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Expression of Alpha Amylase and Osmotin-Like Protein under Drought Stress in Sugar Beet

Youssef, A. B.[1]; Roba M. Ismail[1]; S. E. EL-Assal[2] and Naglaa A. Abdallah[2]*

[1]Gene Transfer Lab, Plant Genetic Transformation Department. Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center (ARC). Giza, Egypt

[2]Department of Genetics, Faculty of Agriculture, Cairo University, Giza, 12613, Egypt

[*]Corresponding author: Prof. Dr. Naglaa A. Abdallah: Professor of Genetics, Faculty of Agriculture, Cairo University, Senior Scientist at the Molecular Plant Virology Lab, Agricultural Genetic Engineering Research Ins. (AGERI), Agricultural Research Center (ARC).

Mobile: +20 (0)1223179109 - Email: Naglaa.abdallah@agr.cu.edu.eg

ABSTRACT

Sugar beet is considered as an essential crop to produce sugar, and its common name in plant breeding is *Beta vulgaris* L. Production of sugar beet is regularly limited by environmental circumstances which result in reduction of photosynthesis rates, sucrose accumulation and effects root development. Therefore research is needed to understand plant response to drought stress at the genomic level, to improve sugar beet crop drought tolerance. Various research efforts aimed to identify water deficit inducible genes by determining gene expression under a stresses abiotic. Our study focused on using qRT-PCR for studying gene expression in sugar beet under *in vitro* drought stress conditions. Treated plants were grown for 30 days on micro-propagation media with 0%, 3%, 5% and 7% PEG. Followed by total RNA extraction from leaves which reverse transcribed into cDNA, that is used as matrix in qPCR reaction using SYBR Green. The glutamine synthetase housekeeping gene, was employed as endogenous control, while alpha amylase and osmotin-like protein were used as target genes. The relative expression quantification values for our genes of interest were measured by the $2^{-\Delta\Delta CT}$ method. Alpha amylase and osmotin-like protein genes under drought stress showed a significant up-regulation expression. Additionally, qRT-PCR protocol provides an accurate and efficient result in studying the potential of expression analysis for the candidate genes under water stress in sugar beet. Our study could help in identifying plant response to abiotic stress at the gene expression level.

Keywords: Drought – qPCR – Amylase – osmotin –Sugar beet

INTRODUCTION

Sugar beet is ranked as the most economic crop for sugar production. According to FAO organization it produces about 25% of sugar worldwide production. Even though it is mainly grown in lands with moderate temperate, where irrigation is not usually operated in many production areas in addition to, summer rainfalls are inadequate and cannot be predicted to encounter crop's water demands. Meanwhile the summer drought can rigorously limit the sugar content, root's value and productivity in sugar beet (Sadeghian and Yavari, 2004). To overcome this problem, one solution is to develop cultivars which can resist drought tolerance. Drought is considered as one of the most abiotic stress which unfavorably affect crop yield and growth (Toker *et al*., 2007). Plant drought tolerance is a complex fact, in which changes occurs for the genotype in accordance to drought interval of time and strength, during plant's developmental stage to be able to resist stress conditions (Micheletto *et al*., 2007). Plant breeders main goal is to investigate plant tolerance process to handle plant's genetic variability to increase tolerant cultivars production (Santiago *et al*., 2009).

A previous study demonstrated that during drought stress there are multiple genetic variations in the genotypic response of sugar beet germplasm (Ober and Luterbacher, 2002). However, several research papers dedicated to this topic (Bloch and Hoffmann, 2005; Hajheidari *et al*., 2005; Hoffmann, 2010; Ober and Rajabi, 2010), breeding for drought tolerance is a complicated problem due to many contributing traits. In vitro culture techniques are determined as convenient and valuable tools for the study of plant stress tolerance processes, by reducing the environmental changes in addition to being able to study high number of samples in a limited period of time and space.

Recent advances rely on the plant molecular responses to recognize water deficiency inducible genes, subsequently to understand how plants react to water stress at the gene level where it is crucial for improving production and crop breeding (Bray, 2004; Stolf-Moreira *et al*., 2010). Microarrays and real-time reverse transcription PCR (qRT-PCR) are the frequent experimental techniques applied to quantify relative levels of gene expression. Microarray is the ideal method for large-scale (e.g., whole-genome) expression profiling, while the qRT-PCR technique is desired for quantifying the gene expression levels in different samples, including limited genes (VanGuilder *et al*., 2008). It offers a precise and perceptive quantification for gene transcript levels, as well as for the genes with relatively low transcript levels (Bustin, 2002; Nolan *et al*., 2006). Alpha amylases hydrolyze starch to glucose which is then altered to sucrose by phosphate synthase. Thus, we recommended alpha amylase gene as one of our targeted genes that could be used in further studies to observe sugar level in sugar beet during in vitro induced water deficiency. In a prior study Alpha amylase play a key role in the recovery of carbohydrates from impaired tissue to healthful plants tissues (Taski-Ajdukovic *et al*., 2012). Osmotins are considered as the members of Pathogenesis-related protein 5 (PR-5) family, which are produced in plants in presence of different abiotic and biotic stresses. Additionally, accumulation of pathogenesis related (PR) proteins are essential for plant defense mechanism. Osmotin and osmotin-like proteins (OLPs) related to the thaumatin-like protein. These proteins were indicated in many plant species, such as soybean, Arabidopsis thaliana and strawberry (Kumar *et al.,* 2015). Our aim of work is to

study the expression of some genes of sugar beet under in vitro induced water deficit conditions using real-time reverse transcription PCR methodology.

MATERIALS AND METHODS

1- Plant material and experimental treatment

In this study, we used palino genotype; which was obtained from sugar crop research institute- Agricultural research center - Giza. First we sterilized the seeds surface then MS medium containing 30 g/l sucrose and 8 g/l agar was used for its germination. After 10 days seedlings were transferred to micro-propagation medium: MS medium pH 5.8 including 0.3 mg/l 6-Benzyl aminopurine (BAP) and 0.01 mg/l Gibberellic acid (GA3) (Taski-Ajdukovic *et al*., 2012). Thereafter seedlings were left for twelve weeks for multiplication, with subsequent cultivation every three weeks, and then placed onto micro-propagation media containing polyethylene glycol (PEG 6000) with the following percentage 0%, 3%, 5% and 7% (w/v) to induce drought stress. 5 glass jars were used for each treatment including 75 ml of media, with four axillary shoots in each. All cultures were incubated at 25 ± 1ºC under 16 hours light. After 30 days, alterations in shoots growth parameters were analyzed. The leaves from plants were combined in duplicate, frozen using liquid nitrogen and stored at -80ºC.

2- RNA isolation and cDNA synthesis

Plant tissues were grinded to fine powder by using liquid nitrogen, followed by total RNA isolation using illustra RNAspin Mini Kit, NanoDrop™ 2000/2000c Spectrophotometers was used for measuring the concentration of total RNA isolated. Polymerase Chain Reaction (PCR) was carried out using glutamine synthetase primres to confirm the absence of contaminating genomic DNA in the isolated RNA by the absence of amplification product. In order to evaluate the quality of RNA, cDNA synthesis from the high-quality RNA was performed by using RevertAid™ Premium First Strand cDNA Synthesis Kit #K1651, followed the producer protocol. Primers oligo (dT)18 primer and random hexamer primer was added to 500 ng of total RNA, followed by 1 µL of 10 mM dNTP Mix, volume was completed to 15 µL by Water, nuclease-free. Then 5X RT Buffer (4 µL Maxima H Minus Enzyme Mixed 1 µL) Total volume (20 µL) Mix gently then centrifuge. The reaction was incubated at 25°C for 10 min followed by incubation for 15 min at 50 °C. Terminate the reaction by heating at 85 °C for 5 minutes.

3- Quantitative PCR

qPCR were carried out using glutamine synthetase specific primers as a reference gene. The PCR amplification reaction mixture of total volume 23µl was used, including 1x reaction buffer (Fermentas), 1.5 mM MgCl2, 0.2 mM dNTP, 0.5 µM primers. (Table 1), 1 unit Taq polymerase (Fermentas) and 2µl of template. Amplifications were carried out in a Biometra T-personal thermocycler using the following PCR profile: initial denaturation for 3 min at 95ºC followed by 30 cycles of denaturation at 95ºC for 30 sec and annealing at 60ºC for 1 min. primer extension was at 72 ºC for 1 min and final extension for 7 min. PCR products molecular size were imagined after electrophoresis on agarose gel (1.5 %). A GeneRulerTM 100 bp Plus and 50 bp DNA Ladder (Fermentas) was used as size reference. Sugar beet genomic DNA was shown on the gel.

Table 1. Primers sequences used in quantitative PCR

Gene Name	Primers	Accession no.	Sequence	Annealing temperature Ta	Molecular size
Alpha-amylase	Alpha-F Alpha-r	12324772	TGATTGGATGAATTGGCTGAAG AGCATATCCCTTGACAAAATCAAAT	62	73bp
Osmotin-like protein	Osmo-F Osmo-R	12324771	GCAAGTGCCCGCAACAC CGTAAGCGGAGTGATCCCTATT	63	65 bp
Glutamine synthetase	GluSyn-F GluSyn-R	(Taski-Ajdukovic *et al*., 2012)	GACCTCCATATTACTGAAAGGAAG GAGTAATTGCTCCATCCTGTTCA	60	110bp

4- Real-time PCR analyses

PCR reactions were prepared in 15µl volume containing 7.5µl iTaq™ universal SYBR® Green Super mix, 2µl primers (Table 1). cDNA template was adjusted in accordance to normalization result, then the volume was completed to 15µ using water nuclease-free, using the following profile: initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 5 sec, and annealing at 60°C for 1 min. Each PCR reaction was repeated three times; therefore, the final number of repetition for each experimental variation was two biological samples and every one was three technical replicates. PCR was carried out using a Real-Time PCR System (Applied Biosystems) in 96-well plates. The housekeeping gene Glutamine synthetase was used to evaluate the different amounts of expressed RNA used for cDNA synthesis (Mazzara *et al*., 2006). Relative quantification for the expression of our targeted genes was measured with comparative cycle threshold (CT) method (Livak and Schmittgen, 2001), using the following equation:

ΔCT = (CT target gene – CT glutamine synthetase) for control and treatment,

ΔΔCT = ΔCT treatment –ΔCT control.

Then Relative expression ratio was measured as $\text{Log}2^{-\Delta\Delta CT}$

The results were expressed as mean ± standard error (SE). Statistical analysis was carried out using the online software Graphpad.

RESULTS AND DISCUSSION

1 -Morphological changes

Several morphological changes were informed by comparing the shoots growth on specific control media using PEG treatment (Figure 1) as artificial condition to study drought tolerance. Generally, water stress effects on growth parameters, Such as reduced vegetative development, decreased shoots number (Table 2). parts of leaves base

were grown on 5 % and 7 % (PEG 600) media, Showed hyper hydration signs, that is look like callous appearance. Researchers stated that water deficiency stress is a crucial factor for the initial phase of plant growth and development, which affects plant growth and elongation (Kusaka *et al*., 2005; Kuykendall *et al*., 2008; Jaleel *et al*., 2009; Anjum *et al*., 2011). Moreover, Moosavi *et al*. (2017) reported that drought effects on dry weight of leaf, root, crown, total dry weight and root crop, Also drought stress effect on leaf temperature characters of different genotypes of sugar beet.

The decreases in the leaf surface area of the plants under water deficit stress due to decreased turgor pressure of cells, causing reduction in leaf growth, development, in addition to shedding of aged leaves, as a response for adaptation to water deficiency. On one hand, lower adapts are built, while leaf production as well as longitudinal, diagonal elongation of roots, yield and dry weight were reduced (Hajheidari *et al*., 2005). On the other hand, stomata closure and cells division sensitivity and growth under water deficiency stress, which can be mentioned as some reasons for lower vegetative growth and dry matter production under water deficit stress conditions. Farooq *et al*. (2012) reported that nutrient uptake decreased under water deficiency stress. Thus, drought stress reduced the leaves growth and development.

Fig. 1. The effect of polyethylene glycol (PEG 6000) with 0 %, 3 %, 5 % and 7 % in MS media on growth parameters.

Table 2. The effect of polyethylene glycol (PEG 6000) concentration 0 %, 3 %, 5 % and 7 % on number of shoot.

Number of shoots	control	3% PEG	5% PEG	7% PEG
	12	7	4	6
	12	7	5	4
	8	6	6	7
	10	10	8	8
	12	9	7	5
	11	10	8	7
Total	53	42	34	31
Average	10.6	8.4	6.8	6.2
SD	1.67	1.8	1.30	1.64
LSD 0.05			1,94	
LSD 0.05			2,64	

RNA isolation and cDNA synthesis validation

The PEG treatment affected the concentrations of total RNA isolated. The A260/A280 ratios were not affected by the PEG treatment in range 2.2 for all of the samples (Table 3).

Table 3. Concentration of isolated RNA (NanoDrop™ Spectrophotometers).

RNA Sample	Concentration (ng\µl)	A260/A280 ratios
control	674.3	2.21
3 % PEG	477.2	2.16
5 % PEG	504	2.18
7 % PEG	417.6	2.20

PCR with glutamine synthetase specific primers was carried out to indicate that the isolated RNA is uncontaminated with the genomic DNA, so that no amplification product was detected. Isolated RNA was reverse transcribed and the cDNA synthesis efficiency was determined by amplifying the housekeeping gene glutamine synthetase specific primers (Figure 4).

Quantitative PCR (qPCR)

The PCR primers specificity was evaluated by multiplex PCR reactions for target genes, after reaction products of expected size were confirmed by using gel electrophoresis. Primers pairs resulted in a unique PCR product of the expected size: Alpha amylse 73 bp and osmatin like protein 65 bp.

Thereafter by comparing gel electrophoresis bands of PCR products of cDNA for control and treated plants, it was shown that the bands of treated plants were sharp and more visible. This means that the drought stress may have an effect on Alpha amylse and osmatin like protein gene expression (Figure 5).

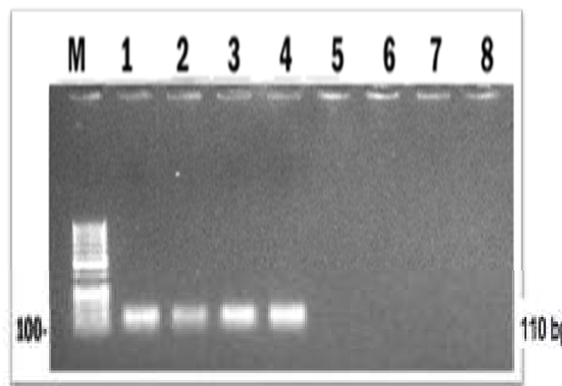

Fig. 4. Glutamine synthetase primers products on isolated RNA and cDNA. (M) 100bp DNA Ladder; Lanes 1 to 4: cDNA as template; Lanes 5 to 8: RNA as template.

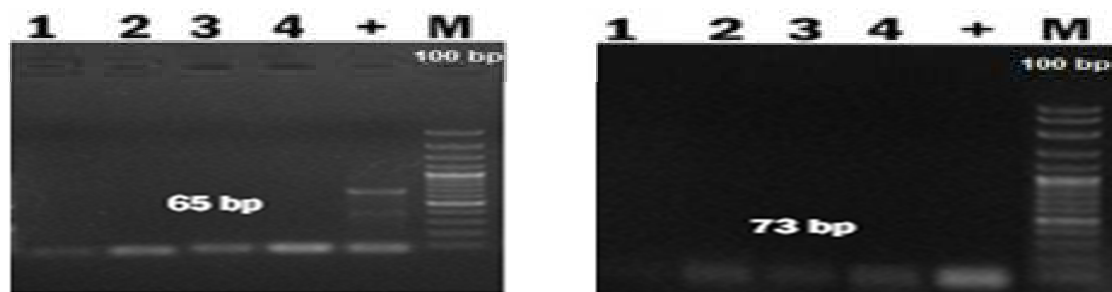

Fig. 5. PCR produced from using osmotin-like protein (65bp) and alpha-amylase (73bp) primers giving the expected band, used as preliminary test before starting real-time PCR analysis.

Results of qPCR analysis for studying the expression of alpha amylase showed that there are changes in expression under in vitro drought stress. Plants treatment with 3% PEG gave an increase in alpha amylase expression of 0.72 folds (P value equals 0.0094) compering to the control, while 5% PEG cause increase of 1.02 folds (P value equals 0.0009) and 7% PEG resulted in 1.13 folds (P value equals 0.0013) increase. In addition, osmotin-like protein gene under drought stress showed there are an over expression in transcription of this gene. The 3% PEG treatment caused an increase of 0.41 (P value equals 0.1715) fold changes comparing with control, 5% PEG resulted in 0.49 (P value equals 0.0311) fold increase while 7% caused an increase of 0.66 fold (P value equals 0.0074) (Figure 6).

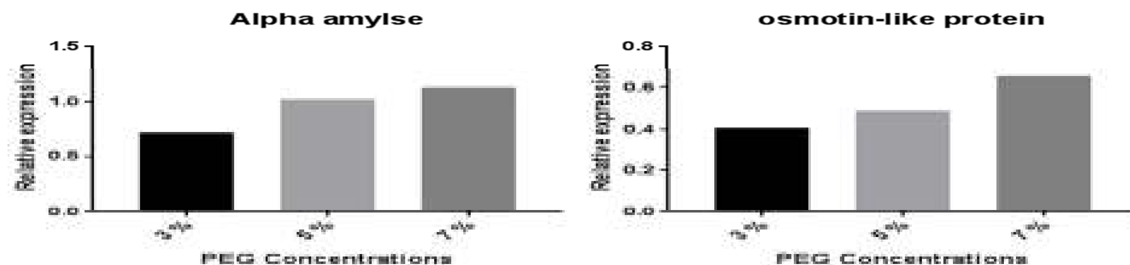

Fig. 6. Effect of water deficit on expression of Alpha amylse and osmatin like protein. Bars represent the fold changes happened in gene expression under different levels of drought stress treatment (charts by Graph prism software).

Alpha amylase and osmotin-like protein genes were selected as investigation genes indicating a significant up regulation in sugar beet transcription levels under stress (Pestsova *et al*., 2008), while osmotin and osmotin like protein play an important role in plant defense and osmotic stress (Datta *et al*., 1999). Furthermore, the over-expression in salt, drought, cold, and disease resistance crops were reported (Suarez-Rodriguez *et al*., 2007; Das *et al*., 2011). Osmotin and osmotin like protein belonged to the gene products group which affects abiotic stresses induced responses that maybe award a direct resistance with chaperones, heat-shock proteins, antifreeze proteins, late embryogenesis abundant (LEA) proteins, mRNA-binding proteins, enzymes accountable for osmolyte biosynthesis, enzymes of detoxification, water channel proteins, saccharides, proline transporters, as well as many proteases. (Yamaguchi-Shinozaki and Shinozaki, 2006; Stolf-Moreira *et al*., 2010). Water deficiency result in starch hydrolyses and participate in increasing soluble sugar level, mostly sucrose in sugar beet leaves (Fox and Geiger, 1986). Sucrose is considered as a common osmolyte in higher plants that significantly participate towards osmo-regulation (Morgan, 1983). Starch is converted into glucose by alpha amylases enzyme, which is then converted into sucrose by phosphate synthase glucose. Therefore, alpha amylase was selected as one of the target genes in further studies with which we would be able to observe the status of sugar level in sugar beet crop throughout induction of water deficiency in vitro. qPCR resulte were predicted, bearing in mind that alpha amylase role in raising the sucrose concentrations, as an important osmo-regulation parameter in higher plants. Additionally, our research proved that osmatin family play an essential role during drought stress.

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التعبير الجينى لإنزيم الألفا أميليز وبروتين شبيه الأوزماتين فى بنجر السكر تحت تأثير الجفاف

أبو بكر الصديق أحمد يوسف[١] ، ربى مدحت اسماعيل[١] ، صلاح الدين العسال[٢] و نجلاء عبدالمنعم عبد الله[٢]

[١]معهد بحوث الهندسة الوراثية الزراعية- مركز البحوث الزراعية - جيزة – مصر

[٢] قسم الوراثة – كلية الزراعة – جامعة القاهرة – مصر

يعتبر محصول بنجر السكر من المحاصيل الهامة اقتصاديا حيث أنه يدخل بشكل أساسي في إنتاج السكر. و يتأثر إنتاج بنجر السكر بالظروف البيئية غير الملائمة مما يؤدي إلي خفض مستوي التمثيل الضوئي و نمو الجذور و تخزين السكر في الجذور. وبالتالي نحتاج (لتحسين قدرة محصول بنجر السكر علي تحمل و تفادي الضغوط الحادثة نتيجة الجفاف) أن ندرس قدرة النبات للتعايش ومواجهة تأثير نقص المياة علي مستوي المادة الوراثية. وقد وجد أن هناك جهوداً من الباحثين خلال الفترة الأخيرة لدراسة التعبير الجيني للنبات تحت الظروف الإجهادات البيئية خاصة لتحديد الجينات المتأثرة بالإجهاد المائى. وقد إرتكز هذا العمل على إستخدام تفاعل البوليميرز الكمى اللحظي لدراسة التعبير الجيني لنباتات البنجرتحت تأثير مستويات من الجفاف من خلال إضافة مادة بولي إيثلين جليكول بتركيزات ٣% و ٥% و ٧% إلي بيئة معملية لنمو النباتات لمدة ٣٠ يوما حيث تم استخلاص الحامض النووي الريبوزي ثم تم تحويله إلي الحمض النووي المكمل (cDNA) باستخدام إنزيم النسخ العكسي ثم إستخدم الحامض النووى المكمل فى تفاعل البوليمريز المتسلسل الكمى المتتبع لحظيا بواسطة التعليم الفلوروسنتى بـSYBR Green . وقد أجريت الدراسة علي الجين المنتج للألفا أميليز وجين بروتين شبيه الأوزماتين و تم استخدام جين تخليق الجلوتامين كجين مرجعي. وتم حساب القيم الكمية للتعبير الجينى النسبى (نسبة ◌ لتعبير الجين المرجعى) بإستخدام طريقة $2^{-\Delta\Delta Ct}$. وقد أظهرت الدراسة زيادة معنوية في التعبير الجيني للجينات محل الدراسة (الفا أميليز وجين بروتين شبيه الأوزماتين) تحت ظروف الجفاف معملياً ، مما يدل على كفاءة تفاعل البوليمريز المتسلسل الكمى اللحظي لدراسة التعبير الجيني لهذه الجينات بالبنجر تحت ظروف الجفاف. وبهذا فإن هذا البحث يساعد على فهم إستجابة النبات للإجهاد البيئى علي مستوي التعبير الجينى.