Universidade de Lisboa

Faculdade de Ciências

Departamento de Biologia Vegetal

MEDICAGO TRUNCATULA AND WATER DEFICIT: EXPLORING A NEW METHOD FOR TREHALOSE 6-PHOSPHATE QUANTIFICATION AND CHARACTERIZATION OF THREE ABSCISIC ACID RESPONSIVE PROMOTERS

André Melão Alcântara

Dissertação de Mestrado em Biologia Celular e Biotecnologia

2011

Universidade de Lisboa
Faculdade de Ciências
Departamento de Biologia Vegetal

MEDICAGO TRUNCATULA AND WATER DEFICIT: EXPLORING A NEW METHOD FOR TREHALOSE 6-PHOSPHATE QUANTIFICATION AND CHARACTERIZATION OF THREE ABSCISIC ACID RESPONSIVE PROMOTERS

André Melão Alcântara

Dissertação Orientada Por:
Professor Doutor Pedro Fevereiro (FCUL)
Doutora Susana Araújo (IICT)

Dissertação de Mestrado em Biologia Celular e Biotecnologia
2011

Acknowledgments

To all the kind people who helped me out in this last year, either in work related subjects or helping me to unwind from work related subjects, I would like to address a few words of gratitude.

To my advisors, Professor Pedro Fevereiro and Doctor Susana Araújo, for the opportunity to develop most part of the present work in the Plant Cell Biotechnology lab and for all the orientation and patience given.

To Professora Anabela Bernardes da Silva, for all the support, orientation and kindness. Also for receiving me at the Plant Physiology group when necessary and for manuscript revision.

To the Mass Spectrometry lab, headed by Doctor Ana Coelho, who kindly allowed me to use the LC-MS equipment and provided all the necessary technical support. A special thanks to Elizabete (aka 'Beta') Pires for the incredible support, help, enthusiasm and patience provided with the T6P assays.

To Inês Trindade for all the help and brainstorming sessions on gateway technology and other molecular biology techniques. Together with Mara Alves, Susana Leitão, Nuno Almeida and Cláudio Capitão, I would like to thank all the occasional practical hints and for all patience they had on all the daily unwinding routines.

To Leonor Tomaz for kindly letting me use almost the complete stock of micropropagated *M. truncatula* plants for the transformation procedures.

To Manuela Lucas for the initial guidance on the lab rules and initiation on lab routines.

To all my friends who occasionally met in an attempt to relax from work, even though 99 % of the time was spent on telling lab stories. A special reference to Susana Silvestre for all the patience demonstrated all year on regular basis.

To FCT for financial support for material and equipment trough the project PTDC/ AGR-GPL/099866/2008.

Finally, I would like to thank my parents for funding my personal life project and, together with my brother, for all the support given.

Resumo

Condições ambientais adversas limitam fortemente o crescimento de plantas, levando a quebras anuais de produtividade em explorações agrícolas. Dos stresses vegetais, a falta de água é o factor que mais limita a produção. Para sobreviver em ambientes adversos, as plantas possuem mecanismos de defesa que aumentam a sua tolerância aos stresses impostos. Um desses mecanismos consiste na acumulação de um dissacárido fosfatado designado por trealose-6-fosfato (T6P). Até há relativamente pouco tempo, julgava-se que a presença de trealose e do seu intermediário na via de síntese apenas ocorria num grupo reduzido de espécies de plantas, conhecidas com "plantas da ressurreição". No entanto, foi provado que acumulação de T6P induz a alteração de expressão de vários genes que estão envolvidos na resposta a esse mesmo stress. A sobreexpressão de genes da via de síntese da trealose, por manipulação genética, levou ao aumento de tolerância de várias espécies de plantas a diversos stresses abióticos, tais como o défice hídrico. No entanto, por também desempenhar um papel no desenvolvimento das plantas, essa mesma sobreexpresssão alterações no fenótipo das plantas transgénicas. Essa limitação foi ultrapassada, essencialmente pela utilização do gene da trealose fosfato sintase (TPS, na sigla inglesa) de *Arabidopsis thaliana* e de promotores induzidos por stress.

Um dos maiores problemas em estudos sobre T6P em plantas é a sua ocorrência em baixas concentrações, sendo necessária utilização de técnicas muito sensíveis para a sua monitorização. Nos últimos anos, muitos trabalhos têm sido elaborados no sentido de detectar e quantificar T6P em extractos vegetais. Foi proposto que a utilização de cromatografia líquida de alta pressão para separação de metabolitos em extractos vegetais acoplado a um espectrómetro de massa para detecção e quantificação (LC-MS, na sigla inglesa) seria o melhor sistema a utilizar para o fim pretendido. No entanto, existe um isómero da T6P, a sacarose-6-fosfato (S6P), que têm uma estrutura molecular muito semelhante ao da T6P e, como tal, interage de forma da mesma forma com as fases estacionárias testadas. Por terem o mesmo peso molecular e padrão de fragmentação, também não são passíveis de serem distinguidas pelo detector de MS.

Neste trabalho foram explorados métodos para extracção e detecção de T6P com o intuito de determinar a concentração de T6P em tecidos de *Medicago truncatula* sujeitos a défice hídrico. Para tal, foram testados três métodos de extracção distintos para avaliar a eficiência de recuperação de açúcares fosfatados, na presença e ausência de material vegetal,

com a adição de glucose-6-fosfato (G6P) no início do processo de extracção. Após ressuspensão em água, foi quantificada G6P por um método enzimático e comparados os três métodos de extracção. Dos extractos em que não foram adicionados tecidos vegetais, a extracção líquido-líquido (LLE, na sigla inglesa) foi a que apresentou melhores resultados, com eficiência de recuperação superior a 90 %. No entanto o mesmo processo de extracção registou valores de recuperação drasticamente mais baixos, da ordem dos 30 %, quando havia adição de folhas de *M. truncatula* maceradas. Essa limitação foi minimizada pela utilização de uma solução de ácido etilenodiamino tetra-acético (EDTA, na sigla inglesa) a 1 % (p/v) na fase de extracção aquosa, subindo os níveis de recuperação para cerca de 70 %. Com o melhoramento do protocolo de extracção LLE, os níveis de recuperação foram comparáveis às recuperações observadas para os outros dois métodos de extracção, as extracções ácida e a etanólica. Foi ainda observado que a principal limitação da recuperação de açúcares fosfatados nos métodos de extracção ácida e etanólica resultava do próprio processo de extracção, uma vez que não havia um decréscimo significativo quando era utilizado material vegetal na extracção. Assim, concluiu-se que o protocolo LLE apresentava maior potencial em termos de extracção de açúcares fosfatados, pelo que foi o escolhido para ser utilizado nos ensaios subsequentes.

Após a aferição dos processos extractivos, seguiu-se para o método de detecção de T6P. Utilizou-se uma coluna de cromatografia de interacção hidrofílica (HILIC, na sigla inglesa), que já tinha sido descrita pela sua capacidade de separação de açúcares fosfatados. Nas condições testadas, não foi possível distinguir entre T6P e S6P por cromatografia nem na detecção por espectrometria de massa. Foi também testado se as moléculas teriam um pico de absorção a comprimentos de onda distintos, possibilitando a sua diferenciação ao nível do Detector de diodos UV-Vis do sistema de cromatografia líquida de alta performance (HPLC, na sigla inglesa). No entanto, essa diferença não foi observada, tendo-se registado um pico de absorção aos 190 nm em soluções de ambas as moléculas.

Por esse motivo, considerou-se a utilização de um processo degradativo, que fosse selectivo para uma das moléculas, possibilitando assim a sua distinção e quantificação. Pela inexistência de enzimas comerciais que fizessem essa distinção, pensou-se na utilização de enzimas que pudessem hidrolisar as moléculas após se proceder à desfosforilação das mesmas. A invertase é um enzima comummente utilizado para a hidrólise da sacarose em glucose e frutose. Os testes em soluções aquosas de sacarose, S6P, trealose e T6P mostraram que o enzima apenas tinha a capacidade de hidrolisar a sacarose, não se tendo registado qualquer degradação nas outras amostras testadas. De seguida foi testada utilização de uma fosfatase alcalina (AP, na sigla inglesa) para a desfosforilação de T6P e S6P. A completa desfosforila-

ção de ambas as moléculas foi registada ao fim de 8 horas de incubação. Assim, concluiu-se que seria possível a distinção de T6P e S6P utilizando um processo enzimático em duas fases, onde seria promovida a desfosforilação de ambas as moléculas seguindo-se a hidrólise da sacarose, possibilitando a quantificação de trealose resultante do processo de desfosforilação. Em alternativa, foi testada a possibilidade de distinguir a sacarose da trealose utilizando trealase. Este enzima provoca a degradação da trealose levando à formação de duas moléculas de glucose. Foi provado que a trealase tinha a capacidade de hidrolisar selectivamente as moléculas de trealose, não provocando qualquer alteração em moléculas de sacarose, pelo que também poderá ser utilizado para a distinção entre T6P e S6P após desfosforilação.

O ácido abscísico (ABA) é uma hormona vegetal cuja síntese e libertação é estimulada em resposta a vários stresses, tanto bióticos como abióticos, provocando respostas fisiológicas e alteração da expressão de genes em resposta a esses mesmos stresses. A sobreexpressão de genes provocada pelo aumento da concentração de ABA nas células vegetais é atribuída essencialmente a dois motivos genéticos presentes nas sequências reguladoras, os promotores, desses mesmos genes, o ABA-*responsive element* (ABRE) e os *coupling elements* (CEs). Num trabalho anterior tinham sido estudados doze promtores que continham ambos os motivos e a expressão dos genes regulados por esses promotores foi quantificada. Para este trabalho, foram escolhidos três promotores cujas sequências foram ligadas à sequência codificante de um gene repórter, o da ß-glucuronidase (GUS).

Folíolos de *M. truncatula* foram infectados com estirpes de *Agrobacterium tumefaciens* que continham as construções promotor-gene repórter e testou-se se a adição de ABA ou a imposição de stress hídrico induzido por polietilenoglicol (PEG) ao meio de cultura levaria a um aumento da expressão do gene *gus*. A análise de expressão transiente, efectuada por contagem dos focos azuis não mostrou diferenças significaitivas na actividade de GUS. O mesmo resultado foi obtido quando se comparou a área azul estimada entre os tratamentos. Estes resultados sugerem que as diferenças ao nível da expressão de genes regulados pelos promotores utilizados poderão ser demasiado baixas para serem analisadas por ensaios de expressão transiente.

Palavras-Chave: *M. truncatula*, T6P, LC-MS, ABA, sinalização de stress

Abstract

A method for extraction and detection of trehalose 6-phosphate (T6P) in *Medicago truncatula* extracts was explored. Three extraction protocols - ethanolic, acid, and liquid-liquid - were tested in order to determine which would have the capacity to recover a greater percentage of phosphorylated sugars. Almost the complete quantity of the glucose 6-phosphate in spiked extracts was detected using the liquid-liquid extraction procedure in samples without plant material. When grinded leafs were added the recovery decreased to 30%, but the addition of EDTA improved the recovery efficiency up to approximately 70%.

The LC-MS detection of standard solutions showed that T6P could not be separated from its isomer sucrose 6-phosphate (S6P). Thus, two two-step enzymatic processes were designed. Invertase and trehalase are two enzymes that were assayed to assess whether they would specifically hydrolyze one of the isomers. Invertase was proven to hydrolyze specifically sucrose molecules, whereas trehalase only hydrolyzed trehalose molecules. The ability of alkaline phosphatase (AP) to dephosphorylate unspecifically S6P and T6P, leading to the assumption that T6P could be quantified by dephosphorylation of plant extracted metabolites with AP followed by specific hydrolysis with either invertase or trehalase, using suitable blank controls.

Abcscissic acid (ABA) is a plant hormone involved in stress signaling, that has been reported to upregulate the expression of genes controlled by promoters that include ABA-responsive element (ABRE) and coupling elements (CEs) sequences. Three reporters with ABREs and CEs were fused to the ß- glucuronidase (GUS) reporter gene and plant transformation was performed. Transient analysis of the reporter gene did not show any differences upon ABA exogenous application nor polyethylene glycol induced water stress, suggesting that changes in gene expression might be too low for detection by transient expression analysis.

Keywords: *M. truncatula*, T6P, LC-MS, ABA, stress signaling

Abbreviations

Δabs – Absorbance variation

35S – Cauliflower mosaic virus constitutional promoter

ABA – Abscisic acid

ABRE – ABA-responsive element

AP – Alkaline phosphatase

bp – Base pairs

bZIP – Basic-domain leucine zipper

CaMV – Cauliflower mosaic virus

CE – Coupling element

de35S – Double-enhanced 35S promoter

dNTP – Deoxyribonucleotide

ε – Molar absorption coefficient

EDTA – Ethylenediamine-tetraacetic acid

EIM – Embryogenic induction medium

ESI – Elecrospray ionization

EtOH – Ethanol

Fru – Fructose

G6P – Glucose 6-phosphate

G6PDH - Glucose 6-phosphate dehydrogenase

GFP – Green fluorescent protein

Glc – Glucose

GUS – ß-glucuronidase

gus:int – *gus* gene with intronic sequence

HILIC - Hydrophilic interaction chromatography

HPLC – High pressure liquid chromatography

HXK – Hexokinase

LB – Luria Broth growth medium

LC-MS – High performance liquid chromatography coupled to mass spectrometry

LLE – Liquid-liquid extraction

m/z – Mass-to-charge ratio

MEP – Methyl erythritol phosphate

MES – 2-(*N*-morpholino)ethanesulfonic acid

MS – Mass spectrometry

MS growth medium – Murashige and Skoog growth medium

MS/MS – Tandem mass spectrometry

MSOA – MS growth medium supplemented with sucrose and microagar

NADPH – Nicotinamide adenine dinucleotide phosphate

OD_{600} – Optical density at 600 nm

OtsA – Microbial genes for TPS

PCR – Polymerase chain reaction

PDA – Photodiode array

PEG – Polyethylene glycol

PVPP – Polyvinylpolypyrrolidone

RCAR – Regulatory Component of ABA Receptor

S6P – Sucrose 6-phosphate

SD – Standard deviation

SnRK1 – Sucrose non-fermenting-Related Kinases 1

SRM – Single reaction monitoring

Suc – Sucrose

T6P – Trehalose 6-phodphate

TEU – Transient expression unit

TFA – Trifluoroacetic acid

TIC – Total ion current

Tm – Melting temperatures

tnos – Nopaline synthase transcription terminator sequence

TOR – Target of Rapamycyn

TPP – Trehalose phosphate phosphatase

TPS – Trehalose phosphate synthase

Tre – Trehalose

TSS – Transcription start site

UDP – Uridine diphosphate

UDPG – Uridine diphosphoglucose

X-Gluc – 5-bromo-4-chloro-3-indolyl glucuronide

Table of Contents

List of Figures

List of Tables

1. Introduction

1.1. *Medicago truncatula*, the Model Legume

The *Leguminosae* family (also referred as *Fabaceae*) is the third largest family among angiosperms, comprising 727 genera with 20 000 estimated species worldwide, with some species of great economic and agricultural importance (Cronk, *et al.*, 2006; Wojciechowski *et al.*, 2004). Legumes represent the major source of protein and oil for both humans and animals, can be used to produce compounds with industrial and economic interest, and its nitrogen-fixing ability contributes significantly to soil improvement (Cook, 1999). Legume plants are widely distributed by different ecosystems but they're more abundant in semi-arid to arid habitats, which is thought to be related to its nitrogen-demanding metabolism (Wojciechowski *et al.*, 2004).

Medicago truncatula is generally recognized as a model organism for legume plants, widely used in research for several characteristics that makes this specie ideal for plant biology and biotechnology studies. It has a relatively short life cycle, with approximately 3 months in long day conditions (16 h of light), it's a diploid and autogamous specie with only 8 pairs of chromosomes, has small genome (500 to 550 Mbp) which is almost completely sequenced (http://www.medicagohapmap.org/?genome), somatic embryogenesis can be easily induced in some strains and efficient transformation and regeneration techniques have been developed (*e.g.*, *Agrobacterium*-mediated transformation) (Barker *et al.*, 2006; Rose, 2008).

The capacity to establish symbiotic interactions with rhizobia, that confers the ability of fixing nitrogen, and the fact that this specie is part of the *Fabaceae* family are key features that defines *M. truncatula* as a better model than *Arabidopsis thaliana* for studies regarding legume plants (Cook, 1999). *Lotus japonicus* is also a nitrogen-fixing legume that shares some characteristics with *M. truncatula*, which is also used in some research studies for having a different nodulation process (Kouchi *et al.*, 2010; Sato & Tabata, 2006). Recent comparisons of genomic data obtained from these model species support the assumption that the discoveries made in these model plants are suitable for extrapolation to other legumes (Cronk *et al.*, 2006; Young *et al.*, 2005). Importantly, several recent works supported the potentiality of using this model to deeply study the response of legumes toward water deficit (Capitão et al., 2011; Nunes et al., 2008; Trindade et al., 2010).

1.2. Water Deficit

Environmental conditions have direct effect in plant development and growth. Plant stress can be defined as the external factor that prevents the organism to reach its full genetic potential, by interfering with its normal functioning and well being (Mahajan & Tuteja, 2005). That external factor can either have a biotic or an abiotic origin. Biotic stresses are consequence of interactions of other organisms with plants, such as predation and infection, whereas abiotic stresses result from adverse conditions present in the plant's environment, which can be influenced by weather conditions (*e.g.*, water availability, temperature, wind, high radiation intensity) or soil properties (*e.g.*, nutrient content, salinity, etc.) (Mahajan & Tuteja, 2005; Mittler, 2006).

Abiotic stress is one of the main causes for crop loss every year, leading to more than 50 % of yield decrease in major crops (Valliyodan & Nguyen, 2006). Among plant stresses, drought is the most common limiting growth condition plants are exposed to, which results in great loss of productivity (Ashraf, 2010; Zhu, 2002). With present concerns about global warming, water availability, deterioration of arable land and increasing world population, it is clear that plants tent to be subjected to more adverse conditions which will lead to further decrease of productivity, and that the demand of food in the coming years will increase. For those reasons, there is a growing scientific interest in understanding the mechanisms of drought perception and resistance in order to improve drought tolerance of economically relevant species (Mittler, 2006; Valliyodan & Nguyen, 2006).

Plants are sessile organisms that perceive and respond to changes in its environment through physiological and biochemical processes (Moore & Sheen, 1999). Plants may have constitutive traits that prevent or delay cellular stress, or they can have very efficient mechanisms to respond to such stresses - adaptive traits. Constitutive traits include characteristics such as bigger root or cuticle thickness, plant size and growth rate, enhanced photosynthesis efficiency, altered carbon metabolism, etc (Kulkarni & Phalke, 2009; Valliyodan & Nguyen, 2006). Adaptive traits are only expressed in the presence of drought stress.

To survive through a drought episode, the gene expression pattern is altered, having a different type and duration of response depending on the type and severity of stress they sense on their environment (Baena-González & Sheen, 2008). Exactly how the stress is perceived and how that perception leads to a specific response is not completely understood yet, but some of the mechanisms were already unveiled. Normally, when water availability decreases, plant cells lose turgor pressure which destabilizes osmotic balance, membrane fluidity and composition, and protein interactions (Barnabás et al., 2008; Chaves et al., 2003; Zhu, 2002).

Such alterations are thought to activate several signaling mechanisms to restore cell homeostasis and prevent cellular (and plant) death.

1.3. Sugar Signaling

Sugars are usually recognized as parts of metabolic pathways, fluctuating passively according to physiological and genetic responses to environmental inputs (Rolland *et al.*, 2002). However, they also play a relevant role in plant development, stress sensing and response modulation. Sucrose (Suc) is the main storage and transport carbohydrate in plants, and its levels in plant tissues determine plant growth and important developmental processes, such as shoot development, time of flowering, seed maturation, etc. Other carbohydrates can function as signaling molecules as well. In fact most processes described involving sugar signal pathways involve phosphorylated sugars pool which are metabolically linked to sucrose biosynthesis and catabolism.

Stresses cause alterations in carbohydrate metabolism which activate signaling pathways leading to stress response. In general, transduction signals in response to plant stress involving metabolites either promote plant growth – such as the hexose signaling systems, trehalose 6-phosphate (T6P) signal and the Target of Rapamycyn (TOR) kinase system – or inhibit it – *e.g.* Sucrose non-fermenting-Related Kinases 1 (SnRK1) and C/S1 bZIP transcription factor network (Smeekens *et al.*, 2010). From all characterized sugar signal transduction system, the T6P signal is the most extensively studied and will be described below in greater detail.

The hexose sensing system involves two distinct pathways: hexokinase (HXK)-dependent and HXK-independent. The HXK-dependent pathway requires sugar phosphorilation and is responsible for photosynthesis inhibition on high sugar content. The HXK-independent pathway recognizes the same molecules from the former one, but does not require sugar phosphorylation, and is responsible for activating a signaling cascade which will lead to gene expression encoding cell wall invertase (CIN), sucrose synthase (SuSy) and phenylalanine ammonia lyase (PAL) (Gupta & Kaur, 2005; Rolland *et al.*, 2002).

The TOR protein kinase signaling pathway is a highly conserved system among eukaryotes, controlling growth, development, and senescence processes (Menand *et al.*, 2004). TOR kinases sense glucose (Glc) levels and TOR–Raptor complex activates the ribosomal protein S6 (rpS6) kinase regulating ribosome biosynthesis and enhancing translation (Smeekens *et al.*, 2010).

SnRK1 is subfamily of protein kinases that regulate plant responses to drought stress modulating directly enzyme activity and gene transcription (Baena-González & Sheen, 2008). A heteromeric complex is responsible for sensing energy and carbon status interacting with genetic regulatory factors involved in response of metabolism and growth to starvation (Halford & Hardie, 1998; Halford, 2003; Zhang *et al.*, 2009). More than 300 genes involved in biosynthetic processes, such as protein, nucleotide, cell wall, lipid, etc, are down regulated by SnRK1 (Baena-González & Sheen, 2008).

1.3.1. Trehalose and Trehalose 6-phosphate

Trehalose (Tre) is a non-reducing disaccharide composed by two glucose monomers linked at the reducing ends. Although other conformations of this disaccharide can be synthesized, such as α,ß-1,1 (neotrehalose) or ß,ß-1,1 (isotrehalose), the only natural occurring form is the α,α-1,1 trehalose) (Richards *et al.*, 2002). There are at least five known trehalose synthesis pathways in living organisms, but only one of them seem to be performed by plant cells - the OtsA-OtsB pathway (Paul *et al.*, 2008). Trehalose phosphate synthase (TPS) binds a glucose-6-phosphate (G6P) molecule to a uridine diphosphoglucose (UDPG) unit, forming T6P and uridine diphosphate (UDP). T6P is then dephosphorylated by trehalose phosphate phosphatase (TPP) to form trehalose and inorganic phosphate. Trehalase can then cleave trehalose, forming two glucose units (Paul, 2007; Wingler, 2002).

The first report of trehalose is generally attributed to H.A. Wiggers in 1832 on solutions of the ergot rye, and ever since its presence has been reported in a wide range of organisms (Richards *et al.*, 2002). The enzymes for the several trehalose formation pathways are present in genomes of almost all groups of organisms, except for vertebrates (Avonce *et al.*, 2006; Paul *et al.*, 2008). This ubiquity among living organisms is often attributed to its function, which varies among species, either functioning as an osmoregulator or as the main storage carbohydrate and transport sugar.

Due to the inability to detect trehalose in most plant species, it was thought to be inexistent among them, except for some marginal species that had the ability to survive through episodes of severe drought stress, known as resurrection plants (Fernandez *et al.*, 2010). It was only in 1997 that trehalose was detected in cultures of potato (*Solanum tuberosum*) supplemented with validamycin A, a specific trehalase inhibitor, showing that plants were able to synthesize trehalose but did not accumulate the metabolite in high concentration (Goddijn *et al.*, 1997). The same authors introduced genes from *Escherichia coli* encoding enzymes of trehalose synthesis pathway in tobacco (*Nicotiana tabacum*) and potato under the control of

the cauliflower mosaic virus (CaMV) constitutional promoter (35S), obtaining plants with altered growth and development, suggesting that trehalose biosynthesis in plants should be regulated according to development stage and tissue identity.

Further developments in plant trehalose research came from complete genome sequencing projects, which lead to the identification of genes involved in trehalose synthesis pathway in most sequenced genomes (Paul *et al.*, 2008). Surprisingly, 11 coding sequences for trehalose phosphate synthase and 10 genes coding for trehalose phosphate phosphatase were found in the *Arabidopsis thaliana* genome, whereas only one gene for trehalase was found (Leyman *et al.*, 2001). This abundance of genes for trehalose synthesis and the conservation of coding sequences among higher plant species showed that this pathway was very important for plant survival and stimulated the research on this area.

Trehalose is known to prevent cell collapse during abiotic stress episodes, particularly in drought and heat stresses (Iordachescu & Imai, 2008). Trehalose can form hydrogen bonds to polar residues preventing protein denaturation and promoting membrane stabilization in plants exposed to abiotic stress (Elbein *et al.*, 2003). Furthermore, trehalose accumulation during drought stress can function as a compatible solute maintaining cell turgor, promoting vitrification in severe drought stress conditions, which preserves dry macromolecules and prevents radical diffusion (Fernandez *et al.*, 2010; Goddijn & Smeekens, 1998). Unlike sucrose, trehalose molecules remain stable when exposed to high temperatures (up to 100 °C) and pH levels from 3.5 to 10. However, it has been shown that high trehalose concentrations are toxic to plant cells, incompatible with chaperone-assisted protein folding (Wingler, 2002).

T6P is an intermediate of the trehalose synthesis pathway, but it has also been proved to be a very important signaling molecule for plant abiotic stress, and its concentration increase in cytosol is known to modulate important physiological and genetic processes, involving carbon storage, development and response to stress. T6P accumulation leads to adenosine diphosphoglucose pyrophosphorylase (AGPase) activation, leading to starch synthesis (Kolbe *et al.*, 2005; Lunn *et al.*, 2006). *A. thaliana* mutants lacking TPS1 cannot survive beyond torpedo stage, which is thought to be related to T6P-deppendent coordination of metabolism and development (Debast et al., 2011; Gómez et al., 2010). Trehalose 6-phosphate is also an inhibitor for hexokinases and SnRK1, which are involved in regulation of transcription of several genes involved in metabolic and developmental regulation processes in response to limiting energy and carbon supply. Thus, T6P accumulation during drought stress leads to activation of biosynthetic and growth processes, and suppresses degradation of cellular compounds, that will result in enhanced tolerance to stress (Debast et al., 2011; Martínez-Barajas et al., 2011).

Considering all known trehalose and T6P functions in plants, several groups have tried to introduce genes from its metabolic pathway attempting to enhance productivity and drought tolerance. Initial transformations were carried out using microbial genes for TPS (OtsA) under the control of the constitutional 35S promoter (Goddijn *et al.*, 1997), which led to phenotypic aberrations and low levels of T6P accumulation. These limitations were overcome using constructions of TPS-TPP fusion proteins, regulation by a stress inducible promoter or more recently, using TPS genes from *A. thaliana* genome (Iordachescu & Imai, 2008). Those alterations led to T6P accumulation and enhanced stress tolerance in several species, such as: *Arabidopsis thaliana*, *Solanum tuberosum*, *Nicotiana tabacum*, *Oryza sativa*, among others (Almeida *et al.*, 2007; Iordachescu & Imai, 2008; Li *et al.*, 2011; Pellny *et al.*, 2004).

As mentioned above, trehalose measurement and characterization in plant tissues were performed in the last few years. However, most important discoveries regarding trehalose relevance in plants were made using molecular biology techniques, with transgenic plants either overexpressing or lacking enzymes from trehalose synthesis pathway. Although important insights were obtained by these techniques, the difficulty to measure trehalose and T6P and considering their important role in plant development led to a limitation of knowledge advance in this area. It was not until 2006, that Lunn *et al.* developed a efficient method for T6P extraction and quantification using anion-exchange liquid chromatography coupled to a mass spectrometer detector (LC-MS). The technique developed was then enhanced and more accurate T6P estimations have been made (Delatte *et al.*, 2011; Delatte *et al.*, 2009; Paul, 2007). However, there still is great difficulty in the separation of T6P from its isomers by the high pressure liquid chromatography (HPLC) system and in their distinction by MS detection. The main problem remains with sucrose 6-phosphate (S6P), which has a very similar structure to T6P, that results in equivalent affinity to the HPLC column, producing the same fragmentation pattern on MS, and existing in levels too low for tandem mass spectrometry (MS/MS) detection. Antonio *et al.* (2007) reported a method for S6P and T6P separation in a hydrophilic interaction chromatography (HILIC) column, however the separation achieved was partial and only resulted in standard solution mixture. More recently, a using capillary electrophoresis coupled to MS, Delatte *et al.* (2011) were able to efficiently separate T6P from its isomers, namely S6P and lactose 1-phosphate (L1P), and quantify it in *A. thaliana* seedling extracts.

Many recent advances have been made in T6P quantification, but a simple method to quantify unambiguously this metabolite in plant extracts is still not available, limiting the knowledge advances about its function in plants. It is therefore urgent to establish a simple and efficient method for T6P determination.

1.3.2. ABA-dependent Response

Abscisic Acid (ABA) is a ubiquitous plant hormone (phytohormone) involved in various stages of plant development and response to several stresses (both biotic and abiotic). ABA is an isoprenoid which is formed by cleavage of C_{40} carotenoids originated from the Methyl Erythritol Phosphate (MEP) pathway (Nambara & Marion-Poll, 2005). The first steps of ABA synthesis are performed in chloroplasts and other plastids from an intermediate in the carotenoid biosynthesis pathway. Zeaxanthin epoxidase (ZEP) converts zeaxanthin to cis-isomers of violaxanthin and neoxanthin, which are cleaved by 9-*cis*epoxycarotenoid dioxygenase (NCED) and form the precursor of ABA (xanthoxal). Then, xanthoxal is converted in the cytosol to ABA-aldehyde by a short-chain dehydrogenase/reductase (SDR/AtABA2), which is finally oxidized by a family of aldehyde oxidases to form the biologically active abscisic acid molecule (Ji *et al.*, 2011; Nambara & Marion-Poll, 2005; Seo & Koshiba, 2002).

ABA is able to regulate plant response to stress both by triggering physiological and genetic responses. One of the most well characterized physiological responses to ABA increase is the loss of turgor by guard cells leading to stomatal closure, which avoids transpiration in water limiting environments and also pathogen entry (Cao *et al.*, 2011). Other abscisic acid well known functions involve root growth, seed dormancy, control of membrane ion transporters, among others (Cutler et al., 2010, Lumba et al., 2010).

ABA-dependent stress response is a very complex and finely regulated process, with several identified ABA receptors and potential targets. Receptors such as FCA (a flowering-time control protein), the Mg-chelatase H subunit, and the G protein coupled receptor GCR2, among others have been extensively characterized (Cutler *et al.*, 2010; Hirayama & Shinozaki, 2007; McCourt & Creelman, 2008; Umezawa *et al.*, 2010; Wasilewska *et al.*, 2008). However, recent discovery of soluble ABA receptors – the PYR/PYL/RCAR – (Ma *et al.*, 2009) provided a more direct link between perception and response through ABA signaling (Melcher *et al.*, 2010). The receptor is usually a heteromeric complex formed by a type 2 protein phosphatase (PP2C); an Abscisic acid Insensitive protein (ABI) and Regulatory Component of ABA Receptor (RCAR) which is present both in the nucleus and in the cytosol. The binding of ABA to the complex leads to phosphatase inhibition activity which results in release of protein kinases, such as sucrose non-fermenting-related kinases (SnRK) and open stomata (OST), and key targets of ABA signaling pathway are activated. Those targets can either be proteins, in which case immediate physiological response is achieved, or transcription factors, that will enable expression of specific genes (Melcher *et al.*, 2010; Raghavendra *et al.*, 2010; Weiner *et al.*, 2010).

Genes expressed after ABA stimulation usually have specific motifs on their promoter sequences such as the ABA-responsive element (ABRE) and coupling elements (CEs) (Chandler & Robertson, 1994; Lee *et al.*, 2010). Transcription factors, such as ABRE-binding factors (ABFs) or ABRE-binding proteins (AREB) have basic-domain leucine zipper (bZIP) and bind to ABRE motifs and leading to ABA-dependent gene expression. It has been proven that a single ABRE motif is not sufficient to induce gene expression in presence of ABA and it is necessary another ABRE or a CE in the promoter to produce ABA-dependent gene transcripts (Kazuko Yamaguchi-Shinozaki & Kazuo Shinozaki, 2005).

Although signaling pathways are characterized independently, most plant development and stress responses are determined by integration of complex environmental stimuli and interaction of signaling processes. Recent research on signaling interaction between plant hormones and sugar sensing pathways showed that these two processes are integrated to regulate seed development, germination and stress responses (Gibson, 2004; León & J Sheen, 2003). Furthermore, combination of stresses leads to differences in signaling and response mechanisms. For that reason, transgenic plants with enhanced tolerance to a particular stress usually do not show the same tolerance level in field experiments, where other factors such as temperature or light, among others, are limiting plant production as well (Mittler, 2006). With technology advances, 'omic' approaches are gaining relevance among the scientific community. In this particular area, transcriptomic, proteomic and metabolomic research may reveal important clues in how plant engineers and/or breeders can enhance plant abiotic tolerance (Stitt *et al.*, 2010; Zhu & Snyder, 2002).

1.4. Plant genetic engineering

Plant biotechnology is, in its broadest sense, the exploitation of plant species for the benefit of mankind, which is in constant development and had registered innumerous technological advances in the past few decades (Flavel, 2004). Plant genetic manipulation technologies allow the introduction of genes from one species to another even if they are not closely related, thus increasing the available gene pool for trait improvement (Chan, 2010).

For the improvement of traits involved in adaptation to environmental conditions, such as drought stress, usually it is only necessary that the gene introduced is expressed when stress conditions are imposed. Furthermore, genes involved in drought stress tolerance are often part of metabolic pathways or can have a role on developmental processes. As mentioned above, when such genes have constitutive expression pattern, phenotypic aberrations

may occur and crop improvement is not achieved (Goddijn *et al.*, 1997). For that reason, researchers are currently concerned in choosing appropriate promoters for genes that improve stress tolerance.

One common approach to analyze promoter functionality is to use the sequence to be analyzed regulating the expression of a reporter gene, *e.g.* ß-glucuronidase (GUS) (Basu *et al.*, 2003). When the promoter is active the reporter gene should be easily detected. In the case of the *gus* gene product, histochemical detection of ß-glucuronidase activity can be detected after infiltration and overnight incubation with its substrate, 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) or 4-methylumbelliferyl-beta-D-glucuronide (MUG) (Jefferson *et al.*, 1987).

2. Objectives

The aim of the present wok was to contribute with new knowledge and methodologies in order to better understand *Medicago truncatula* responses to water deficit. To achieve this broad goal, two specific objectives were defined and each one corresponds to the different parts of this dissertation.

In the first part, the objective of the work done was to establish a method for T6P extraction and quantification towards its variation analysis in *M. truncatula* under water deficit. In order to achieve that, several extraction procedures were tested and adapted to determine which was the most efficient in phosphorylated sugars extraction from plant tissues. Additionally chromatographic and enzymatic methodologies were tested to achieve unambiguous T6P detection.

The main goal of the second part of this thesis was to assess the functionality of three abscissic acid inducible promoters previously identified by sequence analysis in *M. truncatula* genome. Promoter-reporter gene (*gus*) were constructed and inserted into EHA105 strain of *Agrobacterium tumefaciens*. Using a transient expression assay, the inducibility of the promoters was studied by histochemical detection of GUS activity in *M. truncatula* leaf explants after ABA application and polyethylene glycol water stress induction.

3. Materials and Methods

3.1. Plant Material and Growth Conditions

Medicago truncatula cv. Jemalong genotype M9-10a seeds were scarified, sterilized and germinated as described in Araújo *et al.* (2004). Seeds were chemically scarified by immersion in concentrated anhydrous sulfuric acid (Sigma-Aldrich) for approximately 8 minutes and washed with cooled sterile water. Then, the seeds were then surface sterilized by immersion in a solution of 50 % (v/v) commercial bleach and 50 % (v/v) detergent Domestus 3 for 5 minutes, washed three times with sterile distilled water and disinfected again with70 % (v/v) ethanol for 2 minutes. After an additional washing step with sterile distilled water, the seeds were left immersed in water for 20 minutes and plated in Petri dishes with sterile absorbent paper imbibed in a diluted solution (1:2) of growth-regulator-free growth medium Murashige and Skoog (1962) (MS) basal salts and vitamins, 3 % (w/v) sucrose, pH = 5.8 (10 to 12 seeds *per* Petri dish).

Seeds were left for 4 days at 4 ºC in dark conditions and then transferred to a growth chamber at 22 ºC where germination occurred in dark conditions (Heraeus). After 2 days, seedlings were placed at light conditions in a growth chamber (Phytoclima 700 EDTV, Aralab, Portugal) with 16 hour photoperiod of 100 μE m^{-2} s^{-1} and 24/22 ºC of day/night temperature. Two weeks later, seedlings were transferred to a tray, containing watered vermiculite, covered with cling film (Silvex, Portugal) and kept in the same growth chamber for more 3 weeks. During that period, seedlings were watered with tap water when necessary and the cling film was progressively ruptured to achieve plant acclimation.

Finally, the seedlings where transferred to 500 mL plastic pots containing a 2:2:1 (v/v) mixture of commercial soil ("*terra de Montemor*", Horto do Campo Grande, Lisbon, Portugal), vermiculite and sand, grown in a growth chamber with a 16/8 hour light–dark cycle with approximately 45 μmol m^{-2} s^{-1}, and 24/21 ºC day/night. Plants were irrigated every other day with tap water until maximum soil water content was achieved. Fully expanded leafs from eleven week-old plants were excised approximately 3 hours after the beginning of the photoperiod and snap frozen in liquid nitrogen and kept at -80 ºC until metabolite extraction was performed.

3.2. Soluble Sugar Extraction Procedures

In all tested extraction procedures, the recovery efficiencies were monitored by addition

of glucose 6-phosphate solution in early steps of extraction, both in presence and absence of plant material.

3.2.1. Ethanolic Extraction

Extraction of soluble sugars was essentially done as reported by Dickson (1979). Approximately 50 mg of leaf material were grind to a fine powder with liquid nitrogen with a mortar and pestle. Ethanol (EtOH) at 80 % (v/v) with 0.1 % (w/v) ethylenediamine-tetraacetic acid (EDTA) at 80°C was added to the powder and the material was transferred to a polypropylene microfuge tube and heated 80 °C for 5 minutes. Samples were then centrifuged at 16000 x*g* (Centrifuge 5424, Eppendorf) for 5 minutes at room temperature and the supernatant was collected. To remove photosynthetic pigments and macromolecular compounds, active charcoal was added and the resulting mixture was passed through a polyethersulfone membrane with 0.45 µm of pore size (Minisart HF, Sartorius). Then, the supernatant was evaporated to dryness at 80 ºC with a nitrogen (N2) flow and resuspended in 350 µL ultra-pure water.

Aiming to achieve higher recovery efficiency of phosphorylated sugars, a few variations to the protocol were assayed. The adjustments included the use of intact leafs that were boiled for 10 minutes, with an intermediate change of solvent to avoid saturation, and the dilution of ethanol to 50 % (v/v), increasing the available water to solubilize the phosphorylated sugars. Both variations have been reported in (Arrabaça, 1981).

3.2.2. Acid Extraction

Acid extraction procedure was also assayed using part of a protocol described by Almeida *et al.*, (2006). Approximately 50 mg of frozen leaf tissue was grinded using a Retsch TissueLyser MM 300 Ball Mill Homogenizer with liquid nitrogen and a shaking frequency of 30Hz for two cycles of 1 minute each. 3.5 % (v/v) Trifluoroacetic acid was added to the samples (1.5 mL), followed by 30 seconds of vortex. Samples were kept on ice for 10 minutes and shaken again for 30 seconds. Finally, samples were centrifuged at 16000 x*g*, supernatants were collected, snap frozen in liquid N_2 and evaporated to dryness using a centrifugal vacuum dryer (SpeedVac®, Savant) at room temperature. Before metabolite determination, samples were resuspended in 350 µL of ultra pure water.

3.2.3. Liquid-Liquid Extraction

Other metabolite extraction procedure tested was the liquid-liquid metabolite extraction

(LLE) procedure described by Delatte *et al.* (2009) . Approximately 50 mg of plant leafs were grind to powder using the same procedure as in the acid extraction, following addition of chloroform/acetonitrile (3:7, v/v). After vigorous shaking for 30 seconds, samples were maintained at -20 °C for 2 hours, shaking every 10 minutes. Phosphorylated sugars were extracted three times, with addition of 400 µL of ultra pure water at 4 °C, vigorous shaking for 30 seconds and centrifugation at 13,000 x*g* for 5 min at 4 °C. The recovered aqueous phases were then pooled, evaporated to dryness using a centrifugal vacuum dryer at room temperature and reconstituted in ultra pure water before metabolite determination.

To enhance recovery efficiency of phosphorylated sugars the effects of the addition of adsorbents (1 % w/v of polyvinylpolypyrrolidone, PVPP), chelating agents (0.1 % and 1 % w/v of ethylenediaminetetraacetic acid, EDTA), and detergents (1 % v/v of Triton X-100) in the aqueous extraction step were tested. Triton X-100 is a non-ionic detergent commonly used in extraction procedures to avoid adsorption of molecules of interest to plant debris (Deutsher, 1990). EDTA is a polyamino carboxylic acid used in several extraction protocols to chelate divalent ions that are often co-factors of enzymes cause active degradation of other metabolites (Deutsher, 1990). Finally, phenolic oxidation of sugars is a common phenomenon in plant extracts. Usually, insoluble PVP is added to absorb phenolic compounds (Dawson et al., 1986), thus avoiding oxidation and not interfering with metabolite analysis.

3.3. Enzymatic Assays

In an attempt to selectively modify S6P or T6P, aiming for unambiguous T6P quantification, a few enzymatic assays have been performed.

3.3.1. Invertase

Invertase (β-fructofuranosidase, EC 3.2.1.26) is a commercially available enzyme catalyzes the hydrolysis of sucrose into glucose and fructose. Invertase (Sigma-Aldrich) assays were performed as described in the producer's protocol. Briefly, 0.5 mM standard solutions of sucrose, trehalose, S6P and T6P were incubated with 0.034 invertase units in a 0.9 mM solution of sodium acetate buffer pH 4.5 for 20 minutes at 55 °C. Enzyme units ranging from 0.05 to 1, sugar concentration varying from 0.05 to 0.5 mM, and incubation periods from 30 min to 16 h were assayed aiming to obtain full hydrolysis in the sucrose standard solution. Sample hydrolysis was evaluated by high performance liquid chromatography-mass spectrometry (LC-MS).

3.3.2. Alkaline Phosphatase

Calf Intestinal Alkaline Phosphatase (AP, EC 3.1.3.1) (Invitrogen) was tested for the dephosphorylation of T6P and S6P. To assess whether there would be selective catalysis among those metabolites, 0.5 mM standard solutions of each metabolite were inoculated with AP following the producer's instructions and using the supplied buffers. The improvement of the enzymatic catalysis was performed using 0.5 mM of T6P, testing incubation periods from 30 min to 8 h. It was also tested whether AP was able to perform dephosphorylation in acidic solutions, using 50 mM sodium acetate buffer pH 4.5. Enzymatic activity was assessed by LC-MS detection.

3.3.3. Trehalase

Trehalase (α,α-Trehalose glucohydrolase, EC 3.2.1.28) hydrolyzes trehalose into two glucose molecules. Trehalase (Sigma-Aldrich) activity was assayed in 0.5mM standard solutions of trehalose, sucrose, T6P and S6P. Trehalase, 0.01 units, was added to the solutions in 50 mM of 2-(*N*-morpholino)ethanesulfonic acid (MES) pH 5.8 and incubated at 37 ºC for 8 hours. Hydrolysis analysis was performed by LC-MS detection.

3.4 Metabolite detection

3.4.1. Glucose 6-Phosphate Quantification

To determine the recovery of phosphorylated sugars in the tested extraction procedures, a Sucrose/D-Glucose/D-Fructose quantification kit (Boehringer Mannheim, Roche) was used. Glucose 6-phosphate (G6P) levels were determined by measuring the Nicotinamide adenine dinucleotide phosphate (NADPH) formed in the reaction catalyzed by glucose 6-phosphate dehydrogenase (G6PDH):

$$G6P + NADP^{+} \rightarrow gluconate\ 6\text{-phophate} + NADPH + H^{+}$$

The variation of NADPH in solution is stoichiometric with the amount of G6P oxidized and its absorbance was measured at 340 nm (in a Helios Beta UV/Vis spectrophotometer, Thermo Electron Corporation, USA), using 150 µL of plant extract in a final volume of 1 mL. Difference in absorbance before and after the addition of 0.114 U G6PDH was used to determine the variation of NADPH concentration by the Beer-Lambert law:

$$\Delta abs = c \times \varepsilon \times l$$

Where, Δabs represents the absorbance variation caused by G6PDH addition; c is NADPH concentration on solution; ε is the molar absorption coefficient, which for NADPH at 340 nm is $6.22 \times 10^3\ M^{-1}\ cm^{-1}$, and l represents the path length (1 cm).

3.4.2 T6P and S6P Absorbance

To assess whether T6P and S6P had absorption peaks in different wavelengths, the absorbance of 2.5 mM and 5 mM standard solutions of each metabolite, respectively, was measured every 0.2 nm at wavelengths comprised between 190-700 nm (UV500 UV-Visible spectrophotometer, Thermo Scientific, USA). All measurements were performed with simultaneous comparison to absorbance of ultra-pure water as a reference.

3.4.3 LC-MS Assay Conditions

LC-MS sample analysis were carried out on a Thermo Finnigan Surveyor HPLC system consisting of a MS pump, an autosampler, a column compartment unit and a photodiode array (PDA) detector coupled to a Thermo Finnigan LTQ Linear Ion Trap mass spectrometer equipped with elecrospray ionization (ESI) source.

Chromatography was performed using a SeQuant™ ZIC®-pHILIC column (150 x 2.1 mm, 5 μm polymeric beads) at room temperature and using an injection volume of 5 μL. The mobile phase was composed by solvent A, 6 mM ammonium acetate with 0.1 % (v/v) formic acid pH 3.6, and solvent B, acetonitrile containing 0.1 % (v/v) of formic acid, using the gradient program summarized in Table 1, with a flow rate of 200 μL min^{-1}.

Table 1. Gradient program used in metabolite analysis. Solvent A - 6mM ammonium acetate with 0.1 % (v/v) formic acid pH 3.6; Solvent B - acetonitrile with 0.1 % (v/v) formic acid.

Time (min)	Solvent A (%)	Solvent B (%)
0	0	100
5	5	95
7.5	30	70
8	90	10
10	10	90
20	0	100

The MS was operated in negative ion mode with 3 kV ionization potential and 300 ºC heated capillary temperature. Optimization conditions for detection of each metabolite were performed by direct infusion using an infusion pump incorporated in the mass spectrometer. Mass spectra were initially obtained at the mass-to-charge ratio (m/z) scan range 50-1000 for

precursor ion selection. Tandem mass spectrometry (MS/MS) spectra for analysis of the selected precursor ions were generated by collision-induced dissociation (CID) with optimized ion optics parameter settings and using helium as the collision gas at 35 V. Selected reaction monitoring (SRM) was used to detect the most abundant fragment of the selected precursor ions. Mass spectra were obtained using a scan range of 2 *m/z* adjusted to the metabolite to be analyzed (Table 2). All the acquired MS data was used for qualitative analysis purpose only, no quantification of the analyzed metabolites was performed. Comparisons were only made between SRM chromatograms for the fragment ion in study for samples with equal starting concentrations and in experiments carried out at the same day.

The Xcalibur, Processing Setup software (Thermo Finnigan) was used for LC-MS instrument control and data acquisition. Integration of the chromatographic peaks were done manually, at the 12-13 min expected retention time, with adjustments when adequate.

Table 2. Precursor ions and fragments analyzed after collision-induced decomposition for metabolites detected by MS.

Metabolite	Precursor Ion (*m/z*)	Analyzed Fragment (*m/z*)	Mass Range (*m/z*)
T6P	421	241	240-242
S6P	421	241	240-242
Trehalose	341	179	178-180
Sucrose	341	179	178-180
Glucose	225	179	178-180
Fructose	225	179	178-180

3.5. Promoter Isolation and Characterization

3.5.1. Promoter Choice and Primer design

Three previously identified ABA and drought stress inducible promoters (Cordeiro *et al.*, unpublished results) were chosen for analysis of inducibility. Promoter G02 controls the expression of a *myo*-inositol-1-phosphate synthase (CR940309), G03 is the promoter of a protein with glycosyl transferase and trehalose-phosphatase catalytic sites (AC157348), homologous to AtTPS11, and promoter G08 is for a dehydrin (AC141922). Sequences from *M. truncatula* genome were obtained by bidirectional blast in the *Medicago truncatula* HapMap Project website (http://www.medicago.org/genome/), using the 400 bp sequences described in Cordeiro *et al.* (2008), and 1 kb sequences, starting 950 bp upstream and 50 bp downstream of the identified transcription start site (TSS) were retrieved (Annex 1). A ß-glucuronidase (gus) gene with a plant intronic sequence (gus:int) from plasmid pMP2482 was chosen as reporter gene. The coding sequence had also a green fluorescent protein (gfp) fused and a

strong terminator sequence, the nopaline synthase terminator sequence (tnos) (Quaedvlieg *et al.*, 1998).

Primers were designed using Primer3 online software (Rozen & Skaletsky, 2000) using default settings, choosing primer pairs with higher melting temperatures (Tm) and longer polymerase chain reaction (PCR) products. At the 5' end of each primer, gateway recombination sequences were added according to the MultiSite Gateway® Pro booklet (Invitrogen). Complete primer sequences can be found in Annex 2.

3.5.2. Promoter and Reporter Gene Cloning

Genomic DNA from leafs of the above mentioned *Medicago truncatula* cv. Jemalong genotype M9-10a plants and plasmid DNA were extracted using a DNeasy Plant Mini Kit and a MiniPrep Kit (Qiagen) respectively, according to the manufacturer's instructions.

PCR reactions were carried out using 10 ng of *M. truncatula* genomic DNA, for promoter isolation, or pMP2482, for reporter gene isolation, 1x HF Buffer, 200 µM of each dNTP, 0,5 µM specific primers (both forward and reverse), 3 % (v/v) DMSO, 2.5 mM $MgCl_2$ for promoters or 3 mM for gus:int, and 0.4 U of Phusion® Hot Start High-Fidelity DNA Polymerase (Finnzymes). Amplifications were performed at 98 °C for 30 s, followed by 35 cycles at 98 ºC for 10 s, specific primer annealing temperature for 30 s, and 72 °C for 1 min and 45 s, with a final extension step of 72 °C for 5 min in a thermocycler Biometra T-Gradient. Primer annealing temperatures used were: 61 °C for G02, 65 °C for G03, 68 °C for G08, and 64 °C for reporter gene.

PCR amplification products were identified by electrophoresis in a 0.5x TBE, 1 % (w/v) agarose gel stained with SYBR® Safe (Invitrogen) at 100 V for 25 min. Amplified DNA fragments with the predicted size were excised from the agarose gel, illuminated by a UV-light at 280 nm, with a scalpel blade and purified from the agarose gel using a QIAquick Gel Extraction Kit (Quiagen), according to the manufacturer's instructions.

3.5.3. Expression Vector Constructions

Figure 1 schematically represents the process followed for expression promoter construction. Promoter-reporter gene plasmid constructs were developed using a MultiSite Gateway® Pro 2.0 system (Invitrogen) according to manufacturer's guidelines. Briefly, 50 ng of G02 and G03, and 100 ng of G08 of purified PCR products were added separately to 75 ng of pDONR™221 P1-P5r with BP Clonase™ II enzyme mix performing the BP recombination

reaction at 25°C for 1 h. 100ng of reporter gene purified PCR product were added to 150 ng of pDONR™221 P5-P2 along with BP Clonase™ enzyme, performing the recombination reaction at 25 °C for 12 h.

Chemically competent *Escherichia coli* cells were transfected with 2 µl of the resulting recombination reactions. Briefly, 50 µl of competent cells were incubated with recombinant plasmids for 30 min on ice, heat-shocked at 42 °C for 40 s, and ice cooled again. The cells were allowed to recover in 250 µl of Luria Broth (LB) growth medium and incubation at 37 °C for 1 h with agitation at 100rpm. One aliquot of 100 µl of the cell suspension was then spread in solid LB (with 1.5 %, w/v, microagar) supplemented with 50 µg ml^{-1} of kanamycin, and incubated at 37 °C over night.

Recombinant colonies were selected by colony PCR using the M13 primers Annex 2. PCR reactions were performed using 1x green GoTaq® reaction buffer, 200 µM of each dNTP, 0.5 µM M13 primer pair, 3 mM $MgCl_2$, and 1 U GoTaq™ DNA Polymerase (Promega), in a final volume of 25 µL. PCR amplifications were carried out in a T-Gradient thermocycler (Biometra, Germany). After the initial denaturation step at 95 °C for 5 min, 35 cycles of amplification occurred with the following cycling profile: denaturation at 95 ºC for 30 s, annealing at 55 ºC for 30 s, and extension at 72 °C for 1 min (for promoter amplification) or 3 min and 30 s (for reporter gene). A final extension step of 72 °C for 5 min was included. PCR products were identified by electrophoresis in a 0.5x TBE, 1 % (w/v) agarose gel stained with SYBR® Safe (Invitrogen) at 100 V for 25 min, and visualized at a Gel Doc 1000 (Bio-Rad) under UV-light. The colonies with the expected PCR product length Annex 1 were inoculated in 4 mL of LB medium supplemented with 50 µg ml^{-1} of kanamycin and incubated overnight at 37 °C with gentle agitation (100 rpm), for plasmid extraction.

Plasmids were extracted as previously described and relative quantification was estimated through standard agarose gel electrophoresis, by comparison with different concentrations of lambda phage DNA (Invitrogen). To confirm correct sequence selection and check for point mutations, plasmids were sequenced by StabVida Lda using M13 primer pair Annex 2.

After proper sequence confirmation, LR recombination reaction was performed using each of the plasmids with promoter sequences, the plasmid with the reporter gene and plasmid pKGW, the destination vector wit suited recombination sites, purchased from Gent University (http://gateway.psb.ugent.be). LR recombination occurred at 25 °C for 16 h. *E. coli* transformation procedure was performed as described earlier, and colonies were selected by colony PCR using Phusion® Hot Start High-Fidelity DNA Polymerase and specific promoter and

gene reporter primers as described in section 3.5.2. The colonies with the desired PCR products were amplified as described earlier in this section. Plasmid extraction procedure was followed as already stated for *Agrobacterium tumefaciens* transformation.

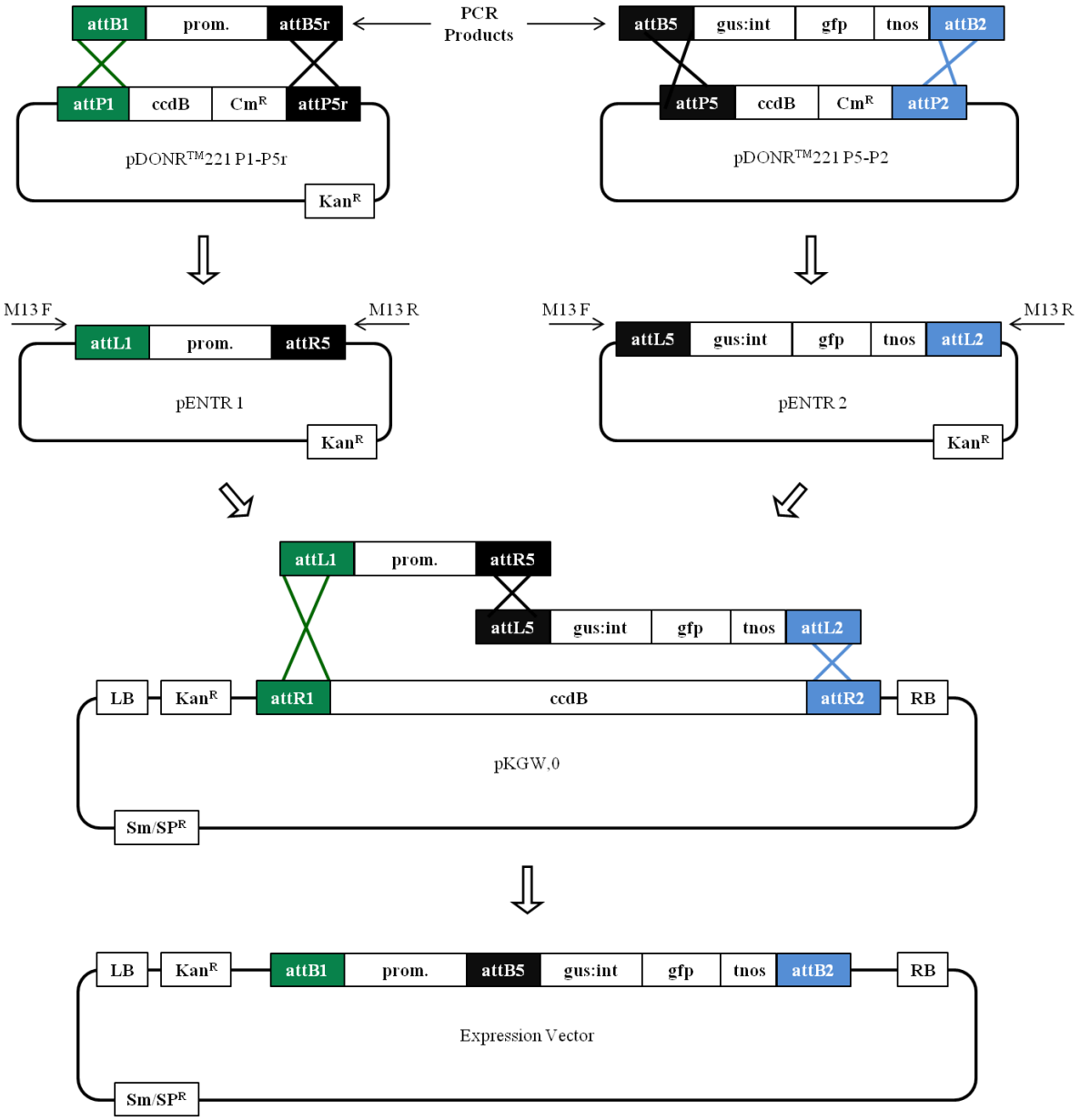


Figure 1. Representative scheme for construction of expression vectors. Promoter entry clones were constructed by the BP reaction using pDONR™221 P1-P5r and attB1-promo.-attB5r PCR products. The reporter gene entry clones were constructed by BP reaction between pDONR™221 P5-P2 and the attB5-gus:int-gfp-tnos-attB2 PCR product. Promoter-reporter gene fusions were inserted by LR recombination reaction between the created entry vectors and pKGW,0. Prom., promoter sequences; gus:int, ß- glucuronidase gene with a plant intronic sequence; gfp – grene fluorescence protein gene; tnos, nopaline synthase terminator sequence; ccdB, negative selection marker; Cm^R, chloramphenicol-resistance marker; Kan^R, kanamycin-resistance marker; Sm/Sp^R, spectinomycin resistance marker; RRB and LB, T-DNA border sequences.

3.5.4. Preparation of *A. tumefaciens* for Plant Transformation

Approximately 150 ng of each expression plasmid, with one of the three promoters to be tested fused o the reporter gene, were added to electro-competent *Agrobacterium tumefaciens* strain EHA105 cell suspension. Cells were electroporated at 2.50 kV, 25 µFD, 400 Ω in a Gene Pulser XCell™ Electroporaion System (Bio-Rad) and recovered for three hours in Super Optimal broth with Catabolite repression growth medium (SOC) (2 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) with gentle shaking at 28 °C. One aliquot of 25 µl from each culture was plated into solid LB growth medium supplemented with 100 µg ml^{-1} of spectinomycin and 50 µg ml^{-1} of rifampicin. As a negative control of ß-glucuronidase detection after plant transformation, a cell suspension of *A. tumefaciens* without carrying any plasmid was used, performing bacterial selection only with 50 µg ml^{-1} of rifampicin. For positive control of plant transformation a previously transformed culture of *A. tumefaciens* containing pMP2482 – which has the coding sequence of ß-glucuronidase gene under control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter – was used, selecting with kanamycin and rifampicin, both at 50 µg ml^{-1}. Bacterial cultures were kept at 28 °C in the dark for 48 hours.

To screen for colonies carrying the plasmids, colony PCR was performed using a pair of primers annealing inside the ß-glucuronidase gene (see attached sequences). PCR reactions were performed at 95 °C for 5 min, followed by 35 cycles at 95 ºC for 30 s, 60 ºC for 30 s, and 72 °C for 1 min, with a final extension step of 72 °C for 5 min. According to colony PCR results, colonies were inoculated in 4 mL of liquid LB medium supplemented with the appropriate antibiotics and incubated overnight at 28 °C, in the dark, with agitation at 150 rpm. In the following day, the cell suspensions were scaled up to 50 mL cultures, using 0.5 mL of the previous culture and grown overnight at the same conditions as described before. On the subsequent morning, culture optical density at 600nm (OD_{600nm}) was measured in an Ultraspec 4000 UV/Visible spectrophotometer (Pharmacia Biotech), cell cultures were pelleted by centrifugation (4500 x*g*, for 5 min at 4 ºC). The supernatant was discarded and bacteria were ressuspended at OD_{600nm} = 0.800 – 0.950 in half concentrated Embryogenic Induction Medium (EIM – MS growth meduim supplemented with 3 % (w/v) sucrose, 0.45 µM of 2,4-dichlorophenoxyacetic acid, 0.91 µM of zeatin, 0.7 % (w/v) microagar, pH 5.8) supplemented with 100 µM of acetoseryngone (Sigma-Aldrich) unless stated otherwise. Cells were incubated at 22 °C at the dark for at least 1 hour before plant transformation procedure for virulence induction.

3.5.5. Plant Transformation

Medicago truncatula cv. Jemalong genotype M9-10a, micropropagated *in vitro* in MSOA medium (MSOA = MS growth medium supplemented with sucrose 3 % (w/v) and microagar 0.7 % (w/v), pH 5.85) as described in Neves *et al.* (2001) were used to perform genetic transformation. Plants were kept a growth chamber (Phytoclima 700 EDTV, Aralab Portugal) with 16 hour photoperiod of 100 μE m^{-2} s^{-1} and 24º/22 ºC of day/night temperature. Approximately 2 months after replication, leafs were excised with a sterile scalpel and plant transformation procedure was followed.

In order to determine the most suitable transformation procedures for the purpose of the present work, 2 types of infection techniques were tested, wounding and infiltration. A disarmed *A. tumefaciens* strain as a negative control, a strain carrying the plasmid pMP2482 as positive control, and the expression vector carrying the promoter G08 fused to the *gus* reporter gene were used for the preliminary analysis.

Plant transformation procedure was performed as described in Araújo *et al.* (2004). Briefly, plants were transformed by wounding leaflets using a scalpel blade contaminated with the mentioned *A. tumefaciens* strains, and placed in Petri dishes containing sterile absorbent paper imbibed in EIM/2 supplemented 100 μM of acetoseryngone. After 3 days of co-culture, leaflets from the positive and negative control groups were transferred to Petri dishes with imbibed absorbent paper with fresh EIM/2, whereas plants transformed with the expression plasmid containing the G08-*gus* promoter-reporter gene fusion were divided in three groups, placed either in fresh EIM/2, EIM/2 supplemented with 100 μM of ABA, or EIM/2 with polyethylene glycol (PEG) 8000 10 % (w/v) to induce water stress. Histochemical detection of GUS activity was performed as described below, 24 h after induction.

This process was repeated to test infection by infiltration, as described in Santos *et al.* (2003). Intact leaflets were placed at *A. tumefaciens* suspension cultures, at OD_{600nm} = 0.650 – 0.700 and vacuum infiltrated at 0.6atm for 15 min. Then the same procedure was repeated as described in the infection by leaflet wounding.

3.5.6. Promoter Characterization

To test the induction of expression of genes controlled by the promoters G02, G03 and G08, the described plant transformation procedure by leaflet wounding was followed. Along with the promoter-*gus* gene constructs, transformations were performed including the *A. tumefaciens* unarmed strain and the strain carrying the plasmid pMP2482 for positive and negative GUS detection, respectively. After 3 days of co-culture at 22ºC in the dark, the explants

from each transformation were divided in two groups (10 leaflets each), to test the influence of water stress in the expression of the reporter gene. The non-stimulated group was transferred to Petri dishes containing sterile absorbent paper imbibed in fresh EIM/2, whereas the second group was transferred to EIM/2 containing PEG 8000 20 % (w/v). Explants were collected 24h after induction to perform the histochemical detection of GUS activity, as described above.

At last, the process was repeated to evaluate ABA-induced gene expression, using a solution of abscisic acid diluted in concentrated methanol. While ABA was added to the treated group, the same amount of methanol, 55 µL, was added in the EIM/2 of the control group.

3.5.7. Histochemical GUS assay

The histochemical detection of GUS activity was performed as described in Jefferson et al. (1987). Briefly, transformed leaflets were transferred to sterile 2ml tubes were GUS staining solution (1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide acid, X-GlcA, 50 mM sodium phosphate buffer pH 7.0, 10 mM EDTA and 0.1 % (v/v) Triton X-100) was added until the explants were covered. Vacuum infiltration was performed with 0.6 atm for 1 h and 30 min and samples were incubated overnight in a humid chamber at 37ºC in the dark. Chlorophyll was removed from stained explants with 80 % (v/v) ethanol for at least 2 days, with gentle mixing and ethanol solution renewal.

3.5.8. Promoter Induction Analysis

GUS activity in transformed leaflets was evaluated by comparison of ABA or PEG treated and non-stimulated explants within explants with the same promoter sequence, using samples transformed with the disarmed *A. tumefaciens* strain and leaflets transformed with pMP2482 as references for negative and maximum expression profiles, respectively. All explants were photographed in a Wild MZ8 stereomicroscope equipped with a DC200 digital imaging system (Leica). Images were acquired with Leica IM50 1.10 release 17 software under a CLS100 light (Leica). GUS activity analysis was carried out by manual foci count and automated blue area measurement. For manual count, in explants presenting confluent foci a count of 50 was randomly attributed. For automated blue area measurement, Adobe® Photoshop® CS4 Extended software was used for background elimination (choosing the upper right background section, option Replace Color with the following settings: 124 fuzziness, 72.43 luminance, 0.79 A, 3.09 B, 0 hue, 0 saturation and 100 lightness) and black and white conversion enhancing contrast in blue-colored areas (Black and white conversion settings: 300 red,

yellow and green, -200 cyan, blue and magenta, without tinting), and using MBF ImageJ for Microscopy software (Rasband, 1997) for threshold definition (pass filter for brightness between 0-200, arbitrary units) and area measurement of the converted photography's.

3.6. Statistical Analysis

Statistical analysis was performed using the Graph Pad Prism® 5 software for windows, version 5.03. The analysis was carried out by mean comparison after evaluation of normality. The Dunns post-test was used to compare all mean pairs, after confirming its significance ($P<0.05$) by the Kruskal-Wallis test.

4. Results and Discussion

4.1. Extraction Processes

Three different extraction procedures have been assayed to evaluate which would extract phosphorylated mono- and dissacharides with greatest recovery efficiency in *M. truncatula* extracts. To assess whether the loss of phosphorylated sugars was caused by the extraction procedure *per se* or from interferences related with other cellular compounds that would interfere with metabolite recovery, all the extraction procedures were tested both in presence and absence of grinded plant leafs. At this stage, glucose 6-phosphate was added at the beginning of the extraction and the recovery efficiency was estimated.

This method for recovery evaluation is relatively simple and efficient. Under the conditions used no G6P present in plant cells could be detected, thus no interferences from G6P present in plant cells would affect the estimation recovery. Even though variation may occur in the recuperation of different phosphorylated sugars within the same extraction process, it is not expected to register significant differences (Antonio *et al.*, 2007). Thus, the analysis of G6P recovery was considered representative of phosphorylated sugars and the method was used for comparison between the three assayed extraction processes.

4.1.1. Ethanolic Extraction

Extraction of metabolites using boiling ethanol is a widely used technique for soluble sugar analysis in plant extracts, commonly used for quantification of both reducing and non reducing sugars (Almeida et al., 2007; Arrabaça, 1981; Dickson, 1979). Depending on the characteristics of the plant tissue ethanol concentration, organic material manipulation and boiling time should be adjusted for optimal recovery. For the present work, 50 % and 80 % (v/v) ethanol solutions both with 0.1 % (v/v) EDTA solutions were tested in intact and grinded leafs. Because leaf powder has a greater surface/area ratio, it is usually left to boil in ethanol for 5 minutes, whereas intact leafs are left at 80 ºC for 10 minutes ethanol exchange in middle of the process to avoid solvent saturation and pooling the two fractions for analysis (Arrabaça, 1981).

Recovery efficiency from extraction processes when no plant material was used are summarized in Table 3. Similar G6P recoveries were achieved among the tested method variations, but a tendency to register slightly higher recoveries and lower variation was noted

when 50 % (v/v) ethanol was used. Thus subsequent analysis, with plant material addition, was carried out using this ethanol concentration.

Table 3. Recovery efficiencies registered in four variation of the ethanolic extraction protocol. Extractions were performed without plant material. Indicated ethanol percentages are in v/v. Minutes correspond to the boiling time.

Extraction	Recovery	SD
EtOH, 80 %, 5 min	79 %	4.7
EtOH, 80 %, 10 min	76 %	8.0
EtOH, 50 %, 5 min	81%	1.4
EtOH, 50 %, 10 min	85 %	3.7

Comparison of the recovery rates registered in extractions performed with plant leafs (Figure 2) show that lower extraction efficiency are obtained when leafs are added, registering 70 % (±3.2) and 46 % (±1.7) recovery for tissue powder and intact leafs, respectively. Furthermore, the 10 minutes boiling of intact plant leafs reduces significantly the recovery of G6P. Altogether these results indicate that the use of 50 % ethanol may slightly improve recovery efficiency and that boiling grinded material for five minutes would be the more appropriate procedure for the extraction of phosphorylated sugars in *M. truncatula* leafs.

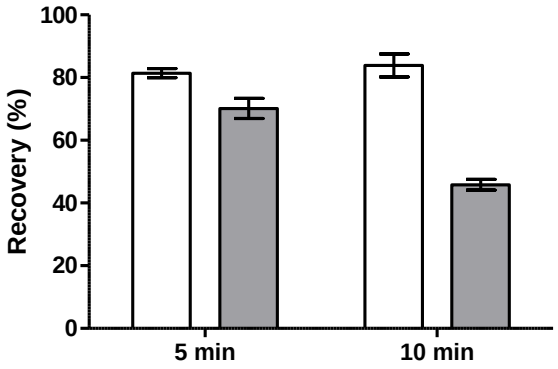


Figure 2. Recovery of glucose 6-phosphate registered in the ethanolic extraction protocol using ethanol at 50 % (v/v) at 80ºC for two different incubation periods. White bars represent extracts without plant material; grey bars represent extractions performed with *M. truncatula* grinded leafs. Values are mean ± SD.

4.1.2. Acid Extraction

Extraction of phosphorylated sugars using a strong acids, such as perchloric acid or trifluoroacetic acid, have been reported for the quantification of T6P in plant extracts (Almeida *et al.*, 2007; Schluepmann *et al.*, 2004). The details of the extraction procedure, however, are not

reported in great detail and the treatment of the samples differs among authors. Therefore, for a simple evaluation of the possibility to use TFA as an extraction solvent a similar procedure to the one described for the ethanolic extraction was used, except that the incubation was made on ice as described in the mentioned reports. As before, the extraction was performed with and without plant material to evaluate the cause for G6P loss during the extraction.

The recovery estimated in samples without the addition of plant material was 73 % (±6.5) and, surprisingly, the recovery in samples with grinded material was the same, 73 % (±1.6). It seems that somehow the limitation of extraction relies on the procedure itself and is independent from the presence of plant material. Although the causes for G6P loss in this process were not evaluated, one can speculate of what might be causing the registered recovery rate. It is possible that the acidic environment and the low temperature at which the extraction is performed would decrease the solubility of G6P, leading to some precipitation in the centrifugation step. Other possibility is that the trifluoroacetic acid could somehow interfere in the enzymatic detection of G6P, thus resulting in a miscalculation in the estimation of recovery. Even though it would be interesting to explore these possibilities, it was decided to test other extraction procedure in order to decide which would be more appropriate to use for the present work.

4.1.3. Liquid-Liquid Extraction (LLE)

The LLE is a relatively recent extraction procedure reported for the analysis of phosphorylated sugars in plant extracts, including T6P. Originally the extraction process was developed using a mixture of methanol, water and chloroform (Nordström *et al.*, 2004), but the later exclusion of water and the substitution of methanol for acetonitrile improved the recovery of phosphorylated sugars (Lunn *et al.,* 2006). Using this protocol, recovery of 97 % (±3.7) from the G6P added at the beginning of the extraction process was achieved in samples without plant material. However the recovery was greatly affected with the introduction of grinded plant leafs in the procedure, registering only 30 % (±4.4) of recovery (Figure 3). Comparison of both recovery efficiencies clearly indicates that plant material interferes considerably with the recovery of G6P.

Considering that almost all G6P could be recovered using this extraction procedure, some alterations in the protocol were made in an attempt to circumvent the loss of recovery caused by the plant material. It was considered that the recovery decrease could be essentially caused by two factors: degradation or adsorption to plant debris. Aiming to improve recovery

in plant extracts, PVP, EDTA, and Triton X-100 solutions were added either in the aqueous extraction phase or at the beginning of the extraction process.

Figure 3 shows the recoveries registered with the LLE protocol alterations. The addition of insoluble PVP at the beginning of the extraction led to a recovery of 33 % (±4.2), which does not represent a significant improvement in G6P recovery in plant extracts, indicating phenolic oxidation is unlikely to be occurring in these plant extracts. On the other hand, the use of EDTA 0.1 % in the aqueous extraction phase seems to improve the recovery up to 54 % (±2.0), demonstrating that the chelation had a positive effect on the recuperation of G6P. However, the addition of Triton X-100 to the EDTA solution did not seem to have any significant effect on the recovery, since the estimated recovery was 55 % (±2.6). This result could indicate that adsorption of phosphorylated sugars to plant debris is not the major factor affecting the recovery rate. However, Triton X-100 is also used in some protein extraction protocols to preserve enzymatic activity (Deutsher, 1990), thus degradation of G6P might have been enhanced after resuspension leading to some loss of recovery. Finally, the increase of EDTA concentration to 1 % (w/v) registered the best recovery efficiency among extracts with plant leafs, leading to 69 % (±1.2) of G6P recuperation.

Altogether, these results suggest that the major limitation for G6P recuperation is its degradation by enzymes that use divalent ions as co-factors. The addition of EDTA greatly improved G6P recovery, obtaining acceptable efficiency and small variation that can be used to metabolite quantification (Antonio *et al.*, 2007).

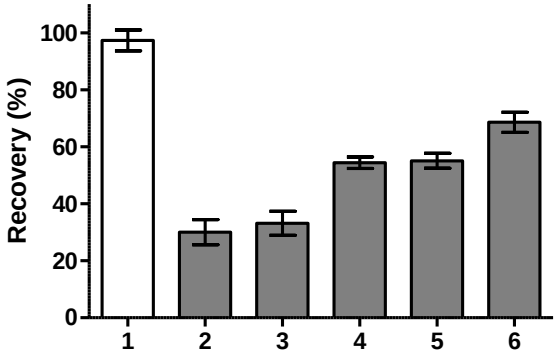


Figure 3. Recovery of glucose 6-phosphate registered in the liquid-liquid extraction procedure. White bars represent extracts without plant material; grey bars represent extractions performed with *M. truncatula* grinded leafs. Different bars correspond to different solutions used in the aqueous extraction phase: 1, water; 2, water; 3, 1 % (w/v) PVP; 4, 0.1 % (w/v) EDTA; 5, 0.1 % (w/v) EDTA with 1 % (v/v) Triton X-100; 6, 1 % (w/v) EDTA. Values are mean ± SD.

4.1.4. Comparison of Protocols

Figure 4 summarizes the results for recovery of G6P for all extraction processes. The liquid-liquid extraction was the protocol with better results in G6P recovery when no plant material was added, but it was registered the worse efficiency when grinded leafs were added, a limitation that was partially solved with the addition of EDTA. In the soluble metabolite extraction protocol a slight difference, although not statistically significant, is noticed when plant material is used, whereas the grinded material seems to have no influence whatsoever in the recovery efficiency for the acid extraction process.

Comparing the different extraction processes (Figure 4), it is possible to notice that similar recoveries are registered for all the extraction protocols when using plant material. However, recovery differences among tested extraction protocols can be observed when plant leafs are not used in the procedure. The extraction using acetonitrile and chloroform seems to have the potential to recover almost the complete amount of phosphorylated sugars if degradation can be avoided, while the other extraction processes appear to have some limitations in the recovery of the phosphorylated sugars which are dependent from the extraction process itself. It is also important to notice that, aside from the advantage of the inability to detect endogenous G6P, the recovery estimations are based on the exogenously added G6P. Consequently, these results represent the loss of phosphorylated sugars resultant from the extraction process, whereas information of the potential to extract endogenous metabolites from the plant material should be assessed by more sensitive techniques.

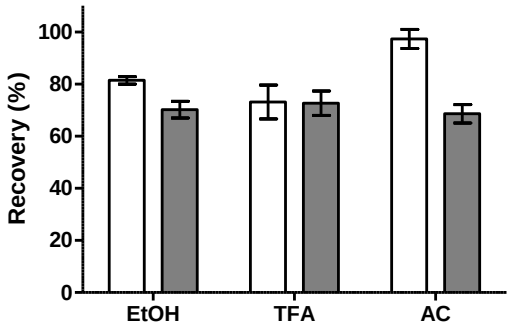


Figure 4. Comparison of the three tested extraction procedures. White bars represent extracts without plant material; grey bars represent extractions performed with *M. truncatula* grinded leafs. Values are mean ± SD.

Overall, the further improvement off the LLE protocol aiming to avoid the phosphorylated sugar degradation would be of great interest, since the recovery efficiency is completely

influenced by the plant material. This is more relevant when the ultimate objective is to quantify molecule that occur in small quantities, such as T6P.

4.2. Metabolite detection by LC-MS

Hydrophilic interaction chromatography (HILIC) columns have been used for chromatographic separation of polar compounds in complex mixtures (Bajad *et al.*, 2006; Schlichtherle-Cerny *et al.*, 2003). Antonio *et al.* (2008) reported a method for separation and identification of highly polar carbohydrate-related metabolites using a ZIC®-HILIC column (SeQuant™). Even though the authors were able to quantify a few metabolites in *A. thaliana* extracts, including some phosphorylated sugars, separation of T6P from its isomer S6P was not achieved, thus T6P content in plant extracts was not measured. For the present work a very similar column, the ZIC®-pHILIC, was used. Its stationary phase is based on porous polymer beads rather than silica like the ZIC®-HILIC column, and a wider range of pH may be used in the chromatographic process.

Standard solutions of T6P, S6P and a mixture of both were used to establish optimal chromatography conditions and confirm whether it would be possible to separate the two isomers. Best chromatography results were achieved using the conditions described in section 3.4.3. (Table 1). However, chromatographic resolution of T6P and S6P was not obtained in any of the assayed conditions. Furthermore, both isomers had the same predominant MS ion at *m/z* 421 and the same intensive MS/MS product ions at *m/z* 241, thus excluding the hypothesis of separation and quantification by MS detection. Some HPLC column manufacturers indicate that a polymer-based stationary phase may not be the ideal choice for the separation of small molecules, such as phosphorylated carbohydrates (Grace Davison Discovery Sciences). Moreover, reported experiments using a ZIC®-HILIC column showed that pH variation of the mobile phase did not affect significantly the retention time in most of the tested molecules (Guo & Gaiki, 2005). It was therefore concluded that, despite of the efforts made, unambiguous T6P identification was unlikely to be achieved in the available LC-MS system. Nevertheless, for its advantages of metabolite separation from other compounds in plant extracts and the sensitivity of the coupled MS detector, it was decided that it would be advantageous to explore alternative methods to distinguish T6P from S6P and use LC-MS for detection and/or quantification of T6P.

4.2.1. T6P and S6P Absorbance

Considering that the used HPLC system was equipped with a PDA detector, the possi-

bility to detect and distinguish S6P from T6P basted on their absorption peak was analyzed. To assess at which wavelength each molecule would have maximum absorbance, standard solutions of both isomers were subjected to absorbance determination for each 0.2 nm at all wavelengths between 190 and 700 nm. Figure 5 shows that both isomers have higher absorbance values at 190 nm, without any defined absorbance peak in the registered spectra. Interestingly, the S6P solution which had lower concentration than the T6P solution registered lower absorbance at 190 nm as well. This result indicates that maximum absorbance of both molecules should occur at a wavelength near or smaller than 190 nm, thus not distinguishable through absorbance monitoring.

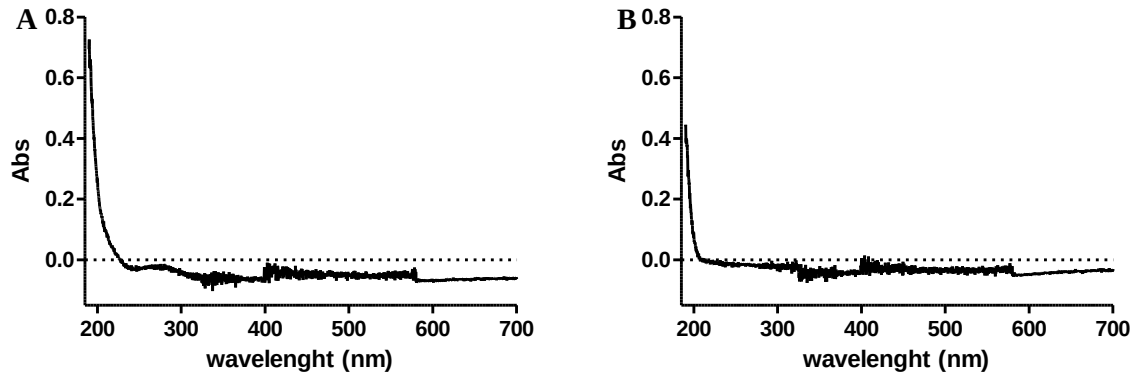


Figure 5. Wavelength spectra from 190 to 700 nm from T6P (A) and S6P (B) standard solutions.

4.2.2. Invertase Characterization

An enzymatic assay to selectively modify one of the metabolites, avoiding the overlap between similar molecules, was then considered. To evaluate the possibility of invertase to hydrolyze the phosphorylated sucrose molecule, the enzyme was added to S6P and sucrose (positive hydrolysis control) standard solutions according to the producer's protocol. Full MS spectra (50-1000 *m/z*) from direct infusion elecrospray mass spectrometry of the enzymatic assays are presented in Figure 6. Due to the high background interference from the buffer in the ionization process, complete hydrolysis of sucrose could not be confirmed. However, the obtained mass spectra suggest that invertase was not able to hydrolyze S6P.

To confirm if complete hydrolysis was achieved and whether invertase specifically hydrolyses sucrose, the enzymatic hydrolysis assay was repeated for sucrose and trehalose standard solutions, acquiring SRM chromatograms after hydrophilic interaction chromatography and using the described single reaction monitoring (SRM) settings (Table 2). Comparison of

chromatograms from sucrose and trehalose incubation showed that invertase was not able to hydrolyze trehalose, whereas only partial hydrolyses of sucrose occurred.

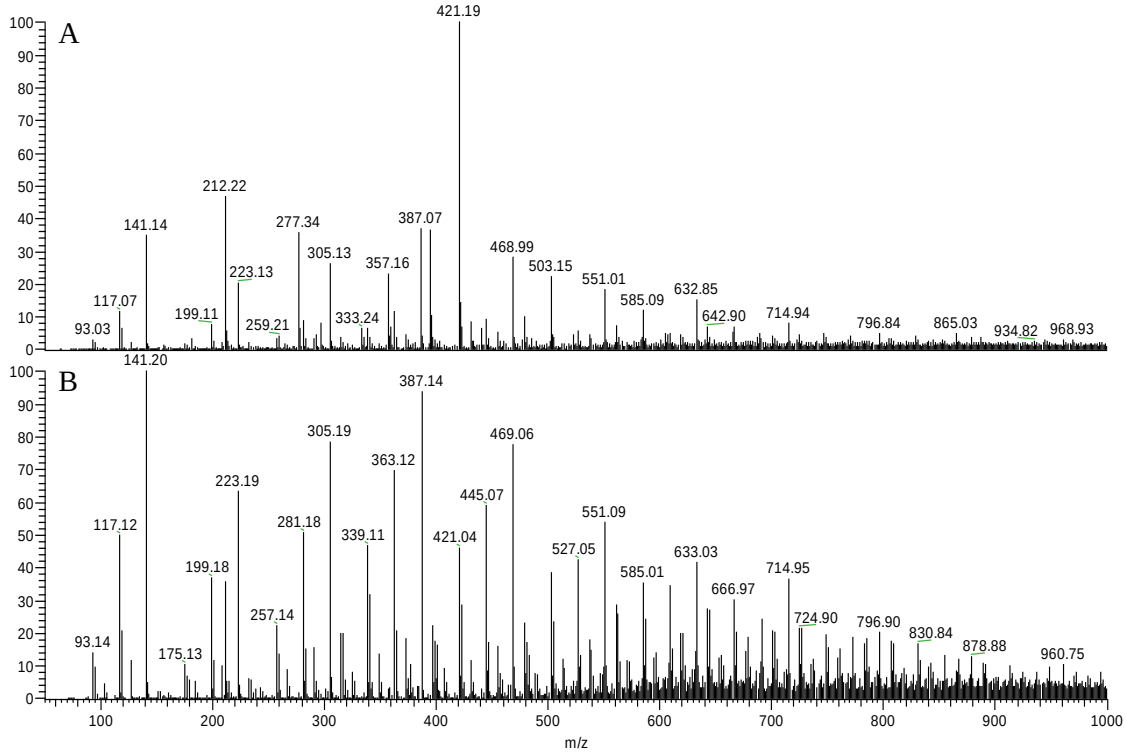


Figure 6. Mass spectra acquired by direct infusion of S6P (A) and sucrose (B) after Invertase hydrolysis. Negative mode ESI-MS; 50-1000 *m/z* mass range.

Aiming for optimization of the hydrolysis reaction, sucrose was used for subsequent assays. The incubation of one unit of invertase for 16 hours led to a significant decrease in sucrose, but no complete hydrolysis was achieved (Figure 7). This apparent inefficiency of the enzyme was attributed to some loss of activity that might have occurred on its storage period. Nevertheless, these results demonstrate that invertase is able to distinguish between sucrose and trehalose, though it is not able to hydrolyze the phosphorylated sucrose molecule. Thus, in order to detect and quantify T6P in a mixture of both isomers through invertase hydrolysis, a preceding dephosphorylation process would be required.

4.2.3. T6P and S6P Distinction Trough a Two-step Enzymatic Hydrolysis

Considering the absence of commercially available enzymes that would selectively modify one of the phosphorylated isomers so that they could be distinguished by mass spectrometry, a two-step enzymatic processing was considered. The plan was to use an unspecific phosphatase that would convert S6P to sucrose and T6P to trehalose, which are still not separ-

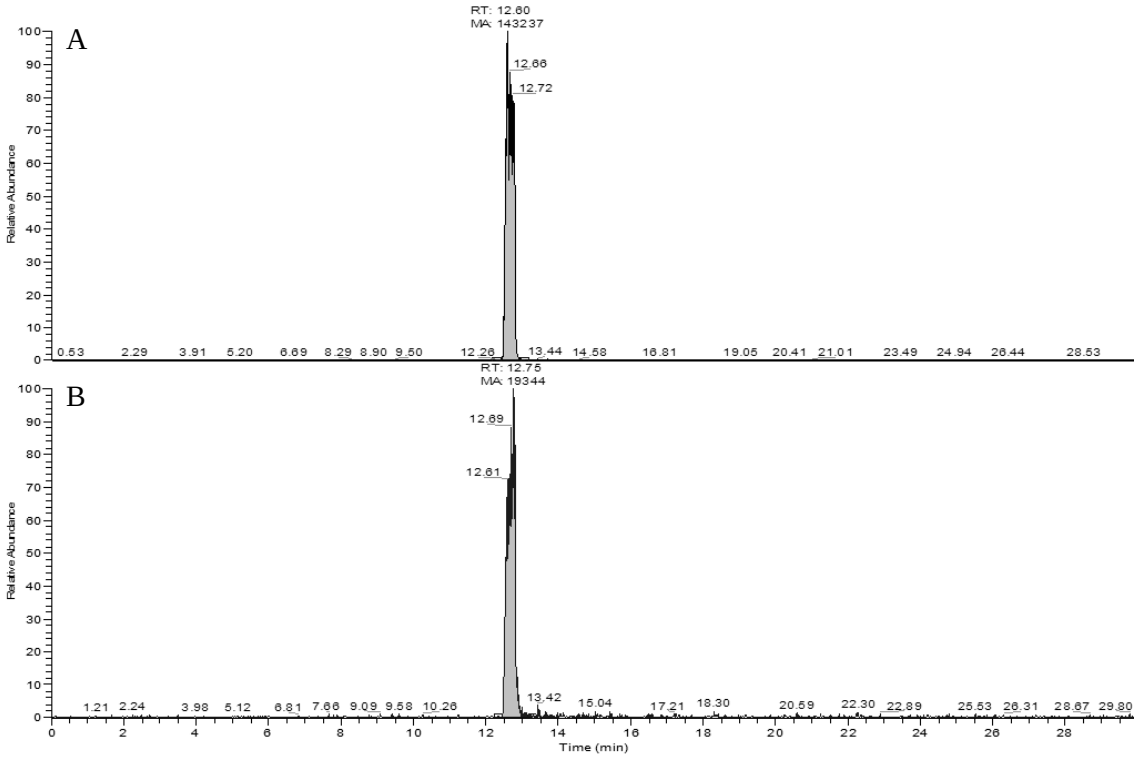


Figure 7. SRM chromatograms for *m/z* 179of standard solutions of sucrose before (A) and after (B) invertase addition. Numbers indicate the respective retention times; MA represents the area of the peak.

able by LC-MS, and then use invertase to hydrolyze sucrose leaving trehalose for quantification. To measure T6P concentration in plant extracts, they would have to be separated in two aliquots. In the first fraction invertase would be added to determine the concentration of trehalose in the extract. In the second fraction the two-step enzymatic assay would be applied and trehalose concentration would be determined. T6P concentration would be estimated by the difference between the two-step enzymatic process and the extract where only invertase was added (Figure 8).

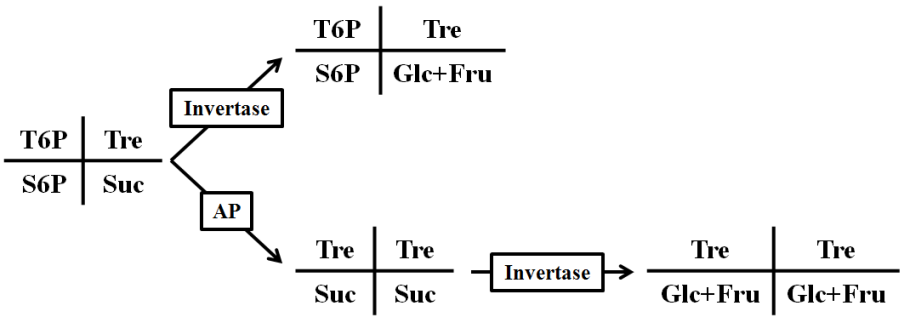


Figure 8. Representative scheme of the two-step enzymatic process for T6P quantification using invertase.

Calf Intestinal Alkaline Phosphatase (AP) is a commercial unspecific phosphomonoesterase produced to remove 3´ and 5´ phosphates from DNA and RNA. To test whether AP was able to dephosphorylate T6P and S6P 0.5 U of this enzyme was added to both sugar standard solutions. After a three hour hydrolyses the samples were analyzed by LC-MS. Decreased T6P and S6P quantities were detected in samples were AP was added (Annex 3 Annex 4). Even though the hydrolysis reaction was not complete, it was possible to observe that both sugars were dephosphorylated by AP. Complete dephosphorylation was achieved using 1 U of AP for 8 hours in a 0.5 mM T6P standard solution (Figure 9).

The buffer used in AP assays (5 mM Tris-HCl, 0.01 mM EDTA), supplied with the enzyme, at pH 8.5. Considering the optimal pH 4.5 for invertase catalyzed hydrolysis, the AP assay was repeated using the sodium acetate buffer, which had been used in the invertase assays. After 8 hours of incubation, complete dephosphorylation was also achieved in a 0.5 mM solution of T6P (Annex 5). These results show that AP is able to hydrolyze phosphorylated sugars, with no evidence of specificity among T6P and S6P. Furthermore, the enzyme is able to catalyze the reaction in acid pH, which indicates that it would be possible to do the two-step sample treatment without the concern to change pH between enzymatic processes.

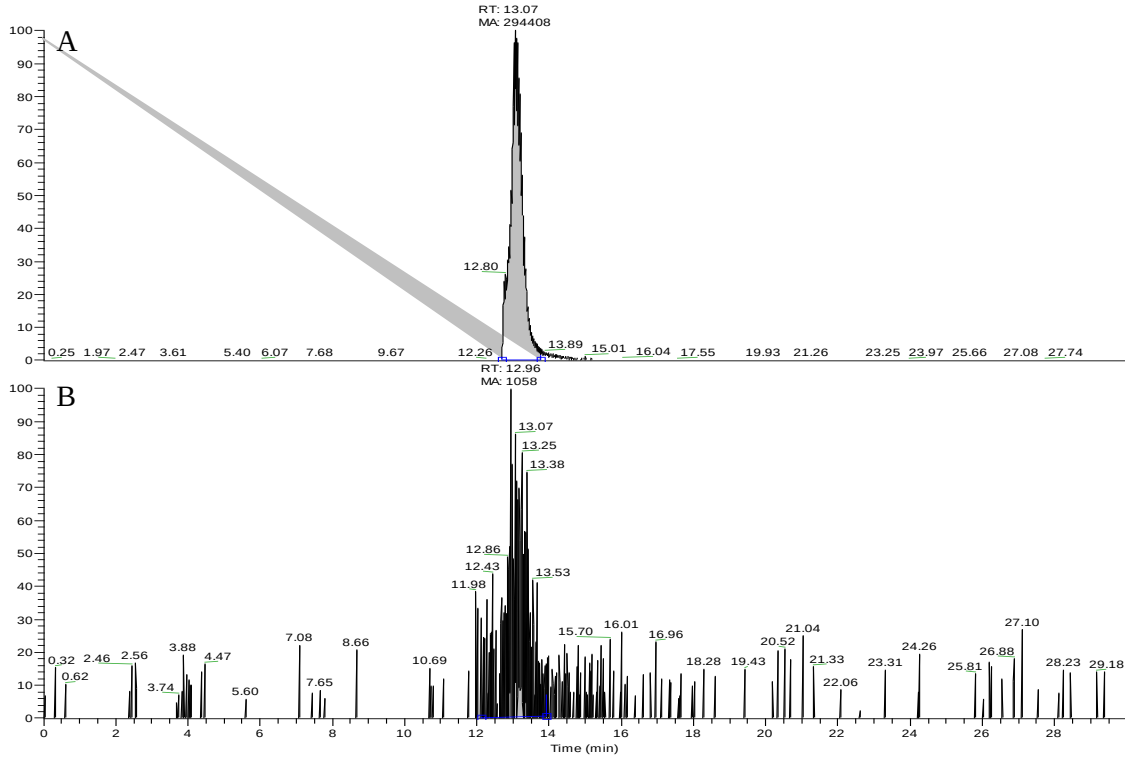


Figure 9. SRM chromatograms for *m/z* 241 of standard solutions of T6P before (A) and after (B) AP addition. Numbers indicate the respective retention times; MA represents the area of the peak.

4.2.4. Alternative Enzymatic T6P and S6P Distinction

An alternative enzymatic determination of T6P was also considered. Taking into account the possibility that trehalase could not hydrolyze T6P molecules a similar strategy to the one described for invertase would be followed. Plant extracts would be divided performing: (a) dephosphorylation followed by trehalose hydrolysis in one part and (b) the inverse procedure (hydrolysis followed by dephosphorylation) in the remaining extract. If the enzymes were successfully inactivated between both catalytic processes, the concentration of T6P could be determined by the difference of quantification of the fragment *m/z* 341 between processed extract (b) and processed extract (a) (Figure 10).

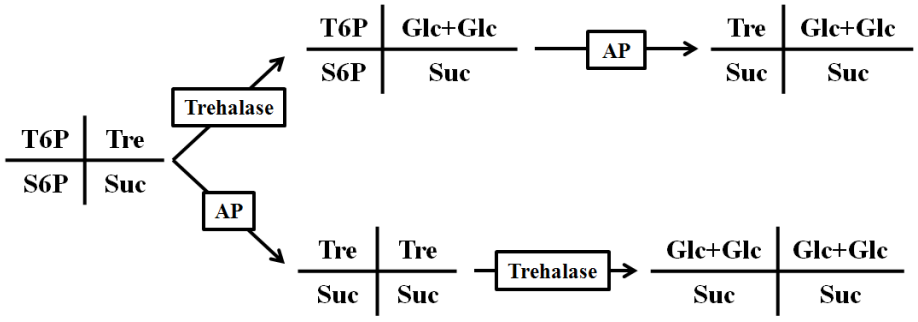


Figure 10. Representative scheme of the two-step enzymatic process for T6P quantification using trehalase.

A specificity test was performed in sucrose and trehalose standard samples following producer's instructions. After 16 hours of incubation, trehalase had completely hydrolyzed trehalose (Figure 11) whereas the chromatogram from sucrose digestion remained with approximately the same intensity and area. Thus, sucrose and trehalose distinction can be made using trehalase to selectively hydrolyze trehalose.

The capacity of trehalase to hydrolyze T6P was also tested. However the acquired spectra from direct infusion were not conclusive due to high background interference from the buffer solution. This information of trehalase activity would be of great interest for the design of the enzymatic assay for T6P quantification. If trehalase does not hydrolyze T6P molecules, the enzymatic process described earlier (and illustrated in Figure 10) should be followed. However, if trehalase does have de ability to hydrolyze T6P molecules, its quantification can be done by detection of either T6P or glucose, a product of the putative degradation, before and after trehalase addition.

It is important to notice that most mentioned parameters used in the enzymatic assays aimed to have complete substrate transformation in fairly concentrated standard solutions. Using different batches of enzymes and lower substrate concentrations (closer to the concentrations expected in plant extracts), incubation periods and enzyme quantity would have to be adjusted. The same applies when using plant extracts, which are complex matrixes that may contain enzyme inhibitors or interfere some other way in the catalytic process.

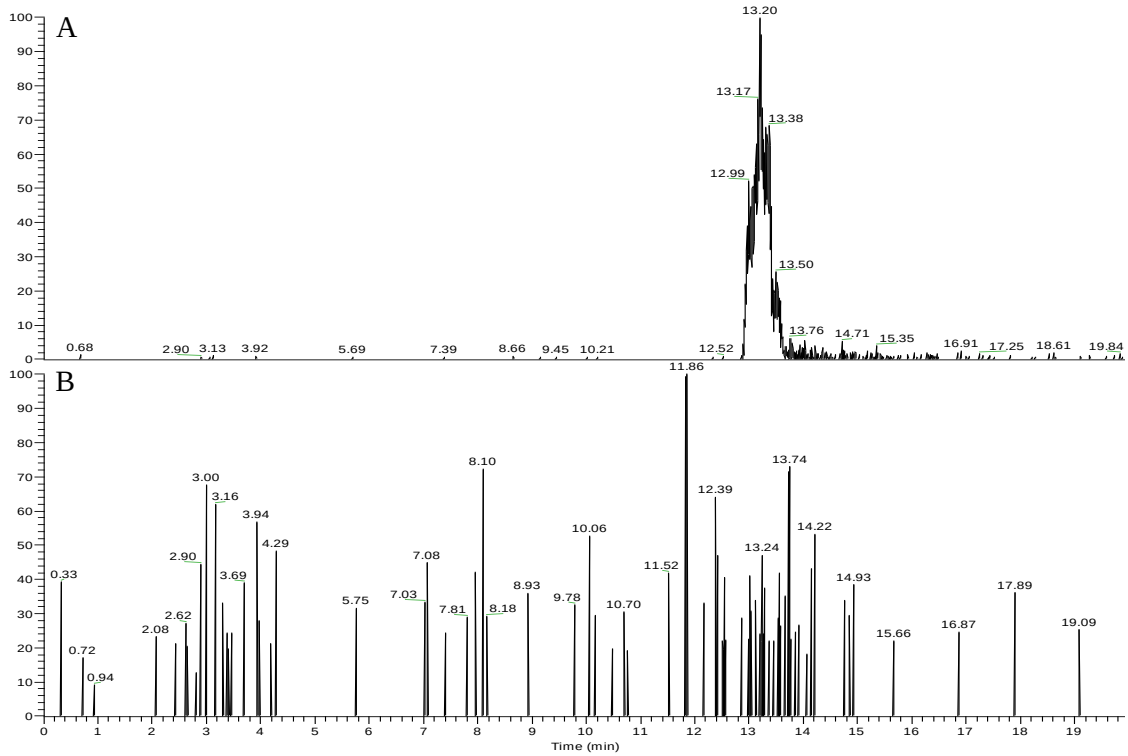


Figure 11. SRM chromatograms for *m/z* 171 of standard solutions of standard solutions of trehalose before (A) and after (B) trehalase addition. Numbers indicate the respective retention times.

4.2.5. Extract Analysis by LC-MS

A few plant extracts obtained by LLE procedure were analyzed using LC-MS. Liquid-liquid extractions were performed using either water or 1 % (w/v) EDTA in the aqueous extraction phase, and in both cases samples spiked with T6P at the beginning of the extraction procedure were used for comparison with non-spiked samples. In addition, a sample where aqueous extraction was performed using 3.5 % (v/v) of TFA and T6P spiking occurred was analyzed by LC-MS. Half of the volume of the non-spiked extracts was used for T6P addition at the final concentration expected in the spiked extracts if full recovery would be achieved. All the extracts were individually resuspended in ultra-pure water or spiked right before LC-MS analysis.

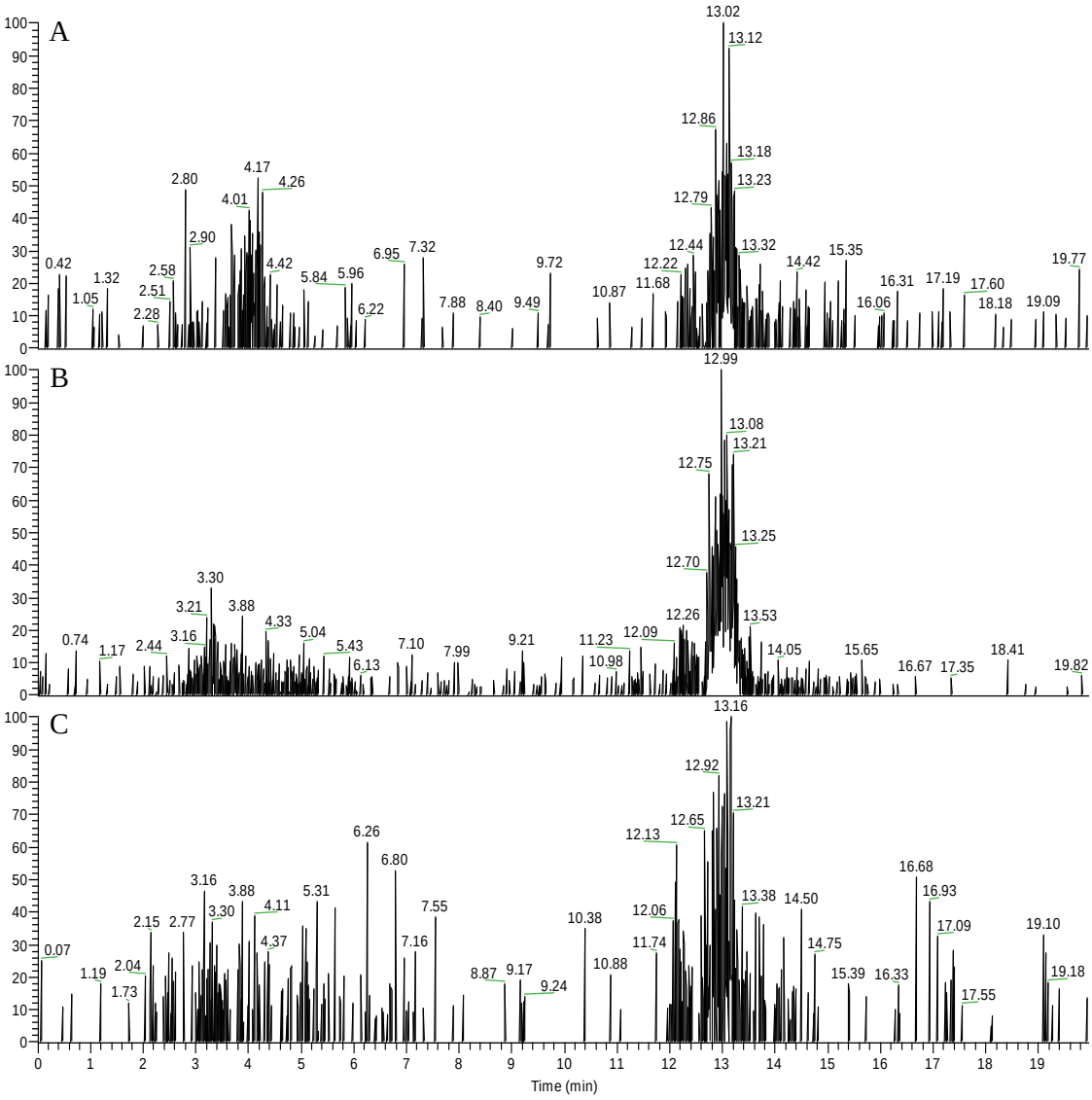


Figure 12. SRM chromatograms for *m/z* 241of extracts where aqueous extraction procedure was performed with water. A – extract without spiking (area = 559); B – spiked extract after resuspension (area = 2192); C – extract 5 hours after spiking (area = 531). Numbers represent retention times.

Figure 12, Figure 13 and Figure **14** show the chromatograms acquired from plant extract samples. The non-spiked sample where aqueous extraction was performed with water did not show any significant T6P/S6P peak above noise-to-signal threshold. However, in the spiked extract a T6P/S6P signal was detected, although not as intense as the signal from the non-spiked extract where T6P was added after resuspension. This last extract was reanalyzed approximately 5 hours after T6P addition and the T6P/S6P signal was no longer detected. Surprisingly, none of the extracts where aqueous extraction was performed with 1 % (w/v) EDTA showed a significant T6P/S6P peak, not even on T6P addition after resuspension. Finally, the chromatogram of the TFA extract presented a peak which was more intense and

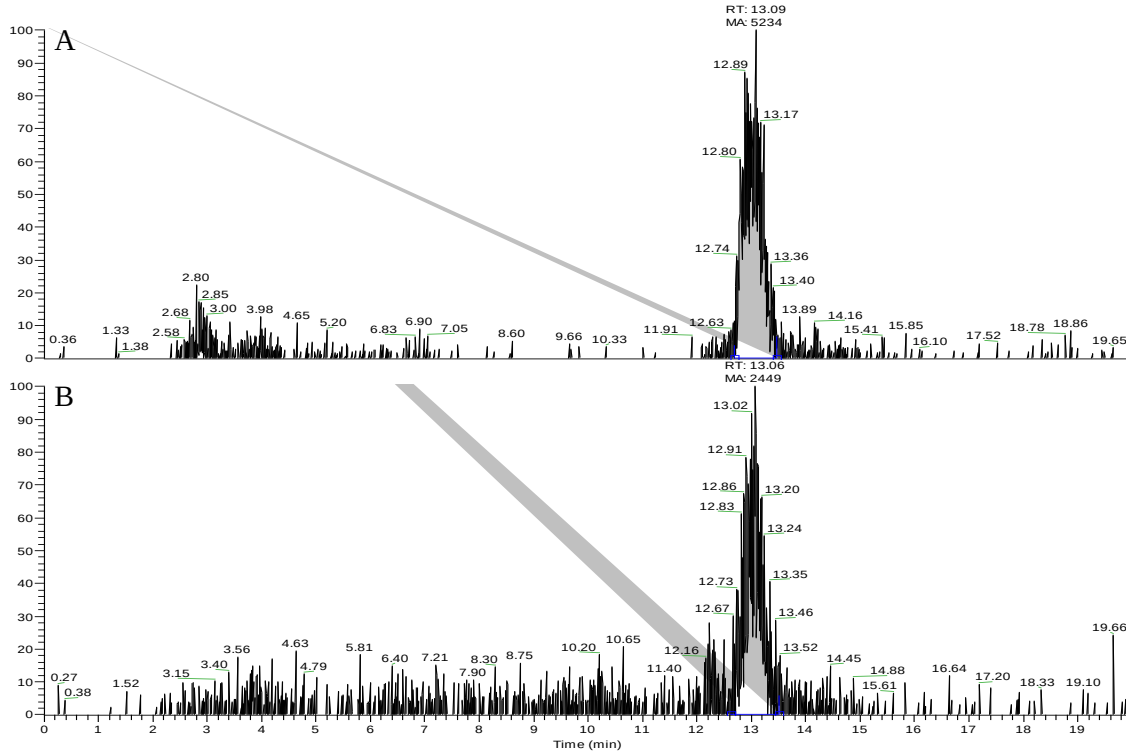


Figure 13. SRM chromatograms for *m/z* 241 of extracts where aqueous extraction procedure was performed with TFA (A) or water (B). Numbers represent retention times.

with superior area when compared with the extract where T6P was added after resuspension (using water in the aqueous extraction procedure).

Even though no replicates were analyzed at this stage and calibration curves were not obtained, it is tempting to speculate the implications of these results in the further development of the extraction and quantification procedure of T6P, if their reproducibility confirmation and metabolite quantification have been made. Firstly, it seemed that the use of 1 % (w/v) EDTA in the aqueous extraction procedure interferes somehow with the detection of T6P/S6P by LC-MS. Although best recovery efficiency of G6P had been determined using EDTA in aqueous extraction, the interference caused is not compatible with MS detection. Secondly, the reanalysis of the spiked extract shows that T6P degradation occurs in plant extracts even after reconstitution in water. Furthermore, the T6P/S6P detected signal in the spiked extract was less intense when compared to the extract where T6P was added after resuspension, which might indicate that recovery efficiency is being limited by T6P degradation. Finally, T6P/S6P detection in the TFA extract revealed a surprisingly intense signal. Even though a non-spiked TFA extract was analyzed, the comparison with the extract where T6P was added after resuspension appears to indicate that the use of TFA in the aqueous extraction may en-

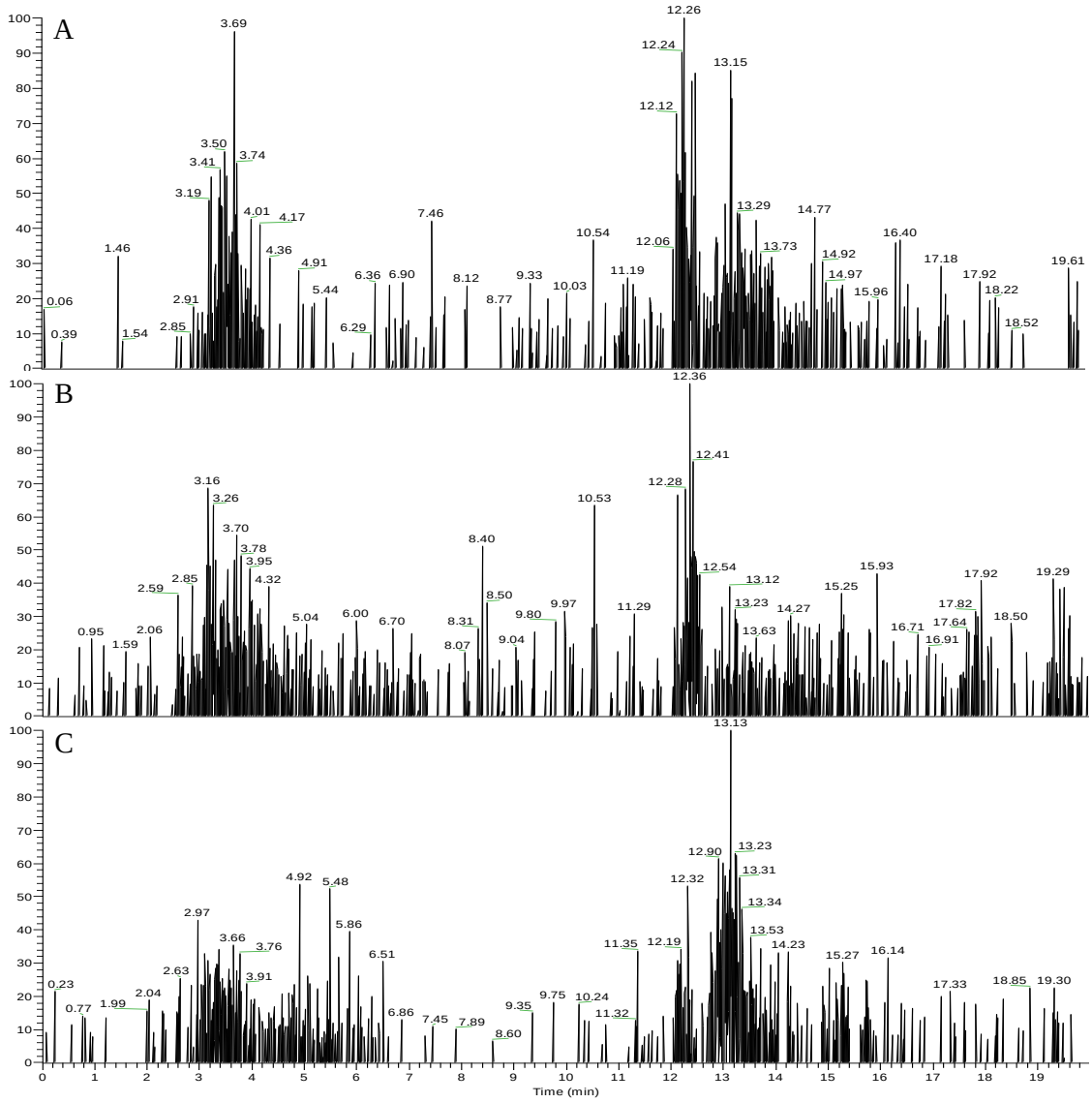


Figure 14. SRM chromatograms for *m/z* 241of extracts where aqueous extraction procedure was performed EDTA. A – extract spiked in the beginning of the extraction procedure; B – non-spiked extract; C – extract spiked after ressuspension. Numbers represent retention times.

hance the recovery efficiency of T6P. A repetition of the LLE with TFA, using suitable controls, a calibration curve, and reanalyzing the sample over time would be at great interest to assess whether degradation occurs in these samples and what is its recovery efficiency for T6P.

4.3. Promoter Isolation and Characterization

4.3.1. Plasmid constructions

The expression of twelve genes which had ABREs and CEs within a range of 50 base pairs downstream and 350 bp upstream the transcription start site (TSS) in the respective

promoter was analyzed in a previous work (Cordeiro *et al.*, unpublished resuts). Three of those promoters (G02, G03 and G08) were chosen, based on the expression profiles of the respective genes in response to water deficit or ABA application, for plant transformation experiments. The objective was to assess whether the previous induction of gene expression would also occur in transformed plants with those promoters controlling the expression of the GUS reporter gene. Promoter G02 controls the expression of a sequence coding for a Myo-inositol-1-phosphate synthase which synthesis a molecule known for its stress signaling function (Abid *et al.*, 2009) and whose expression was greatly enhanced under imposed water deficit conditions. Promoter G03 regulates the expression of a gene annotated in the *M. truncatula* genome as a glycosyl transferase and trehalose-phosphatase, with homology to the *Arabidopsis thaliana* TPS11 gene, whose expression was enhanced after 3 h of exogenous ABA application, whereas under drought stress it seemed to be slightly downregulated. Finally, the promoter G08 is upstream of a coding sequence for a dehydrin, which is known to accumulate at late stages of embryogenesis and in response to dehydration (Rorat, 2006) and which registered an increase of expression upon water deficit.

Abscisic acid-responsive promoters have been reported to have its primary regulatory elements, ABREs and CEs, within the [-350; +50] bp range around the TSS (W. Zhang et al., 2005). However, the full length of most promoters is unknown and can only be determined through genetic manipulation(Guo *et al.*, 2010; Yamaguchi-Shinozaki & Shinozaki, 1994). Previous works on stress-inducible promoters used promoter length varying from 260bp (Marcotte *et al.*, 1989), to 657 bp (Bahramnejad *et al.*, 2010) or even over 1000 bp (Morran *et al.*, 2011). For the purpose of the present work, it was considered that the use of regulatory sequence comprehended approximately [-900; +50] bp around the TSS, would be appropriate for the characterization of the promoters. An exception was made for promoter G03, which had a coding sequence to Heavy metal sensor kinase gene 950 bp upstream of its TSS, thus a 795 bp sequence was characterized.

PCR amplification reactions in *M. truncatula* genomic DNA of G02 and G03 promoter sequences generated DNA fragments around 1000 bp, whereas for G08 the fragment had approximately 800 bp and the reporter gene PCR generated 3000 bp fragments (Figure 15).

After PCR product extraction and BP recombination reaction, colony selection for plasmids containing promoter sequences was based on colony PCR with M13 primers (Figure 16). A slightly longer PCR product was detected on colonies considered to carry plasmids with promoter sequences, which corresponded to a 240bp length fragment located between M13 primers annealing sites and the recombination regions. The same selection was used for

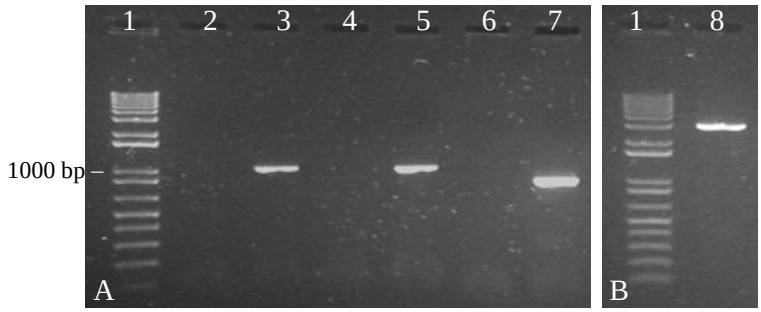


Figure 15. Electrophoresis of PCR products for promoter (A) and reporter gene (B) cloning. 1-kb plus DNA ladder; 2, non-template control for G02 primer pair; 3, G02 PCR product; 4, non-template control for G03 primer pair; 5, G03 PCR product; 6, non-template control for G08 primer pair; 7, G08 PCR product; 8, gus:int-gfp-tnos PCR product.

colonies transfected with products from the BP reaction for *gus* gene, however no PCR products could be detected (Figure 17A). Recombinat plasmids were screened by direct PCR in extracted plasmids (Figure 17B) from two randomly chosen colonies, both with gene specific primers and M13 primers (Figure 17C).

After sequentiation, the second recombination reaction was performed and colony PCRs for promoter and reporter gene were carried to assure that all promoter-reporter gene fusions had occurred. One of the advantages of the Gateway® technology is that it ensures directional insertion of PCR products into plasmids. Thus, it was expected that tandem promoter-reported gene fusion had occurred in the correct order and direction for inducible promoter regulated *gus* gene expression. Colonies with positive PCR reactions for both promoter and reporter gene sequences, *i.e.* the expression vector, were then chosen for plasmid extraction and proceed plant transformation.

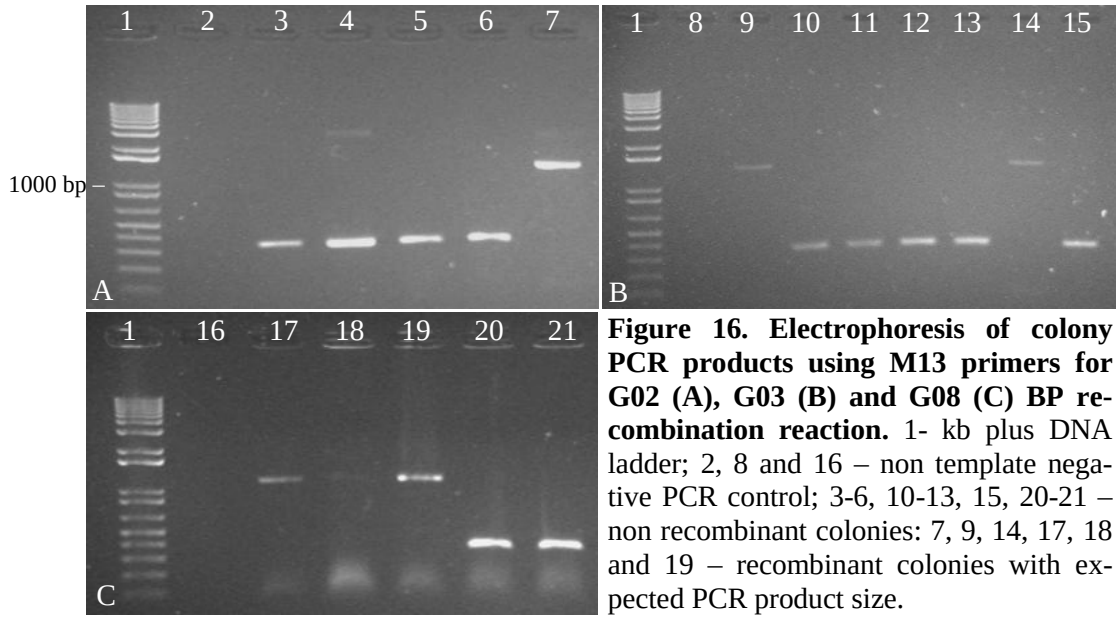


Figure 16. Electrophoresis of colony PCR products using M13 primers for G02 (A), G03 (B) and G08 (C) BP recombination reaction. 1- kb plus DNA ladder; 2, 8 and 16 – non template negative PCR control; 3-6, 10-13, 15, 20-21 – non recombinant colonies: 7, 9, 14, 17, 18 and 19 – recombinant colonies with expected PCR product size.

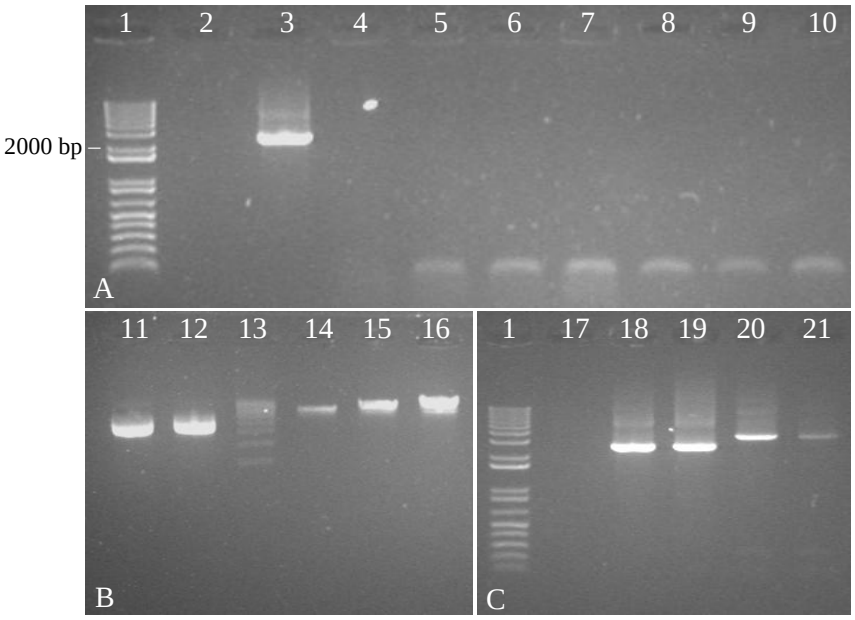


Figure 17. Electrophoresis of colony PCR products for gus:int-gfp-tnos (A), extracted plasmids (B) and PCR with specific reporter gene or M13 primers (C). 1, 1kb plus DNA ladder; 2, non-template PCR control; 3, Positive PCR reaction control; 4-10 – negative PCR reaction for screened colonies; 11-12 – extracted plasmids; 13, super coiled DNA ladder; 14, 25 ng of λ phage DNA; 15, 50 ng of λ phage DNA; 16, ng of λ phage DNA; 18-19, PCR reaction with gus:int-gftnos specific primers in 11 and 12 plasmids, respectively; 20-21, PCR reaction with M13 primers in 11 and 12 plasmids, respectively.

4.3.2. Plant transformation and expression analysis

Agrobacterium tumefaciens mediated plant genetic transformation is widely used technique for its high efficiency and broad range of organisms in which it can be used. The constructed expression vectors were inserted in A. tumefaciens cells and after antibiotic selection, bacteria transfection was confirmed through colony PCR (Figure 16).

Two plant *A. tumefaciens*-mediated genetic infection procedures have been assayed to test which would be more suitable for promoter induction analysis in transient expression detection in this model legume. Transformation induced by leaf wounding is a commonly used transformation technique with high transformation efficiency, which has been used in *M. truncatula* cv. Jemalong genotype M9-10a. However, since the scope of the present work was to characterize the induction of promoters with ABRE and CE motifs and ABA is considered to be involved in broad range of plant stresses, it was thought that part of the induction that might be detected could be a reflection of the wound or the infection itself rather than a response to the stimuli that would be given. Thus, as an alternative, vacuum infiltration of leaflets with *A. tumefaciens* suspensions was also tested.

In both infection procedures, the use of promoter pMP2482 showed that transformation was achieved and *gus* gene expression was successfully detected. As expected, in the negative

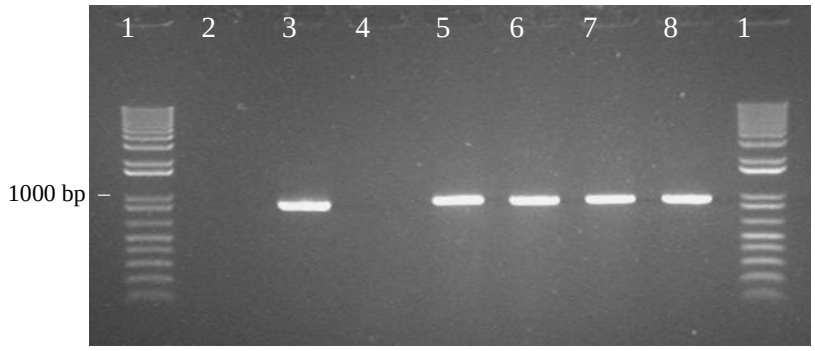


Figure 18. Colony PCR in *A. tumefaciens* using gus-fragment promoter pair. 1, 1Kb plus DNA ladder. 2, non-template PCR control; 3, positive PCR control with pMP2482 as template; 4, unarmed strain; 5, strain carrying G02-gus; 6, strain carrying G03-gus; 7, strain carrying G08-gus; 8, strain carrying pMP2482.

control of transformation (with the disarmed strain) no GUS activity was detected. Overall, in the tested transformation procedures GUS activity could only be detected in wounded areas (Figure 19). In leaflets transformed by infiltration, wounded areas represented only the base of the leaflet, where it had been excised from the leaf, or in occasional regions caused by leaflet manipulation with tweezers. For that reason, it was decided to use the genetic transformation by wounding the leaflets with a scalpel for further transformations and promoter characterization.

In the following transformation procedures, the count of blue foci, or Transient Expression Units (TEUs) (Basu *et al.*, 2003), showed great variability among explants transformed with the same promoter even within the same treatment. Such analysis did not show any significant increase nor decrease in promoter-induced *gus* expression after ABA application or PEG-induced water stress (Figure 20). It was hypothesized that basal expression could be occurring, thus masking the inducibility of the promoters. In that case, promoter induction by stress treatment could not be reflected in the foci number, but in the foci area that would increase upon promoter induction, as a reflection of greater ß-glucuronidase accumulation.

To establish a threshold for area estimation of the blue foci, Adobe® Photoshop® tools were used to enhance blue versus background contrast and convert to black and white images, so that threshold determination and area measurement would not include picture elements other than blue foci (Figure 21). Comparing the results of blue area estimation for explants transformed with plasmid pMP2482 (

Figure 22) show that, aside from the detected variability, similar area means were obtained for GUS expression upon de35S promoter regulation. However, considering that the

areas estimated for leaflets transformed with the putative stress-inducible promoters is significantly lower than the former ones, the variability of the results

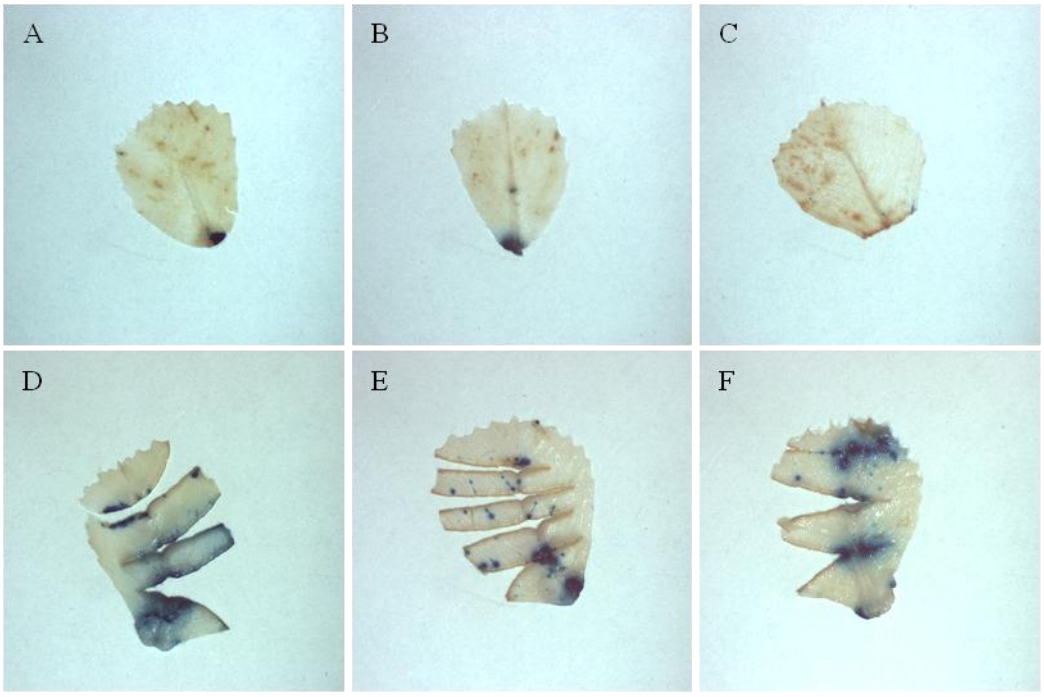


Figure 19. Representative transient expression pattern in leaflets transformed with T-DNA from plasmid pMP2482 in two tested infection procedures. A-C, infection by leaflet wounding; D-F, infection by infiltration.

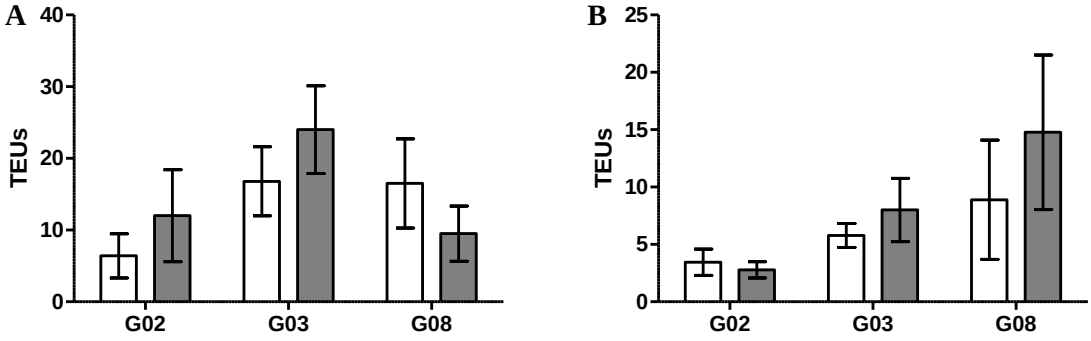


Figure 20. Transient expression units (TEUs) for the tested promoters in non-stimulated groups (white bars) and in groups subjected to ABA (A) or PEG (B) application (grey bars). Values are mean ± SE.

does not allow to find significant differences among leaflets subjected to ABA induction or water stress(

Figure 22). Slight differences were observed in mean areas of explants transformed with G08 promoter when subjected to both treatments, but taking into account the standard error associated it cannot be objectively concluded that the difference is caused by the treatment.

Transient expression has been proven to be a suitable tool for promoter characterization in several plant species, despite the constant limitation of registering great variability among

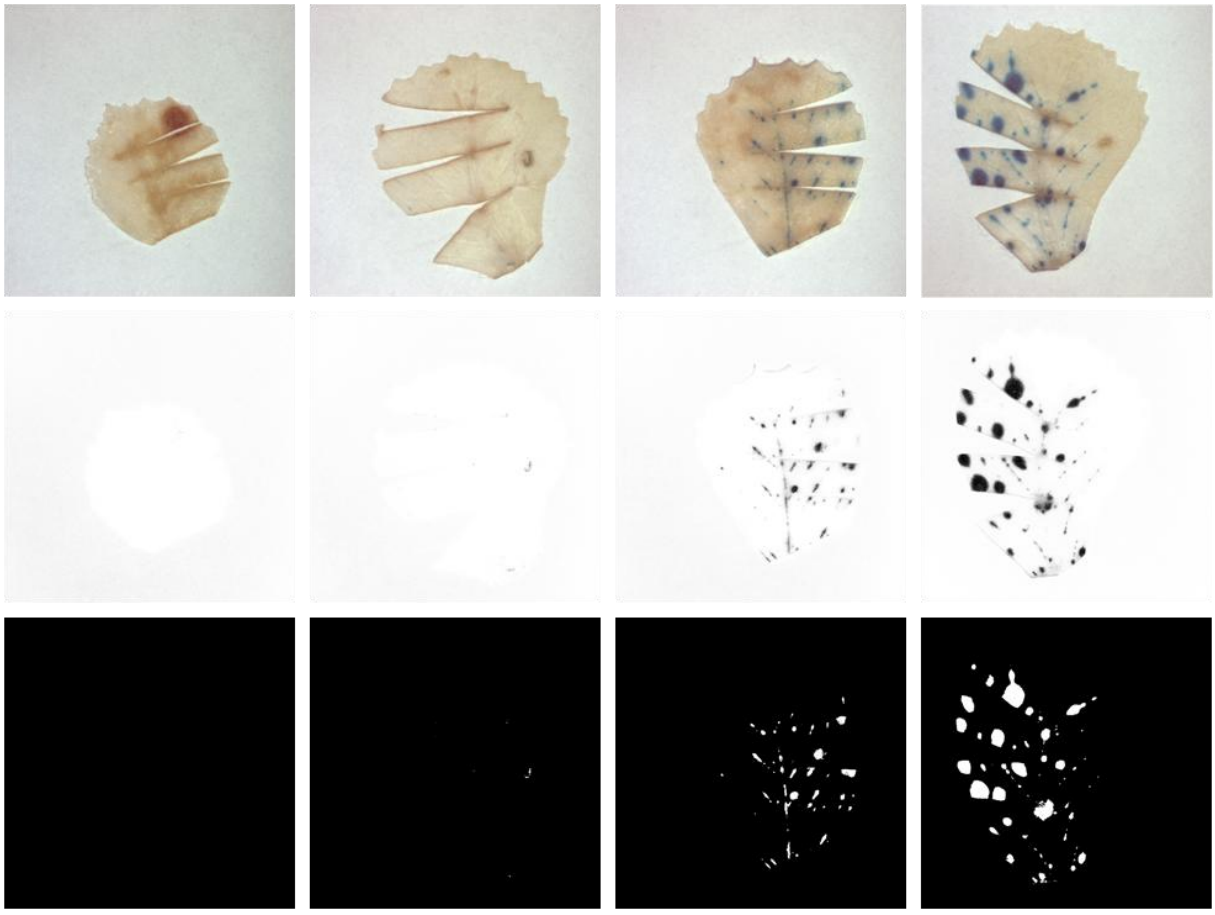


Figure 21. Representative photography manipulation procedure. In the first row non-manipulated photography's are shown; the second row represents the conversion to black and white and; in the last row the established threshold is given.

replicates (Basu *et al.*, 2003; Schenk *et al.*, 1998; Yang *et al.*, 2000). Even thought promoter evaluation by transient expression analysis can be performed in a relatively short period of time, the variability registered in such assays can only be overcome by the analysis of plant stably transformed (Du et al., 2010; Yi et al., 2010). Such variation is mostly attributed to the fact that GUS activity evaluation by transient expression is not normalized by the number of coding sequences that are transferred from bacteria to the plant nucleus. Nevertheless, this method has been used for demonstration of major differences in promoter-induced gene expression (Basu *et al.*, 2003; Yang *et al.*, 2000). Thus, the results obtained could be an indication that small differences in gene expression occurred. In fact, the analysis made by Cordeiro *et al.* (unpublished results), detected around 1.5-fold differences in gene expression.

It should also be noted that the mentioned reports were on characterization of promoters that were either chimerical or involved in developmental processes. The promoters tested in

this report contained ABRE and CE motifs that were expected to induce gene expression in the presence of ABA. Since abscisic acid is a general stress-responsive molecule, the transformation procedure could have led to the release of endogenous ABA, thus increasing the source of variation within the samples.

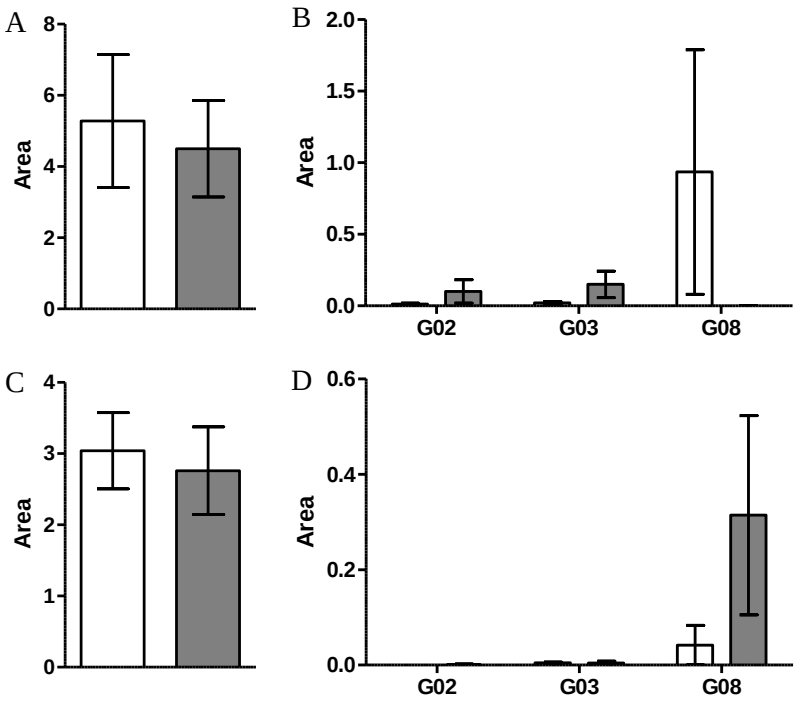


Figure 22. Blue area estimation (in mm^2) in explants transformed with gus gene under regulation of the de35S promoter (A and C) or the promoters to be characterized (B and D). White bars correspond to explants kept in MIE/2 and grey bars represent leaflets that were subjected to PEG induced osmotic stress (A and B) or exogenous ABA application (C and D). Values are mean ± SE.

5. Conclusion and Future Perspectives

In the present work, a method to extract and detect T6P in plant extracts has been explored. The liquid-liquid metabolite extraction procedure has been proven to obtain high recovery efficiencies in plant extracts. Furthermore, the LC-MS detection method for T6P has been described. Due to the impossibility do distinguish T6P from its isomer S6P, two alternative enzymatic assays were designed for unambiguous identification of T6P. The dephosphorylation of both molecules by alkaline phosphatase followed by hydrolysis of either sucrose or trehalose catalyzed by invertase or trehalase respectively enables the unambiguous detection of T6P. The results obtained suggest that the further characterization of the extraction process, namely with the use of trifluoroacetic acid in the aqueous extraction phase of the LLE protocol, is a promising possibility in the enhancement of the recovery efficiency. Furthermore, the tandem two-step enzymatic processes shall be tuned in order to assess T6P variation in extracts from plants under water deficit.

The transient expression analysis performed in leaflets infected with *A. tumefaciens* strains carrying plasmids with promoters containing ABRE and CE motifs fused to the *gus* reporter gene showed that variation within leaflets subjected to the same treatments was comparable to the variation observed between the tested induction media. This result suggests that the increase or decrease in gene expression might occur in ranges that are not detectable trough transient expression analysis. In this context, it will be interesting to establish *M. truncatula* lines with stable expression of these constructs made or eventually access gene expression by using more sensitive techniques, such as quantitative PCR

6. References

Abid, G., Silue, S., Muhovski, Y., Jacquemin, J. M., Toussaint, A., & Baudoin, J. P. (2009). Role of myo-inositol phosphate synthase and sucrose synthase genes in plant seed development. *Gene*, *439*(1-2), 1-10. doi:10.1016/j.gene.2009.03.007

Almeida, A. M., Silva, A. B., Araújo, S. S., Cardoso, L., Santos, D. M., Torné, J. M., Silva, J. M., Paul. M., & Fevereiro, M.P. (2007). Responses to water withdrawal of tobacco plants genetically engineered with the AtTPS1 gene: a special reference to photosynthetic parameters. *Euphytica*, *154*(1-2), 113-126. doi:10.1007/s10681-006-9277-2

Antonio, C., Larson, T., Gilday, A., Graham, I., Bergstro, E., & Thomas-Oates, J. (2008). Hydrophilic interaction chromatography / electrospray mass spectrometry analysis of carbohydrate-related metabolites from *Arabidopsis thaliana* leaf tissue. *Rapid Communications in Mass Spectrometry*, *22*, 1399-1407. doi:10.1002/rcm.3519

Antonio, C., Larson, T., Gilday, A., Graham, I., Bergström, E., & Thomas-Oates, J. (2007). Quantification of sugars and sugar phosphates in *Arabidopsis thaliana* tissues using porous graphitic carbon liquid chromatography-electrospray ionization mass spectrometry. *Journal of chromatography. A*, *1172*(2), 170-8. doi:10.1016/j.chroma.2007.10.011

Araújo, S. S., Duque, A. S., Santos, D. M., & Fevereiro, M. P. (2004). An Efficient Transformation Method to Regenerate a High Number of Transgenic Plants Using a New Embryogenic Line of *Medicago truncatula* cv. Jemalong. *Plant Cell, Tissue and Organ Culture*, *78*(2), 123-131. doi:10.1023/B:TICU.0000022540.98231.f8

Arrabaça, M. (1981). The effect of temperature on photosynthetic and photorespiratory metabolism. PhD thesis, University of London.

Ashraf, M. (2010). Inducing drought tolerance in plants: recent advances. *Biotechnology advances*, *28*(1), 169-83. Elsevier Inc. doi:10.1016/j.biotechadv.2009.11.005

Avonce, N., Mendoza-Vargas, A., Morett, E., & Iturriaga, G. (2006). Insights on the evolution of trehalose biosynthesis. *BMC evolutionary biology*, *6*, 109. doi:10.1186/1471-2148-6-109

Baena-González, E., & Sheen, J. (2008). Convergent energy and stress signaling. *Trends in plant science*, *13*(9), 474-82. doi:10.1016/j.tplants.2008.06.006

Bahramnejad, B., Goodwin, P. H., Zhang, J., Atnaseo, C., & Erickson, L. R. (2010). A comparison of two class 10 pathogenesis-related genes from alfalfa and their activation by multiple stresses and stress-related signaling molecules. *Plant cell reports*, *29*(11), 1235-50. doi:10.1007/s00299-010-0909-6

Bajad, S. U., Lu, W., Kimball, E. H., Yuan, J., Peterson, C., & Rabinowitz, J. D. (2006). Separation and quantitation of water soluble cellular metabolites by hydrophilic interaction chromatography-tandem mass spectrometry. *Journal of chromatography. A*, *1125*, 76-88. doi:10.1016/j.chroma.2006.05.019

Barker, D. G., Pfaff, T., Moreau, D., Groves, E., Ruffel, S., Lepetit, M., Whitehand, S. (2006). Growing M . truncatula : choice of substrates and growth conditions. *Medicago truncatula*, chapter 1 (pp. 1-26).

Barnabás, B., Jäger, K., & Fehér, A. (2008). The effect of drought and heat stress on reproductive processes in cereals. *Plant, cell & environment*, *31*(1), 11-38. doi:10.1111/j.1365-3040.2007.01727.x

Basu, C., Kausch, A. P., Luo, H., & Chandlee, J. M. (2003). Promoter analysis in transient assays using a GUS reporter gene construct in creeping bentgrass (Agrostis palustris). *Journal of plant physiology*, *160*(10), 1233-9.

Cao, F. Y., Yoshioka, K., & Desveaux, D. (2011). The roles of ABA in plant-pathogen interactions. *Journal of plant research*, *124*(4), 489-99. doi:10.1007/s10265-011-0409-y

Capitão, C., Paiva, J. P., Santos, D. M, & Fevereiro, M. P. (2011). In *Medicago truncatula*, water deficit modulates the transcript accumulation of components of small RNA pathways. *BMC plant biology*, *11*(1), 79. BioMed Central Ltd. doi:10.1186/1471-2229-11-79

Chan, S. W. L. (2010). Chromosome engineering: power tools for plant genetics. *Trends in biotechnology*, *28*(12), 605-10. doi:10.1016/j.tibtech.2010.09.002

Chandler, P. M., & Robertson, M. (1994). Gene expression regulated by abscisic acid and i ts relation to s tress tolerance. *Annual Review of Plant Physiology*, *45*, 113-141.

Chaves, M. M., Maroco, J. P., & Pereira, J. S. (2003). Understanding plant responses to drought — from genes to the whole plant. *Functional Plant Biology*, *30*(3), 239. doi:10.1071/FP02076

Cook, D. R. (1999). *Medicago truncatula* - a model in the making! *Current Opinion in Plant Biology*, *2*, 301-304.

Cordeiro, M. A., Freitas, A. T., & Fevereiro, M. P. (2008). In silico discovery and characterization of ABA specific cis-elements and their validation in *Medicago truncatula*.

Cronk, Q., Ojeda, I., & Pennington, R. T. (2006). Legume comparative genomics: progress in phylogenetics and phylogenomics. *Current opinion in plant biology*, *9*(2), 99-103. doi:10.1016/j.pbi.2006.01.011

Cutler, S. R., Rodriguez, P. L., Finkelstein, R. R., & Abrams, S. R. (2010). Abscisic acid: emergence of a core signaling network. *Annual review of plant biology* (Vol. 61, pp. 651-79). doi:10.1146/annurev-arplant-042809-112122

Dawson, R., Elliot, D., Elliot, W., & Jones, K. (1986). *Data for Biochemical Research* (3rd ed.). Oxford, U.K.: Clarendon Press.

Debast, S., Nunes-Nesi, A., Hajirezaei, M.-R., Hofmann, J., Sonnewald, U., Fernie, A. R., & Börnke, F. (2011). Altering trehalose-6-phosphate content in transgenic potato tubers affects tuber growth and alters responsiveness to hormones during sprouting. *Plant physiology*. doi:10.1104/pp.111.179903

Delatte, T L, Schluepmann, H, Smeekens, S C M, de Jong, G J, & Somsen, G W. (2011). Capillary electrophoresis-mass spectrometry analysis of trehalose-6-phosphate in *Arabidopsis thaliana* seedlings. *Analytical and bioanalytical chemistry*, *400*(4), 1137-44. doi:10.1007/s00216-011-4837-2

Delatte, Thierry L, Selman, M. H. J., Schluepmann, Henriette, Somsen, Govert W, Smeekens, Sjef C M, & de Jong, Gerhardus J. (2009). Determination of trehalose-6-phosphate in *Arabidopsis seedlings* by successive extractions followed by anion exchange chromatography-mass spectrometry. *Analytical biochemistry*, *389*(1), 12-7. Elsevier Inc. doi:10.1016/j.ab.2009.03.003

Deutsher, M. (1990). *Guide to Protein Purification* (1st ed., pp. 68 - 89). Connecticut, U.S.A.: Academic Press, inc.

Du, H., Zhang, Z., & Li, J. (2010). Isolation and functional characterization of a waterlogging-induced promoter from maize. *Plant cell reports*, *29*(11), 1269-75. doi:10.1007/s00299-010-0913-x

Elbein, A. D., Pan, Y. T., Pastuszak, I., & Carroll, D. (2003). New insights on trehalose: a multifunctional molecule. *Glycobiology*, *13*(4), 17R-27R. doi:10.1093/glycob/cwg047

Fernandez, O., Béthencourt, L., Quero, A., Sangwan, R. S., & Clément, C. (2010). Trehalose and plant stress responses: friend or foe? *Trends in plant science*, *15*(7), 409-17. doi:10.1016/j.tplants.2010.04.004

Flavel, R. (2004). *Introduction to Plan Biotechnology*. In P. Christou & H. Klee (Eds.), *Handbook of plant biotechnology* (1st ed., pp. 31-38). Wiley.

Gibson, S. I. (2004). Sugar and phytohormone response pathways: navigating a signalling network. *Journal of experimental botany*, *55*(395), 253-64. doi:10.1093/jxb/erh048

Goddijn, O. J., Verwoerd, T. C., Voogd, E., Krutwagen, R. W., de Graaf, P. T., van Dun, K., Poels, J., et al. (1997). Inhibition of trehalase activity enhances trehalose accumulation in transgenic plants. *Plant physiology*, *113*(1), 181-90.

Goddijn, O, & Smeekens, S. (1998). Sensing trehalose biosynthesis in plants. *The Plant journal : for cell and molecular biology*, *14*(2), 143-6.

Guo, L., Yu, Y., Xia, X., & Yin, W. (2010). Identification and functional characterisation of the promoter of the calcium sensor gene CBL1 from the xerophyte *Ammopiptanthus mongolicus*. *BMC plant biology*, *10*(35), 18. doi:10.1186/1471-2229-10-18

Guo, Y., & Gaiki, S. (2005). Retention behavior of small polar compounds on polar stationary phases in hydrophilic interaction chromatography. *Journal of Chromatography A*, *1074*(1-2), 71-80. doi:10.1016/j.chroma.2005.03.058

Gupta, A. K., & Kaur, N. (2005). Sugar signalling and gene expression in relation to carbohydrate metabolism under abiotic stresses in plants. *Journal of Biosciences*, *30*(5), 761-776. doi:10.1007/BF02703574

Gómez, L. D., Gilday, A., Feil, R., Lunn, J. E., & Graham, I. (2010). AtTPS1-mediated trehalose 6-phosphate synthesis is essential for embryogenic and vegetative growth and responsiveness to ABA in germinating seeds and stomatal guard cells. *The Plant journal : for cell and molecular biology*, *64*(1), 1-13. doi:10.1111/j.1365-313X.2010.04312.x

Halford, N G, & Hardie, D. G. (1998). SNF1-related protein kinases: global regulators of carbon metabolism in plants? *Plant molecular biology*, *37*(5), 735-48.

Halford, N. G. (2003). Metabolic signalling and carbon partitioning: role of Snf1-related (SnRK1) protein kinase. *Journal of Experimental Botany*, *54*(382), 467-475. doi:10.1093/jxb/erg038

Hirayama, T., & Shinozaki, K. (2007). Perception and transduction of abscisic acid signals: keys to the function of the versatile plant hormone ABA. *Trends in plant science*, *12*(8), 343-51. doi:10.1016/j.tplants.2007.06.013

Iordachescu, M., & Imai, R. (2008). Trehalose biosynthesis in response to abiotic stresses. *Journal of integrative plant biology*, *50*(10), 1223-9. doi:10.1111/j.1744-7909.2008.00736.x

Jefferson, R. A., Kavanagh, T. A., & Bevan, M. W. (1987). GUS fusions: B-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO Journal*, *6*(13), 3901 -3907.

Ji, X., Dong, B., Shiran, B., Talbot, M. J., Edlington, J. E., Hughes, T., White, R. G., Gubler, F., & Dolferus, R. (2011). Control of abscisic Acid catabolism and abscisic Acid homeostasis is important for reproductive stage stress tolerance in cereals. *Plant physiology*, *156*(2), 647-62. doi:10.1104/pp.111.176164

Kolbe, A., Tiessen, A., Schluepmann, Henriette, Paul, M., Ulrich, S., & Geigenberger, P. (2005). Trehalose 6-phosphate regulates starch synthesis via posttranslational redox activation of ADP-glucose pyrophosphorylase. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(31), 11118-23. doi:10.1073/pnas.0503410102

Kouchi, H., Imaizumi-Anraku, H., Hayashi, M., Hakoyama, T., Nakagawa, T., Umehara, Y., Suganuma, N., & Kawaguchi, M. (2010). How many peas in a pod? Legume genes responsible for mutualistic symbioses underground. *Plant & cell physiology*, *51*(9), 1381-97. doi:10.1093/pcp/pcq107

Kulkarni, M., & Phalke, S. (2009). Evaluating variability of root size system and its constitutive traits in hot pepper (*Capsicum annum* L.) under water stress. *Scientia Horticulturae*, *120*(2), 159-166. doi:10.1016/j.scienta.2008.10.007

Lee, S.-ji, Park, J. H., Lee, M. H., Yu, J.-hyun, & Kim, S. Y. (2010). Isolation and functional characterization of CE1 binding proteins. *BMC plant biology*, *10*(1), 277. BioMed Central Ltd. doi:10.1186/1471-2229-10-277

Leyman, B., Van Dijck, P., & Thevelein, J. M. (2001). An unexpected plethora of trehalose biosynthesis genes in *Arabidopsis thaliana*. *Trends in plant science*, *6*(11), 510-3.

León, P., & Sheen, J. (2003). Sugar and hormone connections. *Trends in Plant Science*, *8*(3), 110-116. doi:10.1016/S1360-1385(03)00011-6

Li, H.-W., Zang, B.-S., Deng, X.-W., & Wang, X.-P. (2011). Overexpression of the trehalose-6-phosphate synthase gene OsTPS1 enhances abiotic stress tolerance in rice. *Planta*. doi:10.1007/s00425-011-1458-0

Lunn, J. E., Feil, R., Hendriks, J. H. M., Gibon, Y., Morcuende, R., Osuna, D., Scheible, W.R., Carillo, P., Hajirezei, M., & Stitt, M. (2006). Sugar-induced increases in trehalose 6-phosphate are correlated with redox activation of ADPglucose pyrophosphorylase and higher rates of starch synthesis in *Arabidopsis thaliana*. *The Biochemical journal*, *397*(1), 139-48. doi:10.1042/BJ20060083

Ma, Y., Szostkiewicz, I., Korte, A., Moes, D., Yang, Yi, Christmann, A., & Grill, E. (2009). Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science (New York, N.Y.)*, *324*(5930), 1064-8. doi:10.1126/science.1172408

Mahajan, S., & Tuteja, N. (2005). Cold, salinity and drought stresses: an overview. *Archives of biochemistry and biophysics*, *444*(2), 139-58. doi:10.1016/j.abb.2005.10.018

Marcotte, W. R., Russell, S. H., & Quatrano, R S. (1989). Abscisic acid-responsive sequences from the em gene of wheat. *The Plant cell*, *1*(10), 969-76. doi:10.1105/tpc.1.10.969

Martínez-Barajas, E., Delatte, T., Schluepmann, Henriette, de Jong, Gerhardus J, Somsen, Govert W, Nunes, C., Primavesi, L. F., et al. (2011). Wheat grain development is characterized by remarkable trehalose 6-phosphate accumulation pregrain filling: tissue distribution and relationship to SNF1-related protein kinase1 activity. *Plant physiology*, *156*(1), 373-81. doi:10.1104/pp.111.174524

McCourt, P., & Creelman, R. (2008). The ABA receptors - we report you decide. *Current opinion in plant biology*, *11*(5), 474-8. doi:10.1016/j.pbi.2008.06.014

Melcher, K., Zhou, X. E., & Xu, H. E. (2010). Thirsty plants and beyond: structural mechanisms of abscisic acid perception and signaling. *Current opinion in structural biology*, *20*(6), 722-9. doi:10.1016/j.sbi.2010.09.007

Menand, B., Meyer, C., & Robaglia, C. (2004). Plant growth and the TOR pathway. *Current topics in microbiology and immunology*, *279*, 97-113.

Mittler, R. (2006). Abiotic stress, the field environment and stress combination. *Trends in plant science*, *11*(1), 15-9. doi:10.1016/j.tplants.2005.11.002

Moore, B., & Sheen, Jen. (1999). Plant sugar sensing and signaling – a complex reality. *Trends in Plant Science*, *4*(1998), 10299-10299.

Morran, S., Eini, O., Pyvovarenko, T., Parent, B., Singh, R., Ismagul, A., Eliby, S., Shirley, N., Langridge, P., &Lopato, S. (2011). Improvement of stress tolerance of wheat and barley by modulation of expression of DREB/CBF factors. *Plant biotechnology journal*, *9*(2), 230-49. doi:10.1111/j.1467-7652.2010.00547.x

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissues cultures. *Physiologia Plantarum*, *15*, 473–497.

Nambara, E., & Marion-Poll, A. (2005). Abscisic acid biosynthesis and catabolism. *Annual review of plant biology*, *56*, 165-85. doi:10.1146/annurev.arplant.56.032604.144046

Neves, L. O., Tomaz, L., & Fevereiro, M P S. (2001). Micropropagation of *Medicago truncatula* Gaertn . cv . Jemalong and Medicago truncatula ssp . Narbonensis. *Plant Cell*, *67*, 81-84.

Nordström, A., Tarkowski, P., Tarkowska, D., Dolezal, K., Åstot, C., Sandberg, G., & Moritz, T. (2004). Derivatization for LC-Electrospray Ionization-MS: A Tool for Improving Reversed-Phase Separation and ESI Responses of Bases, Ribosides, and Intact Nucleotides. *Analytical Chemistry*, *76*(10), 2869-2877. doi:10.1021/ac0499017

Nunes, C., Araújo, S. S., Silva, J. M., Fevereiro, M. P., & Silva, A. B.. (2008). Physiological responses of the legume model *Medicago truncatula* cv. Jemalong to water deficit. *Environmental and Experimental Botany*, *63*(1-3), 289-296. doi:10.1016/j.envexpbot.2007.11.004

Paul, M. (2007). Trehalose 6-phosphate. *Current opinion in plant biology*, *10*(3), 303-9. doi:10.1016/j.pbi.2007.04.001

Paul, M. J., Primavesi, L. F., Jhurreea, D., & Zhang, Y. (2008). Trehalose metabolism and signaling. *Annual review of plant biology*, *59*, 417-41. doi:10.1146/annurev.arplant.59.032607.092945

Pellny, T. K., Ghannoum, O., Conroy, J. P., Schluepmann, H., Smeekens, S., Andralojc, J., Krause, K. P., Goddijn, O., & Paul, M.(2004). Genetic modification of photosynthesis with *E . coli* genes for trehalose synthesis. *Plant Biotechnology Journal*, *2*, 71-82. doi:10.1046/j.1467-7652.2003.00053.x

Quaedvlieg, N. E., Schlaman, H. R., Admiraal, P. C., Wijting, S. E., Stougaard, J., & Spaink, H. P. (1998). Fusions between green fluorescent protein and beta-glucuronidase as sensitive and vital bifunctional reporters in plants. *Plant molecular biology*, *38*(5), 861-73.

Raghavendra, A. S., Gonugunta, V. K., Christmann, A., & Grill, E. (2010). ABA perception and signalling. *Trends in plant science*, *15*(7), 395-401. doi:10.1016/j.tplants.2010.04.006

Rasband, W. S. (n.d.). ImageJ. U. S. National Institutes of Health, Bethesda, Maryland, USA. Retrieved from http://imagej.nih.gov/ij/

Richards, B., Krakowka, S., Dexter, L. B., Schmid, H., Wolterbeek, a P. M., Waalkens-Berendsen, D. H., Shigoyuki, a, & Kurimoto, M. (2002). Trehalose: a review of properties, history of use and human tolerance, and results of multiple safety studies. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*, *40*(7), 871-98.

Rolland, F., Moore, B., & Sheen, Jen. (2002). Sugar Sensing and Signaling in Plants. *The Plant Cell*, 185-205. doi:10.1105/tpc.010455.S186

Rorat, T. (2006). Plant dehydrins-tissue location, structure and function. *Cellular & molecular biology letters*, *11*(4), 536-56. doi:10.2478/s11658-006-0044-0

Rose, R. J. (2008). *Medicago truncatula* as a model for understanding plant interactions with other organisms, plant development and stress biology: past, present and future. *Functional Plant Biology*, *35*(4), 253. doi:10.1071/FP07297

Rozen, S., & Skaletsky, H. J. (2000). Primer3 on the WWW for general users and for biologist programmers. In S. Krawetz & S. Misener (Eds.), *Bioinformatics Methods and Protocols: Methods in Molecular Biology* (pp. 365-386). Totowa, NJ: Humana Press.

Santos, D. M., Araújo, S. S., Duque, A. S., & Fevereiro, M. P. (2003). Reverse transcription-PCR assay to verify gene integrity within plasmid constructs for plant transformation. *Plant Cell Tissue and Organ Culture*, *74*, 293-296.

Sato, S., & Tabata, S. (2006). Lotus japonicus as a platform for legume research. *Current opinion in plant biology*, *9*(2), 128-32. doi:10.1016/j.pbi.2006.01.008

Schenk, P. M., Elliott, A. R., & John, M. (1998). Assessment of Transient Gene Expression in Plant Tissues Using the Green Fluorescent Protein as a Reference. *Plant Molecular Biology Reporter*, 16, 313-322.

Schlichtherle-Cerny, H., Affolter, M., & Cerny, C. (2003). Coupled to Electrospray Mass Spectrometry of Small Polar Compounds in Food Analysis. *Analytical Chemistry*, *75*(10), 2349-2354.

Schluepmann, H., Dijken, A. V., Aghdasi, M., Wobbes, B., Paul, M., & Smeekens, S. (2004). Trehalose Mediated Growth Inhibition of *Arabidopsis* Seedlings Is Due to Trehalose-6-Phosphate Accumulation. *Plant Physiology*,, *135*, 879-890. doi:10.1104/pp.104.039503.1

Seo, M., & Koshiba, T. (2002). Complex regulation of ABA biosynthesis in plants. *Trends in plant science*, *7*(1), 41-8.

Smeekens, Sjef, Ma, J., Hanson, J., & Rolland, F. (2010). Sugar signals and molecular networks controlling plant growth. *Current opinion in plant biology*, *13*(3), 274-9. doi:10.1016/j.pbi.2009.12.002

Stitt, M., Sulpice, R., & Keurentjes, J. (2010). Metabolic networks: how to identify key components in the regulation of metabolism and growth. *Plant physiology*, *152*(2), 428-44. doi:10.1104/pp.109.150821

Trindade, I., Capitão, C., Dalmay, T., Fevereiro, Manuel Pedro, & Santos, D. (2010). miR398 and miR408 are up-regulated in response to water deficit in *Medicago truncatula*. *Planta*, *231*(3), 705-16. doi:10.1007/s00425-009-1078-0

Umezawa, T., Nakashima, K., Miyakawa, T., Kuromori, T., Tanokura, M., Shinozaki, K., & Yamaguchi-Shinozaki, K. (2010). Molecular basis of the core regulatory network in ABA responses: sensing, signaling and transport. *Plant & cell physiology*, *51*(11), 1821-39. doi:10.1093/pcp/pcq156

Valliyodan, B., & Nguyen, H. T. (2006). Understanding regulatory networks and engineering for enhanced drought tolerance in plants. *Current opinion in plant biology*, *9*(2), 189-95. doi:10.1016/j.pbi.2006.01.019

Wasilewska, A., Vlad, F., Sirichandra, C., Redko, Y., Jammes, F., Valon, C., Frei dit Frey, N. (2008). An update on abscisic acid signaling in plants and more... *Molecular plant*, *1*(2), 198-217. doi:10.1093/mp/ssm022

Weiner, J. J., Peterson, F. C., Volkman, B. F., & Cutler, S. R. (2010). Structural and functional insights into core ABA signaling. *Current opinion in plant biology*, *13*(5), 495-502. doi:10.1016/j.pbi.2010.09.007

Wingler, A. (2002). The function of trehalose biosynthesis in plants. *Phytochemistry*, *60*(5), 437-40.

Wojciechowski, M. F., Lavin, M., & Sanderson, M. J. S. (2004). A phylogeny of legumes (*leguminosae*) based on mat k gene resolves many well - supported subclades within the family. *American Journal of Botany*, *91*(11), 1846-1862.

Yamaguchi-Shinozaki, K, & Shinozaki, K. (1994). A novel cis-acting element in an *Arabidopsis* gene is involved in responsiveness to drought, low-temperature, or high-salt stress. *The Plant cell*, *6*(2), 251-64. doi:10.1105/tpc.6.2.251

Yamaguchi-Shinozaki, Kazuko, & Shinozaki, Kazuo. (2005). Organization of cis-acting regulatory elements in osmotic- and cold-stress-responsive promoters. *Trends in plant science*, *10*(2), 88-94. doi:10.1016/j.tplants.2004.12.012

Yang, Y, Li, R., & Qi, M. (2000). In vivo analysis of plant promoters and transcription factors by agroinfiltration of tobacco leafs. *The Plant journal : for cell and molecular biology*, *22*(6), 543-51.

Yi, N., Kim, Y. S., Jeong, M. H., Oh, S. J., Jeong, J. S., Park, S. H., Jung, H., Choi, Y., & Kim, J. (2010). Functional analysis of six drought-inducible promoters in transgenic rice plants throughout all stages of plant growth. *Planta*, *232*(3), 743-54. doi:10.1007/s00425-010-1212-z

Young, N. D., Cannon, S. B., Sato, S., Kim, D., Cook, D. R., Town, C. D., Roe, B. A., Tabata, S. (2005). Sequencing the Genespaces of *Medicago truncatula* and *Lotus japonicus*. *Plant Physiology*, *137*, 1174-1181. doi:10.1104/pp.104.057034.1174

Zhang, W., Ruan, J., Ho, T. H. D., You, Y., Yu, T., & Quatrano, Ralph S. (2005). Cis-regulatory element based targeted gene finding: genome-wide identification of abscisic acid- and abiotic stress-responsive genes in *Arabidopsis thaliana*. *Bioinformatics (Oxford, England)*, *21*(14), 3074-81. doi:10.1093/bioinformatics/bti490

Zhang, Y., Primavesi, L. F., Jhurreea, D., Andralojc, P. J., Mitchell, R. a C., Powers, S. J., Schluepmann, H., Dellate, T., Wingler, A., Paul, M. (2009). Inhibition of SNF1-related protein kinase1 activity and regulation of metabolic pathways by trehalose-6-phosphate. *Plant physiology*, *149*(4), 1860-71. doi:10.1104/pp.108.133934

Zhu, H., & Snyder, M. (2002). "Omic" approaches for unraveling signaling networks. *Current Opinion in Cell Biology*, *14*(2), 173-179. doi:10.1016/S0955-0674(02)00315-0

Zhu, J.-K. (2002). Salt and drought stress signal transduction in plants. *Annual review of plant biology*, *53*, 247-73. doi:10.1146/annurev.arplant.53.091401.143329

http://www.medicago.org/genome/. Accessed during January of 2011.

http://www.medicagohapmap.org/?genome. Accessed in May of 2011.

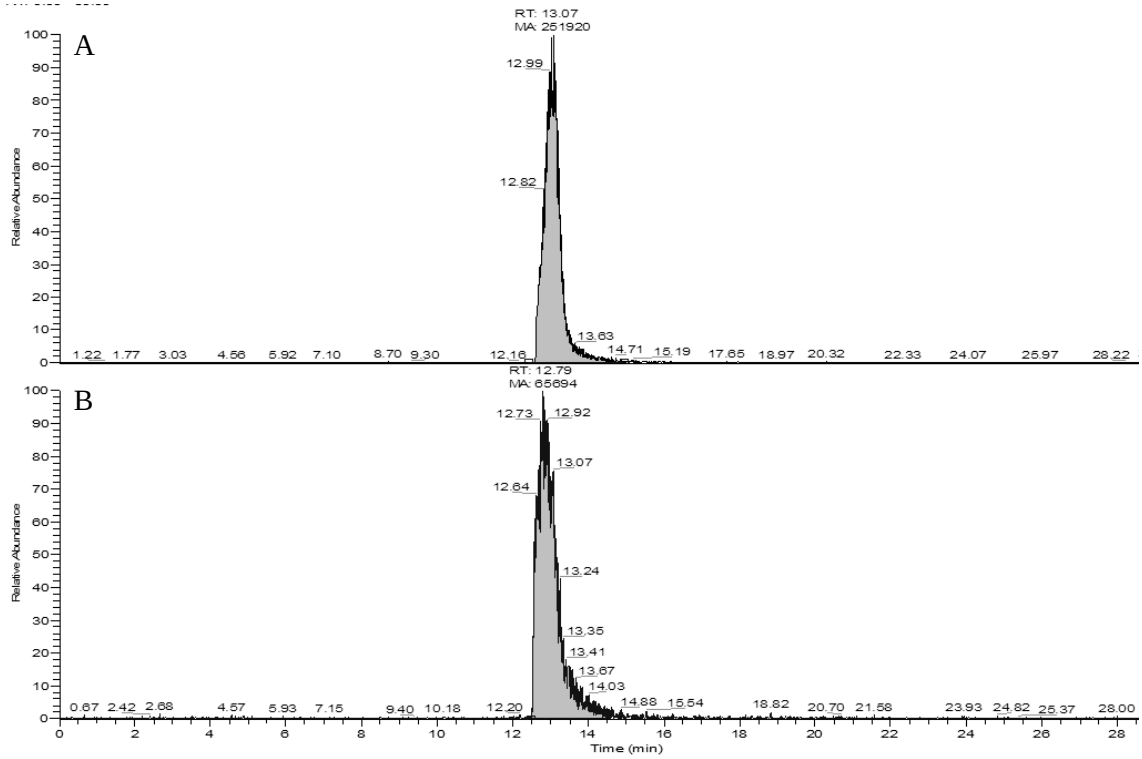
Annexes

Annex 1. DNA sequences from the three promoter segments after PCR reaction.

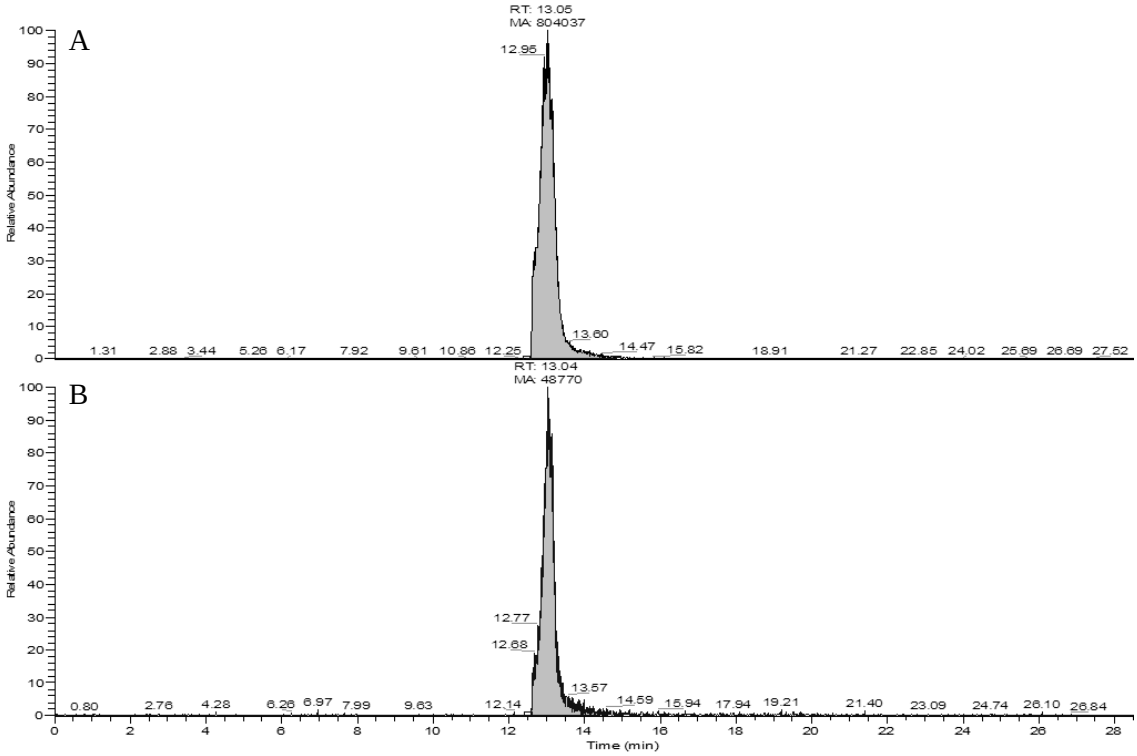
Promoter	Amplified Sequence
G02	TTTGCAAATATACTTTTAGTGTTTGTTTGTTACGTATAAAAAAAATTGTGATTTT TAAATTTAATGTTTTATAGATTCTATTAAAAAAAAAAGAAGCAAAATCAATTTT GTGTTCATTGATCGTAATAAAAATCATTTAGTTTGTTGGATACAACTATATAAA ATTATTATTTTTGTTACGAAAAACTTTTATTTTAAATTTTCTCTCATTAAAAAAA ATACTCTTAACAACTTTTTATTTAAATTTGTTTATAAAATTTCTTTTTGTTTTTTTT TTTTTAAAAGTTGTAGCAAACATATGCAATATTCTTAAAAATGATCTTTTTTCAC TAAAAAAGTTATGAACATGTTACTATAGATTAAAAAAGAAAAAGAATTTTGAA ATTTAATAATTTAAAAATTATTTTATAGAGATTTTTTTAGAAAGTAGAATTTATC TTGTGGTAATTTATTGTAATAAAAATAACTTAAAAAAAATATACTTTAGTTCATT CGAATAAGTGGAGTGTTATTTGAACAACTATTTGATTGACAACTTTTGGGACAA CCATAATTTACAAAGAAAAAATTGATATTTGCACGAAAACCAAAGCAATAGAG AGATAAAGTAAAAAGTAATGTGAGTATGAGAGAGAAAGTTGTTACAAAAATTA TCACAAAATGGATGTTCAAATATCATTTCTTTTTGAATAATTCTTTCCATCATTT AATGCTTATTTTCTCTCCCCTTTCCTTTGAATTGCTCTCTTTTTTTTTTTAAAGTTA CTCTAAACTGCTTTTCATTTCGAAGACATTGTCAAATTTTTTAATTTCAATTTTCT TAAAACGCTAACAAACAAGTACAGCACTCTAAAAGTGATTATTATTTTAAAAAA TGATTATTTTTTCTTACAAAATTATAACAACCTCACTCAATATAAGCCACGTGAC AAGCACAGTGGCAACTTCAGTTATGTTTT
G03	TGTTAACAATTATCCTAAAACCATCCTCAATTATATCACCATTGTGGTATTGATA CCTATTAGGTACTATCATTGTGGATTTTCTTTTTTGGATGCTTAATAAGTTGAGA GTCTCTAATAAACGTTTCTTATTTGTGAGCTCCACACATTTTTAATAATAAAAAT TAAAATATAATGATATAAAATTATTGATATTATTAGTGAGTCTGATCTAAAAAA TTAGTATCAAGTGCTATGTTTGAGAAATTAAATATTTTTTTTTAACAAATTAAAA TGAGATATATTACTAAACCAACAATGTTTCACTCTGCATAAGATGTGTAAAAGG AAAACACCAGAAAATATTTACAAGATTATGGTGCCAAAACAAAAGCACCATCT AGAATAAAAATTACAAGAGAACACACATACGAGGAATGAGGTGTTTCCACCAA TCATGGTAAAAATAGGTAAAATCCATCTATTTCGACCTTAACCATAAGAAAGAA TTAAACTTTACTTTGTCTAAGAGGTTAATAGGATTGAAAACCATATTTTAAAAT CTATGGTTATTCCTATCATTTCAAATGATCCAAAGTGTAGCAAACCAAATAATC GTAAAATATAACTGAGAATAAATATAGTAATAAAATTAGATGTACTATTTGTTA GGAATACTGCAATTTAAAAATAAATTGATGGATTGTTCTATTTTTATGATGTTGC CGTAAAGGCCACGTGATCCAAAGAATTAGCATTGAATGCAAATTTTATACTTTG GTCTATATCCTTATCCATTTTCACCTCACCGGTTATAGCCGGTAACATTTTAAAA TCCAGAAGCAAAGAATAAGCGCATAGTATCTAATGTAGTGATGAGATAAGAAG AAATCTAAACCGTCAGATAAAATTAATTAACATAATATAGAAAACCGGTTCAAT TTCGTTCTTATCCATAGTGGTATAGTCAC
G08	CCGACCATTTCTCCCTCTTTCCAAATTTTGTGAAGGATTTTAGTTTTGTGGTTCAT ATAATTGAGAACATCAAATTGCTCAACTCTTACTTTCGGTGGTGCAATCACTAC TCCTTGTCTACTCTATGCACACTGTGCTTTGAGTAAATTTATTTTAATTCAATCTT AATTTCAATATCTTTCAAGTAACATGACCACTCCCACTCAATCCAGTTGAAAAC ATTATGTCACACTTGGAATGAGAACTTGCAAGTTACAAGGCCACAAAACACAC ATGATAAAAGATAATTTGACGAGTAGGAGCATTGTTTGAAATTCCCAAGAAAA AACAACCATTATACTAACAAAAATAATGATAACCTCCATCTCGTAGTTCTAAAT TTCTTTTACTATCCCGTGAGTTTAGTTTGGTTGATAAAAAAAAAAAAAAAAAT TAGTCCGAGGCTTAAGAATTTTTTTACATCCATACTTAGTCCCAGGCTTATCCGT CTTTATCACCCGTGACGTGACAACAAGGTCACTTTGAAGATAAATGAGTAAAGT TTTCTCATTGCATTTTTTACAAAAAGAGTCATTCATAATCAAACTTCTTTAGAAA TATGTTTTCAATATAATTCATGGAGTTAAAGATAAATGTATTTTTATATAATATT AACTAATAAGTAAAAGTAAAAATAATAATATAATTCTCTTTAAAAAATGGTGTC ATGGTCGCCGCCCTCGAGGTATGGTATTCGCACGTGGCAATCTCTAGTGAAACT TGTCACCGTTTGG

Annex 2. Target sequence, expected PCR product size, forward and reverse primer sequences.

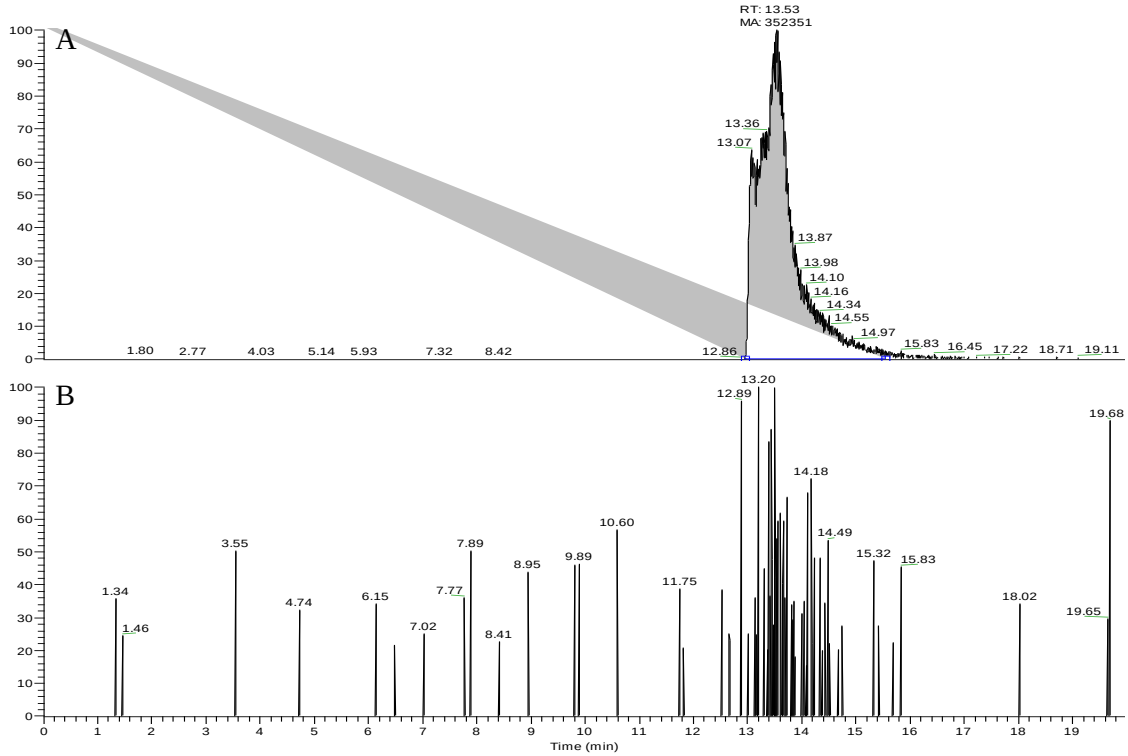
Target	Product Size (bp)	Forward Primer	Reverse Primer
G02	959	GGGGACAAGTTTGTACAAAAA AGCAGGCTTCTTTGCAAATAT ACTTTTAGTGTTTGTT	GGGGACAACTTTTGTATACAAA GTTGTAAAACATAACTGAAGTT GCCACTGTGC
G03	950	GGGGACAAGTTTGTACAAAAA AGCAGGCTTCTGTTAACAATT ATCCTAAAACCATCC	GGGGACAACTTTTGTATACAAA GTTGTGTGACTATACCACTATG GATAAGAACG
G08	774	GGGGACAAGTTTGTACAAAAA AGCAGGCTTCCCGACCATTTC TCCCTCTTTCC	GGGGACAACTTTTGTATACAAA GTTGTCCAAACGGTGACAAGTT TCACTAGA
gus:int-gfp-tnos	~3000	GGGGACAACTTTGTATACAAA AGTTGTCATGACGCACAATCC CACTATCCTT	GGGGACCACTTTGTACAAGAA AGCTGGGTATTGCGCGCTATAT TTTGTTTT
gus fragment	903	CAAGGCACTAGCGGGACTTT	GCCATGCACACTGATACTCTTC
M13	Variable	GTAAAACGACGGCCAG	CAGGAAACAGCTATGAC



Annex 3 SRM chromatograms for m/z 241 from standard solutions of S6P before (A) and after (B) the addition of AP. Numbers indicate the respective retention times; MA represents the area of the peak.



Annex 4. SRM chromatograms for m/z 241 from standard solutions of T6P before (A) and after (B) the addition of AP. Numbers indicate the respective retention times; MA represents the area of the peak.



Annex 5. SRM chromatograms for m/z 241 of T6P standard solutions before (A) and after (B) AP addition in acid (pH 4.5) buffer. Numbers indicate the respective retention times; MA represents the area of the peak.