

Development and application of qRT-PCR for sugar beet gene expression analysis in response to *in vitro* induced water deficit

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Abstract Sugar beet is a significant industrial crop, often grown in the areas where summer drought can severely limit root yield and sugar content. In order to improve development of sugar beet cultivars with increased drought tolerance it is necessary to understand plant response to water stress at the genomic level. Since recent research efforts have focused on the molecular response of the plant in order to identify water deficit inducible genes, the aim of this investigation was to develop qRT-PCR methodology for the quantification of gene expression in sugar beet under conditions of water deficiency *in vitro*. Sugar beet genotypes, selected for different response to water deficit, were grown and multiplied *in vitro*. Axillary shoots were placed on micropropagation media with 0%, 3% and 5% PEG, for 28 days. To determine reaction of sugar beet genotypes to *in vitro* induced water deficit changes in number of axillary shoots, shoot fresh weight and dry matter content were measured. Total RNA was extracted from leaves and reverse transcribed into cDNA, which served as matrix in real-time PCR reaction using TaqMan technology. The housekeeping gene for glutamine synthetase was used as endogenous control, while the genes for alpha amylase and osmotin-like protein were target genes. The relative quantification values for each target gene were calculated by the $2^{-\Delta\Delta C_t}$ method. Selected candidate genes differed in relative gene expression among genotypes and applied PEG treatments. The obtained results indicated that qRT-PCR protocol was efficient and accurate, showing the potential to be used in further expression analysis of candidate genes involved in sugar beet reaction to water stress.

Keywords: *Beta vulgaris*, drought, *in vitro*, real-time PCR.

INTRODUCTION

Drought is one of the most common environmental stress factors that adversely affect plant growth and development. Tolerance to drought is a complex phenomenon, because it changes according to drought intensity and duration, plant's developmental stage during which drought occurs and ability of genotype to tolerate situations of stress (Micheletto et al. 2007). Plant breeders have aimed to understand the tolerance process in order to manipulate the genetic variability for development of more tolerant cultivars (Rodrigues et al. 2009).

Sugar beet (*Beta vulgaris* L.) is a crop of significant economic importance and it accounts for about 25% of worldwide sugar production (<http://www.fao.org>). Although it is primarily grown in countries with temperate climate, there are many production areas where irrigation is not usually applied and summer rainfalls are unpredictable and insufficient to fully meet the crop's water requirements. Since the summer drought can severely limit root yield and quality, as well as sugar content in sugar beet (Sadeghian and Yavari, 2004), it becomes clear that the most economically viable solution for overcoming this problem is the development of cultivars with increased drought tolerance.

There is much genetic variation in genotypic reaction to drought stress within a sugar beet germplasm (Ober and Luterbacher, 2002). Although several papers devoted to this topic (Ober et al. 2004; Bloch and Hoffmann, 2005; Ober et al. 2005; Bloch et al. 2006; Hoffmann, 2010), breeding for drought tolerance is very difficult and complex because of many participating traits. *In vitro* culture techniques can be useful in the study of stress tolerance mechanisms, as they minimize environmental variations and at the same time enable studying large number of samples in a limited space and short period of time.

Since an understanding of how plants respond to water stress at the gene level is essential for crop breeding and improvement of production, recent research efforts have focused on the molecular responses of the plant, in order to identify water deficit inducible genes (Bray, 2004; Stolf-Moreira et al. 2010). Common experimental techniques used to quantify relative levels of gene expression are microarrays and real-time reverse transcription PCR (qRT-PCR). Microarray analysis are the preferred method for large-scale (e.g., whole-genome) expression profilings, while the qRT-PCR has become the method of choice for measuring gene expression levels in multiple samples, involving a limited number of genes (Vanguilder et al. 2008). It provides accurate and sensitive quantification of gene transcript levels, even for those genes with fairly low transcript levels (Bustin, 2002; Nolan et al. 2006).

In order to develop accurate qRT-PCR protocols, it is necessary to establish experimental design and assay validation and optimization. The aim of this research was to develop efficient and reliable real-time reverse transcription PCR methodology for gene expression analysis in sugar beet under conditions of *in vitro* induced water deficiency.

MATERIALS AND METHODS

Plant material and experimental treatment

For this investigation were used four diploid, monogerm inbred lines, here marked as genotypes 7, 8, 10 and 12. They were selected during the field observation test of sugar beet breeding material at Institute of Field and Vegetable Crops, Novi Sad (IFVCNS), for differences in their ability to maintain turgor in the conditions of the summer drought. Genotype 12 showed the smallest loss of turgor, genotype 10 was characterized as very sensitive, whilst genotypes 7 and 8 are considered to be sensitive to drought stress. All of them derived from the same population developed by successive hybridization, where genotypes 8, 10 and 12 had the common parent.

The genotypes were tested for different response to drought stress *in vitro* as described in Nagl et al. (2010). Seeds were surface sterilized and placed on MS medium (Murashige and Skoog, 1962) with 30 g/l sucrose and 7 g/l agar, for germination. After 10 days, the seedlings were transferred onto micropropagation medium: MS medium containing 0.01 mg/l Gibberellic acid (GA₃) and 0.3 mg/l 6-Benzylaminopurine (BAP), pH 5.8 (Mezei et al. 2006). They were multiplied for twelve weeks, with sub-cultivations every three weeks, and then transferred onto micropropagation media containing 0%, 3% and 5% (w/v) polyethylene glycol (PEG 6000) in order to induce water stress. For each treatment there were six plastic boxes 90 x 95 x 95 mm (HxWxD), containing 150 ml of media, with four axillary shoots in each of them. All the cultures were maintained at 23 ± 1°C under 16 hrs illumination. After 28 days, changes in growth parameters of 16 shoots (from 4 containers) were analysed. The leaves from four plants in other two containers were pooled in duplicate, frozen in liquid nitrogen and stored at -70°C.

Growth parameters

To determine the effect of PEG treatment on sugar beet genotypes number of axillary shoots, shoot fresh weight and dry matter content of samples were measured. The shoot fresh weight was determined by dividing fresh weight of the sample and number of shoots. The dry matter content was estimated by dividing dry weight with fresh weight of the explants.

RNA isolation and cDNA synthesis

Total RNA was extracted by the filter-based RNAqueous Small Scale Phenol-Free Total Isolation Kit (Ambion, USA), according to the instructions of the supplier. During the isolation, Plant RNA Isolation

Aid was also used, a reagent containing a high molecular weight polymer to improve isolation of total RNA. To eliminate residual genomic DNA, RNA samples were treated with Turbo DNA-free™ Kit (Applied Biosystems, USA) according to the instructions provided by the supplier. Concentration of isolated RNA was measured using UV-visible Spectrophotometer Evolution 100 (Thermo Electron Corporation, UK), and the ratio of absorbance A260/A280 was used to assess the purity of the isolated RNA. RNA integrity was verified by visualization on 1% agarose gels containing 0.005% ethidium bromide in 0.5 x TBE buffer. RiboRuler™ High Range RNA Ladder (Fermentas, Lithuania) was used as size reference. The visualization was performed in UV transilluminator and the images were captured with DOC PRINT system (Vilber Lourman, USA).

cDNA was synthesized from up to 0.5 µg total RNA using GeneAmp RNA PCR kit components (Applied Biosystems) according to instructions of the provider, in the Mastercycler gradient S thermocycler (Eppendorf, Germany).

In order to evaluate the quality of RNA, qPCR were performed with primer pair for glutamine synthetase (JRC, ISPRA, <http://gmo-crl.jrc.ec.europa.eu>), as a reference gene. For PCR amplification 23 µl of PCR mixture was used which contained: 1x reaction buffer (Fermentas), 1.5 mM MgCl₂, 0.2 mM dNTP, 0.5 µM primers, 1 unit Taq polymerase (Fermentas) and 2 µl of template. Amplifications were carried out in a Biometra Tpersonal thermocycler using the following PCR protocol: denaturation at 95°C for 5 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Positive (sugar beet genomic DNA), negative (fungal DNA) and non-template controls for PCR were used. PCR products were visualized after electrophoresis on 2% agarose gel. A GeneRuler™ 100 bp DNA Ladder Plus (Fermentas) was used as size reference.

Real-time PCR analyses

Primer and probe sequences were designed for genes SF_12-b11 (alpha-amylase, GenBank accession No. FG345587) and SF_59-204R (osmotin-like protein precursor, GenBank accession No. FG343788), based on gene sequences from National Center for Biotechnology Information Entrez Nucleotide Database (www.ncbi.nlm.nih.gov/sites/entrez), using the software Primer Express 3.0. The primers were submitted to NCBI Probe database (Table 1).

Table 1. Nucleotide sequences of the primers and probes used in real-time PCR.

Gene Name	Primer acces. no.	Primers/Probe	Sequence
SF_12-b11	12324772	Forward primer Reverse primer Probe	tgattggatgaattggctgaag agcatatcccttgacaaaatcaaat Fam-cggaaattggtttgacgggtgga-BHQ-1
SF_59-204R	12324771	Forward primer Reverse primer Probe	gcaagtgcccgcaacac cgtaagcggagtgatccctatt Fam-agcccaatttagcattcatg-BHQ-1
GS		Forward primer Reverse primer Probe	gacctcatattactgaaaggaag gagtaattgctccatcctgtca TexRed-ctacgaagttaaagtatgtccgctc-BHQ-2

Real-time PCR, using Taq Man technology, was performed on BioRad iQ cycler (BioRad, USA). Multiplex PCR reaction master mix was prepared with 1x iQ™ Multiplex Powermix (BioRad, USA), 200 nM TaqMan probes and 500 nM each of forward and reverse primers for both reference and target genes (Metabion, Germany) and 5 µl of product from reverse transcription reaction, in a total volume of 25 µl. iQ™ Multiplex Powermix contained: 2x reaction buffer with dNTPs, 12 mM MgCl₂, iTaq™ DNA polymerase and stabilizers. The TaqMan probe consisted of an oligonucleotide with a 5'-reporter dye and a 3'-quencher dye. Reporter dye used for GS was Texas Red. It fluorescence was quenched by Black Hole 2 Quencher Dye (BHQ-2). Target genes (SF_12-b11 and SF_59-204R) were labelled with Fam reporter dye which was quenched by Black Hole 1 Quencher Day (BHQ-1). PCR amplifications were performed using following thermal profile: 95°C for 5 min (initial activation of the DNA polymerase) 40 cycles of 95°C for 15 sec (denaturation) and 60°C for 1 min (annealing/extension) (Taski-Ajdukovic et al. 2011). Fluorescence data were collected during the extension phase of PCR. Three technical replications per biological replication were analysed.

Relative quantification of the target gene expression was calculated with comparative cycle threshold (Ct) method (Livak and Schmittgen, 2001), based on Equation 1:

$$\Delta Ct = (Ct_{\text{target gen}} - Ct_{\text{glutamine synthetase}}); \Delta\Delta Ct = \Delta Ct_{\text{treatment}} - \Delta Ct_{\text{control}}$$

$$\text{Relative expression ratio (R)} = 2^{-\Delta\Delta Ct}$$

[Equation 1]

Glutamine synthetase was used as internal control gene (Mazzara et al. 2006) to correct for different amounts of RNA input for cDNA synthesis. To graphically represent the results, relative expression ratio was transformed in log₂.

Since this method may be used only if efficiencies of the target and reference genes are similar, it was necessary to determine if any competition occurs within the multiplex reaction. Two fold serial dilution of cDNA were amplified using primers and probes specific for both target and internal control gene. A plot of ΔCt versus cDNA dilutions transformed in log₂ was made and the absolute value of slope was determined.

With aim to assess specificity of designed primers and probes, real-time reaction products were separated via electrophoresis on 3% (w/v) agarose gel (Agarose for PCR low melting, SERVA). A GeneRuler™ 50bp DNA Ladder (Fermentas) was used as size reference.

Statistical analysis

All results were expressed as mean ± standard error (SE). Statistical analysis was performed by analysis of variance (ANOVA), and comparisons between means were made by Duncan's multiple range post hoc test. Statistical significance was defined as being at the level $p < 0.05$.

RESULTS AND DISCUSSION

Morphological changes

Many morphological changes were notified by visual comparison of shoots grown on control media and PEG treatments (Figure 1). In general, water deficit caused a decrease in shoot growth, the reduction in the number of shoots, the reduction of the leaflets surface and a gradual loss of chlorophyll, which resulted in a colour change. Lower parts of shoots on 3% and 5% PEG media also showed signs of hyperhydration, which gave the leaves vitreous and callous look. Gradual increase of above mentioned morphological changes corresponding to the increase of PEG concentration were noticed in genotypes 7 and 10, while genotypes 8 and 12 exhibited drastic changes on the media with 3% PEG.

Growth parameters

It is known that water stress in culture can adversely affect plantlet growth and genotypes can differ for their responses (Gopal and Iwama, 2007). When, in our experiment, PEG concentration was increased, all tested genotypes reduced the number of shoots (Figure 2a). Further increase in concentration of PEG, did not affect the number of shoots.

Shoot fresh weight (Figure 2b) on 3% PEG treatment was increased in genotypes 7 and 10. Further increase of PEG concentration provoked a different response: shoot fresh weight did not change in genotype 10, while in genotype 7 there was further increase. In genotype 12 shoot fresh weight increased on 5% PEG treatment while in genotype 8 it was reduced.

The dry matter content on 3% PEG treatment increased in all genotypes (Figure 2c), but further increase of PEG concentration caused no changes.

RNA isolation and cDNA synthesis

Concentrations of isolated total RNA ranged from 0.1 µg/µl to 0.5 µg/µl and were diluted to approximately the same concentration for further analysis. Possible explanations for the difference in extraction efficiencies of RNA could be the amount of the starting material due to different status of the sampled tissues. Some samples, especially on media with 5% PEG were hyper hydrated and thus their handling (homogenization and measurement) was rather difficult. The A260/A280 ratios were lower than 2.0 (the expected value for a pure RNA sample) for all of the samples, ranging from 1.5 to 1.9. As judged by gel electrophoresis isolated RNA was high quality, regardless of water deficiency levels, caused by PEG treatment (Figure 3).

The absence of contaminating genomic DNA in isolated RNA was confirmed by absence of amplification product after PCR with primers for glutamine synthetase (Figure 4, lanes 1-4). Isolated RNA was reverse transcribed and the efficacy of cDNA synthesis was proved by amplification of a 110 bp product after PCR using primer for glutamine synthetase (Figure 4, lanes 5-8 and lane +).

Real-time PCR

Genes SF_12-b11 (alpha amylase) and SF_59-204 (osmotin-like protein), among other candidate genes, were chosen for this investigation as genes showing significantly different transcript levels under stress in sugar beet (Pestsova et al. 2008). Osmotin and osmotin like protein have dual function in osmotic stress and plant defence (Velazhahan et al. 1999), and their overexpression in a number of drought-, salt-, cold- and disease-tolerant crops were reported (Zhu et al. 1995; Zhang and Shih, 2007; Das et al. 2011). They belong to the group of gene products involved in abiotic stresses induced responses that probably confer direct tolerance with chaperones, heat-shock proteins, late embryogenesis abundant (LEA) proteins, antifreeze proteins, mRNA-binding proteins, key enzymes for osmolyte biosynthesis, water channel proteins, sugar and proline transporters, detoxification enzymes, and various proteases (Bray et al. 2000; Yamaguchi-Shinozaki and Shinozaki, 2006; Stolf-Moreira et al. 2010). Water deficit stimulates starch hydrolyses and contribute to increase the level of soluble sugar, particularly sucrose in sugar beet leaves (Fox and Geiger, 1986). Sucrose is universal osmolyte in higher plants which significantly contribute towards osmoregulation (Morgan, 1984). Starch is hydrolyzed to glucose by alpha amylases and is then converted to sucrose by phosphate synthase. Therefore, we chose alpha amylase as one of the candidate genes with which we will be able in the future to examine sugar status in sugar beet during *in vitro* induced water deficit.

Specificity of PCR primers for target genes was assessed by multiplex PCR reactions with glutamine synthetase primers, after which reaction products of expected size were separated. All primers pairs gave a unique PCR product of the expected size: SF_59-204 - 65 bp, GS - 110 bp (Figure 5) and SF_12-b11 - 73 bp.

Ideally, PCR results in an exact doubling of the amount of dsDNA after each cycle. In practice, however, this is generally not the case because the reactions are less than 100% efficient. Since in multiplex PCR both assays are amplified in the same tube and compete for the same reagents (dNTPs and polymerase), it is important that this competition is minimized. Assays can also inhibit each other through interactions among the primers, the probes, the targets or amplicons, or any combination of these (Gibson et al. 1996; Bustin, 2002). Absolute values of the slope for both multiplex PCR reactions were close to zero, 0.04 for SF_59-204 (Figure 6) and 0.064 for SF_12-b11, which meant that the efficiency of target genes and internal standard were approximately equal and the $2^{-\Delta\Delta CT}$ method for relative quantification of target gene could be used.

The effects of *in vitro* induced water deficit on mRNA transcription of genes SF_59-204 and SF_12-b11 in sugar beet genotypes with different response to drought stress was analysed. On 3% PEG treatment SF_59-204 (Figure 7) was significantly induced in genotypes 8 (3.14 fold, P=0.0008), 10 (12.72-fold, P=0.000167) and 12 (3.67-fold, P=0.0009). Exception was genotype 7, where overexpression was detected only on 5% PEG treatment (3.30-fold, P=0.0002). Gene expression in genotypes 8 and 12 decreased (P=0.0015 and 0.0005) on 5% PEG, but still was higher than in control (2.53-fold P=0.0002 and 2.37-fold P=0.0004). Unexpectedly, in the susceptible genotype 10 the transcript levels of osmotin-like protein gene further continues increased on 5% PEG (21.34-fold P=0.00009), suggesting that in this genotype there might be some other mechanisms involved that negated the effect of increased amounts of osmotin-like protein.

Expression analysis of SF_12-b11 gene (Figure 8) on 3% PEG treatment showed that in genotypes 8 and in tolerant genotype 12 there was increase in relative gene expression (4.98-fold $P=0.0001$ and 9.34-fold $P=0.0018$, respectively), while in genotypes 7 and 10 it remained insignificant. Further increase of PEG concentration to 5% resulted in significant induction of SF_12-b11 gene in genotype 7 (5.53-fold $P=0.00017$). On same PEG treatment gene expression in susceptible genotype 10 was 2.68-fold down regulated ($P=0.0002$). These results were expected, bearing in mind the role of alpha amylase in increasing concentrations of sucrose, as an important osmoregulation factor in higher plants.

The presented protocol showed how real-time reverse transcription PCR can successfully determine changes in the genetic expression of SF_12-b11 (alpha amylase) and SF_59-204 (osmotin-like protein) genes over time in response to changes in environmental conditions. It proved itself to be efficient and accurate, showing the potential for further use in expression analysis of other candidate genes involved in sugar beet reaction to water stress.

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Figures

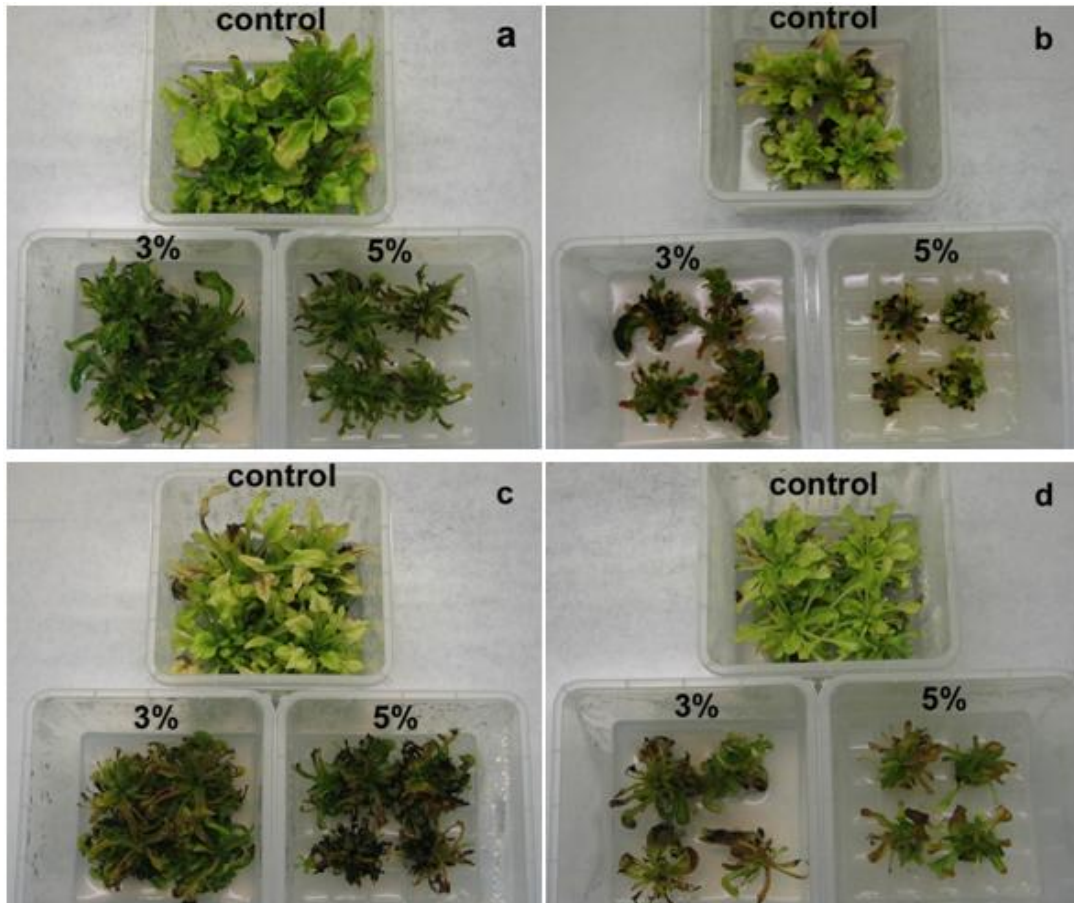


Fig. 1 Reaction of sugar beet genotypes to *in vitro* induced water deficit. (a) Genotype 7; (b) Genotype 8; (c) Genotype 10; (d) Genotype 12 (3%: media with 3% PEG, 5%: media with 5% PEG).

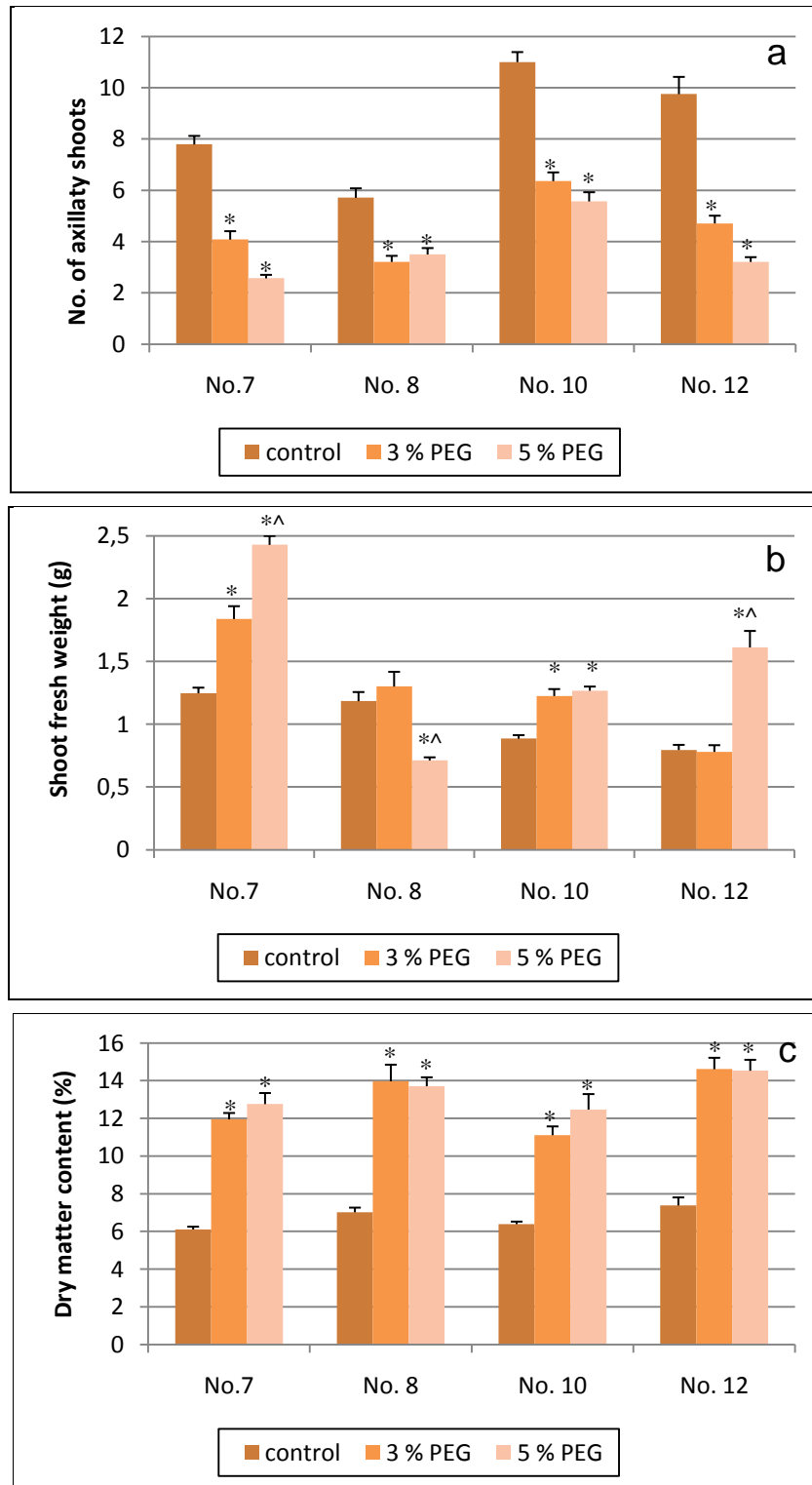


Fig. 2 Effect of *in vitro* induced water deficit on growth parameters in four sugar beet genotypes. (a) number of axillary shoots; (b) shoot fresh weight; (c) dry matter content.

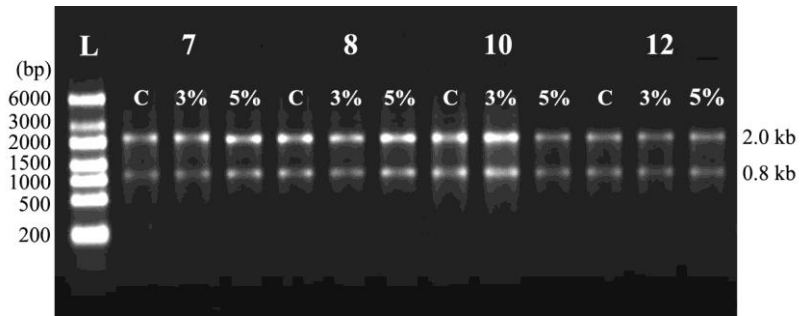


Fig. 3 Agarose gel electrophoresis of RNA isolated from *in vitro* samples of four sugar beet genotypes. Lane L: RiboRuler™ High Range RNA Ladder; Lane 7, 8, 10, 12: sugar beet genotypes; C: control; 3%: 3% PEG treatment; 5%: 5% PEG treatment.

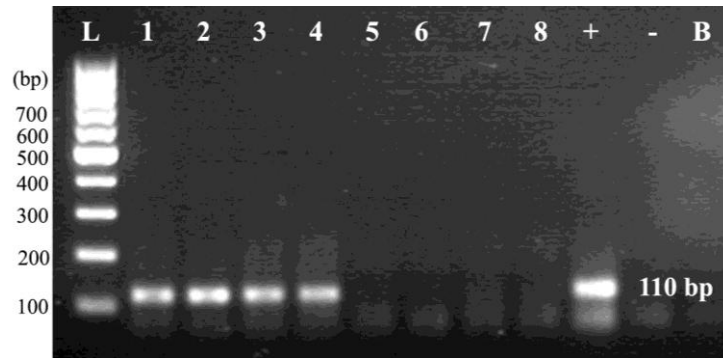


Fig. 4 Products of PCR amplification with glutamine synthetase primers on isolated RNA and cDNA. Lane L: GeneRuler™ 100bp DNA Ladder Plus; Lane 1-4: amplification products with cDNA as template; Lane 5-9: amplification products with RNA as template; Lane +: amplification products using sugar beet DNA; Lane -: amplification products using fungal DNA; Lane B: blank.

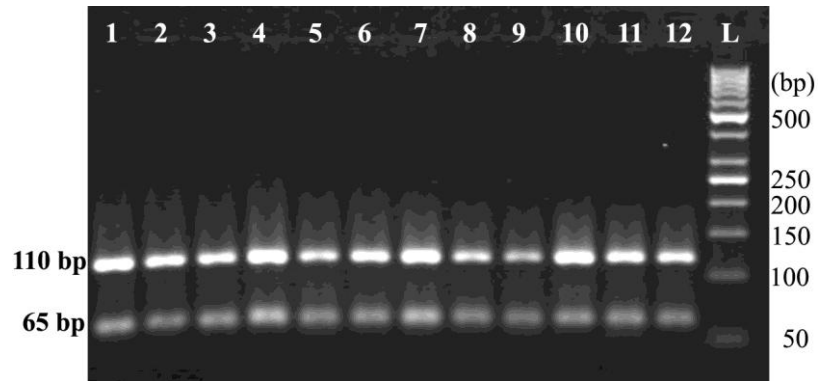


Fig. 5 PCR products of real-time PCR with SF_59-204 (65bp) and GS primers (110bp). Lane 1-12: sugarbeet samples; Lane L: GeneRuler™ 50bp DNA Ladder.

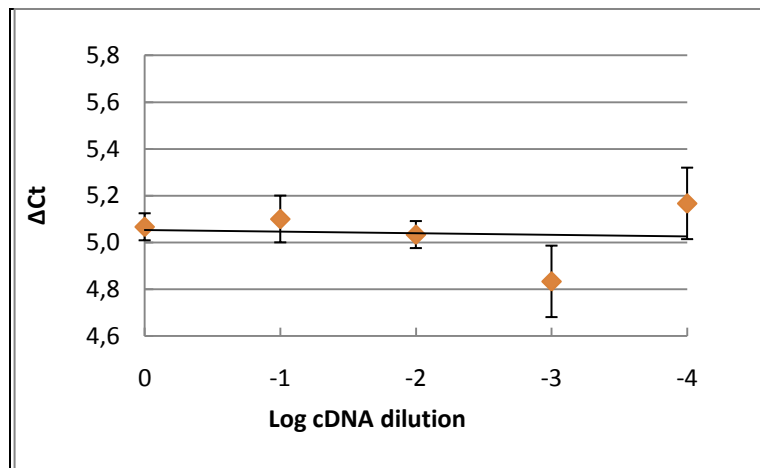


Fig. 6 Relative efficiency plot of amplification of target gene (SF_12-b11) and internal control (glutamine synthetase) examined by real-time PCR. Bars represent the standard error.

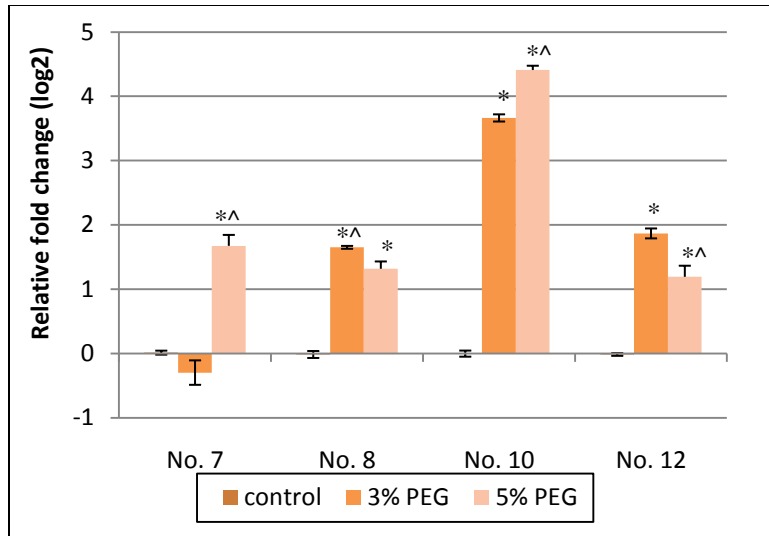


Fig. 7 Effect of water deficit on expression of gene SF_59-204 in four sugar beet genotypes. Bars represent the standard error. Columns labelled with an asterisk are significantly different from the corresponding control, while columns labelled with circumflex accent are significantly different between treatments (Duncan test, $p < 0.05$).

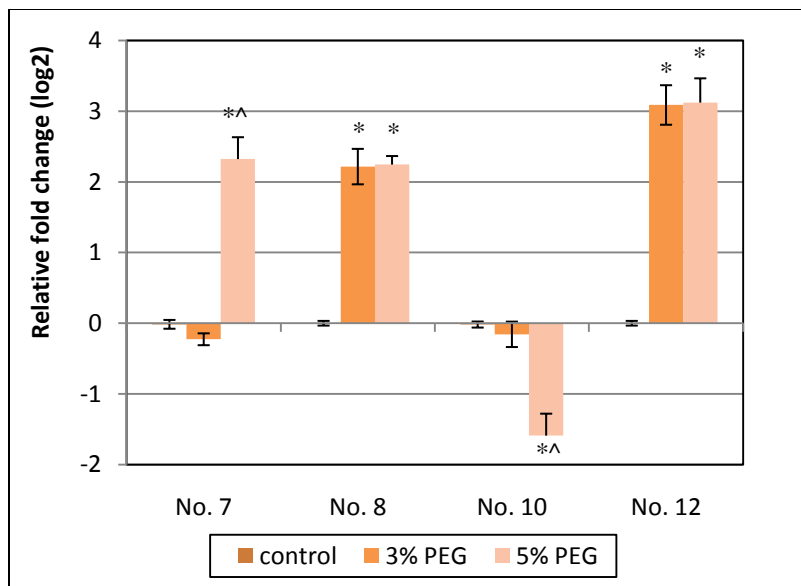


Fig. 8 Effect of water deficit on expression of gene SF_12-b11 in four sugar beet genotypes. Bars represent the standard error. Columns labelled with an asterisk are significantly different from the corresponding control, while columns labelled with circumflex accent are significantly different between treatments (Duncan test, $p < 0.05$).