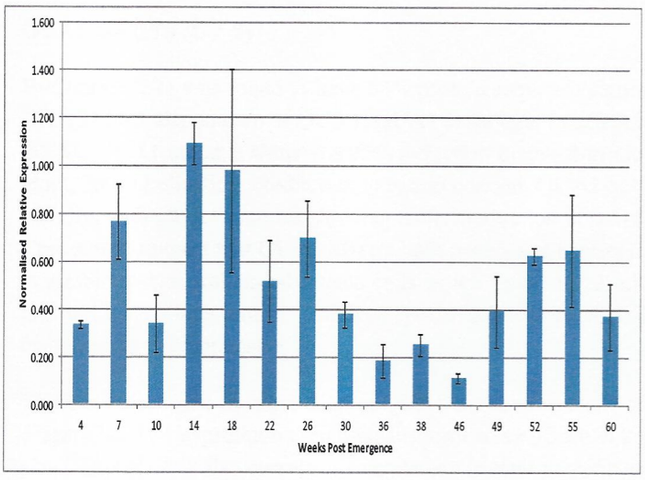


c.

Figure 5. Sugar beet storage roots during early seedling development in the CE room. Error bars indicate the standard error of the mean for both pooled biological replicates.

a. Dry matter composition
b. Fresh weight
c. Root diameter



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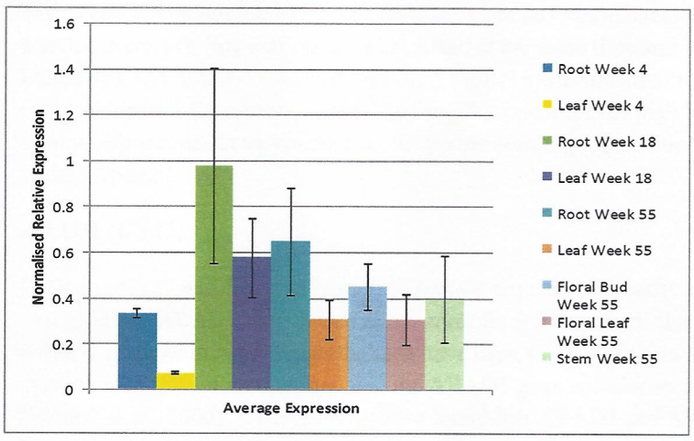

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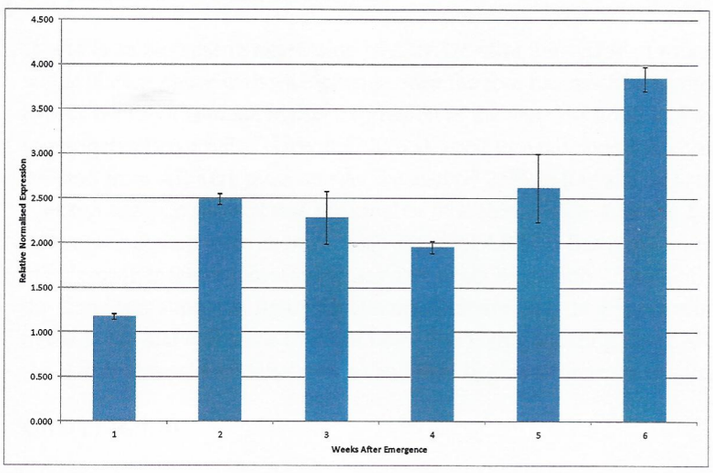


c.

Figure 6. The expression pattern of RGP3 gene homologue in sugar beet

a. Root development in the field. Error bars indicate the standard error of the mean for 3 biological replicates.
b. Different tissues during development. Error bars indicate the standard error of the mean for 3 biological replicates.
c. Early root development in the CE room. Error bars indicate the standard error of the mean for 2 biological replicates.

QUA1/GAUT8 (GT 8)

BvContig07621 was found to have 84% protein sequence similarity with Arabidopsis thaliana GAUT8 (At3g25140) also known as QUA1. QUA1 is thought to affect both pectin and xylan synthesis (Orfila et al., 2005). QUA1 mutants showed a 25% reduction in galacturonic acid levels compared to the wild type (Bouton et al., 2002) indicating a reduction in pectin content. QUA1 has also suggested to be involved in xylan synthase activity, with QUA1 mutants showing a 40% reduction in β-1-4-D-xylan synthase activity (Orfila et al., 2005). This would suggest that QUA1 affects both pectin and hemicellulose synthesis. QUA1 expression is increased in Arabidopsis isoxaben-habituated cells which show increased pectin content (Manfield et al., 2004). Isoxaben is a herbicide which inhibits cellulose synthesis but cultured cells can make changes in other cell wall components to compensate.

There is an increase in expression of QUA1 between weeks 7 and 18, coinciding with the period of rapid growth (Figure7a). The expression then plateaus until week 30 when it appears to drop on the root has reached maximal size. This plateau also correlates to a plateau in total percentage dry matter (Figure 4). This would suggest that QUA1 is involved in cell wall synthesis during periods of cellular expansion and proliferation and may also have a role maintaining the cell wall once the plant has reached maturity. QUA1 expression is higher in the root than the corresponding leaf material harvested at the same time and is expressed in all of the tissues analysed Figure 7b). Orfila (2005) showed that QUA1 mRNA was localised to vascular tissues and subepidermal layers of Arabidopsis inflorescence stems. This could account for the high levels of expression seen in the root and stem which are rich in vasculature. In the young seedling expression of QUA1 increases over the first 6 weeks of development.

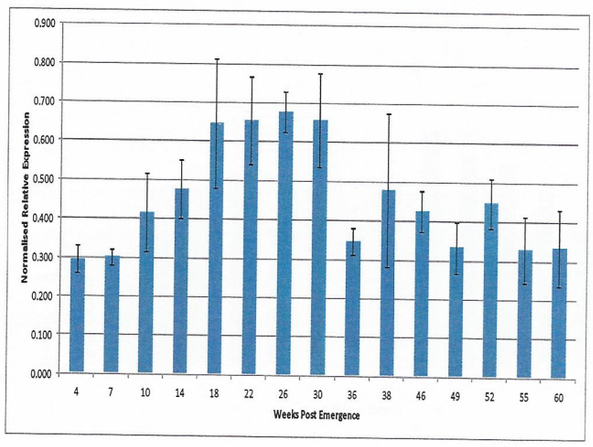
ARAD1 (GT47)

BvContig02127 was found to have 56% protein sequence similarity with Arabidopsis thaliana ARAD 1 (At2g35100). ARAD1 was chosen as a marker for RGI synthesis due to the high arabinose content of RGI. ARAD1 mutants in Arabidopsis thaliana have been show to contain around 30% less arabinose in isolated RGI than the wild type, it is suggested that the ARAD1 gene encodes an arabinan α -1,5-arabinosyltransferase (Harholt et al., 2006). It has recently been found that ARAD1 and ARAD2 proteins form homodimers and heterodimers when transiently expressed in Nicotiana benthamiana (Harholt et al., 2012) and they may function as a complex in arabinan biosynthesis.

There is an increase in expression of ARAD1 after the period of root patterning during the expansion and sugar accumulation phase with little change once the root has reached maturity (Figure 8a). ARAD1 is expressed in all tissues but there is much higher expression in the leaf and floral leaf at week 55 than in any of the other tissues investigated (Figure8b). Harholt (2006) showed in Arabidopsis that the arabinose content of Polysaccharides isolated from ARAD1 mutants was reduced by 25% in leaf and 54% in stems compared to the wild type. Linkage analysis showed that the number of arabinan side chains in the mutant was unchanged compared to the wild type but they were shorter. This suggests ARAD1 has a role in extending pectic arabinan side chains but other enzymes are required to initiate side chain formation. The high expression levels in the 55 wae leaf and the floral leaf shown in figure 8b and the comparatively low expression in the root would indicate ARAD1 plays a role in the leaf cell walls of sugar beet. The young seedling show ARAD1 expression is at a constant level for the first four weeks of development and then begins to increase (Figure 8c).

GAUT1 (GT 8)

BvContig01792 was found to have 83% protein sequence similarity with Arabidopsis thaliana GAUT1 (At3g61130). We chose GAUT1 as marker for pectic HGA synthesis. GAUT1 transfers galacturonic acid residues from uridine 5-diphosphogalacturonic acid onto the pectic polysaccharide HGA (Sterling et al., 2006). The galacturonic acid transferase activity was confirmed by Mohnen (2008) by transiently expressing GAUT1 in HEK293 (Human Embryonic Kidney) cells and yielding galacturonic acid transferase –HGA activity.



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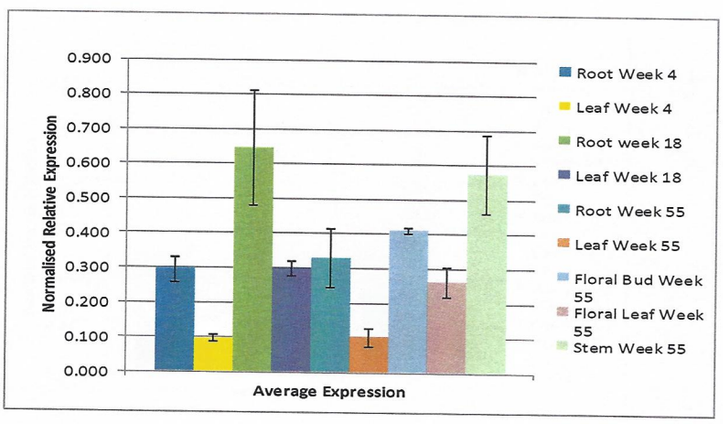

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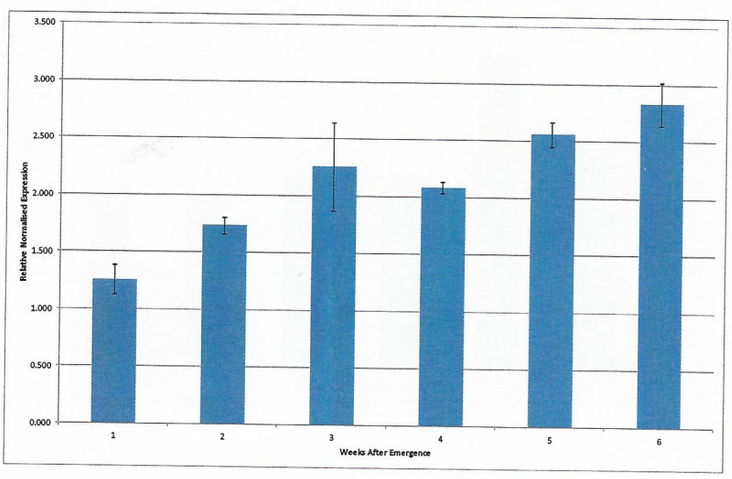
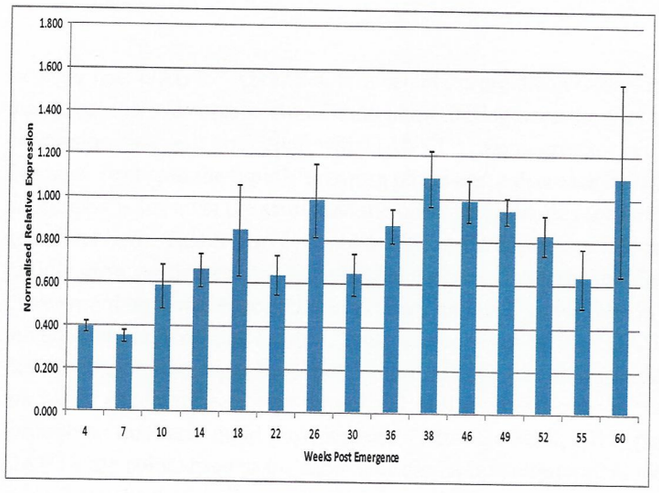


c.

Figure 7. The expression pattern of QUA1 gene homologue in sugar beet

a. Root development in the field. Error bars indicate the standard error of the mean for 3 biological replicates.
b. Different tissues during development. Error bars indicate the standard error of the mean for 3 biological replicates.
c. Early root development in the CE room. Error bars indicate the standard error of the mean for 2 biological replicates.



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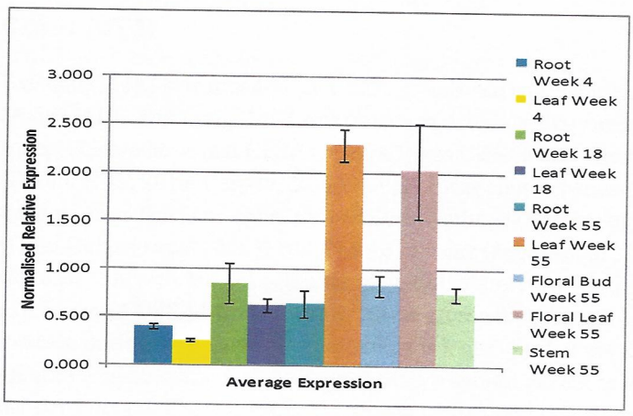

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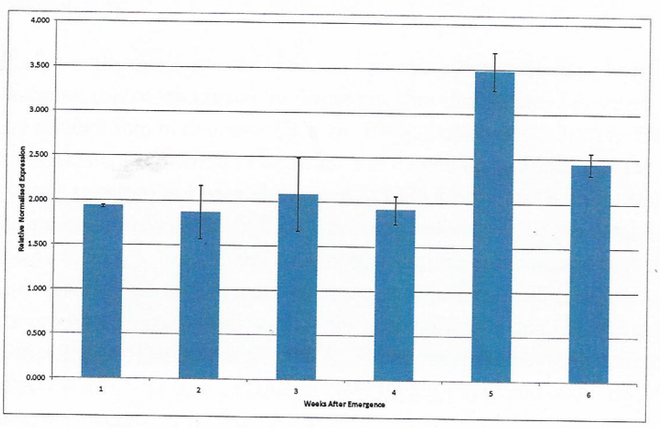


c.

Figure 8. The expression pattern of ARAD1 gene homologue in sugar beet

a. During sugar beet root development in the field. Error bars indicate the standard error of the mean for 3 biological replicates.
b. In various tissues in the field. Error bars indicate the standard error of the mean for 3 biological replicates.
c. During early sugar beet root development in the CE room. Error bars indicate the standard error of the mean for 2 biological replicates.

In sugar beet GAUT1 expression is constant during the period of root patterning and stays constant throughout the expansion and sugar accumulation phase falling once the root has reached maturity (Figure 9a). This expression pattern is consistent with GAUT1's role in primary cell wall synthesis with highest expression when the sugar beet is in the rapidly growing phase and a decrease in expression once it has reached maturity. The expression is lower in the stem than the other green tissues (Figure 9b) harvested at 55 weeks (leaf, floral leaf and floral bud). This is also consistent with a role in primary cell wall synthesis with highest expression in the rapidly growing tissues. As we harvested a portion from the middle of the stem which isn't growing very much there would be lower expression of primary cell wall genes than in the shoot apical meristem which was included in the floral bud sample. There is a constant level of GAUT1 expression during the first 6 weeks of development of the sugar beet root (Figure 9c) this was also seen in the field material (Figure 9a). This is what we would expect to see for a primary cell wall synthesis gene as there would be rapid cell wall synthesis throughout this early rapid growth phase. (Atmodjo et al., 2011) provide strong evidence that GAUT1 and GAUT7 are colocalised to the same specific Golgi compartment. Strong co-expression of GAUT1 and GAUT7 in Arabidopsis thaliana is consistent with their functioning in a protein complex.

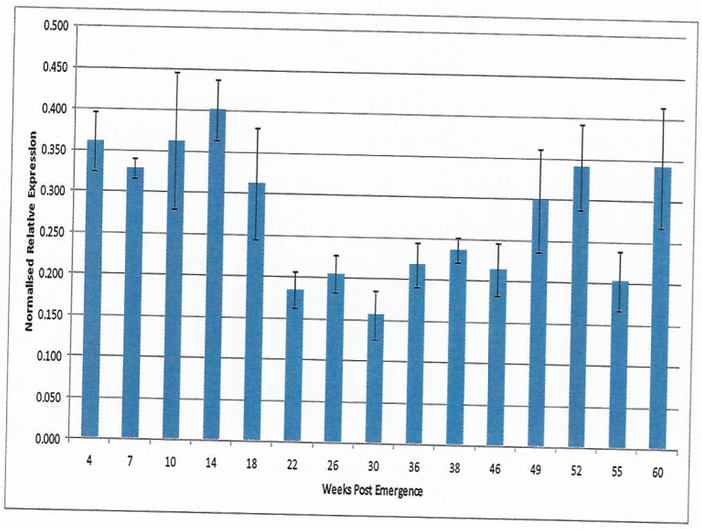
CESA1 (GT 2)

BvContig21182 was found to have 82% protein sequence similarity with Arabidopsis thaliana CESA1 (At4g32410). BvContig21182 was chosen as a marker for primary cell wall cellulose synthesis. It is a widely accepted hypothesis that CESA1, CESA3, and CESA6 proteins function in primary wall cellulose synthesis (Doblin et al., 2010; Carpita, 2011). CESA1 was chosen because it has been identified as the only one of the three proteins that is essential for cellulose synthesis (Beeckman et al., 2002). Arabidopsis CESA3 mutants (Cano-Delgado et al., 2003) and CESA6 mutants (Fagard et al., 2000) have been shown to result in impaired cellulose synthesis but not complete inhibition. After the period of root patterning there is an increase in expression of CESA1 from 7 wae to 18wae after which there is little change in expression (Figure 10a). The increase in expression after root patterning is highlighted in the difference between the 4wae and 18wae root samples (Figure 10b). It also shows there is a similar pattern in the leaves with an increase in expression during the same period. There is lower expression in the floral bud compared to the other green tissues (leaf, floral leaf and stem) harvested at the same time. The young seedlings show CESA1 increasing for the first 5 weeks of development.

Large multi-meric membrane bound protein complexes have been implicated in cellulose synthesis. the complex can be visualised by freeze fracture images as a hexameric rosette structure with six fold symmetry and are about 25nm in diameter (Brown, 1996; Doblin et al., 2002). The larger catalytic domains of the cellulose synthases are estimated to be 50nm and extend up to 35nm into the cytoplasm (Bowling and Brown, 2008). The rosette structure has been show to hold CESA proteins (Kimura et al., 1999). Each of the six globular domains is proposed to hold up to six CESA proteins believed to be the catalytic subunits of the complex (Endler and Persson, 2011). Each of the six globular regions are expected to synthesise four to six glucan chains (Carpita, 2011) which are then assembled into one functional cellulose microfibril (Read and Bacic, 2002) which is then incorporated into the cell wall. It has been indicated that CESA2 and CESA5 have partially redundant functions with CESA6 (Desprez et al., 2007). From our sugar beet transcriptome it appears CESA2 has replaced the function of CESA6 as CESA2 in quite highly expressed and CESA6 is very lowly expressed this may be an interesting area for further research.

Microscopic analysis of early root development

The vasculature of sugar beet roots is arranged in concentric rings around a central stele. All of these rings are laid down during the early part of development and the root expands as the cells in the rings expand and divide. To try and visualise the development of the rings, plants were grown in the CE room and harvested at weekly intervals and the growth stage according to the BBCH scale was recorded. The BBCH scale is a decimal coding system for phenologically similar growth stages in both mono- and dicotyledonous plant species (Hack et al., 1992). The growth stages of Beta vulgaris spp. are divided into 10 macro-stages which are further subdivided into micro-stages (Meier et al., 1993). Each micro-stage has a 2 digit code allowing precise identification, description and comparison of the growth phase.



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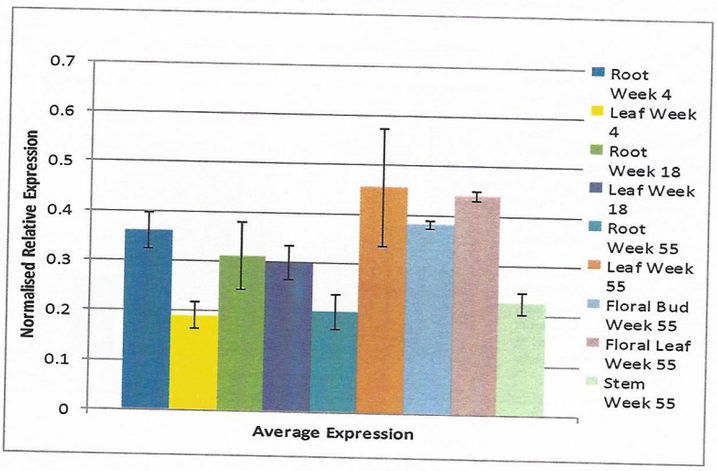

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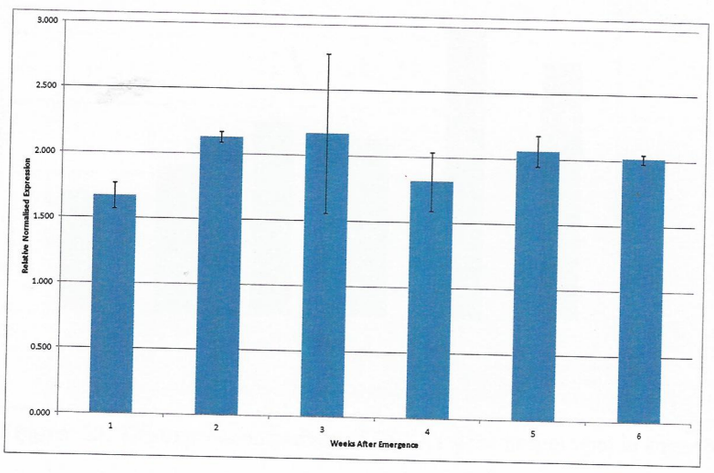
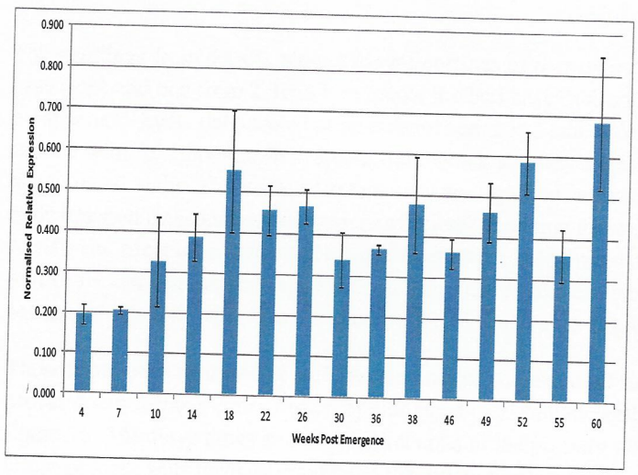


c.

Figure 9. The expression pattern of GAUT1 gene homologue in sugar beet

a. During sugar beet root development in the field. Error bars indicate the standard error of the mean for 3 biological replicates.
b. In various tissues in the field. Error bars indicate the standard error of the mean for 3 biological replicates.
c. During early sugar beet root development in the CE room. Error bars indicate the standard error of the mean for 2 biological replicates.



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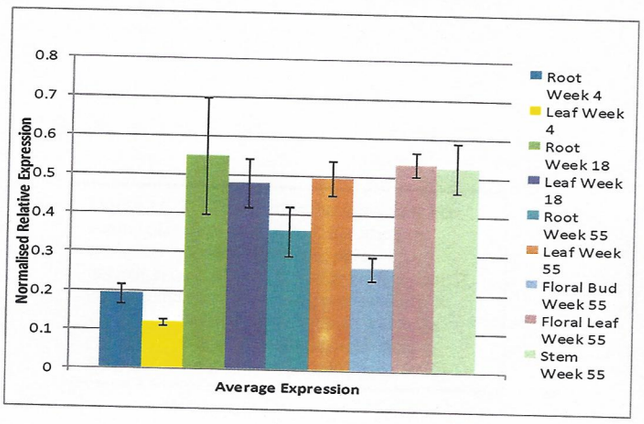

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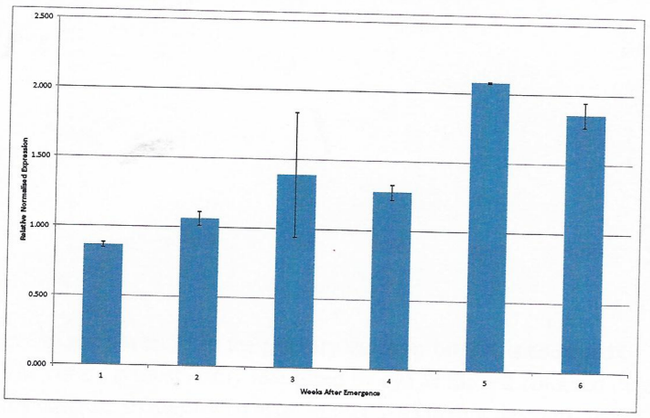


c.

Figure 10. The expression pattern of CESA1 gene homologue in sugar beet

a. During sugar beet root development in the field. Error bars indicate the standard error of the mean for 3 biological replicates.
b. In various tissues in the field. Error bars indicate the standard error of the mean for 3 biological replicates.
c. During early sugar beet root development in the CE room. Error bars indicate the standard error of the mean for 2 biological replicates.

The seedlings from the CE room had two portions of root taken; the first was from 1cm-1.5cm below the leaf base (top) and one from 2.5cm-3cm below the leaf base (bottom). The roots were then fixed in paraformaldehyde, dehydrated in an ethanol series and embedded in Technovit® 7100 resin. The sections were stained with Acriflavin; a fluorescent stain which strongly stains lignified secondary cell walls and poorly stains primary cell walls. The walls of xylem cells are lignified secondary cell walls this enables them to carry out their function transporting water more efficiently and it is these cells which show the most intense staining with Acriflavin. Most other cells in the sugar beet root have primary cell walls containing little or no lignin and so will show less intense staining. Sections from the top and bottom portions of the root both showed the same pattern of development (data not shown). All of the images (figure 12-14) are from the top part of the root.

Three roots were analysed at each developmental stage and all showed similar patterns of development. The procambium forms a dense region of elongated meristematic cells with thin walls which can be seen best in figure 20. The sieve tubes and companion cells of the primary phloem are the first to differentiate. Soon after the initial phloem cells have differentiated the xylem plates begin to form. Artschwager (1926) defines the different regions and cell types of the young sugar beet root (figure 11) that will be referred to hereafter.

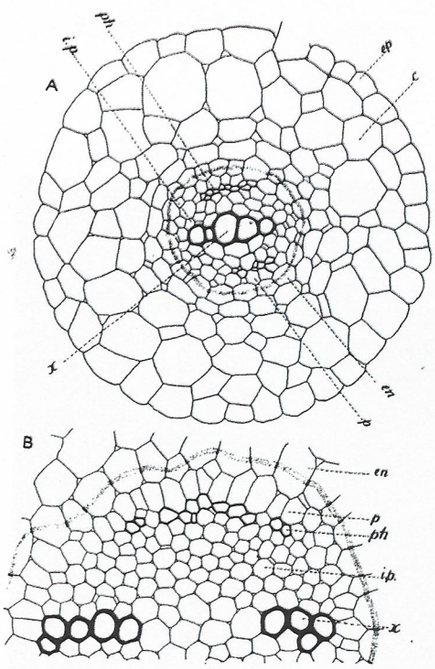


Figure 11. A. Cross section through young beet seedling (10 days old). X 355. c, cortex; ep, epidermis; en, endodermis; ph, phloem; i.p., interstitial parenchyma; p, pericycle; x, xylem. B. Partial cross section of young beet seedling. X 476. The interstitial parenchyma in which the primary cambium originates is much more developed here than in A (Artschwager, 1926).

In figures 12a and 12b the primary vascular bundle is enclosed by a cortex comprising of about 8 cell layers. The cortex is most easily identified by eye as the red coloured outer layer of the hypocotyl and is separated from the vascular tissues by an endodermis. As the root develops the central cylinder inside the endodermis expands, the cortex is stretched and later the cells rupture and collapse as the cortex is sloughed off (Artschwager, 1926) and hence the cortex is not present in the other images. The central cylinder of the root is made up of a diarch protoxylem plate forming the central stele which can be seen in figures 12a and b, 13 c and d one half can be seen in figures 12c and d. This diarch arrangement of the vascular tissues and central stele has also been observed in Arabidopsis thaliana roots (Parizot et al., 2008).There is a band of parenchyma between the xylem and phloem (figure 11). The pericycle forms a concentric ring one cell thick around the endodermis. As the root develops the pericycle cells divide and elongate axially but their cross section remains the same size whereas the cells of the endodermis begin to enlarge.

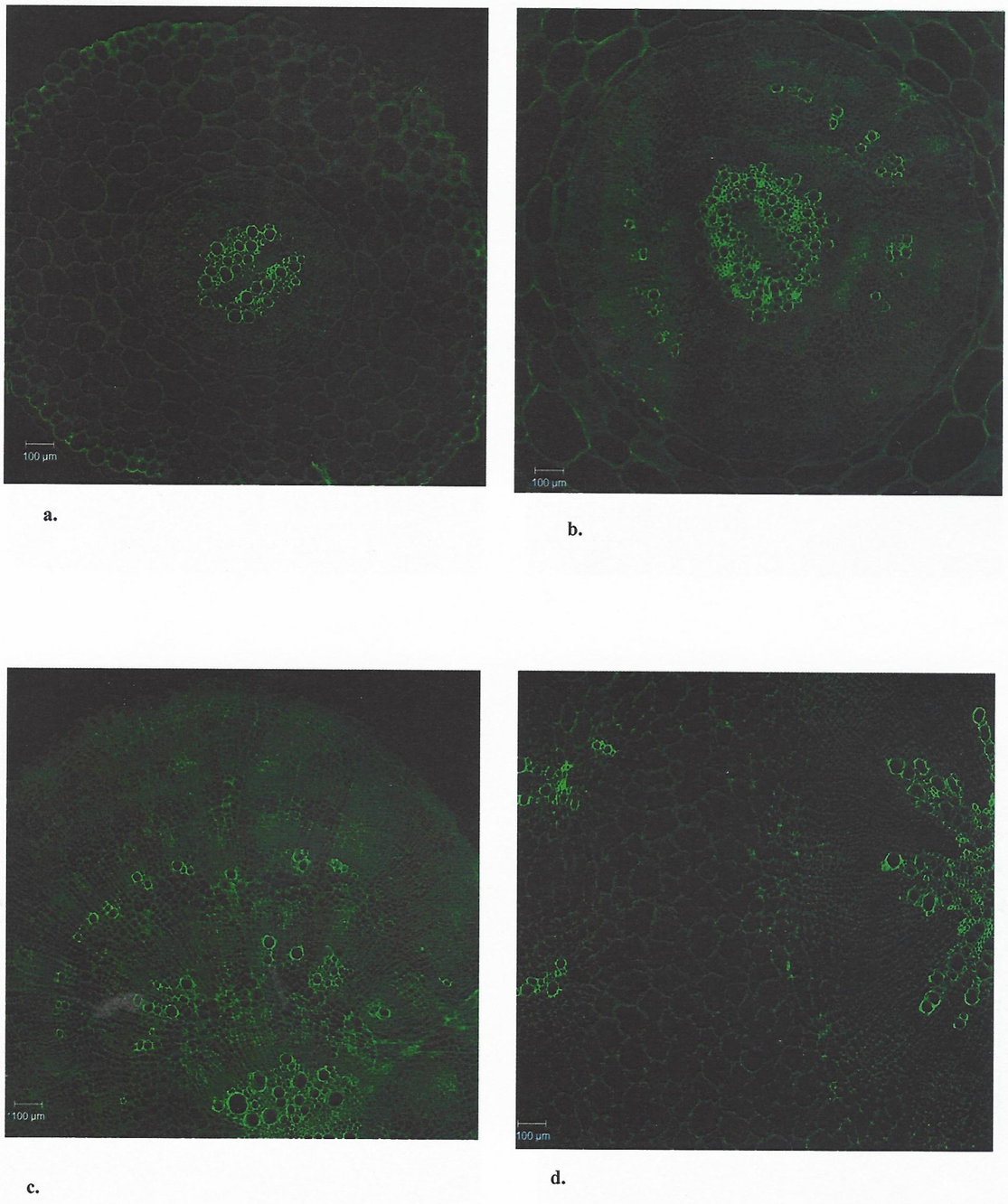
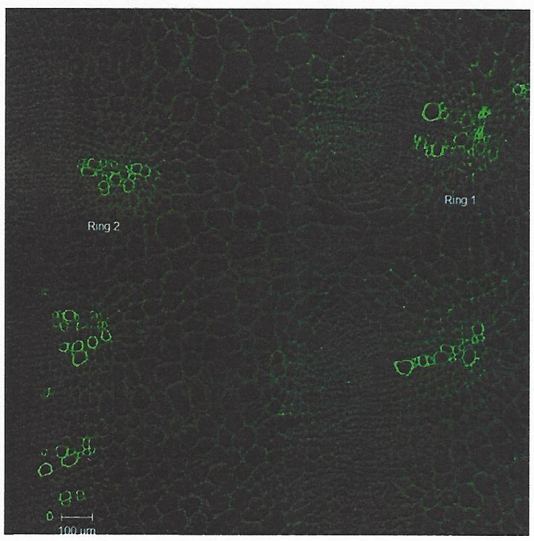
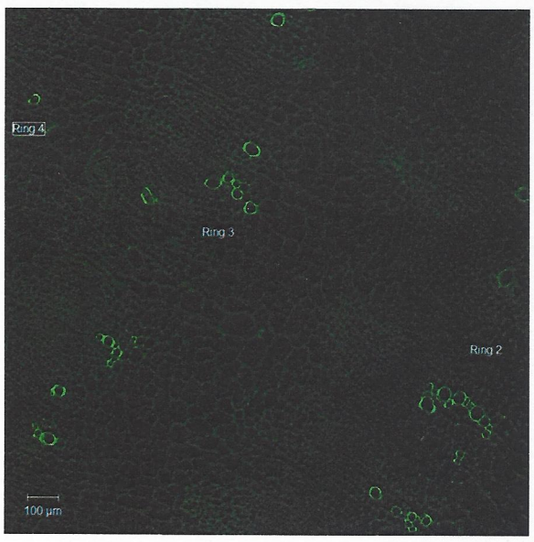


Figure 12. 20µm sections of Technovit embedded seedling roots stained with Acriflavin

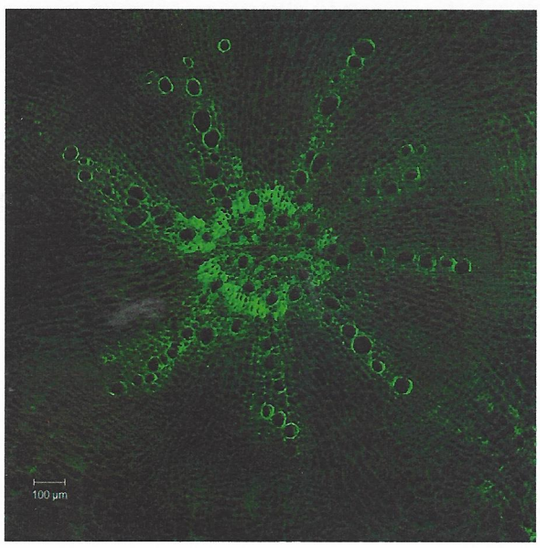
- **a.** 1wae top section (BBCH stage 12) showing a complete cross section of the root.
- **b.** 2wae top section (BBCH stage 14) showing the central stele, first ring and the cortex.
- **c.** 3wae top section (BBCH stage 16) showing half of the central stele, the first 2 rings and the endodermis.
- **d.** 4wae top section (BBCH stage 18) showing the central stele and the first ring.



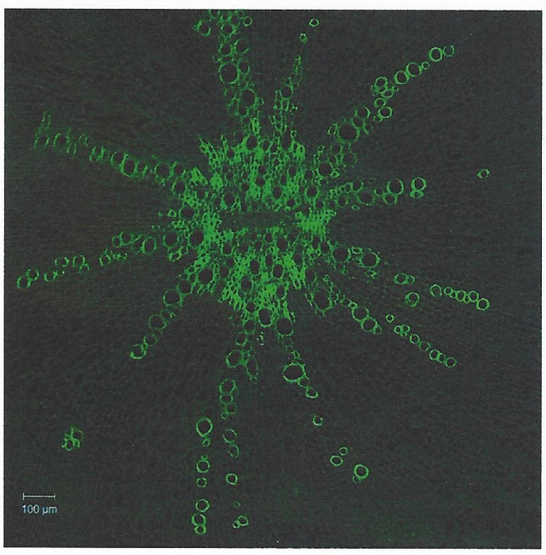
a.



b.



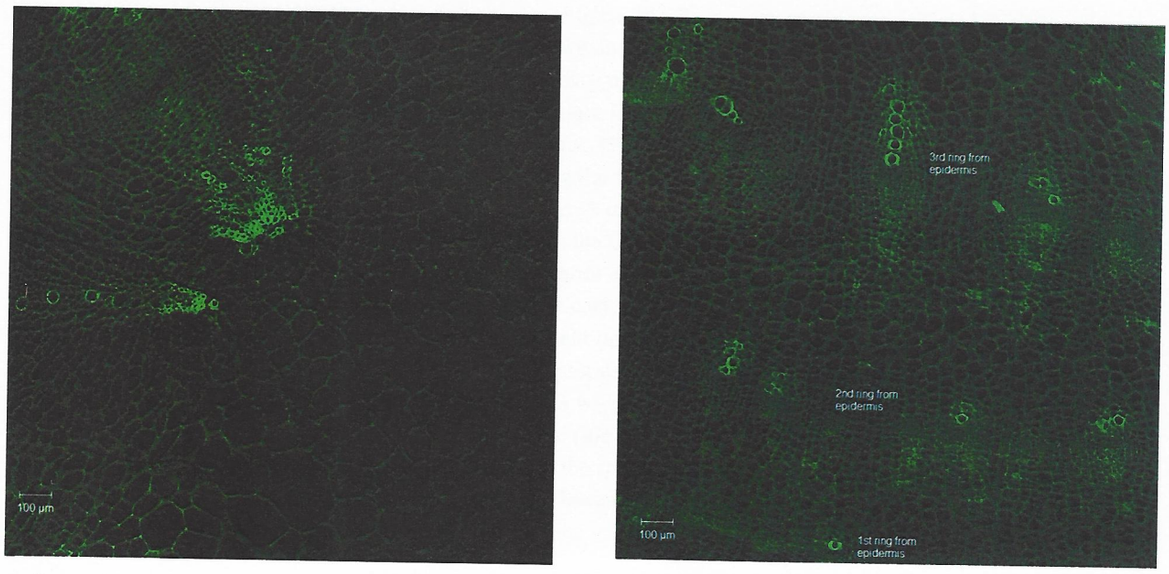
c.



d.

Figure 13. 20µm sections of Technovit embedded seedling roots stained with Acriflavin

- **a.** 4wae top section (BBCH stage 18) showing the first and second rings.
- **b.** 4wae top section (BBCH stage 18) showing the second third and fourth rings.
- **c.** 5wae top section (BBCH stage 20-21) showing the central stele.
- **d.** 6wae top section (BBCH stage 21-23) showing the central stele.



a. **b.**

Figure 14. 20µm sections of Technovit embedded seedling roots stained with Acriflavin
a. 6wae top section (BBCH stage 21-23) showing the first ring.
b. 6wae top section (BBCH stage 21-23) showing the outer 3 rings.

As the second pair of leaves becomes visible (BBCH stage 14) the parenchyma cells between the primary xylem and phloem begin to elongate and divide, giving rise to a meristematic zone which becomes the primary cambium from which develops the xylem and phloem (Figure 15). Secondary cambia are initiated centrifugally but their tissue of origin is still a source of dispute (Cooke and Scott, 1993). Artschwager (1926) suggest the additional cambia arise in the parenchyma of the secondary phloem, (Milford, 1973) reports the inner 2 rings are formed within the pericycle but that the later rings are more likely to develop from the outer region of the previous ring. (Milford, 1973) and (Rapoport and Loomis, 1986) showed that by 2wae the primary cambia are complete the first 2 secondary cambia are formed during the next week. Milford's experiments were carried out on field material which may explain why Figure 12b shows the fully developed primary cambium and the first secondary cambium because the plants develop faster in the CE room than in the field. Milford (1973) also found that ring initiation continues rapidly for 6 weeks after emergence and then the rate of ring initiation began to slow. The CE room plants also showed this pattern of early development and ring initiation but there was no sign of the rate of ring initiation slowing as the experiment only ran for 6 weeks. In figure 12b (2wae) one secondary cambium can be seen and the second ring is just starting to develop on the inner edge of the pericycle. The first and second secondary cambia well formed by 3 weeks after emergence (Figure 12c). In the sections from 4 weeks after emergence (Figures 13a, b and c) the first three secondary cambia can be seen with individual cells beginning to differentiate into xylem in the fourth secondary cambia (marked ring 4). We cannot report how many rings had been formed by 5 and 6wae because only the central (stele and ring 1) and outer (epidermis and outer rings) regions were collected.

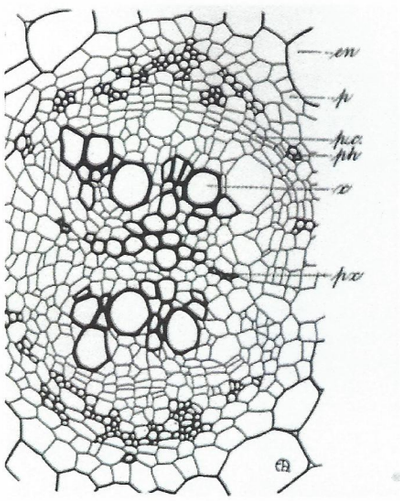


Figure 15. The development of the primary cambium, no supernumerary rings have been formed yet. En, endodermis; p, pericycle; pc, primary cambium; ph, phloem; pr, protoxylem (Artschwager, 1926).

Conclusion

Through qRT-PCR analysis of material harvested from sugar beet grown in the field and of young seedlings grown in a controlled environment room, the temporal expression of five genes homologous to Arabidopsis thaliana primary cell wall synthesis genes has been catalogued throughout development of the sugar beet root and green tissues. As expected for primary cell wall synthesis genes they were all expressed to some extent in all the different tissues analysed. The changes in the expression levels of RGP3 seem to follow changes in growth with higher expression in periods of active cell growth and lowest in the winter when the plant isn't growing. QUA1 expression increases throughout the growth and sugar accumulation phase and falls once the beet has reached maturity. The expression of ARAD1 was highest in the leaf tissue harvested at 55 weeks after emergence. The expression in the root increases throughout the initial expansion phase and then gradually falls. In the root GAUT1 is most highly expressed during root patterning and the early part of the expansion phase. CESA1 expression increases after root patterning and then stays at similar levels throughout the rest of development. Sugar composition analysis of cell wall material may be able to provide further evidence for the role of these genes if an increase in expression correlated to an increase in the corresponding sugars.

Reservations will still remain as to the function of the genes analysed until functional genomics such as transformation and expression in vitro leading to the expected activity confirm the genes role..

Through the development of a new method for the microscopic analysis of sugar beet roots the development of the vascular cambium has been recorded. The development and subsequent differentiation of the meristematic rings responsible for the expansion of the sugar beet root have been visualised. There is more differentiation of the meristematic tissue in the inner most rings and less differentiation in the newer outer rings. The new method developed in this placement provides the basis for a wide range of future work. This includes methods such as in-situ PCR which would allow us to visualise in which cells particular genes are expressed and ELIZA which with antibodies to cell wall epitopes would allow us to link how the changes gene expression affects the composition of the cell wall.

Sugar beet cell walls make up the majority of the pulp left over after the extraction of sucrose. The pulp is the further processed to produce valuable by-products such as bioethanol and betaine. This understanding of some of the fundamental biology controlling sugar beet cell wall composition will aid in the discovery of ways to improve the composition of the cell walls in order to increase the yield of these valuable products or to increase the efficiency of the process.

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