Studying CBF4 Transcription Factor in Arabidopsis Under Drought Stress

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Abstract:

Water deficit is a serious environmental factor limiting the growth and productivity of plants and agricultural crops worldwide. A number of these plants and crops own the ability to survive drought by altering the level of gene expression. Using Arabidopsis in studying gene function and regulation is of crucial importance to plant biotechnology. Our objective was to study the gene expression of C-repeat- Binding Factor 4/ Dehydration Responsive Element-binding Protein 1D (CBF4/DREB1D) (At5g51990) under drought using cbf_4 mutant line (SALK_039486). Two separate experiments were performed. In the first one, plants of homozygous and wild type lines were grown on the same soil, and in the second one; plants of the two lines in parallel with Columbia 8 were grown on separate pots. The molecular analysis showed that the insert is located 52 bases downstream the gene and the mutant were segregated out in a different manner from the mendelian bases. The gene expression of CBF4 was greatly reduced in the mutant comparing to wild type but plant survival showed no significant differences between the different genotypes in both experiments. The mRNA detection indicates the role of CBF4 in drought stress response in Arabidopsis as the mutant showed gene down-regulation but this level was not low enough to affect plant survival.

Keywords: CBF4, DREB1D, drought stress, T-DNA, mutant line, Arabidopsis.

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Introduction:

Drought is a major abiotic stress that impacts plant growth and distribution and causes a reduction of crop productivity and the availability of water is the most significant limiting factor in plants life [24, 26, 30]. Plant production in water-limited environments is very often affected by constitutive plant traits that allow maintenance of a high plant water status; dehydration avoidance[2]. Naturally, plants differ in their susceptibility to drought and the differences referred to the gene expression and genomic content between species [4].

As known, plants absorb water from soil through a process that depends on water potential gradient and flow resistance[19]. This mechanical process could be slow or fast depending on the presence of water and there might be other environmental factors which affect water uptake. During drought stress, plants slow down the water absorption and start to adapt to this stress. To survive, plants tend to close stomata to keep water inside the tissues but under prolonged periods of dehydration, irreversible cellular damage may happen as a result of the production of reactive oxygen intermediates. Also, some plants fold their leaves to provide shading and thus reduce transpiration. Additionally, other components play a crucial role in helping plants to resist dehydration through many adaptive responses [4, 18, 37].

Physiologically, osmotic adjustment is a major cellular stress adaptive response in certain crop plants that enhances dehydration avoidance and supports yield under stress [2, 34]. More precisely, plants respond to this kind of stress by adaptation of the biochemical and physiological processes designed to improve the water status through combined inhibition of water loss by transpiration and more efficient access to the supplies of soil water. Molecularly, the biochemical and physiological reactions are regulated by precise molecular switches operating in a coordinated fashion.This regulation is usually achieved at the level of gene expression (up- and down-regulation), translational modification, modulation of the type and amount of intracellular

solutes and other biochemical changes [34]. In general, these mechanical, biochemical and the other changes are controlled by network signals in which plant phytohormones, in particular ABA plays a crucial role [7, 24, 31, 34].

The patterns of gene expression under water-deficit conditions seem to be complicated [24], and many signalling pathways are involved in drought stress response [5]. Many genes and transcription factors in plants have been reported to be involved in the response to water deficit and usually have a role in resisting other stresses such as cold and salinity [32]. Sakuma et al. [28] reported the drought stress tolerance in transgenic Arabidopsis upon the over expression of DREB2A which is known to interact with cis-acting element DRE/CRT.

Transcription factors are global regulators of gene networks that play a vital role in many cellular processes and represent excellent targets for developing molecular markers [16]. Dehydration and low temperature activate a group of genes that contain C-repeat/dehydration-responsive elements in their promoter [38]. Specifically, CBF_4 transcription factor, a member of CBF/DREB1 family, was found to be up-regulated in Arabidopsis by drought stress through the ABA signalling and not by cold [14]. In wheat, cold hardiness was achieved with high level of CBF_4 gene expression [35]. The over expression of this gene in transgenic Arabidopsis has led to the activation of C-repeat/dehydration-responsive element in the promoter of genes that are involved in acclimation to cold and adaptation to water stress leading to stress tolerance[14]. Fugui et al [11] reported the enhancement of drought tolerance in wheat grass transgenic plant upon transferring an exogenous CBF4 gene which was expressed at a transcription level confirming the involvement of this gene in the response to drought**.** In voz1 (for vascular plant one-zinc-finger) Arabidopsis mutant line, the increased freezing or drought-stress tolerance was found likely to be induced by the constant up-regulation of CBF4 transcription factor without elevated levels of ABA in the mutant [23].

Using T-DNA strategy has become a very useful approach in genomic studies. In Arabidopsis, Salk Institute Genomic Analysis Laboratory (SIGNAL) produces thousands of mutant lines and they are distributed by NASC in Nottingham, UK and ABRC in Ohio, USA [33]. Inserting a T-DNA usually disrupts the expression of the target gene [25]. Accordingly, producing mutants using T-DNA has been proved as an efficient success in plant functional genomics [28] and it is usable in the identification of genes and regulatory elements. Usually, gene knockouts, or null mutations provide a direct approach to determine the function of a gene [22].

Practically and regarding drought stress, there are several ways to perform this kind of stress to plants, and to measure water-deficit stress tolerance. Some would have used withholding water to plants [8, 20], air-dry detached leaves at normal room temperature [39, 40], and others used polyethylene glycol (PEG) in conducting drought stress [21, 36, 40]. The most useful way to perform drought stress to plants grown on soil is withholding water but it needs very obvious control in providing the same conditions to the different phenotypes used in the experiment.

As stated by Gang-Ping et al. [13], CBF_4 has a locus region that corresponds to dehydration tolerance indicating the possible QTL of this transcription factor for drought. My objective was to study the involvement of this transcription factor in the response to dehydration including gene expression and survival after exposing Arabidopsis cbf_4 mutant line (SALK_039486) of CBF_4 gene which carries a T-DNA down stream the gene and the wild type plants to water deficit comparing to Columbia 8 background.

Materials And methods:

Growing Arabidopsis plant:

Two experiments were conducted separately in controlled growth and cold rooms based on the growth room in the School of Biology, Newcastle University and in the Horticulture Department,

University of Tripoli. In the first experiment, plants of homozygous and wild type lines of CBF4 were grown on the same soil in a non-compartmented tray. In the second experiment, plants of Arabidopsis thaliana homozygous and wild type lines in addition to Columbia 8 background were grown in separate pots. T4 (first experiment) and T5 in addition to Columbia 8 (second experiment) seeds, provided by Nottingham Arabidopsis Stock Centre (NASC) ((http://arabidopsis.info/), were used for this study. Seeds of the SALK line (SALK _039486) of CBF4 and Columbia 8 background were sown either on wet John Innes No.2 compost (the first experiment) or on peat moss produced by Floragard Company (the second experiment). After sowing, tray and pots were covered with cling film and put in a cold room (±4ºC and continuous light) for 4-5 days. Tray and pots were, then, transferred to a normal conditioned growth room with 16-22ºC. Short-day conditions (10hrs day - 14hrs night) were provided for vegetation,with light density about 170 µmol m^{-2} s^{-1} (400-700nm), and 50-75% relative humidity. Generally, plants used for the two experiments were grown for a period recognized between stages 3-6 according to Boyes et al. [3].

Performing drought stresses:

After regular irrigation, plants were exposed to initial drought for 5 days then plants were watered again and water was withheld for 17 days (first experiment) and for 11 days (second experiment).Then, the survival was recorded after 5, 14, and 19 days (first experiment) and after 6 and 24 days after recovering (second experiment). Images were taken before and after carrying out drought stress.

RNA isolation:

RNA was isolated according to Helena Bioscience lab protocol using tri-reagent (#T9424, Sigma & Aldrich). Collected RNA pellets were dissolved using DEPC water or ddH_2O and gentle mixing was done by pipetting up and down and starring by

tips. Then, dissolved RNA samples were kept at -80ºC for later use to produce CDNA.

Reverse transcription (RT-PCR) reaction:

The RT- PCR to produce CDNA(DSDNA)was done using Omniscript reverse transpiration kit (#205111) from Qiagene.Then, reverse-Transcription products were stored at -20ºC and then used for thermal PCR to distinguish gene expression of the target gene under drought stress.

PCR reaction:

For amplifying target CDNA (DSDNA) sequence, PCR reactions were performed using a thermo cycler (Px2 Thermal cycler, Thermo Electron Corporation)PCR machine. A certain PCR programwas used to run the reactions using Taq DNA kit (#BIO21070) from BiolineLbs Ltd). Forward [3`AAAGCGCCTCATCATCCATA5`] and reverse [5` CTGCTCGTGCTCATGATGTT 3`] primers were to amplify cDNA.The total volume of each PCR reaction was 20µl and reactions were run according to a specific PCR program (with 30 cycles) and then, PCR products were stored in a -20ºC deep freezer for later use.

Gene expression:

CBF4gene expression was investigated for the mutant line comparing to wild type plants after 11 days of water stress.Samples of unstressed and stressed plants (first experiment) were collected after 11 days of withholding water, when wilting symptoms were clearly seen.

Agarose gel electrophoresis for CDNA(DSDNA):

Different equipment and chemicals were used for running gel electrophoresis. These include an electrophoresis chamber and power supply (Pharmacia GNA 200), gel casting trays with different sizes, well combs, electrophoresis Tris Borate EDTA buffer (TBE buffer # T4415, Sigma Aldrich), Ethidium bromide

(EB) (for visualization),agarose (#300-300, Biogene), 5x DNA loading buffer 'blue dye'(#BIO37045, Bioline labs Ltd), Hyperladder IV quantitative (#BIO33029,BIioline), and gel documentation chamber (BIORAD).

The gel was left to run in a fume cupboard for 1:30-2 hours until cDNA migrated to a proper distance and DNA fragments got stained with ethidium bromide (EB) and then, visualized by UV tranilluminator using gel documentation chamber attached to a computer immediately after running the gel before CDNA diffuses within the gel according to Brown in Ref. [6]. The quality of the CDNA was assessed by observing a clear single illuminated band and smearing was considered sample degradation. RNA gel was only done to check the quality of the extract by loading 2µl of RNA (according to volume) from stock extracts and scanned the same way using gel electrophoresis.

Purification of DNA PCR products:

For PCR products purification, a QIAquick PCR purification kit (50) (#28104) was ordered from Qiagene and used according to the instructions stated. The collected solution was finally transferred to a clean eppendorf tube and stored in a -20°C freezer for further use for CDNA sequencing using 3x diluted primers for the target gene [27].

Sequencing PCR products and DNA alignment:

The purified PCR products of CDNA were sent for sequencing along with diluted primers to the Institute for Research on Environment and Sustainability (IRES) sequencing services at Newcastle University. The resulted sequences were aligned and compared with the known sequences from the database for the target gene using the following software and URLs:

Clustalwhttp://www.ebi.ac.uk/Tools/clustalw2/index.html, [9, 17].
BioEdithttp://www.mbio.ncsu.edu/bioedit/bioedit.html, [15].

Finch TV http://www.geospiza.com/Products/finchtv.shtml.

Results and Discussion:

Molecular analysis of cbf4 mutant (SALK_039486):

In cbf4 mutant line (SALK_039486), the T-DNA is located outside the transcript region (downstream) of CBF4 gene (At5g51990) in chromosome 5 (MSG15_5) as shown in Figure 1. It was not possible to discover the T-DNA at the right border (data not shown), which it has been thought to flank the 3` end of the gene. The conversion of T-DNA sequence to reverse complement allowed to amplify the junction of CBF4**::**T-DNA (the 3` end of CBF4 and T-DNA left border) using F_2R_2 primers as shown in Figure 1. PCRs were performed to amplify 584b of CBF4 gene (for wild type), and 789b for the mutant using specific primers F1 [5'AGTTGGCTGGAATCATAACGA3'], R1 [3' CGTGACTCATGAGAAGCTTGA 5'] and F2 [5' GACTTTGACGGAGTGGGTGA 3'] R2 [3' GGCGTTACCCAACTTAATCG 5'] see gene outline (Figure 1). Sequencing result of the PCR product shows that T-DNA was in the position reported by the database (52bp downstream the CBF4 gene) and also, a part of T-DNA (81bp) at the left border was deleted (Figure 2). Wild type sequence analysis of the amplified PCR product showed complete alignment against the CBF4 gene published by the database (Figure 3). The exact site of the insertion can be of a distance from the expected one due to the overlapping reads of two or more sequences in the T-DNA lines or may be for other reasons [33].

Forty six plants of T3 generation were grown, genotyped and found to be segregating out completely different from the Mendelian bases (1:2:1). The genotypes segregation was as follows; 13, 6, and, 26 of wild type, heterozygous, homozygous, representing 0.28:0.13:0.56 proportion respectively in addition to one unidentified line (Table 1). Chi-square test showed highly significant differences between the observed and the expected proportions (Table 1) where is a big deviation form heterozygous

to homozygous lines was discovered. The obtained proportions of segregation may indicate a second insert as reported by Krysan et al. in Ref. [22] and Feldmann in Ref. [10]. Generally, the majority of the mutants (88%) segregate in a Mendelian manner for the mutant phenotype and the segregation process may depend on the progeny and the number of inserts [10, 12]. When transformants contain one or two inserts, they usually show a clear Mendelian pattern of segregation. In case of more than four inserts the segregation process may differ as measured by the response to KanR,Kan^S and GUS^+ [12]. The reason for this could be explained by the deletion that occurs due to rearrangements during T-DNA integration or due to the inactivation of foreign genes by methylation [12].

To confirm the lack of T-DNA in the wild type genotype of CBF4 or in the nearest region, T-DNA FR primers (within the insert) were used to amplify 665bp. All wild type plants tested showed no products for the insert comparing to the heterozygous lines which were used as a positive control (data not shown).

CBF4/ DREB1D (At5g51990, SALK_039486, N539486)

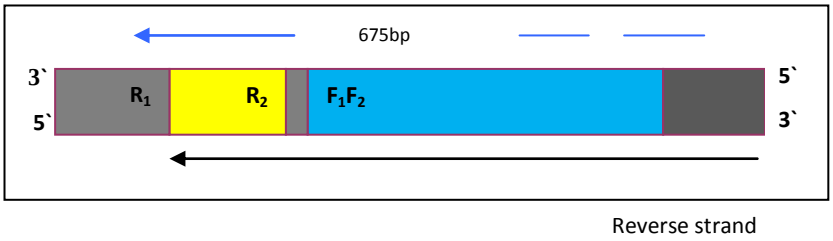

Figure 1: An AtCBF4 transcription factor model diagram showing different regions and the site of forward and reverse primers amplifying either the host sequence of the target gene (for wild type) and the host sequence with T-DNA (for the mutant); upstream sequence (dark gray), downstream (gray), T-DNA (yellow), exon (blue), forward primer (F), and reverse primer (R). No intron in this gene. Modified from (http://arabidopsis.info/).

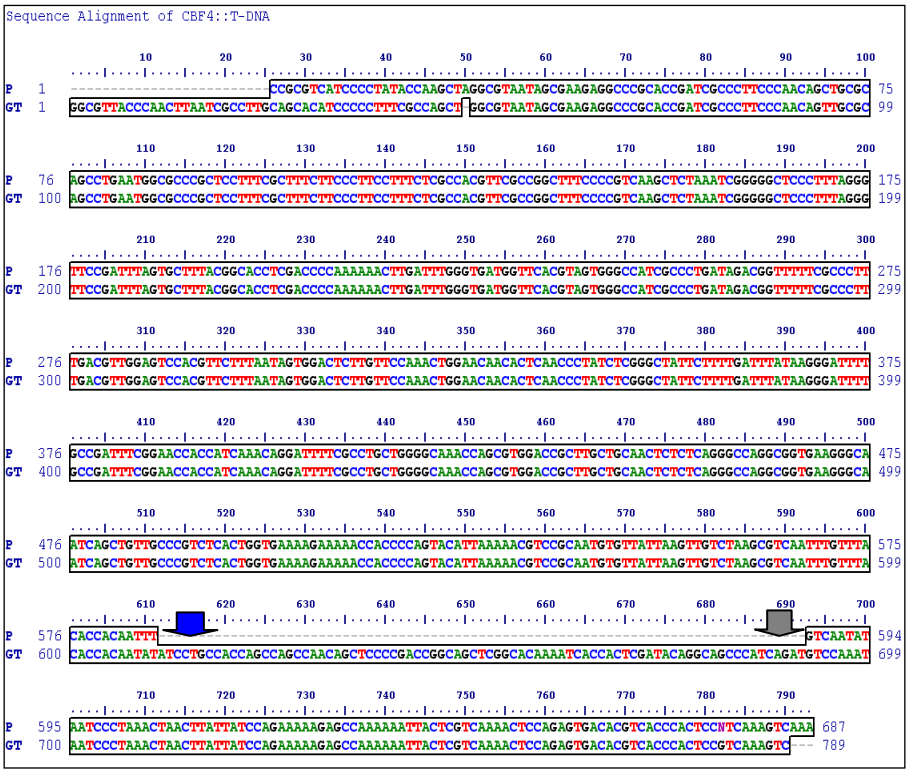

Figure 2: Sequence alignment of the reverse complement of cbf4 homozygous shows the amplified sequence of the junction between the CBF4 gene and the T-DNA using F_2R_2 primers. Upper strand is the PCR product sequence (P). Lower strand is the sequence for the gene and the T-DNA (GT). The blue closed arrow indicates the correct left border of the insert and gray arrow indicates the left border reported by the database. Sequence mismatch in the middle (----) indicates the missing part of the T-DNA.

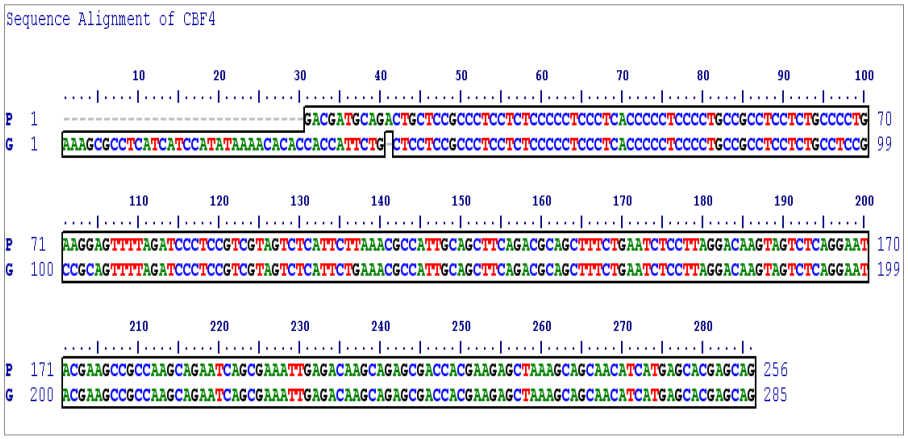

Figure 3: Sequence alignment of the amplified sequence of CBF4 wild type using F_1R_1 primers. Upper strand is the PCR product sequence (P), and the lower strand is the CBF4 gene sequence published by the database (G).

Table 1.A Chi-square (X^2) test for CBF4 genotype segregation; wild type (Wt), heterozygous (Het), and homozygous (Hom).

Type	Population	proportion	Expected	Observed	df	X^2	P-value
Wt	45	0.25	11.25	13			
Het	45	0.50	22.5	6		31.711	0.000
Hom		0.25	11.25	26			

Transcript abundance:

The expression of CBF4 in the wild type and the mutant was investigated. Samples were collected after 11 days of exposing cbf4 mutant and CBF4 wild type plants to water stress. RT-PCR detection revealed that the CBF4 was up-regulated in the wild type and conversely was barely detectable in the mutant under drought stress (Figure 4). This result suggests the induction of CBF4 by drought stress and the T-DNA allowed only very low level of transcript to accumulate in the mutant which is referred to the T-DNA being inserted outside of the coding region (downstream the

gene). Our results are supported by that collected by Haake et al. in Ref. [14] as they found that CBF4 is up- regulated by drought stress confirming that this up- regulation is ABA dependent.

Also, gel electrophoresis showed that some transcripts had been produced of both genotypes at the control condition. CBF4 coding region is slightly bigger (675bp) than that of CBF3 (653) but they share more than 70% identity. Sequence alignment of CBF4 against CBF3 (data not shown) showed high sequence identity between these two genes and indicated about 16 out of 20bp of both forward and reverse primers are with complete match (data not shown). This could explain the transcripts accumulated at the normal (control) conditions which appeared to be smaller in size compared to that under stress conditions (Figure 4).

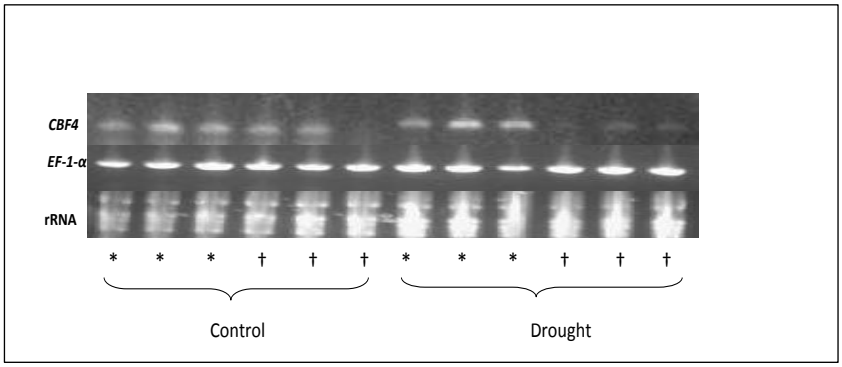

Figure 4: The effect of drought on gene expression of Arabidopsis CBF4 wild type (*) and cbf4 mutant (†) under stress and control conditions (first experiment).

Plant survival:

To ensure better distribution of plants of different lines within similar water content of the soil in trays, and because of the possible differences between plants' ability in absorbing water, I performed drought stress using well-designed experiment outline for plant distribution (Figure 5 D). Thus, both homozygous (H) and wild type (W) lines were alternatively distributed in rows within

non-compartmented trays to eliminate any differences in soil moisture which might affect performing drought to plants. Thirty three plants of each line were distributed on 6 rows within the tray. In the first experiment (data not shown), water was withheld for 17 days and re-watering plants after this period allowed recovering only few plants from both lines. As a result of the poor survival of both phenotypes, data were not subjected to statistical analysis.

Repeating the experiment using four-week-old (Figure 5),water was withheld for 14 days and samples for gene expression were collected after 11 days of exposing to drought stress. Survival of wild type and homozygous plants was recorded after 5, 14, and 19 days from recovering plants (Figure 6). When the experiment was repeated with less drought exposure (only 14 days), better plant survival was obtained with young plants for both genotypes (Figure 5 A, B & C) but no significant difference was obtained (Figure 6).

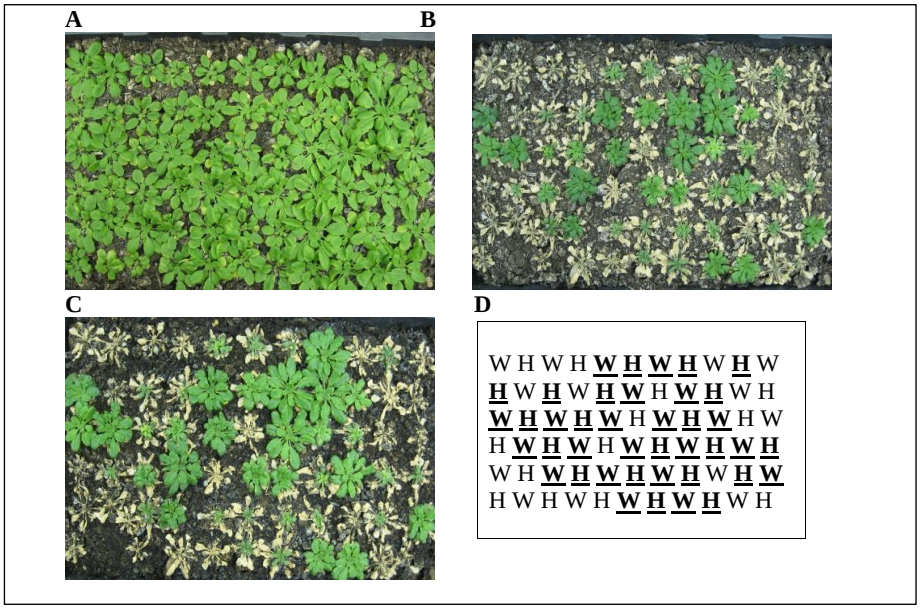

Figure 5: The effect of drought on plant survival of Arabidopsis four-week-old cbf4 mutant (H) and CBF4 wild type (W) plants at 5days after withholding

water (A) and 5 & 9 days from recovering from drought stress B&C respectively) .D= Line distribution within the tray; bold & underlined latters indicate recovered plants.

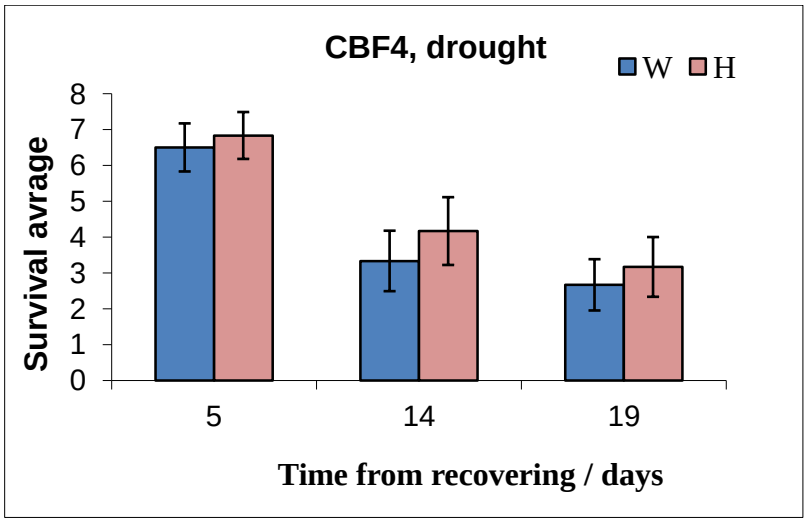

Figure 6: The effect of drought stress on plant survival of cbf4 mutant (pink bars) and CBF4 wild type (blue bars) of Arabidopsis plant as in Figure 5; (first experiment).

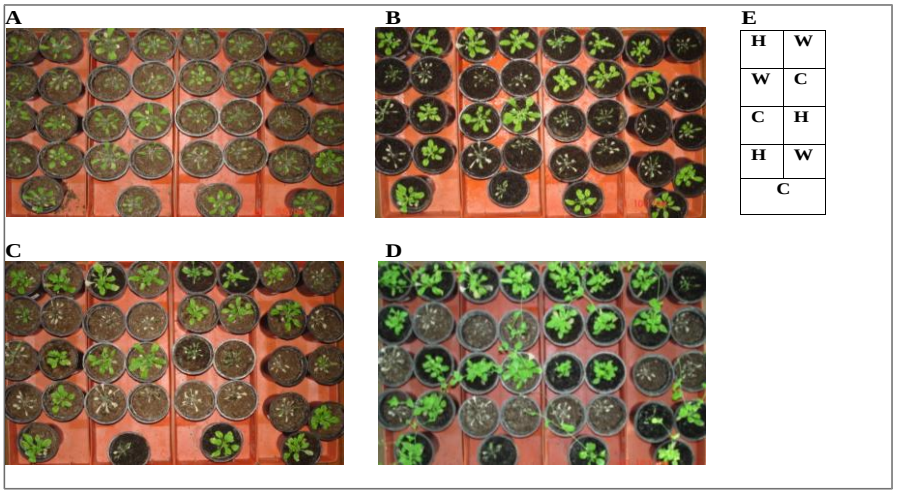

Figure 7: The effect of drought on plant survival of Arabidopsis young cbf4 mutant (H) and CBF4 wild type (W) plants comparing to Columbia 8 (C) at 12 days after withholding water (A) and 4, 6 and 24 days from recovering B,C & D respectively (Second experiment). E=Line distribution within the pots in each separate tray.

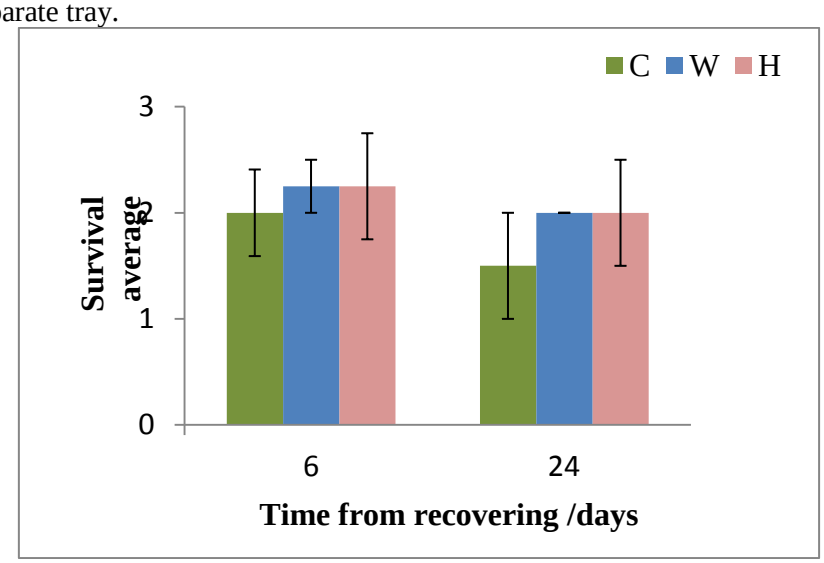

Figure 8: The effect of drought stress on plant survival of cbf4 mutant (pink bars) and CBF4 wild type (blue bars) comparing to Columbia 8 (green bar) of Arabidopsis plant as in Figure7; (second experiment).

In this study, the gene expression result indicated that CBF4 is up-regulated by drought stress. However, even though the T-DNA is located outside the coding region, the expression in the mutant was only slight, and much less than in the wild type. This indicates that CBF4 transcript accumulation in the mutant was reduced by the effect of the T-DNA. This result suggests that the reduced expression would have been resulted from the T-DNA being inserted downstream the gene, the site where it might have affected the polyaddenylation of MRNA reducing its stability, a process reported in the eukaryoticm RNAs [1].

To explain CBF4 gene expression at the control conditions (Figure 4), it appears that one of the other CBFs transcripts was

accumulated for both genotypes. It is unlikely for the mutant to express CBF4 under the control condition and not under drought stress. Thus, for both genotypes at the control conditions, the accumulated transcripts are not for CBF4. CBF4 encodes a protein which is very close homolog to CBF 1, 2, 3 transcriptional activators in Arabidopsis [14]. This information may help to explain the obtained result. As mentioned before, the sequence alignment indicated high identity between CBF4 and CBF3,which I believe to have been amplified instead.

The expression of CBF4 in grapes, the sequence of which is very similar to Arabidopsis CBF1, was extensively studied under different abiotic stresses by Xiao et al. [39]. They reported the transcript production of this gene only by cold and not by drought or salt. Also, they reported the maintenance of its expression for several days under cold; the pattern which is uncommon with the most known CBF genes. On the other hand, Haake et al. [14] studied CBF4 under drought and cold in Arabidopsis. They confirmed the induction of its expression by drought stress and not by cold in plants grown on soil. In transgenic Arabidopsis plants grown in Petri dishes, the over expression of CBF4 activated the downstream cold and drought involved genes leading to increased freezing and drought tolerance. My results are consistent with the results reported by Haake et al. in Ref. [14] as the gene expression was reduced upon exposure to drought.

Together for both experiments, even though there was a reduced expression of CBF4 in the mutant, the plant survival was not affected in both wild type and the mutant and also for the Columbia background (second experiment). Trying to explain the results, I believe that the level of transcript in the mutant was not low enough to affect plant survival and also there might be some other experimental factors in place. Firstly, that is, the plants were exposed to a level of drought stress that possibly was not critical, to allow differences between the phenotypes to be clearly

distinguished. The damage ratio of leaves under water stress could indicate how tolerant a plant is to drought stress. The low plant survival under drought reflects the damage ratio of cell membranes [13]. So, having low survival in the different phenotypes may indicate the extensive damage that happened by severe drought stress. When the period of drought exposure was reduced, better survival was obtained as a result of less damage ratio of plants but this level of drought might still be too severe to detect a difference. Secondly and referring to the segregation results, the possibility for the mutant plants to have a second insert should be taken into account. Thus, there might be another transcript accumulated conferring drought tolerance to the mutant plants.

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دراسة عامل النسخ CBF4 في نبات الارابيدوبسيس تحت إجهاد الجفاف

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وروجر ستيفن بيرس **

المستخلص:

يعتبر نقص الماء عاملاً بيئياً قاسياً ويحد من نمو وإنتاجية النباتات والمحاصيل الزراعية في مناطق شاسعة من العالم. عدد من هذه النباتات يمتلك القدرة على تحمل الجفاف وذلك بتغيير مستوى التعبير الجيني للجينات ذات العلاقة. إن استخدام نبات الارابيدوبسيس في دراسة وظائف وتنظيم الجينات له أهمية كبيرة في مجال التقنية الحيوية النباتية. الهدف من هذا البحث هو دراسة التعبير الجيني لعامل النسخ CBF4 (At5g51990) تحت ظروف الجفاف باستخدام طفرة الـ T-DNA (SALK_039486). أجريت تجربتان منفصلتان وتمت في الأولى زراعة البذور لنباتات الطفرة والنباتات العادية على نفس وسط النمو، وفي الثانية زرعت البذور بشكل منفصل واستخدمت نباتات كولومبيا 8 للمقارنة. أوضح التحليل الجزيئي لنباتات الطفرة أن الـ T-DNA تم إدخاله بعد نهاية الجين بـ52 زوجاً قاعدياً وأن عملية الانعزال الوراثي للجين فيها كانت مغايرة لقواعد مندل للانعزال الوراثي. أظهرت النتائج أن مستوى التعبير الجينيى للجين CBF4 في نباتات الطفرة انخفض بدرجة كبيرة تحت ظروف الجفاف مقارنة بالنباتات العادية غير أنه لم تظهر اختلافات معنوية بين النباتات المدروسة في معدل البقاء في كلتا التجربتين. اتضح من نتائج الكشف عن مستوى التعبير الجيني أن الانخفاض في مستوى الـ mRNA في نباتات الطفرة يشير إلى علاقة هذا الجين بالاستجابة لظروف الجفاف في نباتات الارابيدوبسيس غير أن هذا المستوى المنخفض لم يكن كافيا لإحداث تأثير جلي على معدل البقاء للنباتات.

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