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A Preliminary Study on Genotypic Differences in Transcript Abundance of Drought-Responsive Genes in Sugar Beet

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Abstract: In this study, four sugar beet genotypes of differing responses to drought were selected from a field experiment conducted under well-watered and water-limited conditions in 2004. In addition, two candidate genes: 2-cysteine peroxiredoxin (2-cys prx) and Nucleoside Diphosphate Kinase (NDPK), thought to be associated with drought tolerance, were chosen from a previous proteomics study in sugar beet. An expression analysis of the two drought-regulated genes using semi-quantitative reverse transcription Polymerase Chain Reaction (RT-PCR) indicated that there were genotypic differences in the transcript abundance of the candidate genes with the differences in the expression level of 2-cys prx being likely associated with the drought responses of the genotypes in a two-year field study. However, the expression analysis of the genes has to be investigated at different stages of the stress period on more genotypes.

Key words: Gene expression, drought tolerance, sugar beet

INTRODUCTION

Progress gained from direct selection for yield in water-limited environments has been slow due to high Genotype by Environment (G×E) interactions and hence low heritability of yield. In order to overcome this limitation, indirect selection of secondary traits such as Water Use Efficiency (WUE, total dry matter produced per water used) as estimated by carbon isotope discrimination (Δ) have been successfully used (Condon *et al*., 2002; Nageswara Rao and Wright, 1994). However, measurements of these traits on a large number of genotypes may not be feasible. Some traits have been suggested as alternatives for Δ and WUE. Among these are ash content (Araus *et al*., 1998; Voltas *et al*., 1998; Merah *et al*., 2001), stomatal conductance (White, 1993; Rebetzke *et al*., 2001), canopy temperature (Frank *et al*., 1997) chlorophyll content (Sheshshayee *et al*., 2006) and Specific Leaf Weight (SLW, leaf dry mass per unit leaf area) (Nageswara Rao and Wright, 1994; Araus *et al*., 1997). Although these traits are less expensive and easier to measure than Δ and useful for initial screening of large populations during the early generations of breeding programmes, they may not provide accurate, time-integrated estimates of Δ, WUE and yield. Furthermore, the expression of these traits can be influenced by environmental conditions to a greater extent than Δ (Teulat *et al*., 2002). In order to account for these limitations, molecular markers linked to the Quantitative Trait Loci (QTLs) controlling Δ and hence WUE should be investigated (Condon *et al*., 2002).

Marker-assisted selection was suggested as another indirect selection strategy (Mian *et al*., 1996; Teulat *et al*., 2002; Masle *et al*., 2005). Most water-use related traits such as Δ and WUE are quantitatively inherited as their expression is controlled by several QTLs (Specht *et al*., 2001; Teulat *et al*., 2002). QTLs for Δ have been identified in cotton (Saranga *et al*., 2001), rice (Price *et al*., 2002; Takai *et al*., 2006), barley (Teulat *et al*., 2002) and *Arabidopsis thaliana* (Masle *et al*., 2005). The association between QTLs of Δ and those of productivity found in cotton indicated that high WUE could be associated with either high or low productivity (Saranga *et al*., 2001). This may be due to the fact that variation in WUE and hence Δ, is related to stomatal and/or non-stomatal effects. In barley, 10 QTLs associated with grain Δ were identified, from which 8 QTLs co-located with agronomic and plant water status traits (Teulat *et al*., 2002). In soybean, four Restriction Fragment Length Polymorphism (RFLP) markers associated with QTLs of WUE were identified and when combined they explained 38% of the variability in WUE (Mian *et al*., 1996). In *Arabidopsis thaliana*, a gene known as ERECTA, which regulates transpiration

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efficiency, had a major contribution to a significant QTL for Δ accounting for 21-64% of total phenotypic variation (Masle *et al*., 2005). These studies indicate that Δ may represent a trait which can be effectively traced by molecular markers at the level of DNA.

Finally, candidate gene approach is a promising indirect selection strategy to identify drought-tolerant genotypes. Candidate genes are the genes with known biological function affecting plant development or physiology (Vinod *et al*., 2006). Drought stress induces many morphological, physiological and metabolic changes in plants. At the cellular level, solutes are concentrated, cell volume and membrane shape are altered, turgor is lost and membrane integrity is disrupted (Bray, 1997). At a molecular level, many genes are induced. The products of these up-regulated genes are heat shock proteins, late embryogenesis abundant proteins and proteins involved in regulation of gene expression (Shinozaki *et al*., 2003), metabolism, signalling and transcription (Bray, 2004). These candidate genes can be exploited by creating transgenic plants. However, overexpression of some of these genes has a negative impact on plant growth and productivity (Kasuga *et al*., 1999). The alternative is to use candidate genes as markers for drought tolerance. These genes can be identified by the following methods: (1) QTL mapping can lead to the cloning of major genes (Salvi and Tuberosa *et al*., 2005). However, this is difficult without genomic resources in beet. (2) Forward and reverse genetics in other species have highlighted genes involved in responses to drought (Kathiresan *et al*., 2006). (3) Proteomic studies have highlighted similar proteins in beet to those responding to drought in other species (Hajheidari *et al*., 2005). Whether these drought-related genes can be used as markers for drought tolerance depends on the presence of genotypic differences in their expression level. In the proteomic study quoted above, 11 up-or down-regulated proteins were identified and sequenced as drought-related candidate genes. We chose two proteins and studied the genotypic differences in their expression levels using the corresponding ESTs (Expressed Sequence Tags). The expressed DNA sequences are derived from complementary DNA (cDNA) sequences (thus from sequences which have been transcribed in some tissue at some stage of development). Among the other applications of ESTs are: (1) serving as a source of primers that could be used for amplification of specific genes via Polymerase Chain Reaction (PCR), (2) identifying the presence or absence of a polymorphism and (3) serving as molecular markers for mapping the entire genome (Jones, 2005; Wu *et al*., 2005). These applications indicate that ESTs are a powerful tool, which may facilitate the identification of the corresponding genes and performing further experiments with those genes (Jones, 2005). In this study, the ESTs were used to design primers and measure the expression level of RNA in four sugar beet hybrids. The two candidate genes used in the current study were chosen because of two independent verifications: That they were sequenced in the proteomic study and their corresponding ESTs were available in the sugar beet EST database. The objective of this study was to determine the extent of genetic diversity in the expression levels of the two previously identified candidate genes thought to be associated with drought tolerance.

MATERIALS AND METHODS

A previous proteomics study on sugar beet had highlighted 11 drought-regulated proteins as potential markers for drought tolerance (Hajheidari *et al*., 2005). Two proteins chosen for the current study were: 2-cysteine peroxiredoxin (hereafter referred to as 2-cys prx with accession number Q9FE12) and nucleoside-diphosphate kinase (hereafter referred to as NDPK with accession number S24165). A search in sugar beet EST database (http://genomics.msu.edu/sugarbeet/) found two ESTs similar to 2-cys prx and one EST similar to NDPK. Hereafter, we call these three ESTs as CPx1 (accession number BI096062), CPx2 (accession number AW067625) and NDP (accession number BI543256). The sequence alignments between the candidate genes and the ESTs were conducted using ClustalW programme (www.ebi.ac.uk/clustalw/). The sequences of the ESTs were then used to design the primers (Table 1) for the gene expression analysis.

Plant material: The leaf samples of four field evaluated hybrids (Cinderella, NV8, KWS 1-99 and KWS 04-03) were collected at the end of the stress period in 2004 (Ober *et al*., 2005). After removal of the petioles and midribs, the lamina were stored at 80°C until analysis. Total RNA was extracted from leaf tissues after Davies (2006). The DNase treated RNA samples were then used for determination of RNA quantity and purity with spectrophotometer and subsequent Reverse Transcription (RT) and PCR amplification reactions.

Complementary DNA synthesis via reverse transcription: Before Reverse Transcription (RT), the total RNA extracts were diluted to 0.2 μg μL^{-1}. Twenty microliter of total RNA was mixed with 2 μL of Oligo $(dT)_{12-18}$ primer and 2 μL of 25 mM dNTP mix (Bioline, London, UK). The mixture was incubated at 65°C for

Table 1: Sequences of the forward (F) and reverse (R) primers used in semi-quantitative RT-PCR, the amplicon size and the PCR cycle number. The sequences of the ESTs were used to design the primers. The two candidate genes were 2-cysteine peroxiredoxin (2-cys prx) and nucleoside-diphosphate kinase (NDPK). 18S rRNA was used as internal control

ESTs	Accession No.	Primer sequence	Amplicon size (bp)	PCR cycle No.
CPx1	BI096062	F 5'-AAGCTCATGCTCCAATGGC-3'		
		R 5'-GAGATAGACTTGGTGACATCGG-	449	28
CPx2	AW067625	F 5'-GTCTCATCAACACTACGGCC-3'		
		R 5'-CCTCTCGTCGCACTCAATC-3'	469	28
NDP	BI543256	F 5'-GATCAAGCCTGATGGTGTCC-3'		
		R 5'-GCTCTGCCAGTGGACAACA-3'	361	30
18S rRNA	-	F 5'-TGACGGAGAATTAGGGTTCG-3'		
		R 5'-CCCCAATGGATCCTCGTTA-3'	200	28

5 min. To this mixture, 8 μL of RT buffer, 4 μL of 0.1 M DTT and 2 μL of RNase OUT™ (Invitrogen, USA) were added. The mixture was then incubated at 42°C for 2 min. By adding 2 μL of reverse transcriptase (Invitrogen, Carlsbad, USA), the total volume of the mixture was taken to 40 μL. The RT reaction was conducted at 42°C for 90 min and the RT denaturation step was maintained at 95°C for 5 min. The cDNA was stored at -20°C.

Amplification of cDNA by PCR: The primers used for amplification of cDNA are shown in Table 1. Each PCR reaction consisted of 2 μL of 50 mM $MgCl_2$, 4 μL of each forward and reverse primers (both 100 ng μL^{-1}), 1 μL of 10 mM dNTP mix, 5 μL of 10× reaction buffer, 1 μL of cDNA and 0.5 μL of Taq DNA polymerase (BIOTAQ; Bioline, London, UK). The total volume of each reaction was made up to 50 μL with molecular biology grade water. Following 2 min of denaturation at 94°C, 40 cycles were performed with 30 sec denaturation at 94°C, 30 sec annealing at 55°C and 1 min extension at 72°C. The PCR cycle was followed with a final extension step at 72°C for 5 min. The products were stored at 4°C before visualisation.

Visualisation of PCR products: Agarose gel electrophoresis (1.3% w/v) was carried out to separate the PCR products. 2.5 μL of each PCR product was mixed with 2 μL of loading dye and loaded into the wells in the gel. Five microliter of a molecular weight ladder (Hyperladder I; Bioline London, UK) was loaded to determine the size of the PCR products. The samples were electrophoretically separated at 100 V for 45 min. Then, the products were visualised using a gel imaging system (Biorad, Hercules, USA).

Determination of optimum number of PCR cycles: Linear range analysis was conducted to determine the PCR cycle after which the transcript abundance saturates and the curve reaches a plateau. Separate 50 μL PCR reactions were set up for each PCR cycle (18, 21, 24, 27, 30, 33, 36 and 39 cycles). The band intensity was quantified using image analysis software (Scion Image, Scioncorp, Maryland, USA). The optimum PCR cycle number determined for each primer pair is shown in Table 1.

Quantification of RNA expression level: When the PCR cycle number for each primer pair was determined, the expression level of RNA samples was quantified using image analysis software (Scion Image, Scioncorp, Maryland, USA). The 18S rRNA (Table 1) was used as the internal control.

RESULTS AND DISCUSSION

Gene expression analysis by semi-quantitative RT-PCR was undertaken to assess the differences in transcript abundance among four water-limited (Cinderella, NV8, KWS 1-99 and KWS 04-03) and two well-watered (Cinderella and NV8) sugar beet hybrids. The three ESTs were differentially expressed in the four water-limited and two well-watered hybrids (Fig. 1). However, genotypic differences in transcript abundance were small. The higher level of expression for Cpx2 compared with the other two ESTs may be because CPx2 had been obtained from partially expanded sugar beet leaves, whereas the other two ESTs were derived from sugar beet seedlings (De los Reyes and McGrath, 2000, 2003). It has been shown that the expression level of a gene varies with the tissue sampled, the growth stage and the duration of stress (O'Connell, 1995; Andjelkovic and Thompson, 2006). In water-limited conditions, Cinderella showed the lowest expression for the three ESTs whereas the other three hybrids expressed the three ESTs relatively similarly, except NDP whose expression in KWS 04-03 was lower than that in NV8 and KWS 1-99 (Fig. 1). The ranking of the two well-watered hybrids for the expression of the three ESTs remained constant indicating that the differences are likely to be genotypic, though small. However, data from a larger set of genotypes is needed.

The expression pattern of Cpx2 in the four water-stressed hybrids was correlated with Drought Tolerance Index (DTI) (Fig. 2). Using the data of a 2-y field study, DTI was calculated after Ober *et al.* (2004) as follows:

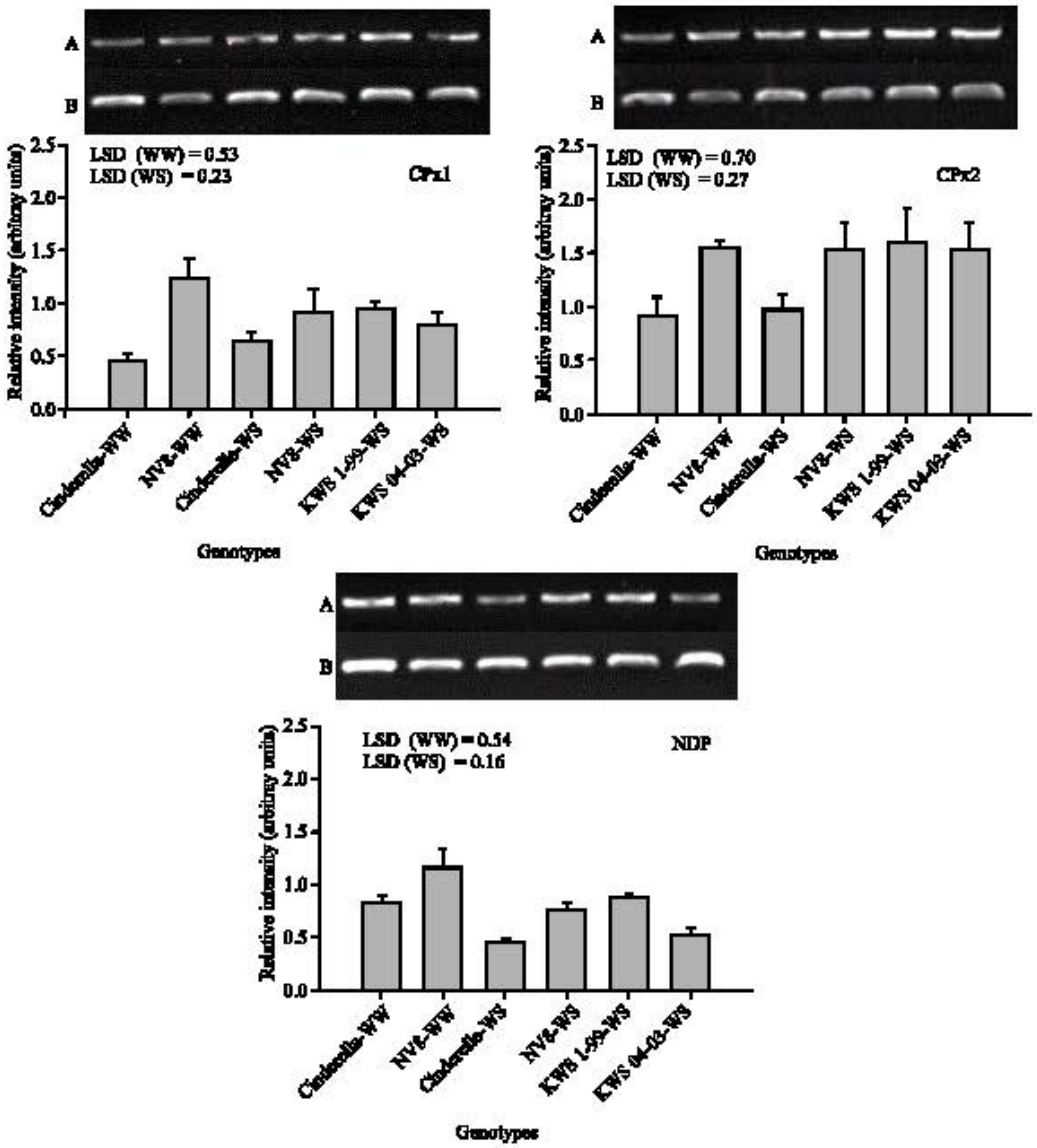

Fig. 1: Expression pattern of cDNAs obtained from leaf tissues of two Well-Watered (WW) and four Water-Stressed (WS) sugar beet hybrids. A representative image of the PCR products is shown above each graph: A represents the relative intensity (expression level) of cDNAs and B represents that for 18S rRNA as internal control. CPX_1, CPX_2 and NDP are the three ESTs corresponding to the three candidate genes used in this study. Leaf samples were taken at the end of stress period for RNA extraction. Vertical bars indicate the standard error of the means. LSD values at the probability level of 5% are shown (n = 3)

$$DTI = \frac{\left(Y_D / Y_I\right)}{\left(\overline{Y}_D / \overline{Y}_I\right)}$$

Where:

Y_D and Y_I = Genotype mean yield under droughted and irrigated conditions, respectively

$\overline{Y}_D$ and $\overline{Y}_I$ = Mean droughted and irrigated yields, respectively, across all genotypes within a trial

In addition, the level of expression of CPx2 in the two well-watered hybrids did not change when compared with the water-limited conditions (Fig. 1). This may suggest that CPx2 is consistently expressed across the two water treatments and that differences observed among

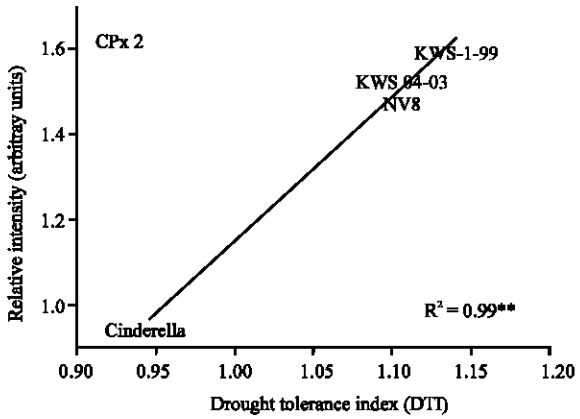

Fig. 2: Relationship of the expression level of CP x 2 with Drought Tolerance Index (DTI) in four hybrids. Drought Tolerance Index (DTI) was calculated based on the relative performance of the four hybrids in well-watered and water-limited conditions in the field over two years (2004 and 2005). The symbol ** represents the significance of the correlation coefficient at the probability level of 1% (n = 3)

the hybrids are real, though small. However, data from a larger set of genotypes is needed.

CONCLUSIONS

In this study, data from well-watered plants were available only for two hybrids (Cinderella and NV8) to compare with water-stressed plants, because in well-watered conditions, leaf samples had been taken only from these two hybrids. Data from this limited number of genotypes, in particular from well-watered plants, indicated that differences in transcript abundance of the genes among the hybrids were small. The possible explanations for the small genotypic differences observed are: The sample size has been small; the method used has not been sufficiently sensitive to detect small differences; real time quantitative PCR would have been a better method had it been available; the genes are constitutively expressed; however, the expression can still be related to drought tolerance and/or WUE, as shown in Fig. 2; the plants have experienced different levels of stress, not different in the level of adaptation or tolerance; this is unlikely because pre-dawn leaf water potential data, obtained on the date of leaf sampling for RNA extraction, do not show significant differences in the level of stress experienced by the four hybrids (data not shown) and differences in the expression level are real but small, which needs retesting with a larger set of genotypes.

The differential expression of these ESTs was studied on the leaf samples taken from a limited number of genotypes at the end of the stress period. Therefore, more investigation is required, using more diverse genotypes, to determine if these ESTs are also expressed earlier in the stress period, i.e., it is necessary to determine how consistently these ESTs are expressed in the course of drought experiment. This is because gene expression is affected by stress duration and intensity (Andjelkovic and Thompson, 2006). The implication is that if sufficient genotypic differences in expression levels exist and the genes show consistent expression throughout the stress period, they could be used as phenotypic markers for mapping, or they could be integrated into the genome map of sugar beet and tested as molecular markers for co-segregation with the QTLs that will be identified for Δ and hence WUE (Schneider *et al.*, 1999; M. Grimmer, personal communication).

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