Nucleotide-Sugar Transporters in Plants

Note for the reader
This report is intended for an audience that has no specialist knowledge in molecular biology. However the use of some technical language is unavoidable. The methods sections should provide the necessary information to allow the results and discussion to be understood.

Introduction
At a biochemical level plants consist of four major groups of macromolecules; proteins, lipids, polysaccharides (long chains of sugars) and nucleic acids. Proteins, lipids and polysaccharides can all be modified by adding sugars as side chains to the molecule in a process known as glycosylation. The great diversity in the chemical structures of sugars means that these modifications can also be very diverse. It is recognised that the process of glycosylation is very important for plant growth and development but little is understood about the specific functions of different types of modification.

The study of sugar modification has important potential applications which are likely to be of great importance to the Chilean economy, in the fruit industry, biofuels and pharmaceuticals production.

Fruit production constitutes the third most important sector of the Chilean national economy (www.cffa.org), and Chile is a global leader in the export of fresh fruit, such as table grapes, apples and peaches. The Chilean economy benefits greatly from the ability to export fruit in the counter season, when fruit production in the northern hemisphere is dramatically reduced in the winter months. However the Chilean fruit that reaches these consumers is often of poor quality due to internal breakdown during long-distance transport in cold storage resulting in a mealy texture and browning of the flesh (Crisostoto et al., 2004). This is thought to be caused, at least in part, by changes in the glycosylation patterns of cell wall components.

Chile possesses minimal fossil fuel deposits within its national boundaries. Consequently around 72% of Chile’s energy is derived from gas, coal and oil imports, representing a large drain on the economy (National Energy Commission, 2006) and an unhealthy dependence on volatile international fuel markets. This means that alternative sources of energy such as biofuels may represent valuable technologies for Chile. Cell wall polysaccharides are an important potential source of bioenergy. An understanding of the sugar modifications that occur to these polysaccharides should further the aim of generating plants with high cell wall biomass and rapid growth which would make ideal biofuel crops.

Sugar modification also relates to technologies aimed at producing pharmaceuticals in plants. Plants could potentially be used to produce human hormones, antibodies and molecules specific to certain pathogens that could be used as vaccines. However for these factors to function correctly in humans they must gain the correct sugar modification patterns. Studying the mechanisms of sugar modification may allow us to manipulate this process in plants so as to produce pharmaceutical products that are correctly modified and thus have biological activity in humans and animals.
Fucose is an important sugar that can be added to macromolecules in the glycosylation process. It is found on various important proteins and polysaccharides in plants, particularly in the cell wall. Fucosylation of proteins and polysaccharides occurs in an intracellular, membrane bound compartment known as the Golgi apparatus. In order to gain a full understanding of the process of fucosylation it is necessary to determine how fucose gets across the membrane that forms the boundary of the Golgi apparatus. Some genes encoding the information to make proteins that are thought to be involved in this transport process have been identified. These are known as GONST3 and GONST4 and are the focus of this study.

The aim of this study was to generate plants containing altered versions of GONST3 and GONST4 and so to determine whether the proteins encoded by the GONST genes are responsible for the transport of fucose into the Golgi apparatus. Specifically we created fusions between the promoter (the region of DNA which controls when and where a gene is expressed) and a reporter gene (GUS) which produces a coloured compound when the gene is turned on. Plants transformed with this construct will facilitate detailed studies of the spacial and temporal patterns of GONST gene expression.

**Methods**

*Polymerase Chain Reaction (PCR)*

To amplify (increase the concentration of) specific sequences within a DNA sample. This technique makes use of enzymes (classes of proteins that catalyse reactions) known as DNA polymerases which are able to make double stranded DNA from single stranded template. This enzyme cannot work on a purely single stranded template however, but can only synthesis DNA from a short double stranded region. This region is generated by including in the reaction shot pieces of single stranded DNA known as primers. These primers bind to the DNA at specific sites dictated by the sequence of the primer. In this way the DNA polymerase can only amplify the region of the DNA between the two primers that are added to the reaction and so high specificity can be achieved. Reaction cycles involved first heating the reaction mix to cause the two strands of the DNA to separate, then cooling it a little to allow the primers to attach to the single stranded DNA. The DNA polymerase can then amplify the region between the two primers. In this manner the concentration of the sequence of interest in the reaction double with each reaction cycle.

*Gel Electrophoresis*

This is a technique for separating fragments of DNA based on their size. It relies on the fact that DNA is a negatively charged molecule. The DNA is loaded into wells at one end of an agarose gel. A voltage is then applied across the gel such that the DNA will move towards the positive terminal at the opposite end of the gel. Larger pieces of DNA move more slowly thought the gel than smaller pieces and so the fragments are separate by size. The DNA is visualised by shining UV light onto the gel which causes fluorescence of a molecule that attaches itself to the DNA. The precise size of a fragment is calculated by comparing its position with the position of pieces of DNA of known size run on the same gel.
Restriction Analysis
Restriction enzymes will only cut DNA at specific sequence sites. If the sequence of the DNA to be cut is known then the sizes of the fragments of DNA produced can be predicted. In a cloning reaction a piece of DNA of interest is inserted into a large circular molecule of DNA. The insertion of this piece of DNA causes the sizes of the fragments produced by the restriction enzymes. This means that restriction analysis can be used to test whether the DNA fragment of interest has been inserted and whether it has been inserted the right way round.

Gateway cloning technology
This is an advanced genetic technique which allows DNA sequences of interest to be joined to another DNA sequence and inserted into the genetic material of the plant which is being studied. In this study we wished to place a promoter sequence (a region of DNA which controls where, when and how a gene is expressed) next to a ‘reporter gene’ called GUS. When the promoter is switched on the protein product of GUS is produced and catalyses a reaction which forms a coloured product allowing the pattern of gene expression to be visualised. Due to the detailed mechanism of this procedure the promoter sequence must first be placed in an ‘entry vector’ (a circular piece of DNA) and then transferred to a ‘destination vector’ which has the necessary properties to allow the transformation of a plant with the DNA. Once each vector has been made it must be put into bacteria such that the bacteria can produce many copies of the vector. The destination vector is placed in bacteria of a particular genus: the agrobacterium. These bacteria have the unusual property of being able to infect a plant and transfer small fragment of DNA into the plant genome. This property is utilised to transfer the promoter:GUS fusion into the plant genome.

Results and Discussion

Amplification of Promoter DNA
DNA was extracted from the leaves of wildtype Arabidopsis thaliana tissue using a Fermentas genomic DNA purification kit. The quality of DNA was assessed using primers to the GAPA gene. Next, specific primers were designed for the GONST3 and GONST4 promoter and PCR conditions were optimised as follows:

GONST4:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
<th>Cycle conditions: (large number are temperature in degrees centigrade, subscripts are time in minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paq DNA polymerase</td>
<td>0.2</td>
<td>[94(<em>{0.30}) 58(</em>{1.00}) 72(<em>{30}) 72(</em>{10}) 4(_{∞}) ]</td>
</tr>
<tr>
<td>10(^{9}) Paq buffer</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>10µM NTP</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>5’ Primer</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>3’ Primer</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>14.4</td>
<td></td>
</tr>
</tbody>
</table>
GONST 3:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
<th>Cycle conditions: (large number are temperature in degrees centigrade, subscripts are time in minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taq DNA polymerase</td>
<td>0.2</td>
<td>[94_{0,30} 57.9_{1.00} 72_{1.30}]^90 72_{10} 4° Extension time was increased by 0.5 seconds every cycle.</td>
</tr>
<tr>
<td>10° Taq buffer</td>
<td>2</td>
<td>Reaction performed with Hotstart</td>
</tr>
<tr>
<td>10µM NTP</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>5’ Primer</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>3’ Primer</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>14.4</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1: Amplification of the GONST 4 promoter. A DNA fragment (indicated by arrow) of the correct size has been produced.
GONST 3 and 4 Promoter DNA was purified from the gels using the invisorb spin DNA extraction kit (invitech).

**Cloning of Promoter DNA into pCR8 entry vector**
The cloning reaction relies on a single adenosine nucleotide overhang at the end of the PCR product to create the following circular molecule represented schematically below:

1µl of promoter DNA (30µg/µl) was incubated at 23ºC for 30 minutes.

*Figure 2: Amplification of the GONST 3 promoter. DNA fragments (indicated by arrow) of the correct size have been produced.*

*Figure 3: A schematic of the pCR8 entry vector used in the process of attaching the GONST promoter to a reporter gene.*

*Transform E.coli with pCR8:GONST*
One Shot Match TR$^R$ E.coli were incubated on ice for 20 minutes in the presence of the pCR8 GONST construct. The cells were then subjected to heat shock at 42ºC for 30 seconds and incubated at 37ºC for one hour. The cells were subsequently spread on agar plates containing 100µg/ml spectinomycin. This means that only the cells which have taken up the pCR8:GONST construct will survive, because only these cells have the spectinomycin resistance gene.

**Sequencing of pCR8:GONST constructs to ensure that the GONST promoter is present, and in the correct orientation.**

Individual colonies were picked from the spectinomycin plates and cultured overnight. Plasmids were then purified from the cultures using an Axygen Mini Prep kit.

DNA sequencing reactions were performed on the plasmids derived from the mini-preps. One forward and one reverse reaction was performed for each construct and each reaction contained around 250ng of DNA.

Good, clean sequence data (eg. Below) was obtained from each reaction.

![DNA sequence data](image)

*Figure 4: A sample of the DNA sequence data obtained from a sample of the pCR8:GONST3 construct. This confirmed the presence of the promoter in the correct orientation.*

The data was used in a BLAST search against the Arabidopsis genome and confirmed the presence of the GONST promoter inserts in the correct orientation.
pGWB3 recombination reaction and E.coli transformation

The GONST promoter in the pCR8 entry vector was transferred to the pGWB3 destination vector via a homologous recombination reaction. The reaction has high site specificity and fidelity and so should always produce a destination vector in which the GONST promoter is in the correct orientation. The destination vector is suitable for expression in Agrobacterium as well as E.coli, and the Agrobacteria are used to transfer the GONST promoter:GUS construct into the plant.

Each GONSTpromoter:pCR8 construct was incubated with pGWB3 and a recombinase enzyme mix for 18 hours. E.coli cells were then transformed with the resulting construct as described above. The cells were spread on agar plates containing kanomycin (100µg/ml) and hygromycin (50µg/ml). Only cells containing the pGWB3 construct which has the hygromycin resistance gene are able to survive on such a media. Cells containing pGWB3 that did not recombine with pCR8 would also die due to the presence of a lethal gene which is removed during the recombination reaction.

Analyses to test for the presence of the GONSTpromoter:GUS fusions in the transformed E.coli

Plasmids were purified from surviving E.coli colonies and subject to restriction analysis and a diagnostic PCR.

Restriction of GONST3promoter:pGWB3 construct with the enzyme HindIII yielded the predicted band for a correct insert at around1800 base pairs:

Figure 5: Restriction analysis of the GONST3p:pGWB3 construct produced a band at 1800 bp, as predicted.
Restriction analysis of the GONST4 construct however failed to provide any diagnostic bands, possibly due to the combination of enzymes that was used.

PCR reactions were also performed with both constructs, using the primers that were used initially to isolate the promoter DNA. This was highly successful for GONST4 (see band at 1500bp below) but not for GONST3:

So there is some evidence for the presence of the GONST promoters in the destination vectors, although this is not as strong as one would wish. Before the project is taken to the plant transformation stage the presence of the promoter should be confirmed by DNA sequencing.

**Agrobacterium Transformation**

A culture of *Agrobacterium tumefaciens* was transformed with the destination vector constructs. The cells were thawed on ice from -80°C before 1500ng of construct was added. The cells were then cold shocked in liquid nitrogen for 10 seconds before being incubated at 27°C for 36 hours. They were then spread on LB agar containing kanomycin (100µg/ml) and hygromycin (50µg/ml).

PCR and restriction analysis failed to establish the presence of the GONST promoter in plasmids extracted from the surviving Agrobacterium colonies. There could have been a problem with the cells used for transformation or a problem with the recombination reaction itself. The sequencing of the constructs from the recombination reaction will be able to distinguish these possibilities. Once Agrobacterium have been successfully transformed it will be possible to transform the Arabidopsis with the promoter:GUS fusions.
Concluding Remarks

During the course of the 6 week project rapid progress was made towards the generation of transgenic plants expressing reporter genes under the control of either the GONST3 or the GONST4 promoter. There is good evidence, in the form of restriction analyses and diagnostic PCRs, that GONSTpromoter:GUS fusions were successfully created. This should be confirmed by sequencing. The Agrobacterium transformation appears not to have been successful. These reactions should be repeated with different cell and enzyme stocks until colony PCRs and restriction analyses give a positive result. The presence of the correct construct should then be confirmed by sequencing.

Once the transgenic plants have been generated they will be a vital experimental tool for understanding the role of nucleotide sugar transporters in-planta. In particular it will be fascinating to see if the expression pattern of these enzymes changes under different environmental condition and at different stages in development. This may provide insights into the precise function of fucosylation in plants and suggest how manipulation of sugar modification patterns could be used to improve food and biofuel crops.

Acknowledgements

I am indebted to the Centre for Latin American Studies, Cambridge for funding this project. As well as making a small contribution to plant sugar biochemistry research, it has been of great personal benefit, both in developing my scientific skills and in allowing me to experience Latin American culture at first hand.

I must also thank Dr Michael Handford and the Universidad de Chile for hosting me and for teaching me the molecular biological techniques necessary to complete the project.

Finally I am grateful to Dr Paul Dupree, my Cambridge director of studies, for initiating this collaboration and for giving me encouragement and support throughout my undergraduate years.