Functional Identification of the Chloroplastic Raffinose Transporter from *Arabidopsis*

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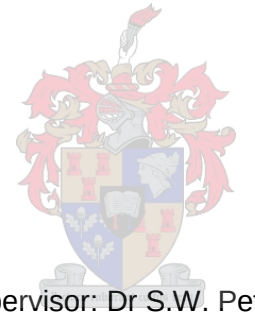

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Abstract

In plants, the raffinose family oligosaccharides (RFOs) have a wide range of functions, ranging from membrane trafficking to participation in signal transduction processes. Their most well characterized role in plants, is the ability to function as compatible solutes that provides protection against abiotic stress factors. Raffinose is a member of the RFOs that have been found to be important in the protection of the photosynthetic apparatus of *Arabidopsis thaliana* under cold-acclimation.

It has been well characterized that raffinose accumulates in chloroplasts under cold-acclimation and is transported via a plastidic membrane transporter. The true identity of this elusive transporter is currently unknown. Presently, the only known eukaryotic transporter ever characterized is Mrt, which is found in the fungus *Metarhizium robertsii*. A protein BLAST analysis comparing the amino acid sequences of Mrt and a well characterized *Arabidopsis* chloroplastic glucose transporter, pGlcT1 (plastidic glucose translocator 1, TAIR accession code: AT5G16150) revealed a 24.7 % sequence homology. This showed significant functional homology between the two transporters.

Our research aimed to identify the chloroplastic raffinose transporter by employing a multipronged approach. Firstly, an *Arabidopsis thaliana* library with full length clones in pBlueScript SK (+) was transformed in *E. coli* BL21 and growth was tested on M9 minimal media supplemented with raffinose to determine whether *pGlcT1* was present. The Gateway® protein expression vector, pDEST17, containing the pGlcT1 gene was also transformed into *E. coli* BL21. The construct was used for heterologous expression of *pGlcT1* in *E.coli* BL21 AI on M9 minimal media supplemented with raffinose to test growth. This approach involved an *in planta* approach which utilized chlorophyll fluorescence to measure the quantum efficiency of Photosystem II photochemistry (F_v/F_m) in *pGlcT1* (SALK_066365) mutant plants. This was to determine if pGlcT1 was essential for raffinose accumulation in *Arabidopsis* chloroplasts under cold-acclimation (4^{o}C) for 7 d.

The results demonstrated that *E. coli* was able to grow on raffinose and sucrose in the presence of pGlcT1. Chlorophyll fluorescence analyses indicated an expected F_v/F_m reduction in the raffinose synthase (RafS) mutant (RS14) plants consistent with previous studies. There was also a notable decrease in F_v/F_m ($P = 0.0006$) within the

pGlcT1 mutant plants while the Col-0 wild type plants maintained normal F_v/F_m values (~0.80) for the duration of cold-acclimation. The decrease in F_v/F_m values for the *pGlcT1* mutant plants were statistically significant (P = 0.0004) based on a repeated measures one-way ANOVA test coupled with a linear trend post-test.

Despite the preliminary nature of our work, it can be deduced from our results that pGlcT1 may facilitate the uptake of raffinose into *E. coli* cells. Raffinose that enters cells is most likely catabolised through inherent α-galactosidase activity. When compared with the F_v/F_m values of the wild type Col-0 plants, the decrease in F_v/F_m values in both the RafS and *pGlcT1* mutant plants indicates that there is a possible raffinose deficiency within the chloroplasts of these plants under cold-acclimation (4^{o}C). This hints at the probable importance of pGlcT1 in transporting raffinose into the chloroplast which safeguards the photosynthetic machinery of PSII under cold-acclimation conditions.

Opsomming

In plante, die raffinose familie oligosakkariede (RVO) het 'n wye verskeidenheid van funksies, wat wissel van membraan handel deelname aan seintransduksie prosesse. Hul mees gekenmerk funksie is die vermoë om as versoenbaar opgeloste stowwe wat plante bied beskerming teen abiotiese stresfaktore te funksioneer. Raffinose is 'n lid van die RVO's wat gevind is belangrik in die beskerming van die fotosintetiese apparaat van Arabidopsis thaliana onder koue akklimatisering te wees.

Dit is goed dat gekenmerk raffinose ophoop in chloroplaste onder koue akklimatisering en via 'n plastidic membraan vervoerder vervoer word. Die ware identiteit van hierdie ontwykende vervoerder is tans onbekend. Tans, die enigste bekende eukariotiese vervoerder ooit gekenmerk is Mrt, wat gevind word in die swam *Metarhizium robertsii*. A proteïen BLAST analise vergelyk die aminosuur volgorde van Mrt en 'n goed gekenmerk *Arabidopsis* chloroplastic glukose vervoerder, pGlcT1 (plastidic glukose locator 1, TAIR toetreding kode: AT5G16150) het ook 'n 27% volgorde homologie. Dit het getoon beduidende funksionele homologie tussen die twee transporters.

Ons navorsing is daarop gemik om die chloroplastic raffinose vervoerder identifiseer deur die gebruik van 'n multipronged benadering. Eerstens, 'n *Arabidopsis thaliana* biblioteek vollengte klone in pBlueScript SK (+) was 'n proteïen uitdrukking te bou met die Gateway® pDEST17 vektor wat die *pGlcT1* gene geskep is. Die konstruk is wat gebruik word vir heteroloë uitdrukking van pGlcT1 in *E.coli* BL21 AI op M9 minimale media aangevul met raffinose toets groei. Hierdie benadering betrokke is 'n *in planta* benadering wat chlorofil-fluoressensie aangewend word om die doeltreffendheid van kwantum Photosisteem II fotochemie (F_v/F_m) in pGlcT1 (SALK_066365) mutant plante te meet. Dit was om te bepaal of pGlcT1 was noodsaaklik vir raffinose opeenhoping in *Arabidopsis* chloroplaste onder koue akklimatisering (4^{o}C) vir 7 d.

Die resultate het getoon dat *E. coli* was net in staat om te groei op raffinose en sukrose in die teenwoordigheid van pGlcT1. Chlorofil-fluoressensie ontledings aangedui 'n verwagte afname F_v/F_m ($P = 0.0006$) in die raffinose sintase (RafS) mutant (RS14)

plante in ooreenstemming met vorige studies. Daar was ook 'n redelik beduidende afname in F_v / F_m (P = 0.0004) binne die *pGlcT1* mutant plante terwyl die kol-0 wilde-tipe plante gehandhaaf normale F_v / F_m waardes vir die duur van die koue akklimatisering.

Dit is duidelik uit die resultate dat pGlcT1 fasiliteer die opname van raffinose in *E. coli* selle. Raffinose wat selle binnegaan is waarskynlik catabolised deur inherente α-galaktosidase aktiwiteit. In vergelyking met die F_v/F_m waardes van die wilde-tipe Kol-0 plante, die afname in F_v/F_m waardes in beide die RafS en *pGlcT1* mutant plante bevestig dat daar 'n raffinose tekort binne die chloroplaste van hierdie plante onder koue akklimatisering (4^{o}C). Dit belig die belangrikheid van pGlcT1 in die vervoer van raffinose in die chloroplast wat die fotosintetiese masjinerie van PSII beskerm onder koue akklimatisering voorwaardes.

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Table of Contents

List of Figures

List of Tables

List of abbreviations

cDNA	complementary DNA
csc	chromosomally encoded sucrose catabolism
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylene diamine tetra acetic acid
gDNA	genomic DNA
kDa	kilo Daltons
L	Litre
M	Molar
MEX	Maltose Exporter
MFS	Major Facilitator Superfamily
mg	milli-gram
µg	microgram
µl	microlitre
ml	millilitre
µM	micromolar
mM	millimolar
PCR	Polymerase Chain Reaction
pGlcT1	plastidic glucose translocator 1
RafS	raffinose synthase
Suc	sucrose
STS	stachyose synthase
Raf	raffinose
WSC	water soluble carbohydrate
WT	Wild Type

Chapter 1: Introduction

Abiotic stress factors such as heat shock, high light, drought, high and low temperatures and salinity negatively influence the biochemistry and physiology of plants (Tuteja *et al*, 2011; Krasensky & Jonak, 2012; Atkinson & Urwin, 2012). Abiotic stress factors have long been selective pressures that have driven plant evolution and environmental adaptation and stringent measures adopted by plants to survive these adverse stress conditions are incredibly complex (Bijlsma & Loeschke, 2005; Pareek, 2010; Ahmad & Prasad, 2012). This complexity is derived from the multifaceted levels of interplay between perceptions, signalling and regulatory mechanisms that elicit plant responses to abiotic stress. These responses result in plants adapting positively to antagonistic growth conditions (Tuteja, 2007; Akpinar *et al*, 2012; Atkinson & Urwin, 2012).

Abiotic stress factors prompt multiple responses within plants. These responses are initiated by stress perception. This is followed by the activation of signalling pathways and consequently the alteration of gene expression levels (Di Toppi & Pawlik-Skowronska, 2003; Gill and Tuteja, 2010; Cramer *et al*, 2011; Ahmad & Prasad, 2012; ElSayed *et al*, 2014). These alterations lead to disparities in plant physiology, growth and development (Yamaguchi & Blumwald, 2005). One of the major mechanisms utilized by plants to counteract these deleterious effects is the synthesis of compatible solutes. Compatible solutes include amino acids, amines, quaternary compounds and sugars such as trehalose and raffinose family oligosaccharides (RFOs, Mahajan & Tuteja, 2005).

Raffinose Family Oligosaccharides such as raffinose, stachyose and verbascose are soluble carbohydrates that are formed when D-galactose units are attached to the D-glucose moiety of a sucrose molecule through an α-(1→6) glycosidic linkage (Avigad & Dey, 1997). They have a diverse range of roles within plants. These include participation in signal transduction processes (Xue *et al*. 2007); membrane trafficking (Thole & Nielsen, 2008), the transport and storage of carbon and they even serve as osmoprotectants during seed desiccation (Hoekstra *et al*, 1997). The most well characterized attribute of RFOs is their ability to function as compatible solutes or osmolytes for defence against abiotic stress (Bachmann *et al,* 1994; Taji *et al*, 2002;

Hannah *et al*, 2006; Skinner, 2006; ElSayed *et al*, 2014). Plants that produce RFOs, generally possess commercial value and include curcubits, mints, legumes, olives, grapes, pines and grains (ElSayed *et al*, 2014). Raffinose Family Oligosaccharides benefit human nutrition even further by functioning as prebiotics, which aid in stimulating the growth of human gut bacteria (Kozlowska *et al*, 2000).

1.1 **RFO biosynthesis**

The biosynthesis of RFOs is the result of the action of a set of galactyltransferases. The first stage, galactinol synthesis as shown in Figure 1.1 is catalysed by galactinol synthase (GolS), from UDP-D-Galactose and myo-inositol (Sprenger & Keller, 2000). The galactinol synthesis reaction is proceeded by the addition of extra galactosyl units donated by galactinol, resulting in sucrose and subsequently, raffinose. This is a reversible reaction and is facilitated by raffinose synthase (RafS) (Lehle & Tanner, 1973). The addition of a further galactosyl unit from galactinol to raffinose in the presence of stachyose synthase (STS) results in the formation of stachyose.

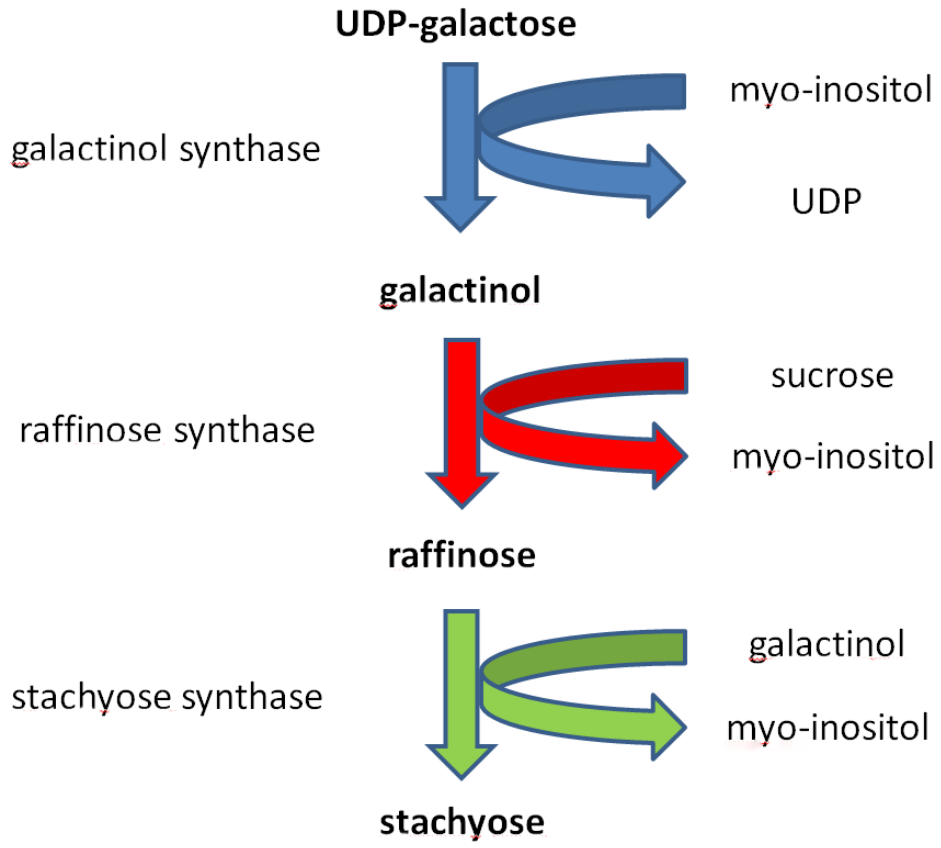

Figure 1.1 Schematic representation of the RFO biosynthetic pathway representing the galactyl-transferases involved in RFO biosysnthesis. (Adapted from Nishizawa *et al*, 2008).

Galactinol synthase, which is localized in the cytosol, plays a critical role in the regulation of carbon separation between sucrose and RFOs (Schneider & Keller, 2009; Saravitz *et al*, 2002). This is corroborated by Taji *et al* (2002), who noted a substantial increase in the levels of endogenous galactinol and raffinose present in transgenic *Arabidopsis thaliana* plants under standard conditions, where the *GolS2* gene was overexpressed. It is possible for RFOs with a higher degree of polymerization (DP) to be produced in the absence of galactinol. In plants such as *Ajuga reptans* and *Coleus blumei*, this is accomplished via the action of galactan: galactan galactosyl transferase (GGT), which is found in the vacuole (Haab & Keller, 2002; Peters *et al*, 2009; Gilbert *et al*, 1997). Despite GGT activity, GolS is still essential to the RFO biosynthetic pathway, especially since it regulates the amounts of specific RFOs that are accumulated (Peterbauer *et al*, 2002).

The second stage of RFO biosynthesis exclusively involves RafS, which transfers a galactosyl unit from galactinol to the C_6 position of the glucose moiety in sucrose. This subsequently forms an α-(1→6) glycosidic linkage that yields the tri-saccharide raffinose (Egert *et al*, 2013). Raffinose is one of the most abundant soluble carbohydrates found in nature, second only to sucrose (Dey *et al*, 1997; Hugouvieux-Cotte-Pattat *et al*, 2009). Raffinose synthases are not well characterized within plants and only a few putative RafS genes have been identified in plants such as pea, cucumber, maize, grape, rice and *Arabidopsis* (Dierking & Bilyeu, 2008). Research regarding RafS is limited, which is not surprising as there are numerous missing links concerning raffinose transport and other raffinose related mechanisms in plants.

Another important RFO that is produced after raffinose in the RFO biosynthetic pathway is the tetra-saccharide stachyose. Stachyose is synthesised by the action of stachyose synthase (SS) and is comprised of 2 α-D-galactose, 1 α-D-glucose and 1 α-D-fructose subunits respectively. Stachyose, like raffinose has been found to accumulate in plants under cold-acclimation conditions (Bertrand *et al*, 2006; Iftime *et al*, 2011). Raffinose and stachyose are both able to interact with membranes, conserving them in a liquid crystal-like state once the hydration shell of the lipid head-groups is misplaced during drying or freezing of the membrane in question (Hincha *et al*, 2003). Subsequently membrane damage is prevented (Hincha, 2003).

Stachyose has been demonstrated to possess superior membrane protection capability to raffinose *in vitro* (Santarius, 1973; Iftime *et al*, 2011). However, despite its greater protective ability, Iftime *et al* (2011) found that stachyose does not accumulate in the chloroplast and is confined to the cytosol whereas raffinose is transported into the chloroplast. This fact supports the theory that raffinose does not play a role in freezing tolerance of plants but rather it is more integral to protecting the thylakoid membrane of the chloroplast (Zuther *et al*, 2004; Knaupp *et al*, 2011).

1.2 **Role of RFOs as a stress protectant**

Drought, salinity and heat stress are all environmental factors that can drastically affect plant growth on a biochemical and physiological level. Plants have adapted to adverse environmental factors by synthesizing protective compounds that allow them to survive these harsh environmental changes. The protective compounds often react by stabilizing membranes and proteins or by mediating osmotic adjustment (Bohnert *et al*, 1995; Hoekstra *et al*, 2001). These compounds include water soluble carbohydrates (WSC) like glucose, sucrose as well as Raffinose Family Oligosaccharides, for instance raffinose and stachyose (Amiard *et al*, 2003).

RFOs, namely raffinose and stachyose have numerous functions regarding abiotic stress defence such as osmo-protectant capabilities that confer desiccation tolerance in certain plant seeds (Saravitz *et al*, 1987; Blackman *et al*, 1992). Additionally, RFOs are responsible for the protection of uni-lamellar liposomes from dehydration via direct *in vitro* sugar membrane interactions (Hincha *et al*, 2003). According to Santarius (1973), raffinose, in particular has been found to reduce the inactivation of electron and cyclic photophosphorylation during photosynthesis, in the thylakoid membrane of chloroplasts found in spinach (S*pinacea oleracea*) under freezing, desiccation and high temperature conditions.

Taji *et al* (2002) reported that galactinol synthase over-expressing *Arabidopsis* plants that had increased levels of galactinol and raffinose, exhibited increased drought stress tolerance. This suggests that galactinol and raffinose may function as osmo-protectants under high salinity stress levels. Nishizawa-Yokoi *et al* (2008) have

demonstrated that high intracellular levels of galactinol and raffinose accumulate within *Arabidopsis* plants overexpressing the *GolS* gene. This accumulation was associated with increased tolerance to methyl-viologen treatment (induces oxidative stress within plants) and chilling stress. The galactinol and raffinose that accumulated was found to effectively protect salicylate from attack by hydroxyl radicals *in vitro* (Nishizawa-Yokoi *et al*, 2008). The importance of these results, imply that galactinol and raffinose do not only function as osmo-protectants, but they also serve as antioxidants within the leaves of *Arabidopsis* plants.

The antioxidant function of RFOs such as raffinose, is necessary to counteract the accumulation of reactive oxygen species (ROS) under abiotic stress conditions (Nishizawa *et al*, 2008; Peshev *et al*, 2013). These ROS are necessary for various physiological processes occurring within plants (Bolwell *et al*, 2002; Bailey-Serres, 2006; Tripathy & Oelmüller, 2012; Baxter *et al*, 2013). They are constantly produced via mitochondrial respiration and photosynthesis. Plants start undergoing stress when the ROS concentration increases as a result of stresses such as drought, chilling, heat and high light irradiation (Bowler *et al*, 2002). This increase in ROS levels if unquenched by antioxidants results in oxidative damage at the cellular level of plants.

Arguably, the most harmful form of ROS is the hydroxyl radical derived from hydrogen peroxide (ElSayed *et al*, 2013). This hydroxyl radical has the potential to react with all biological molecules and increased levels will eventually cause cell death as cells have no method of removing the hydroxyl from their system. Within recent years, RFOs have been shown to play essential roles in oxidative stress defence mechanisms in plants (Nishikawa *et al*, 2008; Peshev *et al*, 2013). As stated previously, the fact that raffinose has been identified in protecting photophosphorylation and electron transport within chloroplast thylakoid membranes of spinach (Santarius *et al*, 1973), convincingly supports the idea that raffinose might function as a ROS scavenger. This is further supported by Nishizawa *et al* (2008), who observed increased levels of raffinose present in *Arabidopsis* plants, where the *RafS* gene was over-expressed. This increased accumulation of raffinose led to a proportional increase in ROS scavenging as well as oxidative stress tolerance.

Raffinose's almost ubiquitous presence in nature is arguably derived from its function as an important stress protective molecule in plants. Raffinose is the only RFO to accumulate in *Arabidopsis* vegetative tissues such as leaves and roots and is only detected under abiotic stress conditions (Nishizawa *et al*, 2008). High raffinose concentrations have been associated with freezing tolerance in plants as varied as *Arabidopsis thaliana* (Klotke *et al*, 2004; Rohde *et al*, 2004) and woody conifers (Strimbeck *et al,* 2007).

Arabidopsis accumulates high concentrations of raffinose in the leaves, under conditions of cold-acclimation (4^{o}C, Santarius & Milde, 1976; Taji *et al*, 2002; Zuther *et* al, 2004; Strimbeck *et al*, 2007; Schneider & Keller, 2009). However, Zuther *et al* (2004) first observed that in *Arabidopsis* plants (RS14 line) containing a knock-out mutation of the only endogenous *RafS* gene present, there was no difference in the freezing tolerance of cold acclimated wild-type and mutant plants. This suggested that despite an almost ubiquitous response in raffinose mass increase during cold-acclimation in many plants, raffinose's occurrence was not responsible for freezing protection as was previously thought (Schneider & Keller, 2009; Knaupp *et al*, 2011; Iftime *et al*, 2011). Subsequently, reports described a novel transport mechanism where a portion (up to 20% in *Arabidopsis*) of raffinose accumulated in leaves during cold-acclimation appeared in the chloroplast (Zuther *et al*, 2004, Klotke *et al*, 2004; Schneider and Keller, 2009; Knaupp *et al*, 2011; Iftime *et al*, 2011).

Schneider & Keller (2009) conducted compartmentation analyses on leaf mesophyll protoplasts which led them to the conclusion that up to 20% of raffinose accumulates in the chloroplasts of the common bugle (*Ajuga reptans*), spinach (*Spinacia oleracea*) and *Arabidopsis* under cold acclimated conditions. They deduced that since *GolS* & *RafS*, both essential for raffinose synthesis were localized to the cytosol, raffinose must enter the chloroplast via a raffinose transporter located in the chloroplast envelope, most likely via an active uptake process.

Knaupp *et al* (2011) conducted an elegant experiment utilizing chlorophyll fluorescence imaging and electrolyte leakage analyses to determine the role of raffinose in stabilizing PS II during freeze-thaw cycles. Chlorophyll fluorescence analysis is a powerful and widely used tool that allows researchers to analyse the photosynthetic

performance of plants under a variety of environmental conditions (Maxwell & Johnson, 2000). Measuring the yield of fluorescence allows researchers to monitor and gather data on any changes in photosynthetic processes which provides useful data on a plant's photosynthetic performance. Additionally, chlorophyll fluorescence is able to provide valuable information on a plant's ability to tolerate environmental stresses. It can in particular, determine the extent to which these stresses have affected the photosynthetic machinery of the plant (Maxwell & Johnson, 2000). The basic principle underlying chlorophyll fluorescence involves the absorption of light energy by chlorophyll molecules present in leaves. This energy can be used in three different paths, namely; to drive photosynthesis, excess energy is dissipated as heat and finally the light energy can be emitted as a photon resulting in fluorescence (Maxwell & Johnson, 2000).

Knaupp *et al* (2011) confirmed that raffinose is not essential for freezing tolerance through electrolyte leakage and chlorophyll fluorescence imaging analyses conducted on the same *Arabidopsis thaliana* RafS mutant previously used by Zuther *et al* (2004) along with wild type Col-0 plants. From the electrolyte leakage studies, they discovered that a lack of raffinose plays no role in electrolyte leakage from leaf cells following freeze-thaw cycles. This finding verifies the fact that raffinose is not a pre-requisite for plasma membrane protection. Alternatively, Knaupp *et al* (2011) confirmed via the chlorophyll fluorescence analysis that under cold-acclimation conditions, the maximum quantum yield of PSII (F_v/F_m) along with other fluorescence constraints, were significantly lower as opposed to the wild type plants.

This is an important distinction with regards to the role of raffinose as a cryo-protectant molecule in that it is not responsible for the protection of the plasma membrane but rather, it is more likely to be involved in preserving the chloroplast thylakoid membrane (Iftime *et al*, 2011). Hence, raffinose aids in maintaining the functional and structural integrity of the photosynthetic machinery within the chloroplasts. Under cold-acclimation conditions, the raffinose that accumulates within chloroplasts has been shown to protect thylakoid membranes with functions such as stabilizing the chloroplast coupling factor CF1, which is essential for photophosphorylation (Lineberger & Steponkus, 1980a) and also membrane attachment of plastocyanin

which is responsible for electron transfer between cytochrome f of the cytochrome b_6f complex from Photosystem II (PS II) and P700+ from Photosystem I (Hincha, 1990).

1.3 **The molecular identity of the chloroplastic transporter is unknown**

Although the biosynthesis and protective roles of raffinose have been studied in depth, there is a major gap regarding its transport and localization in the chloroplast. For many years, raffinose has been understood to be synthesised in the cytosol (Bachmann *et al*, 1994). Raffinose has mainly been found to occur in seeds as well as in the leaves of plants such as *Arabidopsis* (Zuther *et al*, 2004). An interesting observation that ties in with the compatible solute capabilities of raffinose, is its increased concentration levels in the chloroplasts of plants such as *Arabidopsis* and *Ajuga reptans*, under cold-acclimation conditions (Santarius & Milde, 1977; Bachmann *et al*, 1994; Zuther *et al*, 2004; Schneider & Keller, 2009). Even though raffinose is synthesized in the cytosol, approximately 20% of total cellular raffinose accumulated within chloroplasts, during exposure to cold stress (Schneider & Keller, 2009, Knaupp *et al*, 2011; Iftime *et al*, 2011).

Raffinose is not synthesized in the chloroplast of *Arabidopsis*. This is supported by Schneider & Keller (2009), who found that raffinose biosynthetic enzymes, GolS and RafS activities are not present within the chloroplast but rather in the cytosol. They also discovered that galactinol, which is a GolS product and a RafS substrate, was only present extra-chloroplastically. Emanuelsson *et al* (2007) determined that no chloroplastic transit peptides were linked to all known GolS and RafS protein sequences when different sequence based predictors were utilized.

Past studies have utilized aqueous (Schneider & Keller, 2009) and non-aqueous fractionation techniques (Iftime *et al*, 2011; Voitsekhovskaja *et al*, 2006; Benkeblia *et al*, 2007; Nadwodnik and Lohaus, 2008) to determine water-soluble carbohydrate distribution patterns between the leaf chloroplasts of a multitude of cold acclimated plants such as *Ajuga reptans, Apium graveolens, Arabidopsis thaliana, Brassica oleracea and Plantago sylvestris* among others. Raffinose was found to accumulate at higher levels than the compartment size fraction in the chloroplasts but only in cold acclimated plants. This further substantiates the finding that raffinose is synthesised in

the cytosol and only accumulates in chloroplasts to fulfil its stress protectant role. Raffinose is in fact degraded in the chloroplast during de-acclimation by *AtDIN10* (DARK INDUCIBLE 10, AT5g20250), which has been recently categorized as a stroma-localized α-galactosidase involved in raffinose degradation during stress recovery (Christ, 2013).

Schneider & Keller (2009) determined that raffinose uptake in *Ajuga* and *Arabidopsis* is an active process as opposed to the facilitated diffusion of glucose into the chloroplast. The presence of raffinose within the chloroplast along with its rapid and active uptake is indicative of some form of transport mechanism that allows raffinose to enter the chloroplast. In *Ajuga* chloroplasts, the transport kinetics of the raffinose transporter were very similar to the only known RFO transporter, the stachyose/H^+ antiporter found in Japanese artichoke (*Stachys sieboldii*) tuber vacuoles (Keller, 1992; Niland & Schmitz, 1995; Schneider & Keller, 2009) This transport mechanism is most likely a chloroplastic membrane transporter that allows raffinose to pass through across the chloroplast envelope into the chloroplast. Furthermore, the transport system in *Arabidopsis* is known to be selective only for raffinose and not stachyose (Hannah *et al*, 2006; Iftime *et al*, 2011). Despite extensive characterisation of the actual chloroplastic transport system, the molecular identity of the chloroplastic raffinose transporter in *Arabidopsis* remains unknown.

Numerous raffinose transporters have been characterized in prokaryotes (Aslanidis & Schmitt, 1990; Benz *et al*, 1992; Ulmke *et al*, 1997). However, the first eukaryotic raffinose transporter was only recently functionally characterized (Fang & St. Leger, 2010). Metarhizium Raffinose Transporter (MRT) represents the first oligosaccharide transporter ever discovered in a fungus, *Metarhizium robertsii* and more importantly in any eukaryote, signifying the extensive importance of RFO transport in nature (Fang & St. Leger, 2010).

Fang & St. Leger (2010) screened over 20 000 transformants in a *M. robertsii* genome-wide random DNA insertion library. They found a mutant that exhibited poor growth on root exudate was disrupted in the MRT sugar transporter gene. They later found that disrupting the *Mrt* gene led to the mutants exhibiting diminished growth as opposed to the wild type fungi on media supplemented with sucrose, lactose, raffinose, stachyose

and verbascose separately. However, on media supplemented with simple monosaccharides and oligomers composed entirely of glucose subunits, both the wild type and *Mrt* mutants grew equally well. This suggested that MRT was the sole transporter for heterologous disaccharides as well as oligosaccharides such as raffinose (Fang & St. Leger, 2010).

A BLAST analysis conducted with the *Mrt* gene CDS (GenBank accession no. GQ167043) against TAIR (The *Arabidopsis* Information Resource) curated sequence database, yielded a match with a significant homology of 36.1%. Additionally, protein BLAST analysis, established that the pGlcT1 transporter shared a 24.7 % amino acid identity similarity with the raffinose transporter Mrt found in *Metarhizium robertsii*. This match was for a well characterised *plastidic glucose translocator 1* gene (*pGlcT1*, GenBank accession no.: AT5G16150).

The *pGlcT1* gene was first characterized by Weber *et al* (2000), in spinach (*Spinacia oleracea*) leaves. It was found that in higher plants, pGlcT plays a significant role in the export of starch degradation products (Cho *et al*, 2011). As photosynthesis occurs, a portion of the fixed carbon is directed towards the synthesis of transient starch, which functions as an intermediate carbon storage resource in chloroplasts. This transient starch is then transported at night time. Hydrolytic enzymes degrade the starch into glucose and this particular pathway requires a glucose translocator to facilitate the export of the glucose from the chloroplasts (Weber *et al*, 2000).

After discovering *pGlcT1*, Weber *et al* (2000), re-assessed the kinetic properties of the glucose translocator utilizing a differential labelling approach. They identified a 43-kDa protein present in the chloroplast inner envelope membrane which matched *pGlcT1*. They later cloned homologous cDNAs from tobacco, potato, *Arabidopsis*, and maize. Weber *et al* (2000) conducted *in vitro* protein importation experiments and found that the pGlcT1 protein is imported into the chloroplast inner envelope membrane.

The aim of the present study was to determine whether the pGlcT1 glucose transporter also functions as a raffinose transporter. A two-pronged approach was utilized. Firstly, a heterologous screening system (*E.coli*) was used to determine if recombinant *pGlcT1* expression allows *E. coli* to utilise raffinose as the sole carbon source for growth.

Laboratory strains of *E. coli* are deficient in this ability due to the absence of a raffinose transporter.

Secondly, using an *in planta* strategy and the known physiological response of the RS14 mutant line (RafS and raffinose deficient, Zuther, *et. al*, 2004; Knaupp *et al*, 2011) during cold-acclimation (4^{o}C) the quantum efficiency of PS II was determined in pGlcT1 T-DNA insertion mutants. We reasoned that under cold-acclimation one should recover comparable F_v/F_m values between the RS14 and the pGlcT1 mutants, since if pGlcT1 genuinely encoded for a raffinose transporter then both mutants would be unable to accumulate any raffinose in the chloroplasts (during cold-acclimation), compromising PSII quantum efficiency under stress.

Chapter 2: Materials and Methods

2.1 Plant material

2.1.1 Seed stocks

Two *Arabidopsis thaliana* SALK line (SALK_051876 and SALK_066365) seed sets for T-DNA insertions within the *pGlcT1* gene were obtained from the Nottingham *Arabidopsis* Stock Centre (NASC). Plants were grown under standard *Arabidopsis* growth conditions as described in 2.1.2.

2.1.2 Plant propagation

The SALK line *Arabidopsis* seeds were placed on Jiffy-7®-peat pellets, with sterile forceps (Jiffy International AS, Kristiansand, Norway) and stratified at 4^{o}C overnight before being transferred to an isolated *Arabidopsis* growth room. All plants were subjected to long photoperiods of 16 h (500 µmol.m^{-2}.s^{-1}, 25^{o}C, 8 h dark and approximately 60-70 % relative humidity) in order to induce rapid flowering. Each plant was supplemented with 5-10 ml (1.44 g/L) of Phostrogen All Purpose Plant Food (Bayer CropScience Limited, Cambridge, United Kingdom) at 7 and 14 d after germination respectively, to provide the plants with additional nutrients.

2.1.3 Seed harvest

Seeds were harvested by first bagging the mature plants with paper bags and then allowing them to dry out. The bagged inflorescences were threshed by hand and the seeds are separated from chaff through a fine sieve onto clean paper. The seeds were sieved multiple times to remove any excess debris. Seeds were then split equally into three adequately labelled, threaded cryo-vials with rubber seals and stored indefinitely at room temperature (22-25^{o}C).

2.2 General experimental methods

All major buffer and media compositions are shown in Appendix A1. All major protocols that were used in the experimental procedures that follow are shown in Appendix A2.

2.2.1 Genomic DNA extractions

All genomic DNA (gDNA) extractions on *Arabidopsis* plants were performed using a simple and rapid genomic DNA extraction protocol adapted from Edwards *et al* (1991). Each extraction process required one large *Arabidopsis* leaf which was macerated. The Edward's extraction buffer [200 mM Tris base (pH 7.5), 250 mM NaCl, and 25 mM EDTA, 10% SDS (w/v)] was used in conjunction with isopropanol and 70% (v/v) ethanol to isolate genomic DNA. The gDNA is was re-suspended in TE buffer (pH 8.0).

2.2.2 Polymerase chain reaction (PCR)

Standard PCR conditions were used, including an initial denaturation temperature of 94^{o}C for 50 s. This was followed by 35 cycles at 94^{o}C for 50 s, primer annealing temperature for 50 s, 72^{o}C initial elongation at 1min/kb and a final elongation step at 72^{o}C for 10 min completes the PCR. All primer-specific annealing temperatures are mentioned in their respective experimental outlines.

Each PCR amplified approximately 150 ng template DNA in a 50 µl total reaction volume. Every PCR constituted 1x GoTaq® Green Reaction Buffer (Promega Corporation, Wisconsin, USA), 0.30 mM deoxy-nucleotide triphosphates, 0.30 µM primers and 0.50 U GoTaq Taq polymerase (Promega Corporation, Wisconsin, USA). All primers, unless stated otherwise were designed using Oligo-Explorer v1.5 (Teemu Kuulasma, 2002).

PCR reactions that required the Roche Expand™ High Fidelity PCR system (Hoffman-La Roche, Basel, Switzerland) were conducted as per the manufacturer's instructions. These PCR reactions had an initial denaturation temperature of 94^{o}C for 2 minutes followed by 35 cycles of 94^{o}C for 30 s, primer annealing temperature for 30 s, 72^{o}C initial elongation at 40 s/kb with a final elongation step at 72^{o}C for 7 min.

All colony PCRs were performed using the same reagent specifications as mentioned above with the exception of the template DNA being replaced by single colonies. Also, colony PCR conditions were slightly different and were as follows. An initial denaturation temperature of 95^{o}C for 5 min, followed by 35 cycles at 95^{o}C for 1 min, primer annealing temperature for 1.5 min , 72^{o}C initial elongation at 1min/kb and a final elongation step at 72^{o}C for 5 min.

2.2.3 Gel electrophoresis and gel visualization

Gel electrophoresis for visualization of PCR products involved the use of 1 % (w/v) agarose gels with 1x TBE buffer as the solvent. All gels were pre-stained with non-toxic PronaSafe®(Laboratorios CONDA, Madrid, Spain) stain for visualization under UV light. Restriction digests that required gel excision and purification were run on 0.75% low gelling temperature agarose gels (Sigma-Aldrich, Missouri, USA). BenchTop 1kb ladder (Promega Corporation, Wisconsin, USA) was the molecular marker of choice for gel electrophoresis, unless otherwise stated. Gels were visualized at 260 nm UV wavelength in an Alpha-Innotech Illuminator (Alpha-Innotech, California, USA).

2.2.4 Restriction digests and ligations

All diagnostic restriction digests used 500 ng template DNA with a total reaction volume of 20 µl. All digests were conducted using Thermo-Fisher Scientific (Massachusetts, USA) restriction enzymes and buffers. Digests which required gel excision and purification for ligations used 1-5 µg of template DNA per reaction, with a total reaction volume of 20 µl. Ligations were performed at an insert and vector DNA ratio of 4:1, within a total reaction volume of 20 µl. The reaction mixture contained 2x Rapid Ligation Buffer (Promega Corporation, Wisconsin, USA) and 2U T4 DNA ligase (Promega Corporation, Wisconsin, USA).

2.2.5 *E.coli* transformations, mini-preparations, plasmid recovery & DNA purification from agarose gels

All vector constructs were transformed into chemically ultra-competent *E.coli* cells. Table 3.1 indicates the various *E. coli* strains and vectors used in the study. *E. coli* cells were rendered ultra-competent using a modified Inoue *et al* (1990: 23-28) protocol (Refer to Appendix A2). Transformation involved using a standard heat shock protocol (Refer to Appendix A2). Plasmids were isolated from 5 ml of the *E. coli* transformant cultures grown in 50 ml BD Falcon tubes containing liquid LB medium, overnight at 37^{o}C with 200 rpm vigorous shaking. Antibiotic selection was dependant on the plasmid to be isolated.

Plasmid recovery was performed with the Promega Wizard® Plus SV Minipreps DNA Purification System kit (Promega Corporation, Wisconsin, USA) as per the manufacturer's detailed protocol.

Plasmid recovery was facilitated through the use of the Wizard® Plus SV Minipreps DNA purification (Promega Corporation, Wisconsin, USA). For DNA purification, the required bands were excised from the gel using a sterile scalpel. The subsequent purification of DNA from the agarose gel pieces as well as from PCR products were done via the Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, Wisconsin, USA) as per the manufacturer's detailed protocol.

Table 2.1 Vectors and bacterial strains used, including relevant characteristics.

Vector/Strain	Specificity
pGEM T-Easy	Cloning vector, ampicillin resistance
pBlueScript (SK +)	Cloning and sub-cloning vector, ampicillin resistance
pCR8™/GW/TOPO	Gateway® recombination Entry vector, high efficiency cloning
pRSET C	Bacterial vector for high-level expression of proteins with a cleavable 6xHis tag and ampicillin resistance
pDEST17	Gateway bacterial expression vector with a 6xHis tag, ampicillin resistance and a T7 promoter
E. coli DH5α	Bacterial strain with reduced endogenous nuclease activity, often used for plasmid propagation
E. coli BL21 AI	Tight regulation and strong expression of toxic proteins from any T7 promoter-based expression systems, Gene expression is regulated by the addition of L-arabinose to culture
E. coli TOP10®	For high-efficiency cloning and plasmid propagation
E. coli OmniMax®	For transformation of Gateway® and TOPO® reactions, ccdB sensitive
E. coli DB 3.1®	Gateway cloning strain. Contains the gyrA462 allele which renders the strain resistant to the toxic effects of the ccdB gene

2.2.6 Sequencing analysis

Plasmid constructs and PCR products were sequenced at the Central Analytical Facilities (University of Stellenbosch, Stellenbosch, South Africa) using a 3730XL DNA Analyzer (Thermo-Fisher Scientific, Massachusetts, USA) in conjunction with the

BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo-Fisher Scientific, Massachusetts, USA) which is centred on the original dideoxynucleotide chain termination method established by Sanger *et al* (1977). All sequences were aligned and edited *in silico* using BioEdit v7.2.5 (Hall, 2013) and Addgene Analyze Sequence (https://www.addgene.org/analyze-sequence/).

2.3 **Cloning**

2.3.1 Isolation and cloning of pGlcT1 cDNA

Total RNA was extracted from fresh leaves of *Arabidopsis thaliana* Col-0 plants using the QIAGEN RNeasy Mini Kit (QIAGEN, Hilden, Germany) as per the manufacturer's instructions. cDNA was generated via reverse-transcription PCR with the Promega M-MLV RT, RNase H Minus, Point Mutant kit (Promega Corporation, Wisconsin,USA) according to the manufacturer's instructions. PCR using the Roche Expand™ High Fidelity PCR system (Hoffman-La Roche, Basel, Switzerland) was performed (annealing temperature of 58^{o}C), with the previously generated cDNA as template as per PCR conditions mentioned in 2.2.2. The forward primer 5'-CAGCAATCACAATCTATCTG-3' and reverse primer 5'-TAGTGGTCATGAAAAGATTTC -3' were used. The resulting PCR product was electrophoresed in a 1.0 % agarose gel as described in 2.2.3 to visualise the amplified PCR product.

2.3.2 TOPO cloning of *pGlcT1*

The PCR product from 2.3.1 was purified with the Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, Wisconsin, USA) as per the manufacturer's detailed protocol.

The purified pGlcT1 PCR product was then TOPO cloned using the pCR™8/ GW/ TOPO® TA Cloning Kit (Invitrogen, Carlsbad, USA) to generate the pcr8:: *pGlcT1* entry clone. The correct orientation of the *pGLcT1* gene within the construct was verified for downstream cloning purposes with the gene specific forward primer and the T7 reverse primer. Additionally the pcr8:: *XhLEA* construct was obtained from Erik Denkhaus

(University of Stellenbosch, Stellenbosch, South Africa) to serve as an entry clone for the creation of an expression construct that would serve as a control for the heterologous expression of *pGLcT1*.

2.4 **SALK line genotyping**

2.4.1 Isolation of genomic DNA

Seeds from the two SALK lines previously mentioned in 2.1.1 were planted and fresh leaves were used for genomic DNA extraction. Initially genomic DNA extraction was conducted for 6 plants for both lines using a modified protocol described by Edwards *et al* (1991). Large scale genotyping of many plant leaf samples was conducted using a Harris Uni-Core 0.5mm punch (Sigma-Aldrich, Missouri, USA) with 6 leaf disc cuttings substituting buffer extracted gDNA per PCR.

2.4.2 PCR genotyping

PCR conditions were as described in 2.2.2. Six plants from each SALK line were individually genotyped via the modified Edwards *et al* (1991) method mentioned in 2.2.1. The Harris punch method was used for large scale genotyping of many SALK_051876 plants, due to the difficulty experienced in identifying homozygous plants. Primers for genotyping both SALK lines were acquired from the SALK Institute's primer database (http://signal.salk.edu/tdnaprimers.2.html).

For SALK_051876 plants, the left-border primer (LP)
5'-TCTGCACACTCTGAGCTGTTG-3' and the right-border primer (RP)
5'-ATTTCGTGATGTTGCGTCTTC-3' (annealing temperature of 58^{o}C) were used.
For SALK_0663665 plants, LP 5'-TTTTCTTTTGCGAAGACGTTG-3' and RP
5'-TTCCTTTAACCGCATACGTTG-3' (annealing temperature of 58^{o}C) were used.
For both SALK lines, a universal internal border primer (LBb1.3)
5'- ATTTTGCCGATTTCGGAAC-3' was used in combination with each line's respective RP (annealing temperature of 52^{o}C).

2.5 *Arabidopsis* cDNA library screening

An *Arabidopsis* cDNA library, where full length clones were available in the pBlueScript SK (+) vector (Stratagene, Anatech, South Africa) was obtained from Dr James Lloyd (University of Stellenbosch, Stellenbosch, South Africa). It was transformed into *E.coli* One Shot TOP10® chemically competent cells (Invitrogen, Johannesburg, South Africa) and serial dilutions (between 10^{-2} and 10^{-10}) were plated onto M9 minimal media plates containing D-Glucose (Sigma-Aldrich, Missouri, USA) or Raffinose-pentahydrate (Sigma-Aldrich, Missouri, USA) supplemented with ampicillin (100 µg/ml). All plates were incubated at 37ºC with agitation.

Colonies which had grown on raffinose supplemented M9 plates were then grown in 5 ml starter cultures (37ºC, 14 h, with agitation) and plasmid DNA isolated using the Wizard® Plus SV Miniprep DNA purification system (Promega Corporation, Anatech, South Africa) following the manufacturer's instructions. Plasmid isolates were digested with the *Bam HI* and *XhoI* restriction endonucleases. Additionally, plasmid isolates were also re-transformed into TOP10® competent cells and plated onto raffinose supplemented M9 plates. Plasmids inserts were fully sequenced using a rolling-sequencing approach (Macrogen Inc., Amsterdam, Netherlands).

2.6 Preliminary growth testing of E.coli strains on M9 minimal media

The common laboratory strains of *E. coli*, namely DH5α, OneShot® OmniMax 2-T1R (Invitrogen, Carlsbad, USA), BL21 AI (Invitrogen, Carlsbad, USA) and OneShot® TOP10 (Invitrogen, Carlsbad, USA) were tested for growth on M9 minimal media. M9 minimal media plates (Refer to Appendix A1 for M9 minimal media preparation protocol) were prepared separately with 100 mM glucose, 100 mM raffinose, 100 mM sucrose and a combination of 100 mM glucose and 100 mM raffinose, plain M9 minimal media lacking a carbon source and finally Luria Agar (LA) plates. No antibiotics were added to these plates. The carbon source stock solutions in combination with the M9 minimal media were tested for reducing hexose sugar contamination via a Benedict's reagent [1.14 M sodium carbonate, 0.7 M sodium citrate dehydrate, 0.7 M copper(II) sulphate pentahydrate] test as described by Simoni *et al* (2002). The aforementioned

E. coli strains were streaked separately onto the various M9 plates and incubated at 37ºC for approximately 10 d before recording the results.

2.7 **Heterologous expression in *E. coli***

2.7.1 Sub-cloning *pGlcT1* and *XhLEA*

Plasmid DNA mini-preparations were set up from *E.coli* (One Shot® OmniMAX™ 2-T1R) 5 ml cultures transformed with the pcr8:: *pGlcT1* and pcr8:: *XhLEA* construct, which had been grown overnight. Mini-preparations were conducted as described in 2.2.5. Two Gateway® LR Clonase II reactions (Invitrogen, Carlsbad, USA) were performed according to the manufacturer's specifications in order to the generate pDEST17:: *pGlcT1* and pDEST17:: *XhLEA* constructs using the pcr8:: *pGlcT1* and pcr8:: *XhLEA* entry clones respectively along with the pDEST17 (Invitrogen, Carlsbad, USA) protein expression vector. The pDEST17:: *XhLEA* construct was used a control as the pDEST17 empty vector could not be used. The empty pDEST17 vector contains the lethal *ccdB* gene which encodes the ccdB toxin that targets the gyrase enzyme preventing the growth of *E. coli* (Afif *et al*, 2001). Hence, the pDEST17 as an empty control was not a viable option. The *XhLEA* gene is a known gene from our previous studies and when cloned into pDEST17 acts as a sufficient control.

The pDEST17:: *pGlcT1* and pDEST17:: *XhLEA* constructs were subsequently transformed into *E.coli* (One Shot® OmniMAX™ 2-T1R) competent cells via a standard heat shock transformation protocol. Transformed colonies were selected for on M9 minimal media comprising different carbon sources (100 mM glucose, sucrose and raffinose).

2.7.2 M9 minimal media growth testing with pDEST17:: pGlcT1 construct

The pDEST17:: *pGlcT1* and control pDEST17:: *XhLEA* constructs were tested for growth on M9 minimal media plates supplemented separately with 100 mM glucose, 100 mM sucrose, 100 mM raffinose, plain M9 with no carbon source and plain LA. All plates were supplemented with 1 µg/ml ampicillin. Both constructs were transformed

into BL 21 Arabinose Inducible (AI) (Invitrogen, Carlsbad, USA) *E. coli* competent cells via a standard heat shock transformation protocol.

Transformed cells were then spread plated onto the M9 plates containing the different carbon sources. Plates were incubated at 37^{o}C for 10 d to allow sufficient growth. Colonies were sub-cultured onto fresh M9 minimal media supplemented with the appropriate carbon sources to the confirm growth phenotypes.

2.7.3 Recombinant pGlcT1 expression

A sterile 50 ml Erlenmeyer flask containing 5 ml of Luria Broth (LB) with 1 µg/ml ampicillin was inoculated with a single colony of *E. coli* BL 21 AI containing pDEST17:: *pGlcT1* overnight at 37^{o}C with agitation. Additional sterile Erlenmeyer flasks under the same conditions were inoculated with the controls namely, *E. coli* BL 21 AI containing an empty pRSET C vector and pDEST17:: XhLEA. The cultures were then inoculated into separate 1 L Erlenmeyer flasks containing 200 ml of pre-warmed LB (37^{o}C with agitation) with 1 µg/ml ampicillin.

The cultures were grown until an OD_{600} of 0.40 was obtained, then 13.32 mM L-Arabinose (Sigma-Aldrich, Missouri, USA) was added. After 4 h of growth, 200 ml of each culture was transferred to a 250 ml polycarbonate copolymer (PPCO) bottle (Nalge Nunc International, New York, USA). *E.coli* cell pellets were collected by centrifugation (Sorvall RC6 Plus Centrifuge, 5500xg, 10 min at 4^{o}C) and stored at -20^{o}C for protein purification.

Prior to centrifugation, 5 ml of each induced culture was used for growth testing on M9 minimal media with different carbon sources. The cultures were centrifuged (Heraeus Biofuge Pico, 5000xg) for 1 min at room temperature. The supernatant was aspirated and the pellet was re-suspended in distilled water. These steps were repeated two more times so as to remove any residual L-Arabinose present in the cultures. The re-suspended cultures were then plated onto new corresponding M9 minimal media plates containing the appropriate carbon sources as described earlier.

2.7.4 Protein purification

All proteins to be purified were expressed by genes cloned into vectors containing 6xHis tags. Therefore, immobilized metal ion affinity chromatography (IMAC) purification was performed using Protino® Ni-TED 1000 gravity flow Packed Columns (Macherey-Nagel, Düren, Germany). The *E. coli* cell pellets stored at -20°C as mentioned in 2.6.3 were thawed on ice and thoroughly re-suspended in 2 ml of the Protino® Ni-TED 1000 kit's supplied LEW (Lysis, Equilibration, Wash) buffer.

1 mg/ml lysozyme (Sigma-Aldrich, Missouri, USA) was added to cell suspension samples and incubated on ice with agitation for an hour. The cell suspension samples were each sonicated on ice (10 x 15 s bursts) with a Virtis Virsonic 100 Ultrasonic Cell Disruptor (Virtis, Massachusetts, USA). The sonicated samples were centrifuged (Sorvall RC6 Plus Centrifuge, 10 000xg, 30 min at 4°C) to remove cellular debris and the supernatants were transferred to sterile 10 ml tubes on ice. Subsequently, the detailed manufacturer's instructions were followed for protein purification under native conditions.

2.7.5 SDS-PAGE

The pGlcT1, empty pRSET C and XhLEA purified proteins obtained in 2.6.4 were separately re-suspended in 10 µl of 5x SDS loading sample buffer [250 mM Tris-HCl (pH6.8), 10% SDS (w/v), 30% Glycerol (v/v), 10 mM 2-mercaptoethanol, 0.05% bromophenol blue (w/v)] and boiled at 100°C for 5 min. Samples were then electrophoresed in a SDS-PAGE gel consisting of a 5% stacking gel [30% Acrylamide mix (v/v), 1.0 M Tris (pH 6.8), 10% SDS (w/v)] and a 12% resolving gel [30% Acrylamide mix (v/v), 1.5 M Tris (pH 8.8), 10% SDS (w/v)]. SDS-PAGE gels were electrophoresed at 130 V. Gels were stained overnight in Coomassie brilliant blue R-250 staining solution [0.05% Coomassie brilliant blue R-250 (w/v), 50% methanol (v/v), 10% glacial acetic acid (v/v), 40% H_2O (v/v)]. De-staining was performed with Coomassie de-staining solution [50% methanol (v/v), 40% glacial acetic acid (v/v), 10% H_2O (v/v)].

2.7.6 Bacterial total RNA extraction and transcript detection

Starter cultures (5 ml) containing single colonies of *E. coli* BL21 AI transformed with pDEST17:: *pGlcT1*, the empty pRSET C vector and pDEST17:: *XhLEA* were inoculated (37^{o}C with agitation). The starter cultures were added to 50 ml pre-warmed LB in sterile 250 ml Erlenmeyer flasks. The cultures were then induced as described in 2.6.3. The induced cultures were monitored until the OD_{600} values reached 1.0 / ml of culture. This value was the approximate equivalent to 1 x 10^9 *E. coli* cells/ ml of culture. For each sample, 1 x 10^9 bacterial cells were processed for total RNA extraction with the QIAGEN RNeasy Mini Kit (QIAGEN, Hilden, Germany) as per the manufacturer's detailed instructions.

Once total RNA was purified, cDNA was generated for each of the samples with the Promega M-MLV RT, RNase H Minus, Point Mutant kit (Promega Corporation, Wisconsin, USA). The random hexa-deoxynucleotide primers supplied in the kit were used instead of the oligo $(dT)_{15}$ primers. PCR reactions were performed using the previously generated cDNA for each sample under the PCR conditions described in 2.2.2 to determine transcript levels.

2.8 **Chlorophyll fluorescence analysis**

Six confirmed homozygous SALK_066365 plants were planted out as described in 2.1.2. Six wild type Col-0 and six RSS5 (RS14 line from Zuther *et al*, 2004) mutant plants were also planted to serve as controls for the experiment. The six plants from each line were planted out and stratified over the same period of time in order to make sure all seeds germinated at the same time. This step was taken to ensure that all plants were growing under standardized conditions, so as to avoid any discrepancies with regards to downstream chlorophyll fluorescence experiments. After 6 weeks, when the plant leaves were strong enough to withstand repetitive physical stress, chlorophyll fluorescence parameters were measured with a Hansatech FMS-2 field portable chlorophyll fluorometer (Hansatech, Norfolk, UK).

Prior to the chlorophyll fluorescence measurement experiment, sqPCR was performed on cDNA generated from total RNA isolated from the leaves of 6 SALK_066365 plants

and one Col-0 wild-type plant that were planted out, to confirm that the *pGlcT1* gene was knocked out. sqPCR gene specific primers amplifying a 550bp fragment were used to detect *pGlcT1* expression while Actin2 (*ACT2*) sqPCR forward and reverse primers were used as a control.

F_v/F_m was determined consistently after 8 h dark-adaptation every day over the course of one week with cold-acclimation (4^{o}C) in a Micro Clima-Series Economic Lux micro-climate chamber (Snijders Labs, Tilburg, Netherlands). The plants were first allowed to acclimate to the new light and temperature conditions (8 h light and 16 h dark at 22^{o}C) in the growth chamber for one week before cold-acclimation was induced. For each line, three leaves from each of the 6 plants were continuously measured. The Modfluor32 and Parview32 software (Hansatech, Norfolk, UK) was used to consistently record the chlorophyll fluorescence parameters and also to plot comparative analyses curves.

Statistical analyses were performed on the observed F_v/F_m values for all six SALK_066365 and RSS5 plants over the 7 d period of cold acclimation (4^{o}C) included a repeated measures one-way ANOVA coupled with a post test for linear trend between mean and F_v/F_m values for all 6 plants. The linear trend post-test was most appropriate for repeated measures data sets.

Chapter 3: Results

3.1 *In silico* analysis of pGlcT1

```
   1 ATGCAGTCGT CAACGTATGC GGTTAAAGGA AACGCTGCGT TTGCGTTTCA
  51 GAGACGGACC TTCTCTTCTG ACAGATCGAC GACTTCTACC GGAATTCGCT
 101 TCGCTGGTTA TAAGAGCTTA GCCACCACCG GGCCACTCTA CTGTTCTGGT
 151 TCTGAAGCCA TGGGAGCGAC GCTTGCTCGT GCTGATAACG GGATCCAGAG
 201 CGTTATGAGT TTCTCTTCTG TCAAAGCTCG ATCGGTCAGA GCTCAAGCCT
 251 CATCTGATGG AGATGAAGAA GAAGCTATAC CTCTGAGATC TGAAGGGAAA
 301 AGCTCTGGAA CAGTTTTGCC TTTTGTTGGT GTTGCTTGTC TTGGTGCTAT
 351 ACTCTTTGGT TATCATCTCG GGGTGGTTAA TGGTGCTCTT GAATATCTTG
 401 CTAAGGATCT TGGGATCGCC GAAAATACTG TTTTGCAAGG ATGGATTGTT
 451 AGTTCTCTGC TTGCTGGTGC TACGGTAGGT TCATTCACTG GAGGTGCATT
 501 AGCTGACAAA TTTGGACGAA CAAGAACTTT TCAATTGGAT GCTATCCCGC
 551 TTGCCATTGG AGCTTTCTTA TGTGCAACAG CTCAGAGTGT GCAGACTATG
 601 ATTGTGGGAC GTCTGCTCGC TGGAATTGGA ATTGGAATCT CATCAGCGAT
 651 TGTACCACTT TACATATCTG AGATATCACC AACTGAAATC CGTGGAGCAC
 701 TCGGATCTGT GAACCAGTTG TTCATCTGTA TAGGAATACT TGCAGCCTTG
 751 ATAGCTGGAT TACCCCTTGC AGCAAACCCT CTATGGTGGA GGACGATGTT
 801 TGGTGTTGCA GTTATCCCTT CCGTTCTATT GGCCATAGGA ATGGCTTTTT
 851 CTCCAGAAAG CCCAAGGTGG CTCGTTCAGC AAGGAAAAGT CTCTGAAGCT
 901 GAAAAGGCGA TCAAAACTTT GTATGGTAAA GAAAGAGTGG TTGAACTAGT
 951 TCGCGACTTA TCAGCCTCTG GCCAAGGTTC TTCTGAGCCG GAGGCAGGAT
1001 GGTTTGATCT ATTCAGCAGC CGCTACTGGA AAGTTGTAAG CGTAGGTGCG
1051 GCTCTCTTCT TGTTTCAACA GTTAGCCGGG ATAAACGCAG TTGTGTATTA
1101 CTCCACATCG GTATTCCGTA GTGCGGGAAT CCAATCAGAT GTTGCAGCCA
1151 GTGCTCTCGT TGGAGCATCA AATGTCTTTG GCACTGCTGT TGCTTCATCG
1201 TTGATGGATA AAATGGGAAG GAAAAGTCTT TTACTGACAA GCTTTGGTGG
1251 AATGGCTTTG TCAATGCTGT TACTCTCCTT GTCCTTCACA TGGAAGGCTC
1301 TTGCTGCCTA TTCTGGAACC CTTGCCGTTG TTGGAACTGT TCTATATGTC
1351 CTGTCATTCT CACTTGGTGC TGGCCCGGTA CCGGCTCTTC TTCTTCCAGA
1401 GATATTTGCA TCCCGAATCA GAGCAAAAGC CGTCGCTCTT TCTCTCGGCA
1451 TGCACTGGAT ATCAAACTTT GTGATCGGAC TATACTTCTT AAGCGTTGTG
1501 ACTAAATTCG GAATCAGCAG TGTCTACTTG GGTTTTGCTG GAGTCTGCGT
1551 CCTTGCGGTC CTCTACATTG CAGGAAACGT CGTCGAGACT AAAGGTCGAT
1601 CACTGGAGGA AATAGAGCTT GCTCTTACAT CTGGAGCTTG A
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The nucleotide sequence of *pGlcT1* from generated cDNA was discovered to have an open reading frame (ORF) of 1641bp. It encodes a polypeptide that is predicted to have an average mass of 56.97 kDa (UniProtKB accession code: Q56ZZ7) and a total of 546 amino acid residues. Upon BLASTp analysis, the pGlcT1 transporter was found to have a 25% amino acid identity similarity with the raffinose transporter Mrt found in *Metarhizium robertsii*.

3.2 pGlcT1 SALK lines genotyping

Initially 5 plants from both the SALK_051876 and SALK_0666365 lines were genotyped. All 5 plants genotyped for the SALK_066365 line were confirmed as homozygous mutants for the *pGlcT1* gene (Figure 3.1). The initial 5 plants for the SALK_051876 line were genotyped as wild type (Figure 3.2). More plants for the SALK_051876 line were genotyped and observed as wild type plants. An additional pair of primers suggested by the Salk Institute Genomic Analysis Laboratory (SIGnAL) were used to genotype the SALK_051876 plants. The new results (Figure 3.3) confirmed that the plants were wild type.

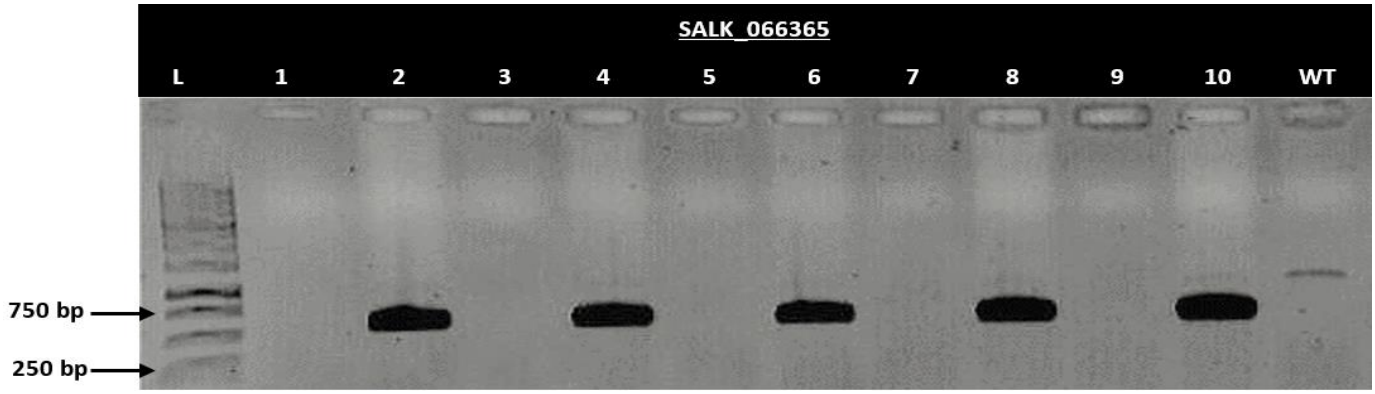

Figure 3.1 PCR genotyping results for the SALK_066365 plants. Lanes 1, 3, 5, 7, 9 and WT represent the LP & RP primer combination (wild-type allele) whereas lanes 2,4,6,8 and 10 represent the LBb1.3 & RP primer combination (mutant allele) for genotyping analyses performed on 5 individual plants**.**

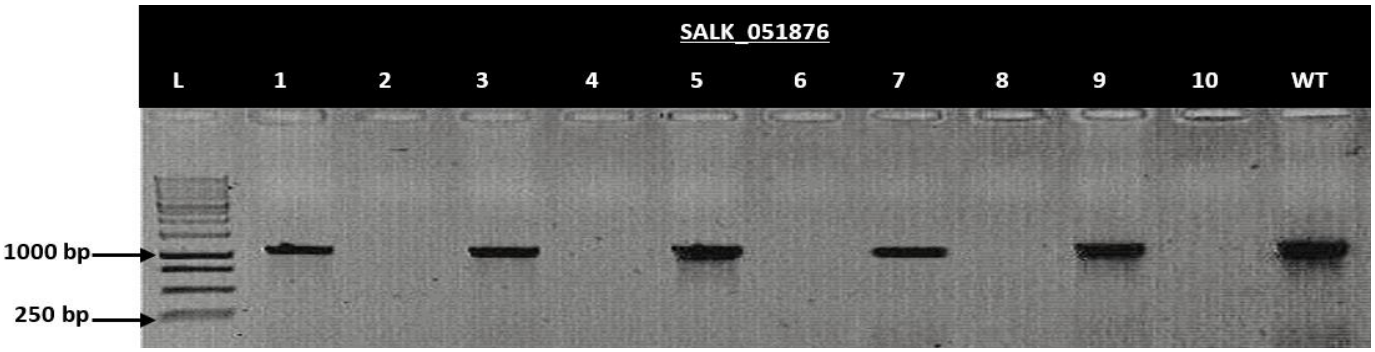

Figure 3.2 PCR genotyping results for the SALK_051876 plants. Lanes 1, 3, 5, 7, 9 and WT represent the LP & RP primer combination whereas lanes 2,4,6,8 and 10 represent the LBb1.3 & RP primer combination for genotyping analyses performed on 5 individual plants**.**

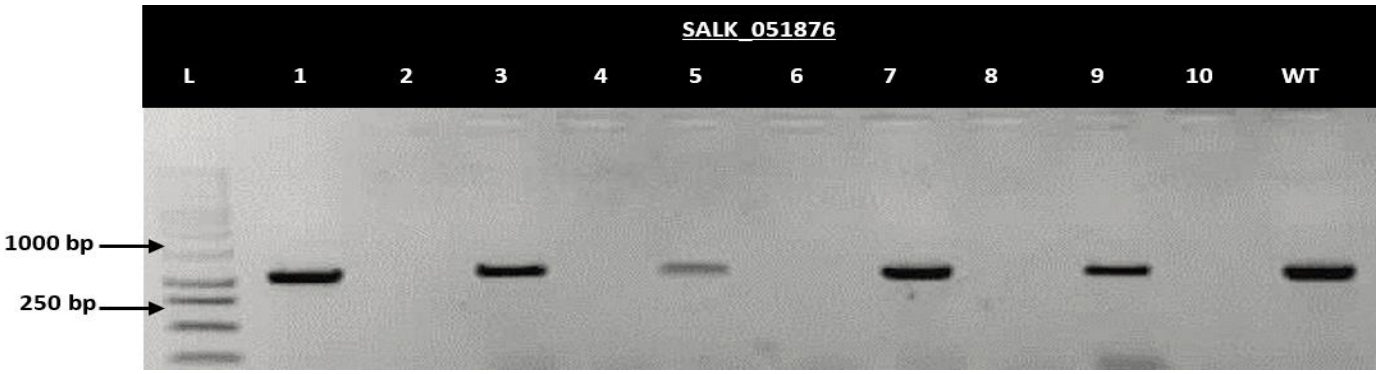

Figure 3.3 PCR genotyping results for additional SALK_051876 plants using a different primer set for the wild-type and mutant alleles. Lanes 1, 3, 5, 7, 9 and WT represent the LP & RP primer combination whereas lanes 2, 4, 6, 8 and 10 represent the LBb1.3 & RP primer combination for genotyping analyses performed on 5 individual plants**.**

3.3 sqPCR analyses of SALK_066365 plants

The sqPCR performed on cDNA generated from total RNA of the SALK_066365 plants grown for measuring chlorophyll fluorescence were confirmed to be knockout mutants as shown in Figure 3.4. cDNA integrity was confirmed by performing PCR with on all 6 cDNA samples using *Actin2* gene specific primers. The *Actin2* sqPCR control was performed on plant 1 cDNA (Figure 3.4, lane 2). When compared to the *Actin2* (~500bp) and WT (~550bp), no amplification occurs with the *pGlcT1* gene specific primers, confirming that the plants are indeed homozygous knockout mutants.

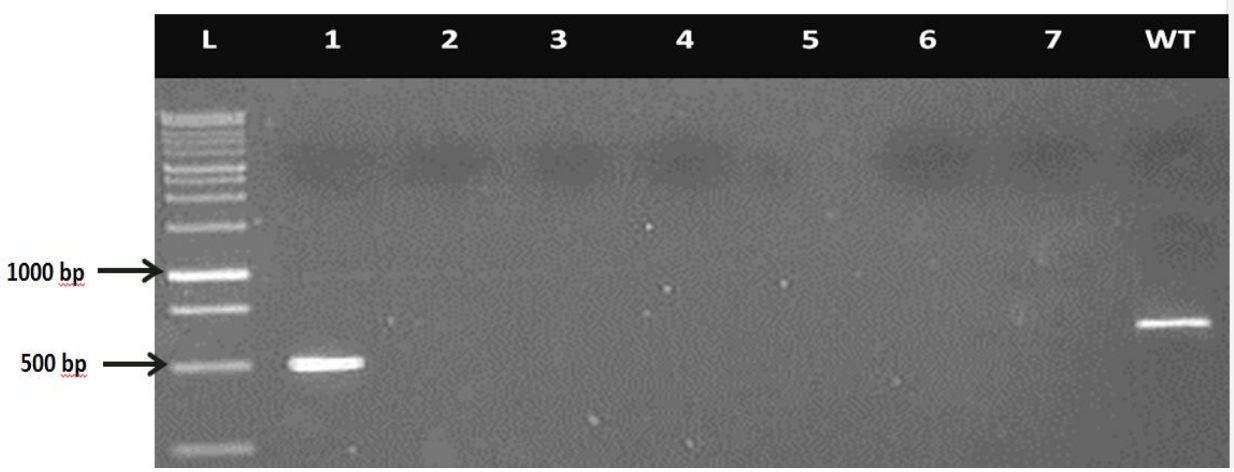

Figure 3.4 sqPCR performed on cDNA generated from SALK_066365 plants. Lane 1 represents the *ACT2* housekeeping gene transcript, lanes 2-7 represent the SALK_066365 plants and the last lane shows *pGlcT1* amplification in the wild type positive control

3.4 Preliminary *E.coli* growth tests on M9 minimal media

To ensure that our carbon supplements (raffinose and sucrose) were not contaminated by hexose sugars such as glucose, fructose and galactose, they were subjected to a classical Benedict's test for reducing sugars (hexose). The sucrose and raffinose solutions in combination with the other M9 minimal media reagents did not change colour in the presence of Benedict's reagent, however the glucose stock solution did change colour as expected (Figure 3.5).

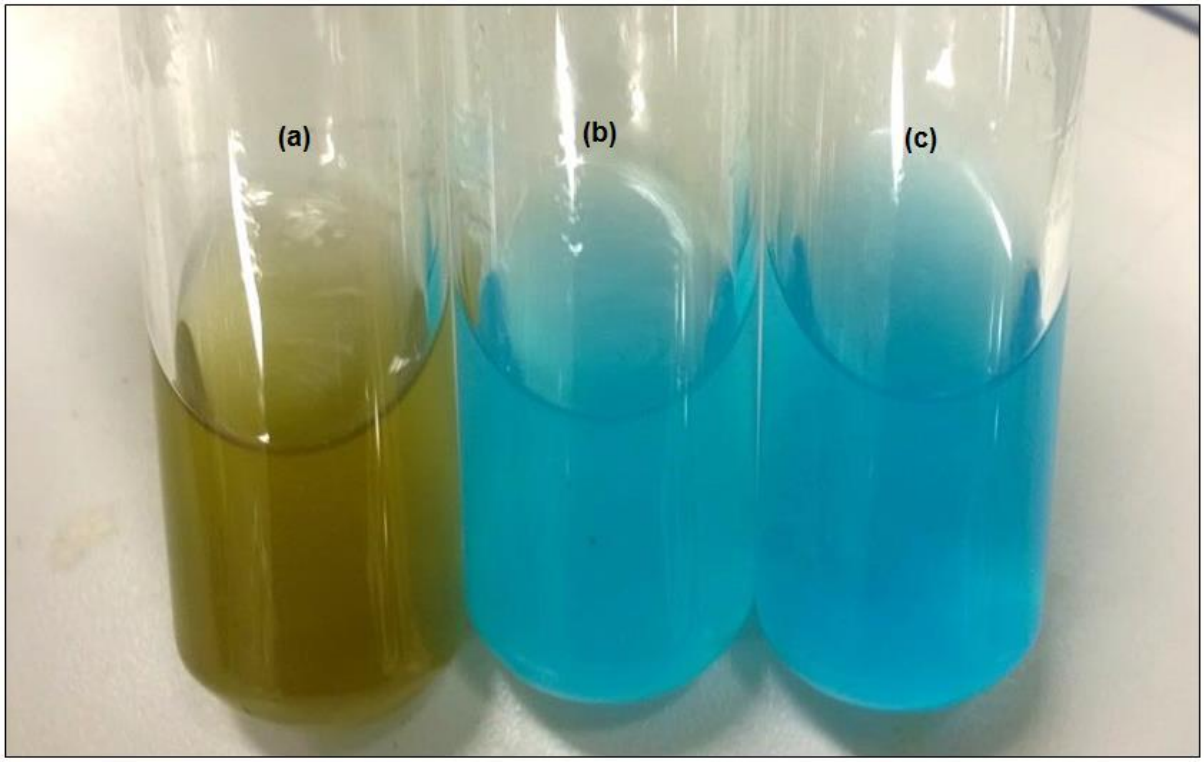

Figure 3.5 Benedict's reagent test for reducing sugar contamination within M9 minimal media components with the carbon sources (a) 100 mM glucose, (b) 100 mM sucrose and (c) 100 mM raffinose.

Colony growth for the *E. coli* lab strains DH5α, TOP10®, OmniMax® and BL21 AI was observed on Luria Agar (LA) and M9 minimal media plates supplemented with 100 mM glucose as well as on a combination of 100 mM glucose and 100 mM raffinose after 10 d (Figure 3.6). No growth was observed on M9 plates supplemented with 100 mM sucrose, 100 mM raffinose and a plain M9 plate lacking a carbon source.

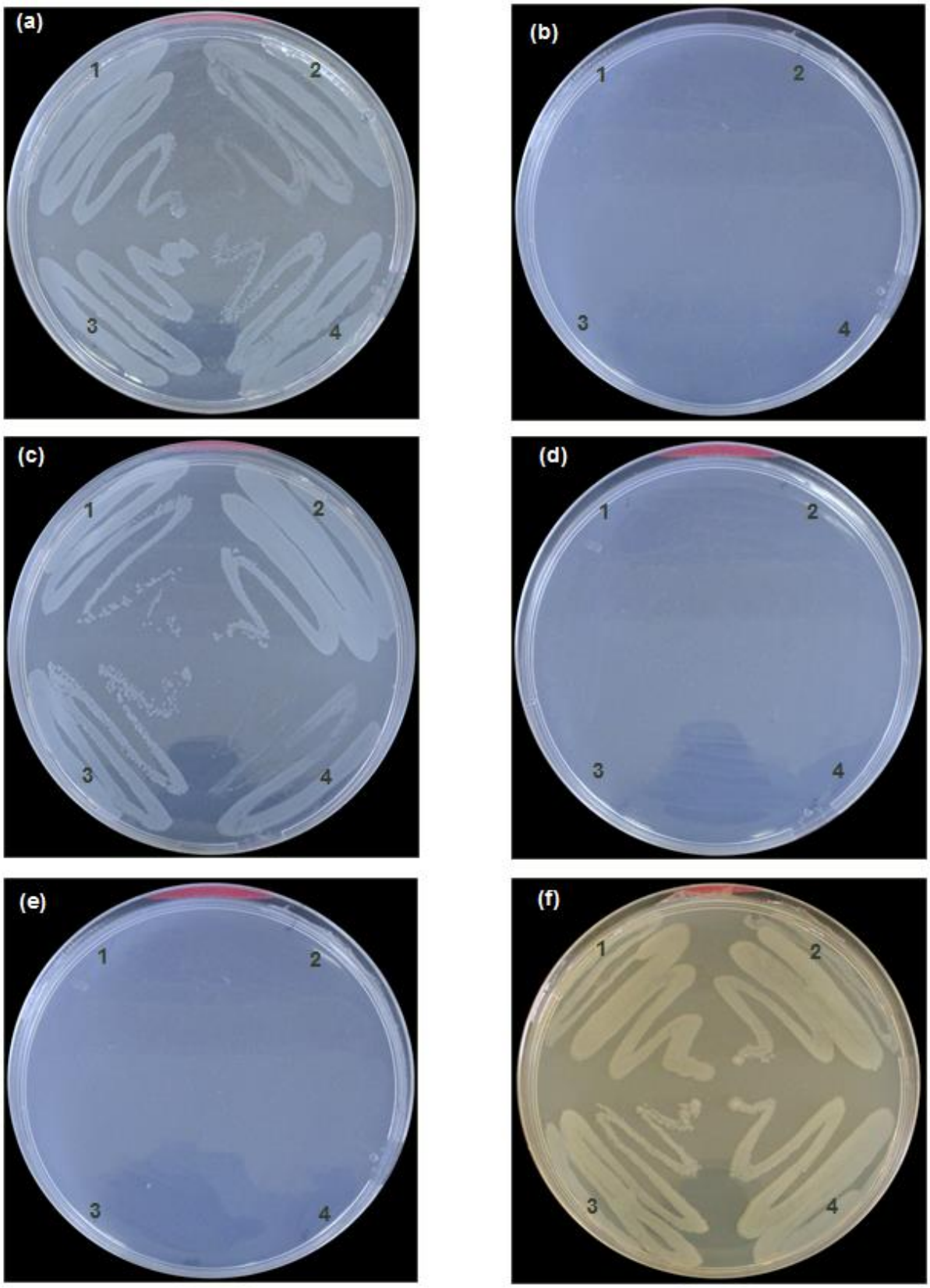

Figure 3.6 Laboratory strains of *E. coli* (1) DH5α, (2) BL21 AI, (3) OmniMax® and (4) TOP10® streaked onto M9 minimal media plates supplemented with (a) 100 mM glucose, (b) 100 mM sucrose, (c) 100 mM glucose and 100 Mm raffinose in combination, (d) 100 mM raffinose, (e) M9 with no carbon source and (f) on LB. Plates were incubated at 37°C for 10 d.

3.5 *Arabidopsis thaliana* cDNA library screening

An *Arabidopsis thaliana* cDNA library (commercially purchased) in pBlueScript II SK (+) was used to screen for *E.coli* (TOP10®) growth on M9 minimal media plates supplemented with either 100 mM glucose or 100 mM raffinose after 6 or 10 d, respectively (Figure 3.7a & Figure 3.7b). Fourteen colonies were recovered from this approach and sub-cultured onto LA and M9 minimal media plates (100 mM raffinose supplemented).

Following the sub-culturing, twelve colonies showed positive growth after 1 d of incubation (37^{o}C) on the LA plates while all 14 colonies showed positive growth after incubation for 3 d. These colonies were marked and sub-cultured onto a fresh LA (Figure 3.8a) and M9 minimal media plates with raffinose (Figure 3.**Figure 3.8**8b).The growth period was faster with visible colonies forming within 3-4 d.

After confirming that growth consistently occurred on M9 minimal media with raffinose as the sole carbon source, plasmid DNA was isolated from these positive colonies. Only 8 of the 14 colonies yielded viable plasmid DNA (concentration ≥ 100 ng/µl). Subsequent restriction enzyme digestions of these plasmids using *Bam HI* and *XhoI*, exhibited similar banding patterns (Figure 3.9) as well as different fragment sizes when run on a 1% agarose gel. A band in each sample lane on the gel seemed to correlate with the pBlueScript II SK (+) backbone size of 3000 bp.

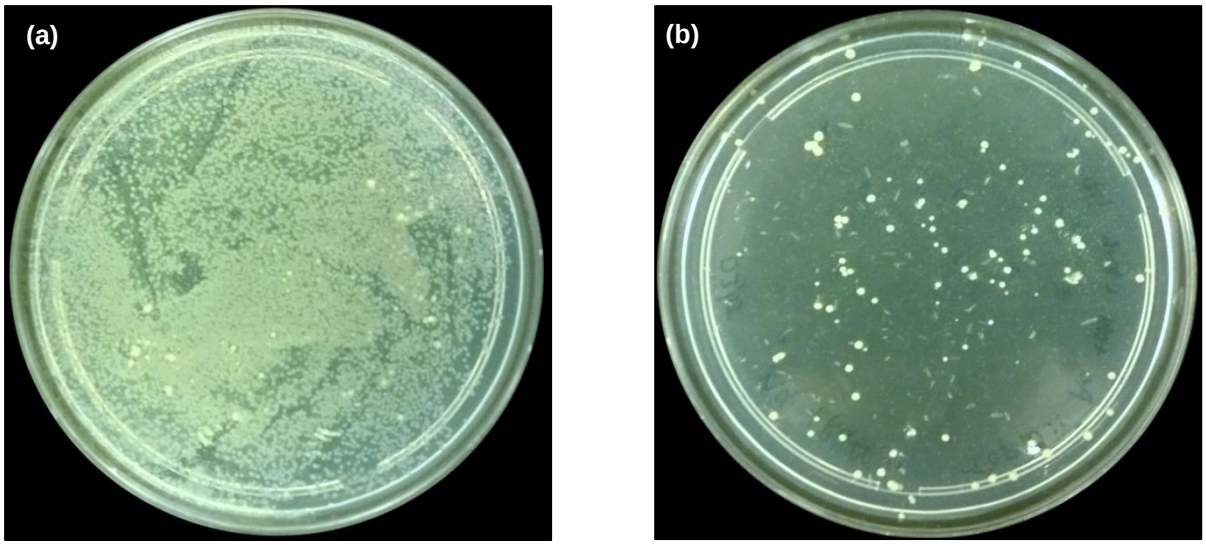

Figure 3.7 *Arabidopsis thaliana* cDNA library in pBlueScript II SK (+) transformed into *E. coli* (TOP10®) and plated onto M9 minimal media plates supplemented with (a) 100 mM glucose and (b) 100 mM raffinose**.** Plates were supplemented with 100 µg/ ml Ampicillin and incubated at 37°C for (a) 10 d or (b) 6 d

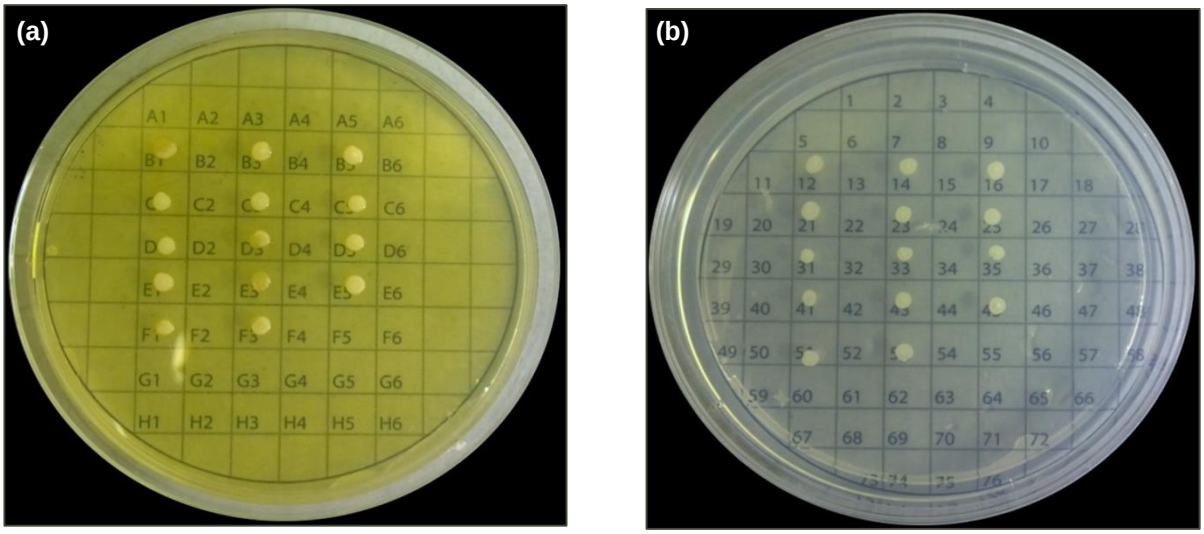

Figure 3.8 *Arabidopsis thaliana* cDNA library in pBlueScript II SK(+) transformed into *E. coli* (TOP10®) and sub-cultured from initial screening plates onto (a) M9 minimal media and (b) LB plates supplemented with 100 mM raffinose and 100 µg/ ml Ampicillin. Plates were incubated at 37°C for 2 d.

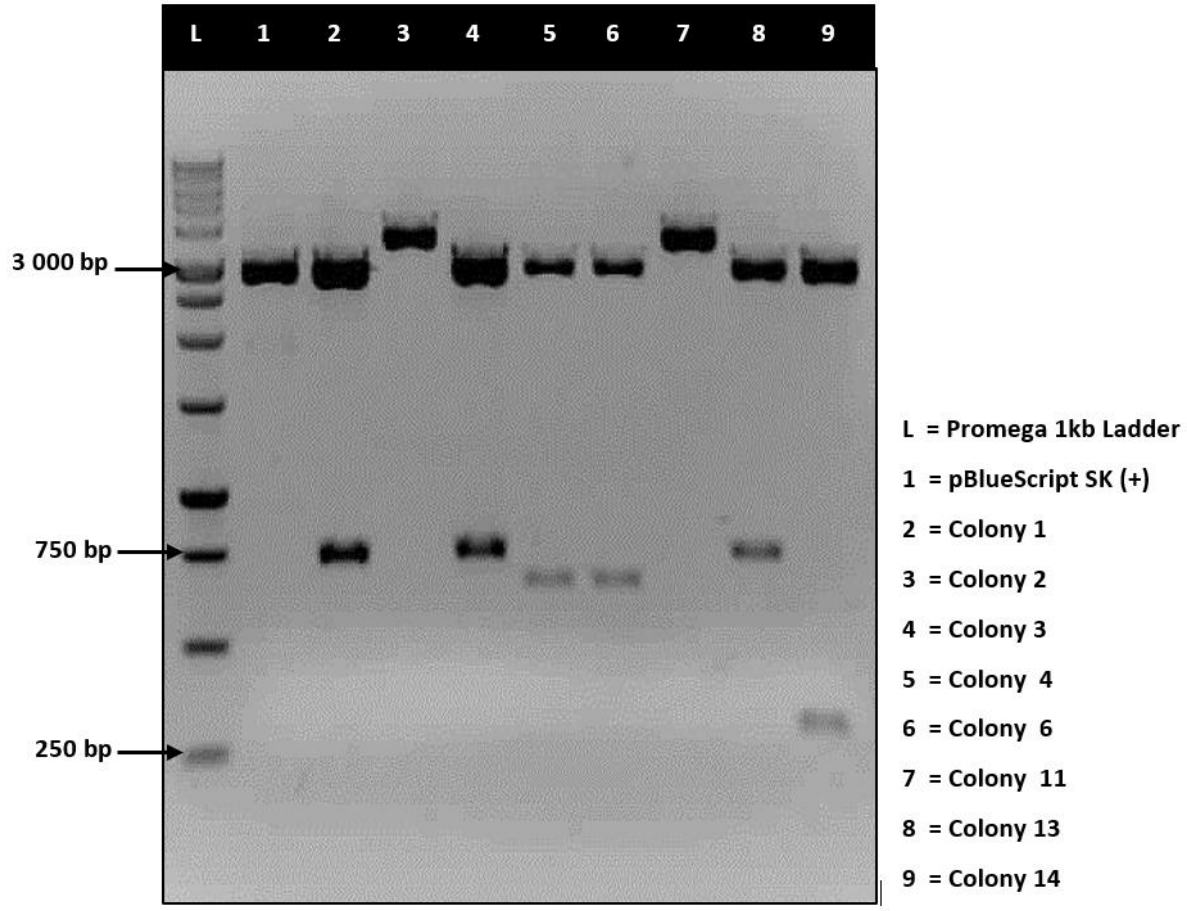

Figure 3.9 An agarose gel representing plasmid DNA isolated from *E. coli* colonies growing on M9 minimal media supplemented with 100 mM raffinose following transformation with an *Arabidopsis* cDNA library in pBlueScript II SK (+). Plasmid samples were digested with *BamHI* and *XhoI*.

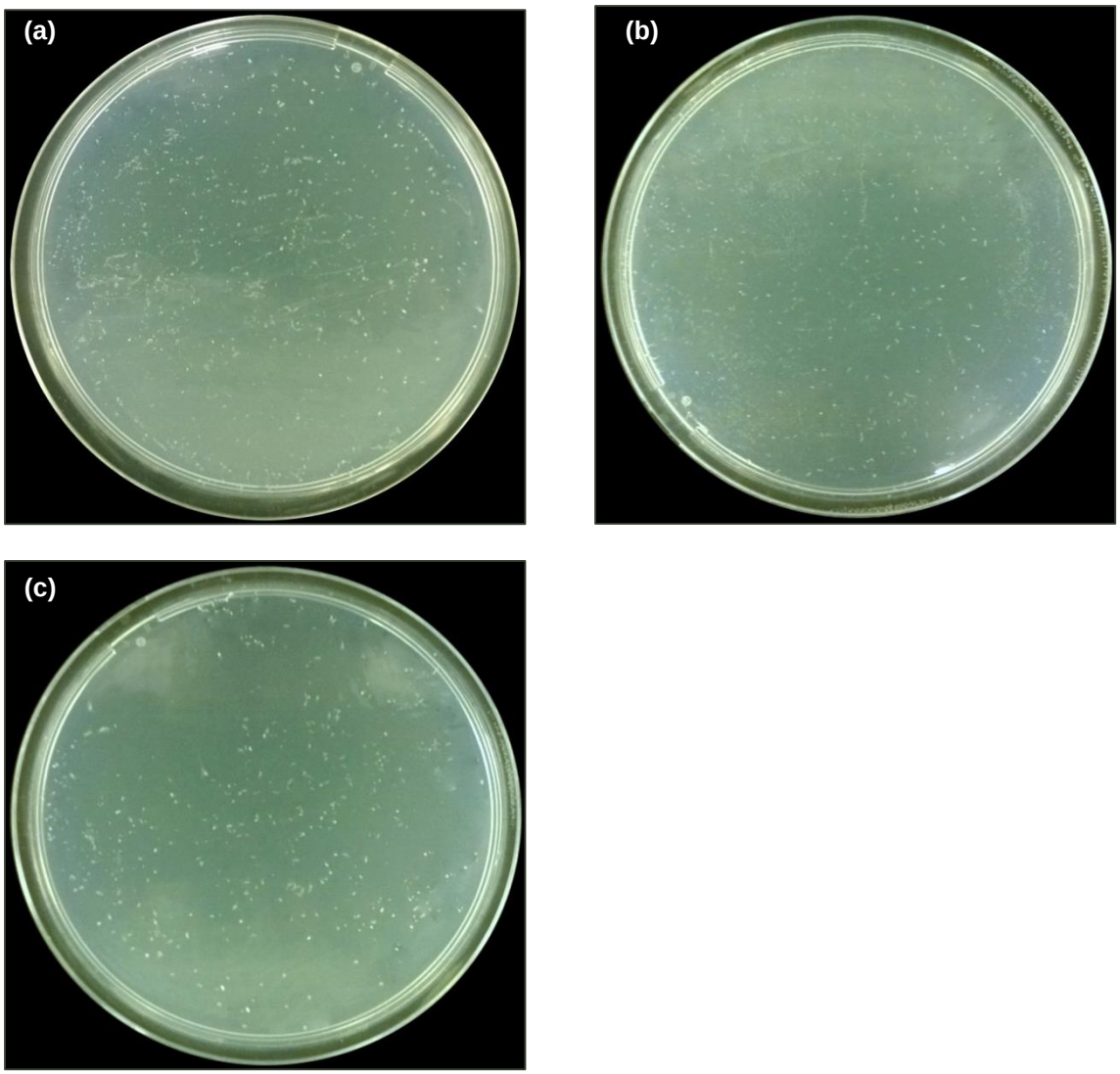

Figure 3.10 Three plasmid isolates from *E. coli* colonies transformed with the *Arabidopsis thaliana* cDNA library in pBlueScript SK (+) that demonstrated positive growth on M9 minimal media supplemented with 100 mM raffinose were then re-transformed into *E. coli* (TOP10®) for (a) colony 3, (b) colony 11 and (c) colony 14 and plated onto M9 minimal media plates supplemented with 100 mM raffinose. All plates were supplemented with 100 µg/ ml Ampicillin and incubated at 37°C for 3 d.

The plasmid isolates were sent for sequencing (Macrogen, Amsterdam, Netherlands) returned sequences identical to 5 different genes present in the annotated *Arabidopsis thaliana genome* (Table 3.1).

Table 3.1 Genes derived from sequencing of plasmid isolates of the *Arabidopsis thaliana* cDNA library in pBlueScript that allowed for growth of *E. coli* (TOP10®) on M9 minimal media supplemented with 100 mM raffinose.

Gene	TAIR Accession no.	Function
adenosine kinase 1 (ADK1)	AT3G09820	Involved in the salvage synthesis of adenylates and methyl recycling
Ribulose bisphosphate carboxylase (small chain) family protein	AT5G38410	Encodes a member of the Rubisco small subunit (RBCS) multigene family. Functions to yield sufficient Rubisco content for leaf photosynthetic capacity
UDP-D-gluconurate 4-epimerase 6 (GAE 6)	AT3G23820	Catalyses the conversion of UDP-gluconurate to UDP-D-galactouronate which is a major sugar in plant cell walls
Ascorbate peroxidase 1 (APX1)	AT1G07890	Scavenges hydrogen peroxide in plant cells. Two chloroplastic types have been described for *Arabidopsis*. Part of the induction of heat shock proteins during light stress in *Arabidopsis* is mediated by H_2O_2 that is scavenged by APX1
Photosystem II reaction centre protein M (PBSM)	ATCG00220	Anchored in the thylakoid membrane of plants and are main protein components of plant photosynthetic reaction centres

3.6 **Heterologous expression of pGlcT1 in *E. coli***

3.6.1 Sub-cloning of *pGlcT1* into pDEST17

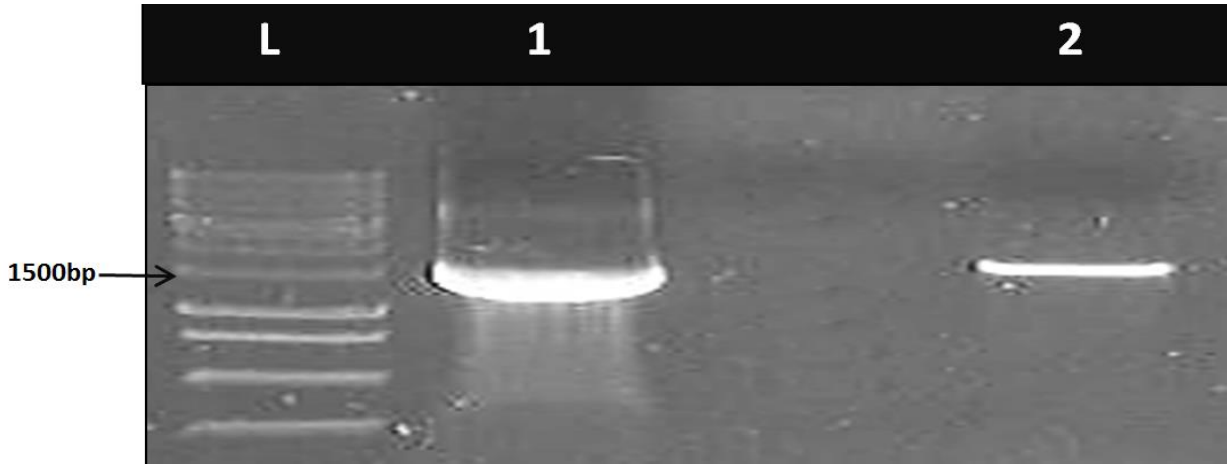

Figure 3.11 Colony PCR confirmation of pDEST17:: *pGlcT1* construct with lane 1 representing pGlcT1 fwd and rev primers and lane 2 indicating T7 fwd an pGlcT1 rev primers

3.6.2 Recombinant pGlcT1 expression (SDS-PAGE Gel)

Colony PCR performed on 6 BL21 AI colonies were tested for the presence of the *pGlcT1* gene within the bacteria. Figure 3.12, shows that the gene was present in all 6 colonies. Results for PCR performed on cDNA generated from L-arabinose induced BL21 AI cultures containing the pDEST17:: *pGlcT1* and the pDEST17:: *XhLEA* constructs with gene-specific primers is shown in Figure 3.13. The presence of mRNA transcripts is verified.

The SDS-PAGE gel conducted with proteins purified from L-arabinose post-induction cultures yielded the ~ 57 kDa pGlcT1 protein (Figure 3.14, lane 1). The XhLEA positive control also yielded the correct ~25 kDa protein (Figure 3.14, lane 2).The negative control pRSET C did not show any significant band in the gel, as expected (Figure 3.14, lane 3).

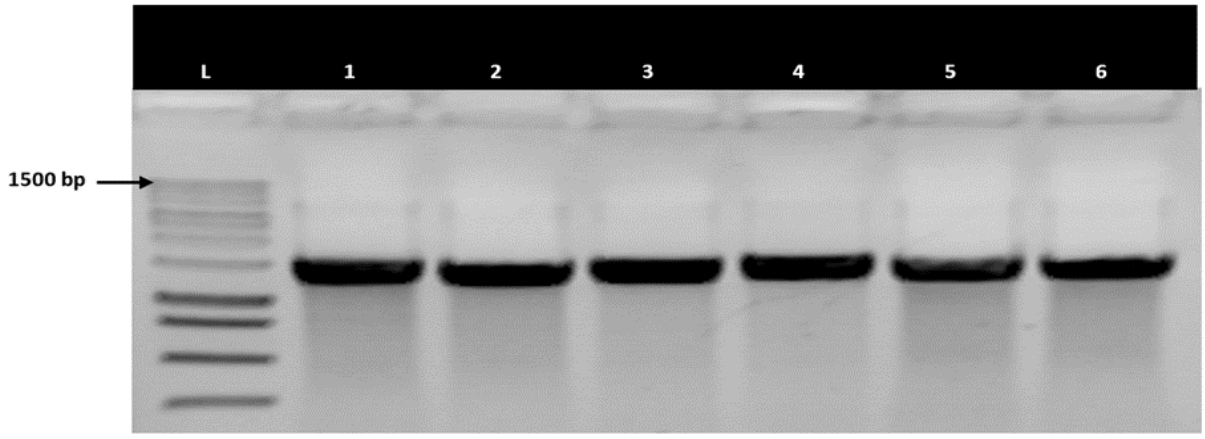

Figure 3.12 Results for colony PCR performed on 6 Bl21 AI colonies containing the pDEST17:: *pGlcT1* construct with *pGlcT1* gene-specific primers positively confirming the presence of the *pGlcT1* gene.

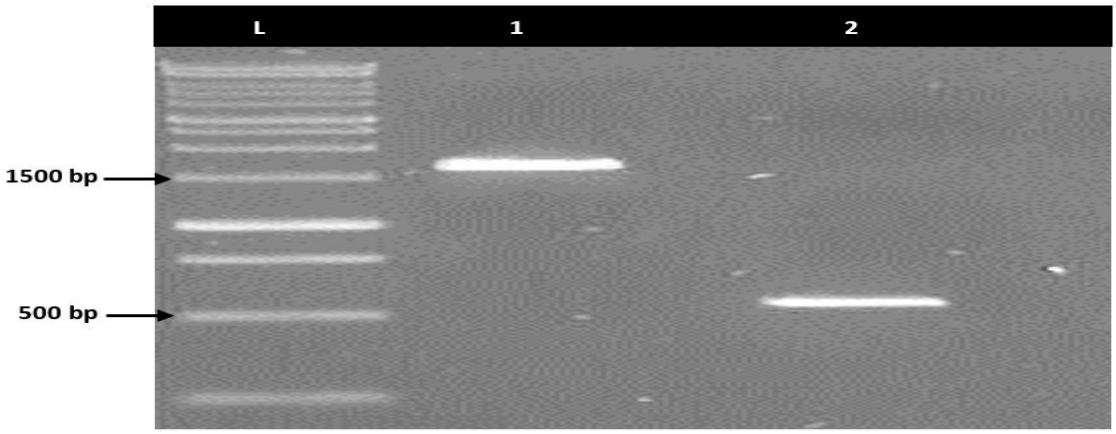

Figure 3.13 PCR performed on cDNA generated from total RNA isolated from L-arabinose induced cultures, confirming the presence of the *pGLCT1* mRNA transcripts. Lane 1 represents the pDEST17:: *pGlcT1* construct and Lane 2 represents the control, pDEST17:: *XhLEA*.

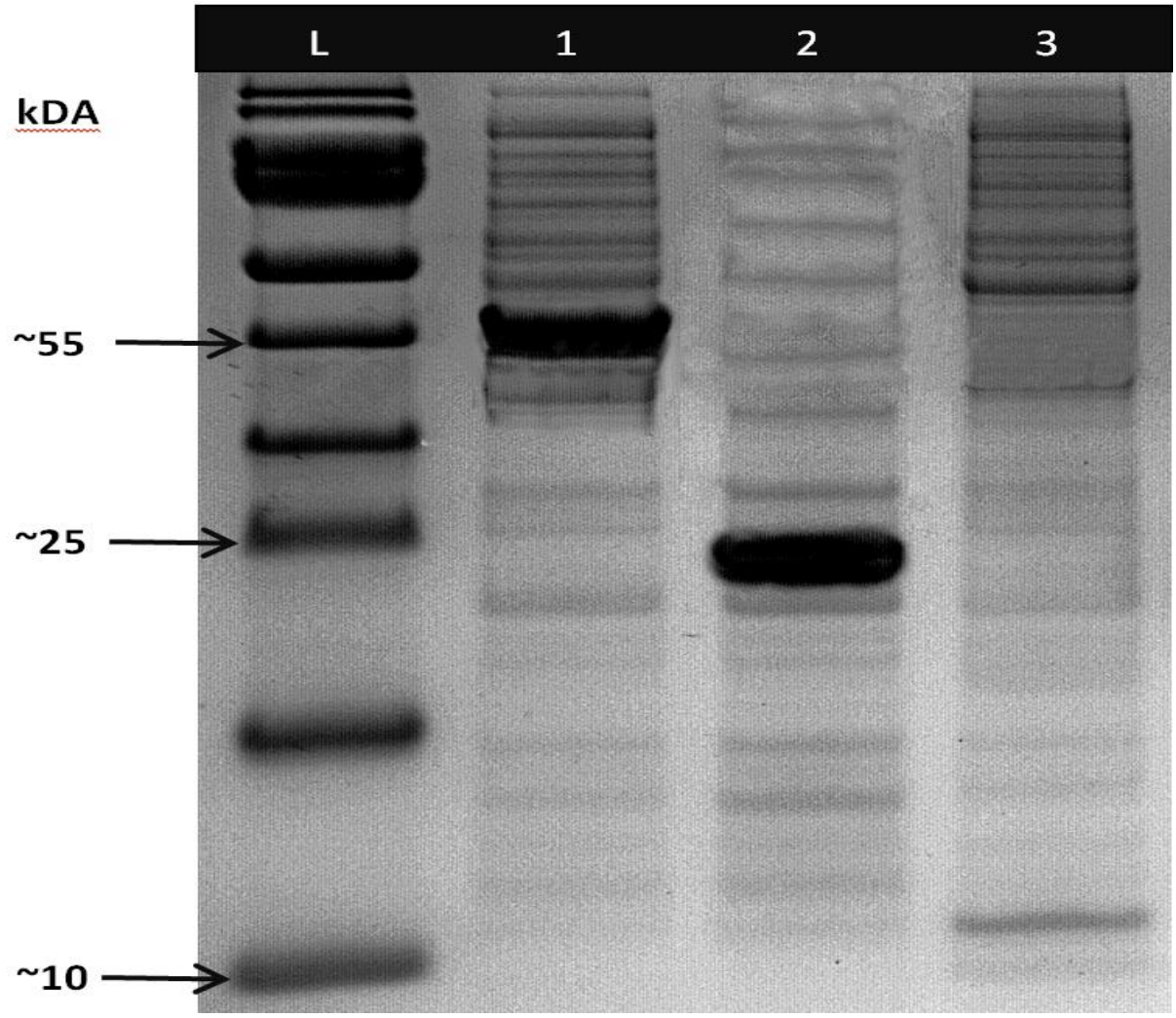

Figure 3.14 SDS-PAGE gel depicting proteins purified after culture induction with L-arabinose. Lane L represents the PageRuler Plus marker, lane 1 represents recombinant *pGlcT1* (~57 kDa) obtained from arabinose induction of transformed into *E. coli* (BL21 AI), lane 2 represents recombinant XhLEA (~25 kDa, positive control for recombinant protein induction) obtained from an L-Arabinose induction of pDEST17:: *XhLEA* and lane 3 represents empty pRSET C vector control.

3.6.3 M9 minimal media growth testing with pDEST17:: *pGlcT1* construct

BL21 AI colonies containing the pDEST17:: *pGlcT1* construct were induced for recombinant protein expression with 13.32 mM L-arabinose. Four centrifuge rinsing steps were conducted before cell pellets were re-suspended and plated onto M9 minimal media plates supplemented with either 100 mM sucrose (Figure 3.16Figure 3.14 c) or 100 mM raffinose (Figure 3.1**Error! Reference source not found.**6e). Growth was observed after 6 to 10 d, respectively. Growth on the raffinose M9 plate was more prominent compared to the sucrose M9 plate.

The controls, namely BL21 AI containing the pDEST17:: *XhLEA* construct (Figure 3.16d & Figure 3.16f) as well plain BL21 AI with no construct (Figure 3.15Figure 3.14 b & Figure 3.15c) showed no growth on the 100 mM sucrose or 100 mM raffinose M9 plates after 6 and 10 d, respectively. Subsequently, BL21 AI colonies containing pDEST17:: *pGlcT1* and showing positive growth on M9 minimal media supplemented with either sucrose or raffinose were sub-cultured onto fresh M9 plates supplemented with 100 mM sucrose and 100 mM raffinose. Much faster growth was observed with colonies forming within 3 d as shown in Figures 3.17a & b.

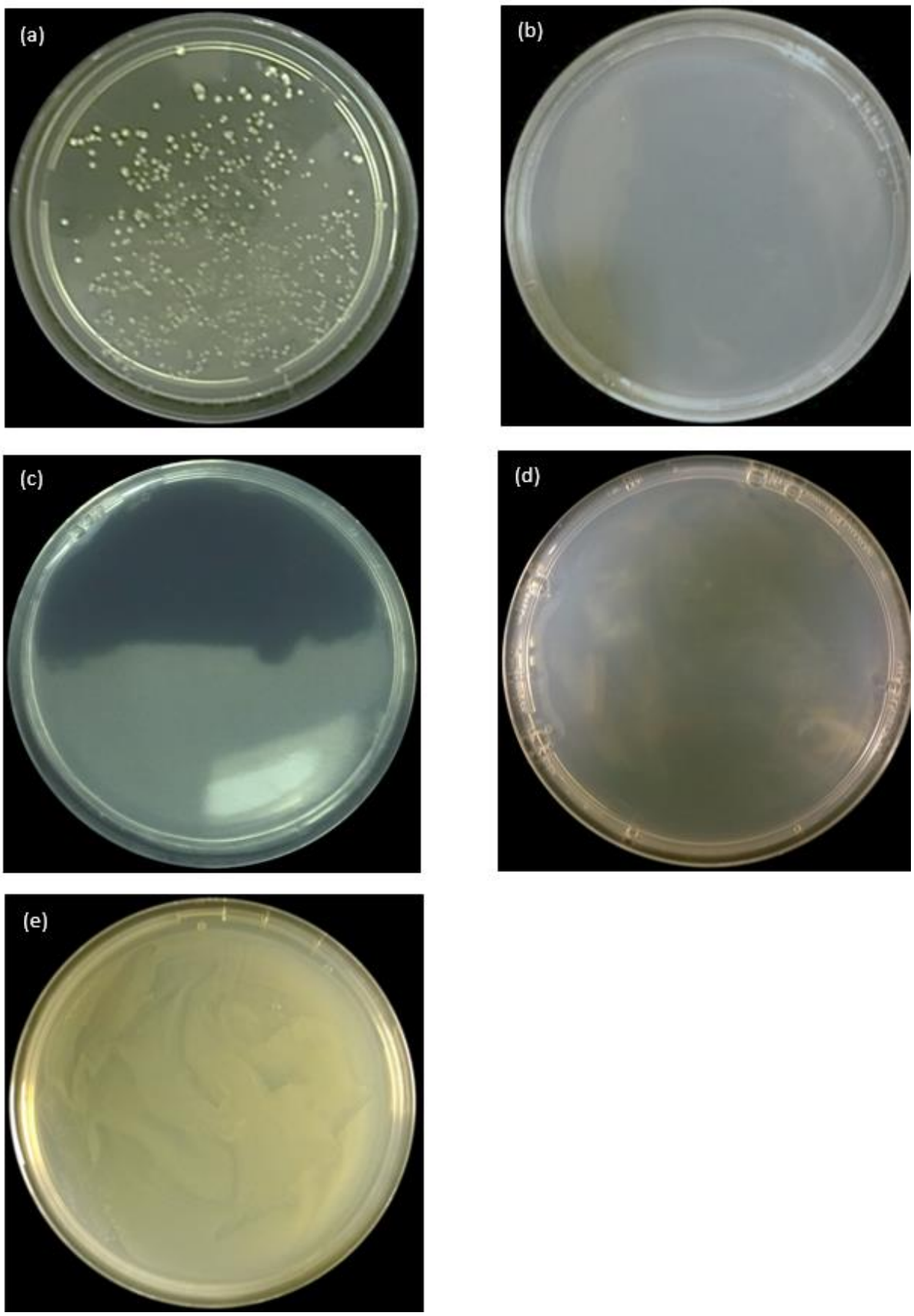

Figure 3.15 Growth profiles of *E. coli* (BL21 AI) without a construct when plated onto M9 minimal media plates supplemented with (a) 100 mM glucose, (b) 100 mM sucrose, (c) 100 mM raffinose, (d) plain M9 only (e) LA, following L-arabinose induced-induction for recombinant protein expression for a period of 4 h. Plates were incubated at 37°C for 10 d**.**

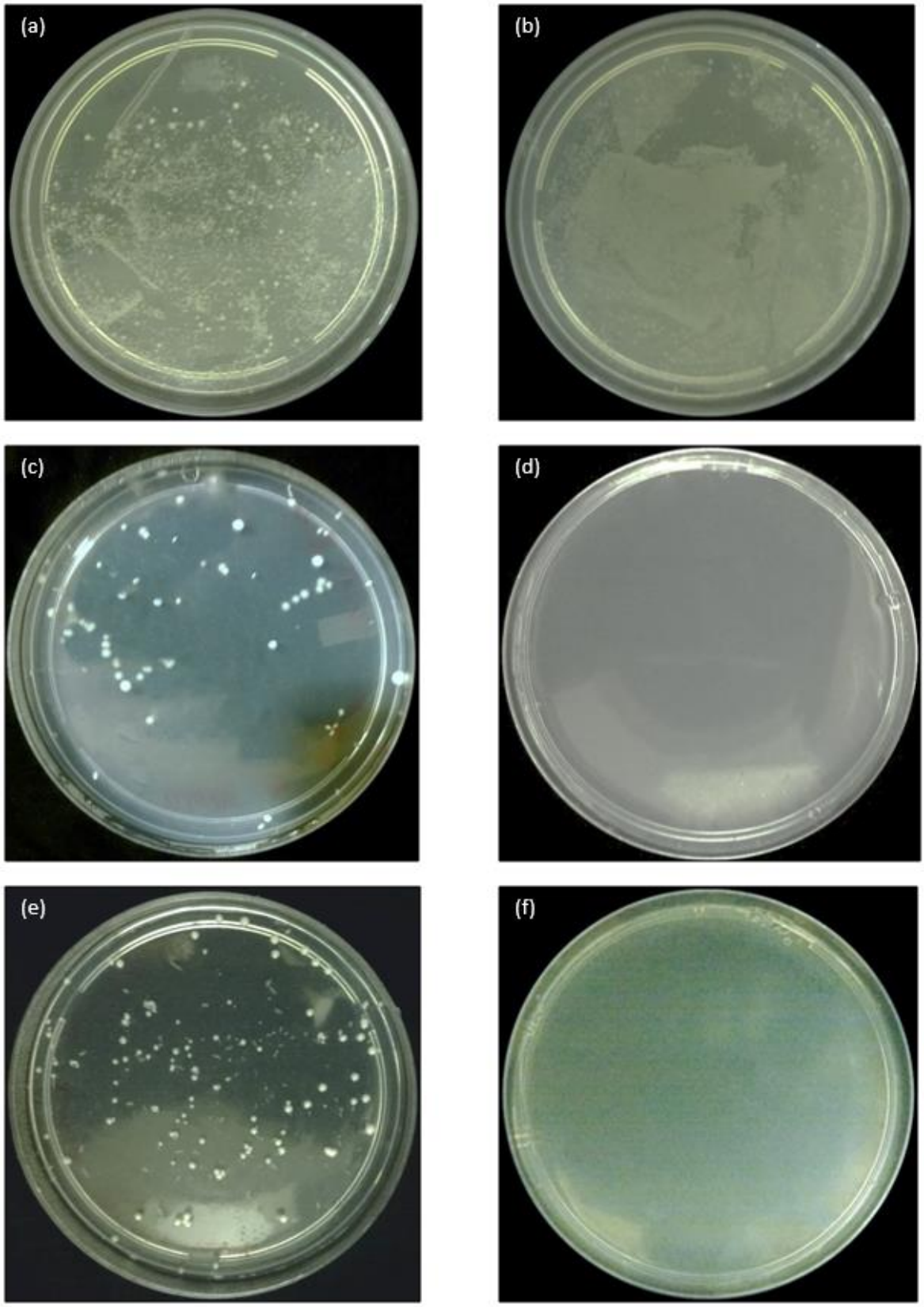

Figure 3.16 Growth profiles of E. coli (BL21 AI) with pDEST17:: pGlcT1 plated onto M9 minimal media supplemented with 100 mM glucose, 100 mM sucrose, 100 mM raffinose, M9 only and LA (a, c , e, g and I respectively) and with pDEST17:: *XhLEA* plated onto M9 minimal media supplemented with 100 mM glucose, 100 mM sucrose, 100 mM raffinose, M9 only and LA (b, d, f, h and j respectively) following L-arabinose induced-induction for recombinant protein expression for a period of 4 h. Plates were incubated at 37oC for 10 d.

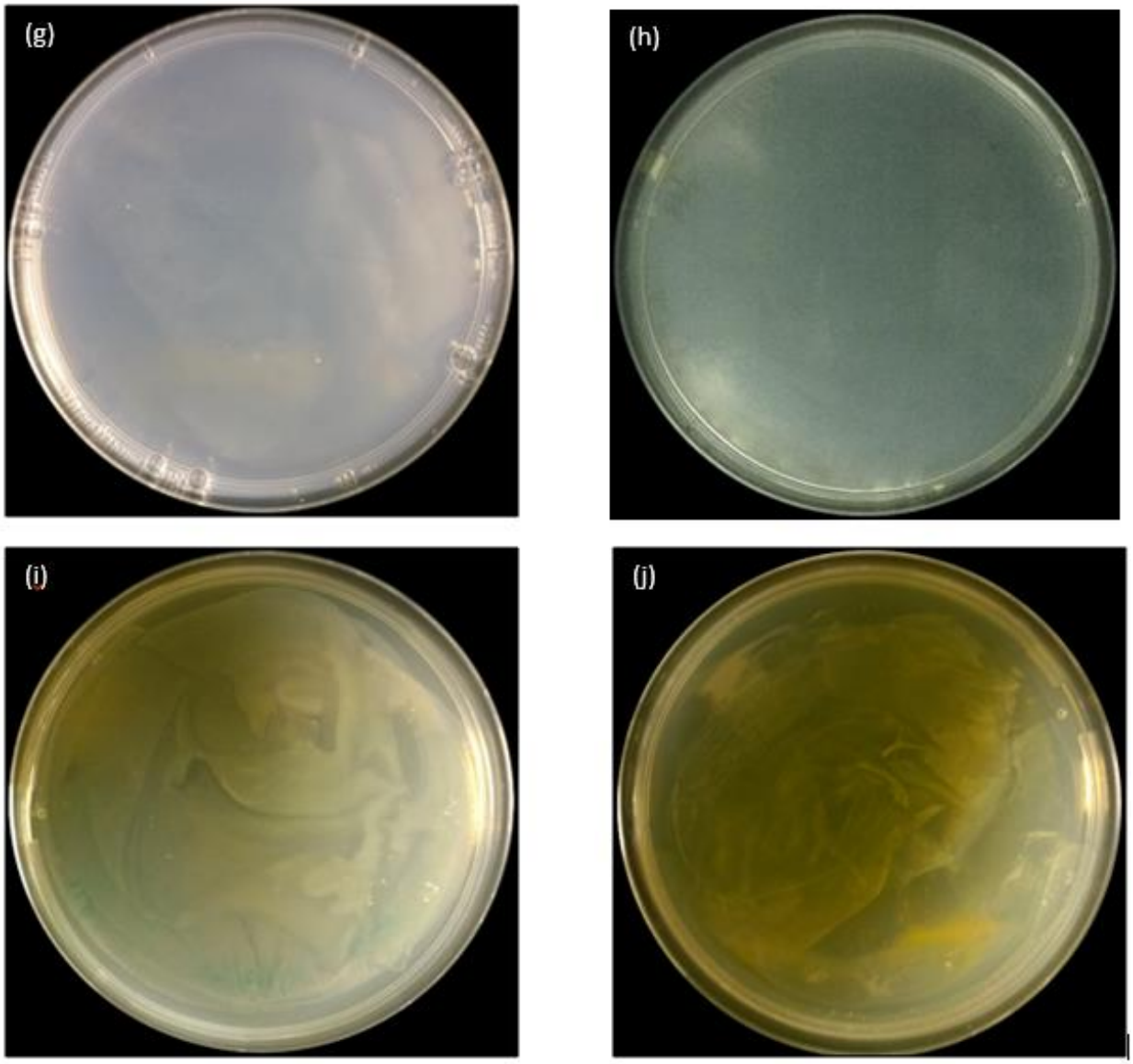

Figure 3.16 Growth profiles of *E. coli* (BL21 AI) with pDEST17:: *pGlcT1* plated onto M9 minimal media supplemented with 100 mM glucose, 100 mM sucrose, 100 mM raffinose, M9 only and LA (a, c , e, g and I respectively) and with pDEST17:: *XhLEA* plated onto M9 minimal media supplemented with 100 mM glucose, 100 mM sucrose, 100 mM raffinose, M9 only and LA (b, d, f, h and j respectively) following L-arabinose induced-induction for recombinant protein expression for a period of 4 h. Plates were incubated at 37^{o}C for 10 d**.**

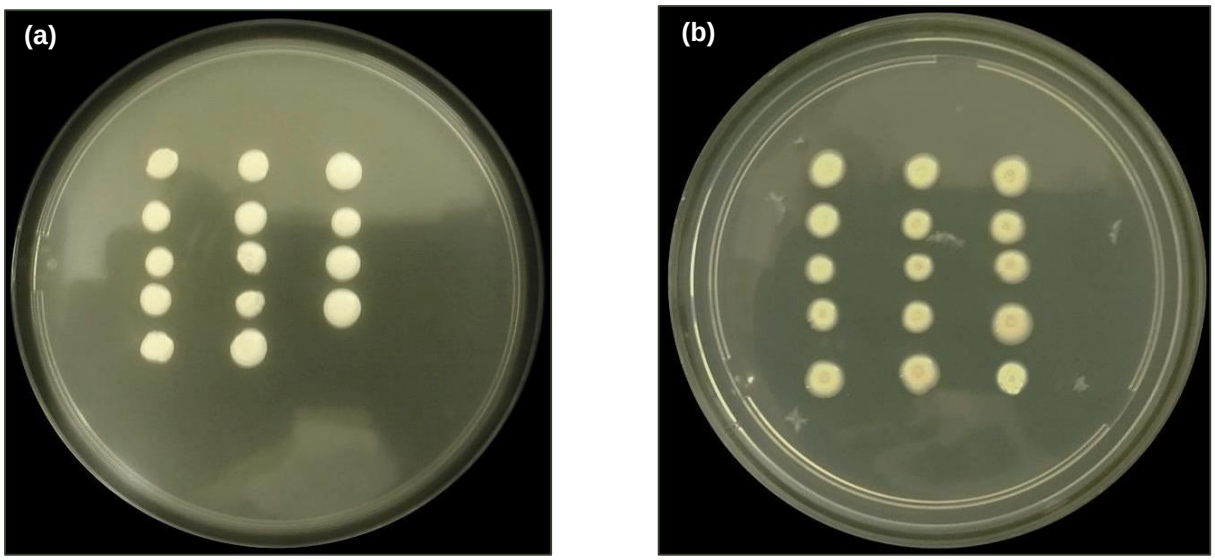

Figure 3.17 Growth profiles of BL21 AI colonies containing pDEST17:: *pGlcT1* sub-cultured onto M9 minimal media plates supplemented with (a) 100 mM sucrose and (b) 100 mM raffinose. All plates except the M9 plate with the BL21 AI were supplemented with 100 µg/ml Ampicillin and incubated at 37ºC for 10 d.

3.7 Evaluation of the quantum efficiency of photosystem II in the *pGLCT1* mutant exposed to cold stress

The F_v/F_m growth curves for the *Arabidopsis thaliana* Col-0 wild type, RSS-5 and SALK_066365 plant lines is shown in Figure 3.18. The F_v/F_m value for plants for all three lines at day 0 under normal conditions (25ºC) was found to be ~0.835, which is within the expected range for *Arabidopsis* leaves under optimal plant growth conditions. The chlorophyll fluorescence analysis showed a fairly constant F_v/F_m for the Col-0 wild type plants which averaged 0.82 over the duration of 7 d under cold-acclimation (4ºC). The RSS-5 and SALK_066365 plants exhibited a consistent decrease in F_v/F_m over the 7 d period under cold-acclimation. The decrease in F_v/F_m was more pronounced for the RSS-5 line as opposed to the SALK_066365 line. However, both the RSS-5 and SALK_066365 lines had a clearly visible decrease in F_v/F_m values as opposed to the Col-0 wild type plants.

The repeated measures one-way ANOVA test (Appendix A2, Table A2.1 & Table A2.2) performed on observed F_v/F_m values between replicate measurements of three leaves per plant for all six SALK_066365 ($P = 0.0006$) and RSS5 ($P = 0.0004$) line plants were determined to be statistically significant when compared to the Col-0 WT plants. The

linear trend was also found to be significant for the post test for linear trend involving the SALK_066365 (P = 0.0032) and RSS5 (P = 0.0001), respectively.

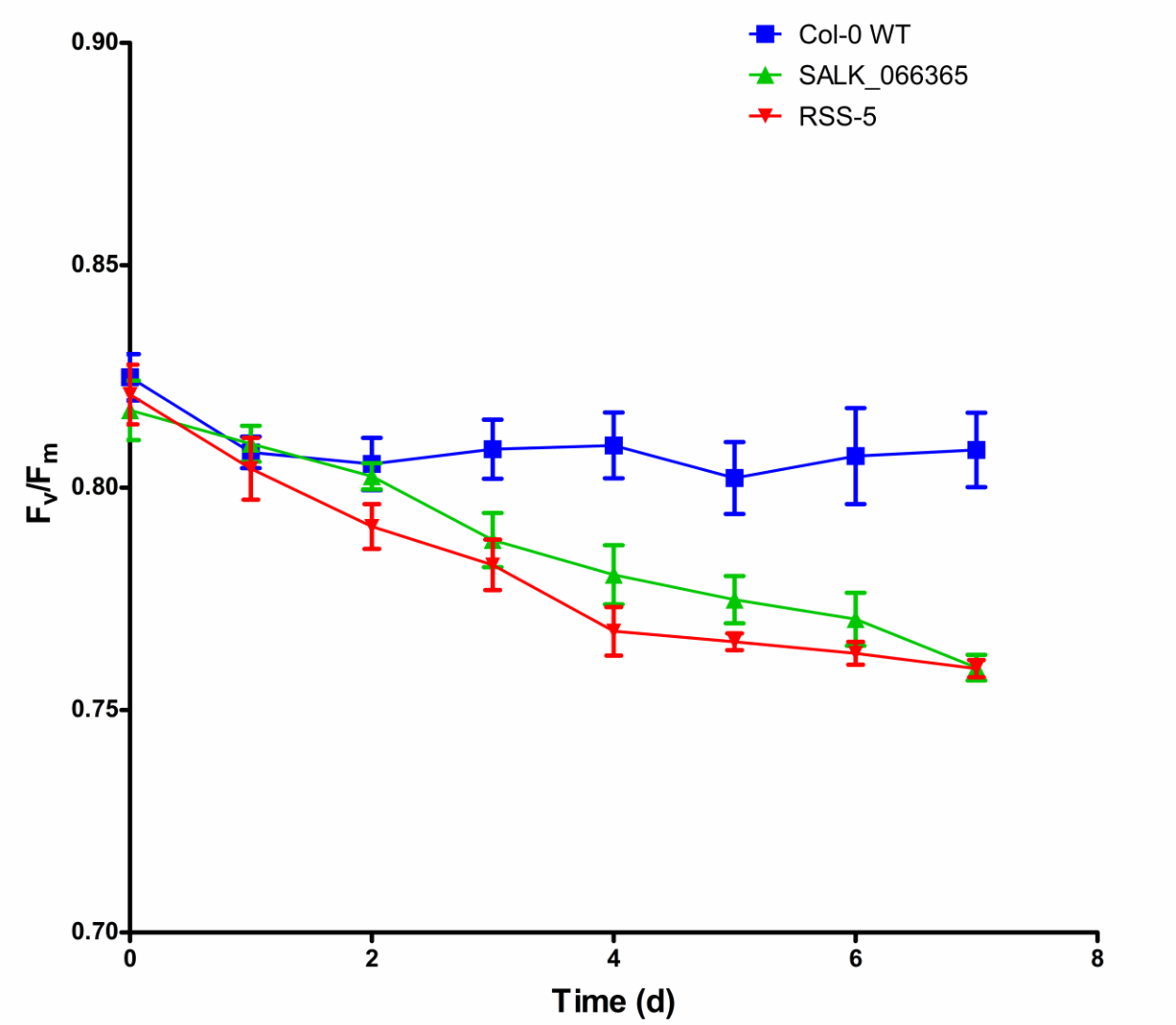

Figure 3.18 Graph showing the Fv/Fm values obtained for Col-0, the RSS-5-1 T-DNA insertion mutant (Zuther *et al*, 2004, Knaupp *et al*, 2011; Egert *et al*, 2013) and *pGlcT1* T-DNA insertion mutant (SALK_066365) plants under cold-acclimation conditions (4^{o}C, 7 d). Measurements were taken prior to the day cycle of the controlled environment chamber and represent plants that were thus dark adapted for a period of 8 h. Statistical significance for SALK_066365 (P = 0.0006) and RSS5 (P = 0.0004) were determined through a repeated measures one-way ANOVA test with a linear trend post-test.

Chapter 4: Discussion

Raffinose family oligosaccharides (RFOs) have been extensively described in their roles in phloem translocation and carbon storage (roots and tubers, reviewed in Keller and Pharr, 1986). However they have also been implicated in the general responses to abiotic stress in plants, where their role has been linked to osmotic adjustment as compatible solutes (Taji *et al*, 2002; Zuther *et al*, 2004; Hannah *et al*, 2006; Nishizawa *et al*, 2008; Egert *et al*, 2013; ElSayed *et al*, 2014).

The tri-saccharide raffinose (Suc-Gal_1) is the primary RFO reported to have increased concentrations in vegetative tissues (leaves and roots, Pessarakli, 2002; Amiard, 2003; Sengupta *et al*, 2015) during various abiotic stress-events. The exact mechanism(s) which underpin any protective functions remain elusive. However, under cold-acclimation, a fraction of raffinose accumulated in the leaf mesophyll tissue has been described to accumulate within the chloroplasts. This was first described in cold-treated cabbage and wheat, respectively (Heber, 1959; Santarius and Milde, 1977). Lineberger and Steponkus (1980) suggested the protective function of raffinose on proteins and membranes within isolated chloroplast thylakoid membranes. This was explicitly demonstrated in the *Arabidopsis* model, where the chloroplastic raffinose accumulated during cold-acclimation was shown to improve photosynthetic efficiency under low temperature (4°C, Knaupp *et al*, 2011).

Furthermore, a recent study demonstrated a novel raffinose transport system that actively transports raffinose that is synthesised in the cytoplasm into the chloroplast lumen (Schneider and Keller, 2009). That study demonstrated that RFO biosynthetic enzymes do not occur in the chloroplast. Subsequent studies using transgenic *Arabidopsis* which overexpressed a stachyose synthase (SS) isoform clearly showed that this transport system was selective only for raffinose, since mesophyllic stachyose was located exclusively in the cytosol (Keller & Schneider, 2009; Iftime *et al*, 2011)

While raffinose transporters are quite well characterised in microbes (Ulmke *et al*, 1997; Aslandis and Schmitt, 1990; Benz *et al*, 1992), the molecular identity of the transporter involved in chloroplastic raffinose transport in plants is still unknown. The first eukaryotic raffinose transporter (MRT, Genbank accession no.: GQ167043) was

only recently described from the fungus *Metarhizium robertsii* (Fang & St. Leger, 2010). This discovery has spurred on a greater research focus on putative raffinose transporters in plants, especially in *Arabidopsis*.

These findings led us to consider a multipronged strategy that sought to identify the chloroplastic raffinose transporter from Arabidopsis. Firstly, using a functional screening approach, we hoped to identify cDNAs from an *Arabidopsis* library that allowed laboratory strains of *E. coli* to grow on M9 minimal media supplemented with raffinose. Secondly, using rudimentary bio-informatics, we identified *pGlcT1* (TAIR accession code: *AT5G16150*) as a potential homologue to MRT since it was a known mono- and disaccharide transporter with a specific plastidial (chloroplast) sub-cellular localisation. A reverse-genetic approach was adopted to characterise *Arabidopsis* T-DNA insertion mutants for their ability to accumulate chloroplastic raffinose by indirectly comparing their photosynthetic performace against the RS5 (TAIR accession code: *AT5G40390*) mutant line which is completely deficient in raffinose accumulation (Zuther *et al.,* 2004; Knaupp *et al*., 2011; Egert *et al*., 2013).

Another approach to identify the chloroplastic raffinose transporter was to utilise E.coli as a functional screening system.

Since laboratory strains of *E. coli* are well described to lack raffinose transport systems, a simple yet efficient method to deduce what genes play a role in the raffinose transport mechanisms of *Arabidopsis* was used to screen an entire *Arabidopsis thaliana* cDNA library in the pBlueScript SK (+) vector. The ability of *E. coli* (wild-type strains) to utilise raffinose is plasmid-borne. Conjugative plasmids such as pRSD2 (which possess a Raf operon and encodes a peripheral raffinose metabolic pathway) have been found in the *E.coli* K-12 strain, allowing for growth on only raffinose as a carbon source (Ulmke *et al*, 1997). In that study, the pRSD2 plasmid was described to contain a raffinose permease (transporter), an invertase (sucrose degrading enzyme) and an α-galactosidase (raffinose degrading enzyme), facilitating the growth of certain enterobacteria such as *E.coli* K-12 on raffinose.

All readily available *E.coli* laboratory strains namely DH5α, OneShot® OmniMax®, OneShot® TOP10 and *BL21 AI* were tested for growth on M9 minimal media plates

supplemented with 100 mM raffinose (Figure 3.6d). None of the strains proliferated when raffinose was provided as the sole carbon source.

When an *Arabidopsis thaliana* cDNA library was cloned and expressed in *E.coli* (OneShot®TOP10) we recovered a small number of colonies from raffinose supplemented M9 minimal media plates. We reasoned that laboratory *E.coli* strains transformed with this library would show growth in the presence of raffinose as the sole carbon source only if genes from *Arabidopsis* facilitated raffinose transport. Plasmid DNA was obtained for eight cDNAs that consistently facilitated growth with raffinose as the sole carbon source when these plasmids were re-transformed. Surprisingly, when we conducted restriction digests on these plasmids we obtained inserts of various sizes (Figure 3.9), implying that more than one gene (cDNA) was imparting the ability to utilise (grow) on raffinose.

The five genes identified from sequencing the above mentioned plasmid isolates (Table 3.1) seem to possess no currently known function relating to raffinose uptake. Further, we did not identify the *pGlcT1* gene from these results. It is currently unclear how these particular genes facilitated the growth of *E. coli* on M9 minimal media supplemented with raffinose. Future studies need be conducted on these particular genes to determine whether these genes do indeed play a critical role in raffinose uptake. An alternative rationalization of these results may be that the *Arabidopsis* library screening system was leaky. This may have resulted in the selection of false positive colonies that allowed greater growth of *E.coli* (TOP10®) on 100 mM glucose as opposed to 100 mM raffinose. This explanation, however, does not support the continuous growth observed of the isolated colonies that were sub-cultured onto fresh M9 minimal media supplemented with 100 mM raffinose.

Since the results from the experiment involving the *Arabidopsis thaliana* cDNA library did not confirm the *pGlcT1* gene's role in transporting raffinose, an alternative experimental strategy was used to exploit the inability of *E. coli* to grow on raffinose (due to the missing uptake system). This involved creating a construct that allowed for the expression of the *pGlcT1* gene within a tightly regulated and highly inducible environment. The BL21 AI *E. coli* strain afforded an ideal heterologous expression background, especially for potentially toxic eukaryotic membrane proteins such as

pGlcT1. It offers one of the most tightly regulated high-level expression systems available, greatly reducing basal expression, due to the arabinose-inducible *araBAD* promoter upstream of the T7 RNA polymerase gene. This meant that the *pGlcT1* gene could be cloned into a T7 promoter-based vector such as the pDEST17 Gateway® expression vector.

Upon confirmation of the pDEST17:: *pGlcT1* construct (Figure 3.11), growth of *E. coli* BL21 AI transformed pDEST17:: *pGlcT1* onto M9 minimal media supplemented with 100 mM glucose, sucrose and raffinose was investigated. As expected favourable growth of BL21 AI containing pDEST17:: *pGlcT1* was observed on the glucose M9 plate (Figure 3.16 a). However, this growth on glucose was much more prolific when compared to BL21 AI controls (raffinose and sucrose). We suggest that this is due to pGlcT1 actually being a well characterized glucose transporter in a variety of plant species including *Arabidopsis thaliana* (Weber *et al*, 2000; Froelich *et al*, 2003; Ferro, 2003, Lu *et al*, 2006; Cho *et al*, 2011; Chaparro *et* al, 2013). Therefore, recombinant pGLCT1 could supplement the natural glucose uptake system in *E.coli* and enhance growth under our conditions.

The reduced number of colonies observed on M9 minimal media supplemented with 100 mM sucrose and 100 mM raffinose (Figure 3.16 c and e respectively) as opposed to prolific growth on glucose can also be attributed to the fact that co-transporters, particularly symporters facilitate the transport of certain molecules at a slower rate than other molecules (Lodish *et al*, 2000; Alberts *et al*, 2002). This is due to it being an active transport system which requires continuous amounts of energy to facilitate transport activity against concentration gradients. Consequently, M9 plates supplemented with 100 mM glucose allowed easier growth as opposed to M9 supplemented with sucrose and raffinose, which *E. coli* is not able to metabolize as easily, if at all. Additionally, glucose is a much smaller molecule than both sucrose and raffinose (Lodish *et al*, 2004), hence the uptake of glucose would be much faster, allowing faster metabolism as opposed to sucrose and raffinose.

pGlcT1 was first characterized by Weber *et al* (2000) via a procedure which involved differential labelling with glucose. The localization of pGlcT1 to the chloroplast envelope was further supported through mass spectrometry analyses (Froelich *et al*,

2003; Ferro, 2003). Amino acid sequence similarity analyses conducted on pGlcT1 and the glucose transporter from spinach corroborated that pGlcT1 can be categorized into the sugar porter family which is a member of the large Major Facilitator Superfamily (MFS) (Saier, 1998; Weber *et al*, 2000).

Although pGlcT1 is responsible for glucose transport within chloroplasts, it is not entirely limited to this function. This is supported by the finding that despite pGlcT1 being localized in the chloroplast, a fair amount of pGlcT1 transcript was found in *Arabidopsis* roots, where starch degradation occurs. This infers that pGlcT1 most likely participates in non-starch related metabolism (Cho *et al*, 2011). Additionally, Butowt *et al* (2003) determined that pGlcT1 is not only expressed in starch-containing tissues but it is also expressed in tissues containing no starch, such as ripe olives. This implies that pGlcT1 not only functions in chloroplasts but in chromoplasts and plastids as well (Jaiwal *et al*, 2008).

An alternate function that pGlcT1 may possess is the ability to transport sucrose. This is supported by one of our findings, where growth of the Bl21 AI strain (containing the pDEST17:: *pGlcT1* construct) on M9 minimal media supplemented with 100 mM sucrose (Figure 3.16c) was observed. The controls, BL21 AI (containing the pDEST17:: *XhLEA* construct) as well as BL21 AI without any plasmid did not grow on M9 supplemented with sucrose at all (Figure 3.16d and Figure 3.15b respectively). This suggests that in the presence of pGlcT1, *E. coli* is able to utilise sucrose as a carbon source.

An initial concern with regards to screening for growth via M9 minimal media supplemented with different carbon sources, was potential reducing sugar contamination in either the carbon sources or in the M9 minimal media components. A Benedict's reagent test was performed to verify whether there was any reducing sugar contamination present in the M9 minimal media with glucose, sucrose and raffinose (Figure 3.5). The M9 minimal media with 100 mM glucose (Figure 3.5a) changed colour as expected since all monosaccharides are categorized as reducing sugars (Simoni *et al*, 2002). The M9 minimal media containing 100 mM sucrose and 100 mM raffinose (Figure 3.5b & Figure 3.5c, respectively) did not change colour in the presence of Benedict's reagent, confirming that there was no reducing sugar contamination.

The Benedict's reagent test may not be as accurate as results obtained from HPLC methodologies, however, it is very effective as both a qualitative and quantitative test for the presence of reducing sugars (Simoni *et al*, 2002). Figure 3.15 (b and c) shows that there is no growth of *E. coli* BL21 AI (lacking a construct) at all on the M9 plates supplemented with 100 mM sucrose and 100 mM raffinose. This positively confirms that contamination of the sucrose and raffinose stock solutions was highly unlikely as there was significant growth of BL21 AI (with pDEST17:: *pGlcT1*) on M9 plates supplemented with sucrose and raffinose. Hence, it can be argued that growth of BL21 AI (with pDEST17:: *pGlcT1*) witnessed on the M9 minimal media supplemented with sucrose and raffinose was not a result of hexose sugar contamination.

In plants sucrose is the foremost transportation form for photo-assimilated carbon together with additional functions such as a source of carbon skeletons as well as energy for plant organs unable to perform photosynthesis better known as sink organs (Kuhn, 1999; Lalonde, 1999; Lemoine, 2000; Ayre, 2011). Sucrose is synthesised in the cytosol of photosynthesizing cells and is translocated over relatively long distances in solution via the phloem sap and as such it has to pass through a variety of membranes (Hammond & White, 2007). Hence, sucrose membrane transport has for a long time been considered as a fundamental aspect of determining plant productivity (Lalonde, 1999; Lemoine, 2000).

Presently, nine sucrose transporter genes (SUTs) have been characterized in *Arabidopsis* (Kühn & Grof, 2014). Almost all known plant sucrose transporters have been described as sucrose/H^+ symporters (Zhou *et al*, 2007). In *Arabidopsis*, multiple sucrose transporter cDNA sequences were discovered to be highly conserved and contain the characteristic 12 transmembrane α-helices associated with the MFS (Riesemeier *et al*, 1992; Lemoine, 2000; Kühn & Grof, 2014), which is the same superfamily that pGlcT1 belongs to. This is an interesting observation as it suggests a high likelihood that plant glucose transporters such as pGlcT1 may possess the innate ability to transport sucrose.

E. coli is generally unable to metabolize sucrose with only two known natural occurring strains capable of utilizing sucrose as a major carbon source namely; the *E. coli* W and

E. coli EC3132 strains (Jahreis *et al*, 2002; Sabri *et al*, 2013). Both these strains are able to utilize sucrose via the collaborative action of chromosomally encoded sucrose catabolism (*csc*) genes. The csc regulon encompasses three genes, namely a sucrose permease (*cscB),* a frucokinase (*cscK*) and a sucrose hydrolase or invertase (*cscA*). These genes are organized into two operons and negatively controlled at the transcriptional level via the repressor CscR (Jahreis *et al*, 2002; Sabri *et al*, 2013).

There is often uncertainty associated with the transport of sucrose via lactose permease (LacY) in the lac operon of *E. coli*. This is attributed to the fact that both LacY and cscB (sucrose permease) fall under the oligosaccharide/H^+ symporter sub-family of the Major Facilitator Superfamily (Vadyvaloo *et al*, 2006; Sugihara *et al*, 2011). LacY and cscB only share an overall homology of ~51%, however many of the amino acid residues responsible for transport activity in LacY are highly conserved in cscB (Vadyvaloo *et al*, 2006). Despite this shared homology, there is no common substrate for these two permeases (Sugihara *et al*, 2011). Hence, LacY is unable to transport sucrose and cscB is unable to transport lactose.

In our experiments we consistently did not recover any growth on sucrose supplemented M9 minimal media in our controls (Figure 3.15b & Figure 3.16d) but did observe growth when the *pGLCT1* construct was present. This uptake of sucrose in the *E. coli* BL21 AI cells (containing pDEST17:: *pGlcT1*) could be explained by the presence of pGlcT1, which shares the superfamily associated with sucrose transporters in *E. coli*.

Sabri *et al* (2013) recently discovered that the sucrose utilizing *E. coli* strains require only the invertase (*cscA)* and the sucrose permease (*cscB*) which is a sucrose-H^+ symporter, to catabolize sucrose. The CscB has been proven to show similarity to other well-studied sucrose transporters categorized as members of the MFS (Jahreis *et al*, 2002, Sabri *et al*, 2013). This also implies a certain level of similarity to pGlcT1, further supporting the theory that pGlcT1 may play a role in sucrose transport.

pGlcT1 appears to confer certain traits to the BL21 AI strain, that allows it to catabolize sucrose into glucose and fructose. The fact that the controls, B21 AI (pDEST17:: *XhLEA*) and the empty BL21 AI cells did not grow under identical

conditions on sucrose, further re-affirms that the only contributing variable is pGlcT1. At present, the underlying mechanism of sucrose utilization in the presence of pGlcT1 is not clearly understood but we suggest that the presence of non-specific alkaline and/or acidic α-galactosidases may very well result in the cleavage of both raffinose and sucrose when they enter the *E.coli* cell, thereby making glucose accessible for metabolism (and growth).

BL21 AI (containing pDESt17:: *pGlcT1*) was able to consistently grow on M9 minimal media supplemented with 100 mM raffinose (Figure 3.16e). The controls pDEST17:: *XhLEA* and the empty BL21 AI cells (Figure 3.16f & Figure 3.15c respectively) were unable to utilize raffinose at all for growth. This provides strong evidence that pGlcT1 may be responsible for raffinose transport into the cell. Colony PCR on randomly selected colonies show the presence of the *pGlcT1* gene which confirms that the transporter is being expressed and that the growth on raffinose is not the result of an esoteric variable. Sub-culturing colonies after multiple centrifugation and rinsing steps negate the possibility that residual L-arabinose from induction might be facilitating growth on the M9 media supplemented with raffinose. The Benedict's test (Figure 3.5c) also confirmed that no other external factors such as monosaccharide contamination were influencing the results in any way.

The results are encouraging as BL21 AI along with most *E. coli* strains do not possess any inherent ability to transport raffinose into the cell (Ulmke *et al*, 1997). This is a plasmid facilitated ability that allows raffinose to enter the cell through the action of a raffinose porin (RafY) where it is metabolized (Ulmke *et al*, 1997). In the absence of plasmid-mediated raffinose uptake, BL21 AI must be using pGlcT1 to facilitate raffinose uptake into the cell.

Once the raffinose has entered the cell, it is hydrolysed into galactose and sucrose by α-galactosidase. Sucrose is then cleaved into glucose and fructose by an invertase (Schmid & Schmitt, 1976). This entire process is part of the raffinose (Raf) catabolism operon encoded by conjugative plasmids such as D1021, pRSD1 or pRSD2 (Schmid & Schmitt, 1976; Ulmke *et al*, 1997). Common *E. coli* strains do not seem to possess an invertase or α-galactosidase which is responsible for raffinose catabolism (Burkhardt *et al*, 1978; Aslanidis *et al*, 1989; Ulmke *et al*, 1997).

Interestingly enough the plasmid induced uptake and catabolism of raffinose is highly similar to uptake and breakdown of sucrose. Both these systems are plasmid mediated in only certain enteric *E. coli* strains (K-12, W and EC3132) that are able to break down WSCs such as sucrose and raffinose. This essentially demonstrates that *E. coli* in general, has not evolved a method that allows it to catabolize raffinose under normal circumstances. The exact way in which this is accomplished needs to be studied in greater detail.

Although the results from the M9 minimal media raffinose screening system were promising, a more targeted approach in plants was required to substantiate our conclusions that pGLCT1 could facilitate raffinose transport, in addition to its reported functions in glucose and sucrose export. To this end we looked to the recently described study which confirmed a function for chloroplastic raffinose protecting thylakoid membranes thereby improving photosynthetic efficiency under low temperature (Knaupp , 2011). In that particular study, a raffinose synthase mutant for the *RS5* gene (RS14 line, Zuther *et al*, 2004) clearly displayed a perturbation in photosynthetic efficiency under low temperature.

We reasoned that since the raffinose deficient RSS-5 loss-of-function mutant accumulated no raffinose at all, then the T-DNA insertion mutant line for *pGLCT1* (SALK_066365) would accumulate mesophyllic raffinose but not chloroplastic raffinose (based on the assumption that pGLCT1 was responsible for chloroplastic raffinose transport). This would result in similar decreased photosynthetic efficiency of both mutant lines under cold-acclimation since neither would contain any chloroplastic raffinose.

The targeted *in planta* approach employed was based on chlorophyll fluorescence detection in *Arabidopsis* leaves under dark-adapted cold-acclimation (8h dark, 4^{o}C) conditions. Under standard growth conditions for *Arabidopsis,* the F_v/F_m parameter provides an approximation of the maximum quantum efficiency of PS II photochemistry and its value for undamaged plant leaves is approximately 0.83 (Schreiber, 2004). Any impairment of PS II photochemistry efficiency due to environmental stress, such as cold stress decreases the F_v/F_m value (Krause & Weise, 1991; Barbagallo *et al*, 2003).

Our findings concerning the maximum quantum efficiency of PS II photochemistry within Col-0 WT, RS14 (RSS-5) and SALK_066365 mutant plants yielded similar results as Knaupp *et al* (2011), to a certain extent. Figure 3.18, shows that the F_v/F_m in Col-0 WT plants remained fairly constant with an average of 0.82, which is consistent with the values Knaupp *et al* (2011) observed for WT plants. Knaupp *et al* (2011) demonstrated that the F_v/F_m values measured for plants from the RS14 (RSS5) line were significantly reduced when compared to the Col-0 WT plants. Our results followed the same trend with the greatest reduction in F_v/F_m values observed in the RS14 (RSS5) line as opposed to the *pGlcT1* mutant and Col-0 WT lines.

Knaupp *et al* (2011) found that the average F_v/F_m values in the RS14 mutants, first used by Zuther *et al* (2004) were lower than the F_v/F_m values obtained for Col-0 WT plants under cold-acclimation conditions. This denotes the lack of chloroplastic raffinose due to a knocked-out *RafS* gene, further confirming that although raffinose is not necessary for freezing tolerance or cold-acclimation in *Arabidopsis* (Zuther *et al*, 2004), it is implicated in the protection of photosynthetic machinery of the chloroplasts.

The *pGlcT1* loss-of-function mutant plants also demonstrated reduced F_v/F_m values ($P = 0.0006$), although not as low as the RS14 line ($P = 0.0004$) but lower than the Col-0 WT plants. This suggests that the *pGlcT1* mutant plants were experiencing reduced photosynthetic efficiency under cold-acclimation (4^{o}C). The only attributable factor is the lack of the *pGlcT1* gene in the mutant plants. Although the results do agree with Knaupp *et al* (2011), only one physiological experiment was conducted within the time period of the study and this may have affected the results to a certain extent.

An alternative explanation for the reduced F_v/F_m values in the SALK_066365 plants is the possible starch degradation impairment present in the *pGlcT1* loss-of-function mutant plants. Since *pGlcT1* has been implicated in starch degradation (Weber *et al*, 2000; Cho *et al*, 2011), the mutant plants may have significantly compromised starch degradation.

Glucose along with maltose, is an end product of hydrolytic starch degradation and needs to be exported from the chloroplast stroma into the cytosol (Wise & Hoober, 2006; Cho *et al*, 2011). Studies have demonstrated that starch-laden chloroplasts transport this glucose and maltose at night in the dark (Servaites, 2002; Ritte & Raschke, 2003; Weise *et al*, 2004). While pGlcT1 exports glucose out of the chloroplast stroma to the cytosol in the dark, maltose is simultaneously exported via a chloroplastic maltose exporter known as *maltose exporter 1* (*MEX1*, TAIR accession code: AT5G17520). Cho *et al* (2011) have demonstrated that when both *pGlcT1* and *MEX1* in *Arabidopsis* Col-0 were mutated, a more significant growth reduction phenotype was observed as opposed to when *MEX1* was mutated alone. This reduction in growth does suggest that mutations in *pGlcT1* can affect starch degradation adversely, which may have a negative impact on protection of PSII photochemistry machinery in the chloroplasts.

Our results indicate a definite reduction in photosynthetic performance of the *pGlcT1* ($P = 0.0006$) mutant plants when compared to our wild-type controls (Col-0), thereby possibly confirming our claim that the well characterised plastidial glucose transporter pGlcT1 is also able to transport raffinose. We need to verify our results with additional replicate experiments. The functional characteristics of raffinose within the chloroplasts of the *pGlcT1* loss-of-function mutant plants could have been investigated further through the use of transmission electron microscopy (TEM). This could be used to identify any differences that exist between Col-0 WT and *pGlcT1* mutant plants' chloroplast ultrastructure with regards to thylakoid membrane protection conferred by raffinose under cold acclimation conditions.

General conclusion and future recommendations

As discussed previously, raffinose is known to accumulate in chloroplasts under cold-acclimation conditions the presence of a raffinose transporter localized to the chloroplast envelope has been well characterized. The identity of this transporter has, however, remained a mystery. Recent findings have justified that raffinose is not explicitly involved in freezing tolerance within plants. However, it was determined that raffinose plays a major role in protecting the photosynthetic apparatus of chloroplasts. Our work was conducted to determine whether a well characterized plastidic glucose

transporter (pGlcT1) possessed the ability to transport raffinose into chloroplast of *Arabidopsis thaliana.*

Our preliminary analyses showed only a ~25% similarity between the amino acid sequences of the only characterized eukaryotic raffinose transporter (Mrt) and the pGlcT1 transporter. However, from our findings, it is clear that the ability of *E.coli* containing a plasmid encoding the *pGlcT1* gene to grow on M9 minimal media supplemented with raffinose, is indicative of raffinose being transported into the cells. This raffinose is most likely catabolised via the action of an intrinsic and non-specific α-galactosidase.

Our chlorophyll fluorescence analysis findings corresponded to a large extent with previously studied data (Knaupp *et al*, 2011) concerning the Fv/Fm values of *Arabidopsis* Col-0 and RS14 ($P = 0.0004$) lines under dark-adapted cold-acclimation conditions (4ºC). Most importantly, the F_v/F_m values ($P = 0.0006$) we obtained for the *pGlcT1* T-DNA insertion mutant plants (SALK_066365), indicated reduced quantum efficiency of Photosystem II photochemistry when compared to wild-type controls. This could be attributed as a function for pGlcT1 (*in planta*) in chloroplastic raffinose transport. However, our interpretation is based on only one physiological experiment.

Future studies involve repeat experiments to measure F_v/F_m under cold-acclimation in the *pGlcT1* loss-of-function mutant. Furthermore, on the basis of the findings being reproducible, we will further conduct non-aqueous subcellular fractionation analyses on the *pGlcT1* T-DNA insertion mutant plants to determine the amount of raffinose that accumulates in the chloroplasts under cold-acclimation conditions. A consideration is also the functional rescue of the mutant line where *pGlcT1* expression is restored under the control of the native *pGlcT1* promoter. Additionally, an electrophysiological procedure such as the patch-clamp technique may be applied to *E. coli* vesicles where recombinant pGlcT1 occurs, to further understand the active raffinose transport activity related to pGlcT1 at the molecular level and conclusively determine whether pGlcT1 is irrefutably the raffinose transporter in *Arabidopsis*.

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Appendix

Appendix A1

M9 Minimal Media

- 64g Na_2HPO_4-$7H_2O$
- 15g KH_2PO_4
- 2.5g NaCl
- H_2O to final 1L volume and autoclave

To prepare 500mL M9 minimal media;

- 100mL of 5xM9 salts
- 1 mL 1M $MgSO_4$
- 50 uL 1M $CaCl_2$
- 5 mL 100x Basal Medium Eagle Vitamin Solution (Gibco)
- 2.5 mL filter sterilized NH_4Cl (0.2 g/mL) or 0.5g dry
- 10 mL 20% d-glucose or 2g dry
- glass distilled & autoclaved H_2O to final volume of 500mL

1. pH solution to 7.3 and filter sterilize (0.2u filter).

2. Introduce media to a pre-autoclaved, wide-bottom (baffled) 2L flask and add ampicillin to a final concentration of 70-100 ug/ml.

3. Grow 5mL overnight culture in same media to inoculate 500mL M9.

4. Shake culture at 37^{o}C until an OD_{600} of 07+/-0.2 then induce protein expression with the addition of IPTG (0.01-0.1 mM final concentration).

Appendix A2

Simple and rapid method of plant genomic DNA isolation for PCR analysis

(Adapted from Edwards *et al* (1990) NAR 19: 1349)

METHOD:

1. Grind tissue (one leaf generally; either fresh or pre-frozen in liquid N_2) in 1.5 ml Eppendorf tubes on liquid N_2 with plastic pestle until well macerated
2. Add 400 µl of buffer (200 mM Tris-Cl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS)
3. Vortex vigorously for approximately 1 min (place samples on ice until all preps are ready)
4. Centrifuge (full speed, 13000 x *g*) at RT for 10 min
5. Transfer 300 µl of supernatant to a fresh tube (keep on ice)
6. Add 300 µl of isopropanol, mix by inversion, incubate at -20^{0}C for 60 min
7. Centrifuge (full speed, 13000 x *g*) at RT for 15 min, remove and discard supernatant
8. Rinse the pellet with 1 ml of 70% ETOH, drain and air-dry pellet (approximately 1 hr until all residual ETOH has evaporated)
9. Resuspend pellet in 100 µl TE (pH 8.0). **NOTE: Pellet will not dissolve completely, gently pipette up and down for 1 min, centrifuge at full speed for 1 min and use the supernatant for PCR)**
10. Use 5 µl in a 50 µl PCR reaction

EXTRACTION BUFFER:

For 200 ml:

- 4.844 g Tris base (Fw 121.1) dissolving in 180 ml ddH_2O. Adjust Ph to 7.5 with concentrated HCl
- Add 2.922 g NaCl (Fw 58.44)

- Add 1.861 g EDTA (Fw 372.2), alternatively 10 ml 0.5 M EDTA (pH8.0)
- Bring to volume of 190 ml with ddH_2O
- Autoclave
- Add 10 ml 10% SDS to make up 200 ml final volume

Preparation of ultra-competent *E. coli* cells for transformation

(Modified from Inoue *et al* (1990), Gene, 96:23-28)

METHOD:

1. Culture bacterial cells on LB agar plates at 37^0C overnight
2. Pick a colony and grow starter culture in 3 ml LB at 37^0C overnight, with vigorous shaking (200 rpm)
3. Inoculate 200 ml LB with the starter culture and grow cells at 37^0C, shaking at 200 rpm, until it reaches OD_{600} = 0.5 (takes approximately 4 hrs)
4. Place the flask in ice for 10 min
5. Pellet cells by centrifugation (4000 rpm) for 10 min at 4^0C (cells can be divided into four 50 ml tubes)
6. Resuspend each pellet in 20 ml ice-cold TB (hereafter, combine appropriately so that there are two tubes containing 40 ml TB) and store on ice for 10 min
7. Centrifuge (4000 rpm) for 10 min at 4^0C
8. Gently resuspend each pellet in 10 ml ice-cold TB and 700 µl DMSO (DMSO needs to be stored at -20^0C overnight before use)
9. Aliquot the cells in volumes of 50 µl, snap-freeze in liquid nitrogen and store at -80^0C
10. Use aliquots for transformation as per “The magic *E. coli* transformation protocol” instructions.

TB solution:

- 10 mM PIPES
- 15 mM $CaCl_2$
- 250 mM KCl
- Dissolve in milliQ water and adjust pH to 6.7 with KOH or HCl
- Add $MnCl_2$ to final concentration of 55 mM
- Adjust to final volume
- Sterilize by filtration with a 45 µm filter and store at 4^0C

Standard heat-shock transformation method

1. Take competent cells out of -80^0C freezer and thaw on ice
2. Add 5 µl of ligation reaction to cells. **DO NOT MIX BY PIPETTING UP AND DOWN**
3. Incubate mixture on ice for 30 min
4. Set heating block to 42^0C
5. Heat-shock the cell mixture at 42^0C for 45 s
6. Place back on ice for 2 min
7. Add 250 µl LB to cells and incubate at 37^0C, with 200 rpm shaking, for 30 min
8. Plate 50 µl and 100 µl of each transformation onto selective plates (LB+antibiotic)
   Culture cells overnight at 37^0C

Table A2.1: Statistical analyses conducted on observed F_v/F_m values obtained for SALK_066365 plants. Repeated measures 1-way ANOVA (P = 0.0006) was used with linear trend between means and all plants were used.

Table Analyzed	**Data 1**		
Repeated Measures ANOVA			
P value	**0,0006**		
P value summary	***		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	18		
F	2,794		
R square	0,2853		
Was the pairing significantly effective?			
R square	0,8757		
F	167,6		
P value	< 0,0001		
P value summary	***		
Is there significant matching? (P < 0.05)	Yes		
ANOVA Table	SS	df	MS
Treatment (between columns)	0,002116	17	0,0001244
Individual (between rows)	0,05224	7	0,007463
Residual (random)	0,0053	119	0,00004454
Total	0,05966	143	
Post test for linear trend			
Slope	-0,0001614		
R square	0,006768		
P value	0,0032		
P value summary	**		
Is linear trend significant (P < 0.05)?	Yes		

Table A2.2: Statistical analyses conducted on observed F_v/F_m values obtained for RSS5 plants. Repeated measures 1-way ANOVA (P = 0.0004) was used with linear trend between means and all plants were used.

Table Analyzed	Data 1		
Repeated Measures ANOVA			
P value	0,0004		
P value summary	***		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	3		
F	14,74		
R square	0,678		
Was the pairing significantly effective?			
R square	0,5099		
F	6,462		
P value	0,0016		
P value summary	**		
Is there significant matching? (P < 0.05)	Yes		
ANOVA Table	SS	df	MS
Treatment (between columns)	0,003325	2	0,001662
Individual (between rows)	0,005101	7	0,000729
Residual (random)	0,001579	14	0,000113
Total	0,01001	23	
Post test for linear trend			
Slope	-0,01373		
R square	0,3014		
P value	0,0001		
P value summary	***		
Is linear trend significant (P < 0.05)?	Yes		