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Research article

Identification and expression analysis of CBF/DREB1 and COR15 genes in mutants of *Brassica oleracea var. botrytis* with enhanced proline production and frost resistance

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ABSTRACT

Frost resistant mutants of *Brassica oleracea var. botrytis* were investigated for the presence of CBF/DREB1 and COR15a gene products and induced frost resistance. Total RNA of clones was isolated after 3 h, 6 h, 24 h and 14 d acclimation at 4 °C and proteins and free proline were isolated after 14 d acclimation. cDNA was produced using RT-PCR and the first CBF gene in *B. oleracea* detected and did quantify. Through SDS-PAGE and Western blotting, the COR15a protein was detected for the first time in *B. oleracea*. The results confirmed the first report of the presence of BoCBF/DREB1 in *B. oleracea* and this only appeared under cold acclimation. The sequence analysis of predicted amino acids revealed a very high homology (90%) with CBF sequences of other Brassica species (*BnCBF5*/*DREB1*, *BrDREB1* and *BjDREB1B*) and homology reduced to 67% when compared to plants other than Brassicas. *BoCBF*/*DREB1* transcript levels increased up to 24 h acclimation and then declined. Some mutants showed *BoCBF*/*DREB1* expression at 3 h while others only after 6 h and 24 h acclimation. The genotypes showed positive significant correlation between *BoCBF*/*DREB1* expression and frost resistance ($R^2 = 0.9343$). The proline level under acclimation increased about 8 fold and demonstrated positive and significant correlation with *BoCBF*/*DREB1* expression. Proline also showed positive and significant correlation with frost resistance under cold acclimation but very not under non-acclimation. All clones were positive for COR15a protein after 14 d cold acclimation and expression correlated with frost resistance. Under non-acclimation COR15a was constitutively expressed in 3 mutants.

1. Introduction

Frost, drought and salt are environmental stresses that have major limitations for plant growth and crop productivity globally. In the case of economically important plants, frost can cause significant losses to crop production. The major damaging effect of freezing is that it induces acute cellular dehydration and eventually severe cell membrane damage [1]. Many plants on exposure to non-freezing low temperatures increase their freezing tolerance, a process known as cold acclimation [2]. Explanation of the mechanisms of acclimation and the development of freezing tolerance is of vital importance for the design of novel crop varieties to allow the use of marginal lands for agricultural production. A concerted effort through physiology, molecular biology and biochemistry is required to understand the complex quantitative trait of low temperature and freezing tolerance [3]. Cold acclimation plays an important role in membrane freezing tolerance as shown by early research that cold-acclimated cabbage and spinach synthesized proteins that protected thylakoid membranes against freeze damage and the cryoprotective activity of these proteins was 10,000 times higher than sucrose in protecting against freeze–thaw damage [4]. Analysis of gene expression in plants during cold acclimation has revealed the existence of low temperature responsive genes with complex regulatory mechanisms, pathways and products that assist plant cells to resist and survive freezing [5]. Several of the low temperature responsive genes contain in their promoter regions one or more copies of a cis element C-repeat/low temperature responsive element/dehydration responsive element (CRT/LTRE/DRE) [6] with a conserved core sequence of CCGAC, which is the binding site for the cold specific transcriptional activators CBFs/DREBs [7] that induce the expression of cold responsive (COR) genes which subsequently enhance cold/frost resistance in plants [3]. The CBF/DREB proteins are characterized by an AP2 (Apetala2) domain and the CBF signature motif DSAWR [8].

In many higher plants the existence of a transcription factor ICE (Inducer of CBF expression), acts at the promoter of CBF/DREB

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(C-repeat/dehydration responsive element binding factor) genes upon cold acclimation. The ICE stimulates the transcription of *CBF/DREB* genes followed by induction of the CBF regulon, a set of co-expressed genes that share a similar expression profile across multiple temporal, spatial, genetic and environmental conditions and under the common transcriptional regulations [9,10]. Even though the whole cold induced regulon is still not well understood, various specific profiles of gene expression have been observed during cold acclimation [11] and the expression of COR15 protein is used to indicate the up-regulation of the cold induced regulon.

Physiologically, cell membranes and other important macromolecules need to be protected during freezing induced dehydration and compatible solutes and sugars can provide important osmoregulatory protection. Proline is considered to be an important compatible solute and this amino acid could be a part of the adaptation mechanism to several abiotic stresses [12]. Proline accumulation under stress was reported for the first time in plant tissues of rye grass [13] and subsequently this has been shown to be a common phenomenon of plants on exposure to cold acclimation [14]. Proline, COR proteins, and sugar biosynthesis are regulated co-ordinately and the *CBF* gene plays an essential role in this regulation [15].

Cauliflower (*Brassica oleracea var. botrytis*) is one of the most popular vegetable crops worldwide and originated in the temperate Mediterranean coastal area where it gets exposed to freezing temperatures [16,17]. Cauliflower is grown for its white curd, which consists of inflorescence and floral meristems. It is a low-calorie vegetable, a rich source of vitamins C, K, and A (beta-carotene), folic acid, fiber, and flavonoids which give the cauliflower anti-inflammatory and antioxidant proprieties [18]. Cauliflower is a potentially cancer preventative vegetable [19]. It is also a carbohydrate source that could be an efficient fuel for energy production [20]. Cauliflower belongs to the highly phenotypically diverse species *B. oleracea* which also includes Broccoli, Cabbage, Brussel Sprouts, Kale and Kohl rabi. *B. oleracea* is one of the diploid cornerstones of the Brassica triangle [21]. Presently, the molecular mechanism by which the cauliflower perceives and tolerate freezing is poorly understood. Through chemical mutagenesis the cauliflower mutants were produced using N-nitroso-N-ethyleurea (NEU) and N-nitroso-N-methylurea (NMU) followed by selection in the presence of hydroxyproline [22]. These mutants and control clones were maintained under the in-vitro condition for three years by continuous subculturing after each 3–4 months. These clones were reassessed and some of these mutants were shown to be proline over-accumulators and were variously more resistant to frost, salt or drought or combinations of these stresses [23]. However the molecular bases of these resistances have not hitherto been determined in cauliflower.

In previous investigation on these mutants [23] the symbol 'S' had been used for mutants, while in the present investigation these mutants were regenerated and then indicated by symbol 'K' throughout this paper. In this paper we report the investigation to determine whether CBF/DREB1 and COR15 genes from these cauliflower mutants show any variation in expression and whether or not such variation is correlated with the expression of frost resistance and the level of proline. Since the presence of CBF/DREB1 and COR15 genes in cauliflower have not yet been reported in the literature it was necessary firstly to determine whether these genes were present in cauliflower.

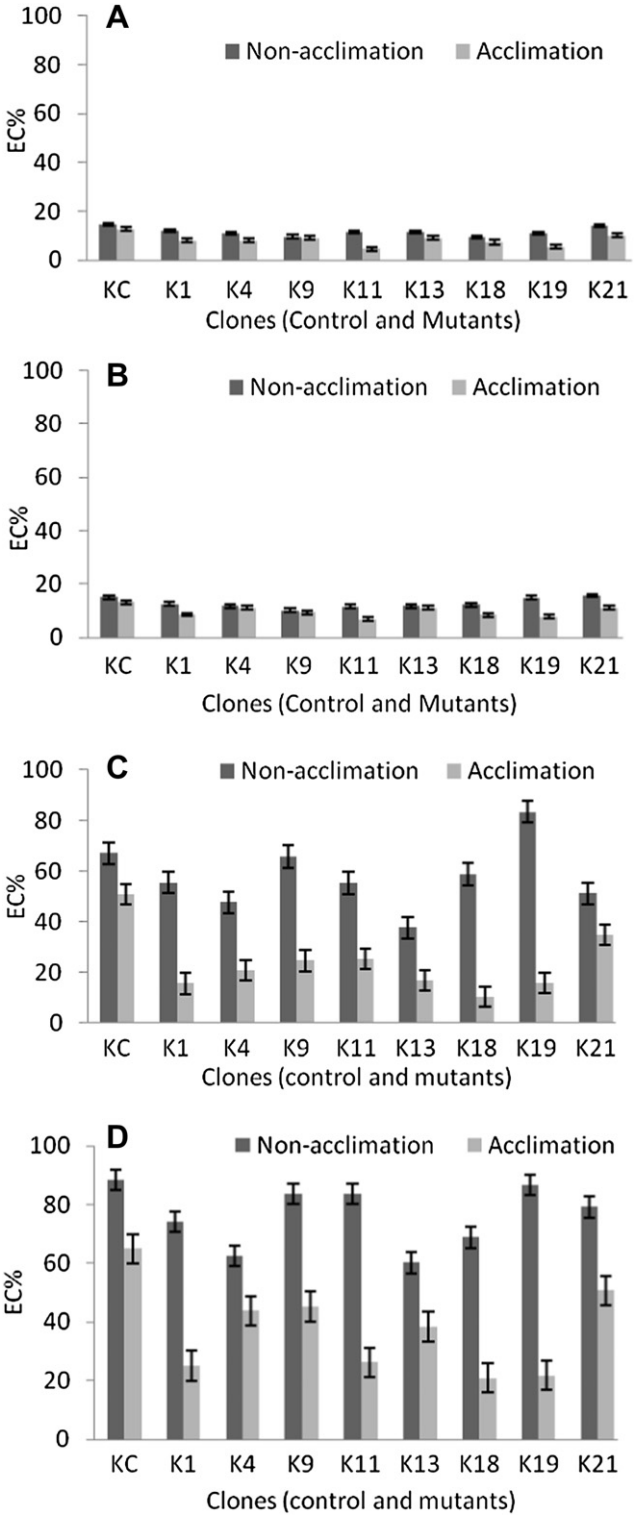

Fig. 1. Frost damage (Electrical conductivity EC%) of cauliflower clones at +1 °C (A), −2 °C (B), −4 °C (C) and −6 °C (D). KC is control and K1–K21 are mutant clones. The bars indicate standard error of the mean values of replicates.

2. Results

2.1. Frost resistance analysis of the clones (mutants and control)

The electrolytes leakage increased with lowering the temperature (Fig. 1A–D). Non-freezing +1 °C temperature was used to compare with the effect of freezing temperature at −2 °C, −4 °C and −6 °C. But at +1 °C and −2 °C showed almost similar effect and no clear difference was observed as shown in Fig. 1A and B. The leakage of electrolytes from cells sharply increased at −4 °C (Fig. 1C) and the highest damage in terms of highest relative electrical conductivity (REC) was observed at −6 °C as shown in Fig. 1D.

The acclimation process was found to be very effective in increasing frost resistance but was more effective in the mutant genotypes. Clear differences existed among acclimated mutants at −6 °C with some mutants like K1, K11, K18, and K19 showing high resistance where the EC% was less than 40% whilst the mutants K4, K9, K13, and K21 showed moderate resistance as compared to the control (Fig. 1D).

2.2. Expression analysis of BoCBF/DREB1

The *CBF/DREB1* gene sequence of *B. oleracea var. botrytis* was identified and designated as *BoCBF1/DREB1*. The PCR product of cDNA and genomic DNA showed similar fragment sizes assuming that the isolated sequence is an exon (Fig. 2A–E). Among the mutants there were apparent differences in expression level observed under cold acclimation. Such as after 3 h cold acclimation only K1, K11, K13, K18 and K19 (represented by lane 1, 4, 5, 6, 7 respectively in Fig. 2A) showed RT-PCR product. After 6 h acclimation, two more clones K21 and KC (lane 8 and 9 in Fig. 2B) also showed the product. After 24 h acclimation the clones showed maximum product of RT-PCR (Fig. 2C) whilst after 14 d cold acclimation, only very weak bands were present (Fig. 2D) indicating that the transcript level had declined. Similar and optimized RT-PCR reactions were made for both of acclimated and non-acclimated clones. But all the mutants and control clones under non-acclimation condition showed no RT-PCR product as shown in Fig. 2F.

2.3. Sequence analysis

The cDNA sequence and the amino acid sequence of the predicted protein encoded by the isolated *BoCBF1/DREB1* was subjected to multiple sequence alignment using clustalW2 EMBL-EBI [24]. The cDNA sequence (Fig. 3A) and the amino acids sequence (Fig. 3B) of the isolated BoCBF1/DREB1 gene posses all the important structural domains and regions that distinguish CBF such as the highly conserved AP2 domain and the CBF signature motif 'DSAWR' immediately downstream of the AP2 domain (Fig. 3B). The AP2 domain has been suggested to play a crucial role in recognition of the DNA binding sequence in the promoter of cold responsive genes [25]. The alignment of *BoCBF1/DREB1* with cold induced CBF's of Brassica species *Brassica napus* (GeneBank, AAM18958), *Brassica juncea* (ABX00639), and *Brassica rapa* (ACL12046) showed ~90% sequence homology (Fig. 3B). But when the *BoCBF1/DREB1* amino acids sequence was compared in a broad way with members of the Brassicaceae family other than Brassicas, then the consensus reduced to 67% as shown in Fig. 3C. These findings indicate that the sequence isolated here from *B. oleracea var. botrytis* is more highly conserved in Brassica species than in the other members of Brassicaceae family but homology with other members was still high.

2.4. Correlation between CBF expression and frost resistance in clones (mutants and control)

There was a positive correlation between frost resistance and apparent CBF expression, with increased CBF expression associated with decreased % relative electrical conductivity. This correlation was significant after 14 d (Fig. 4A) and after 24 h (Fig. 4B) cold acclimation, but after 6 h (Fig. 4C) and 3 h (Fig. 4D) cold acclimation the correlation was also positive but not statistically significant. The highest association was found between CBF expression at 14 d and EC% at −6 °C (R^2 = 93.43%) and the second highest between CBF expression at 24 h and EC% at −6 °C (R^2 = 82.57%).

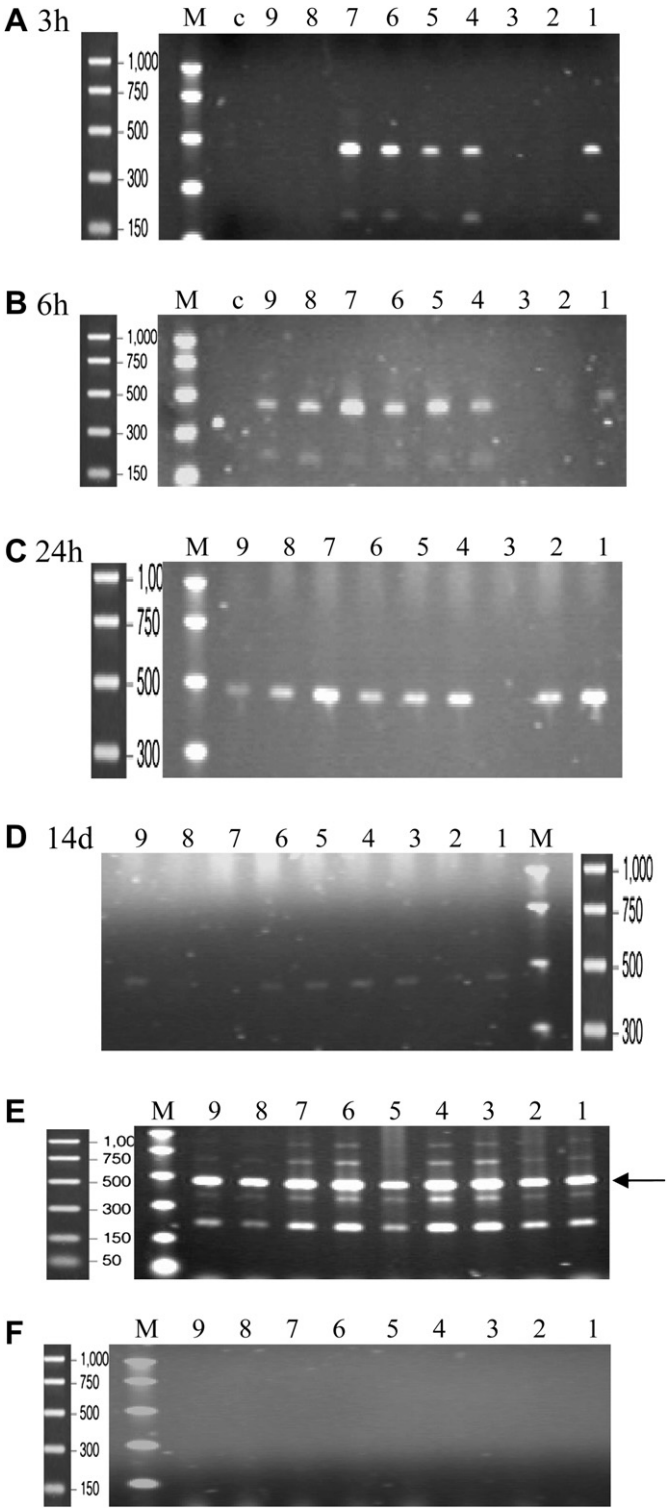

Fig. 2. RT-PCR products of cauliflower clones after 3 h (A), 6 h (B), 24 h (C), and 14 d (D) cold acclimation. The PCR product of genomic DNA (E) where the arrow indicates the bands of interest. RT-PCR under non-acclimation (F). M = Marker, lane 1 = K1, 2 = K4, 3 = K9, 4 = K11, 5 = K13, 6 = K18, 7 = K19, 8 = K21, 9 = KC (control).

2.5. CBF expression and proline production under cold acclimation

Cold acclimation increased proline production (Fig. 5A) and a significant positive correlation was found between CBF expression and proline production after 14 d cold acclimation (Fig. 5B) and no relationship was found under non-acclimation.

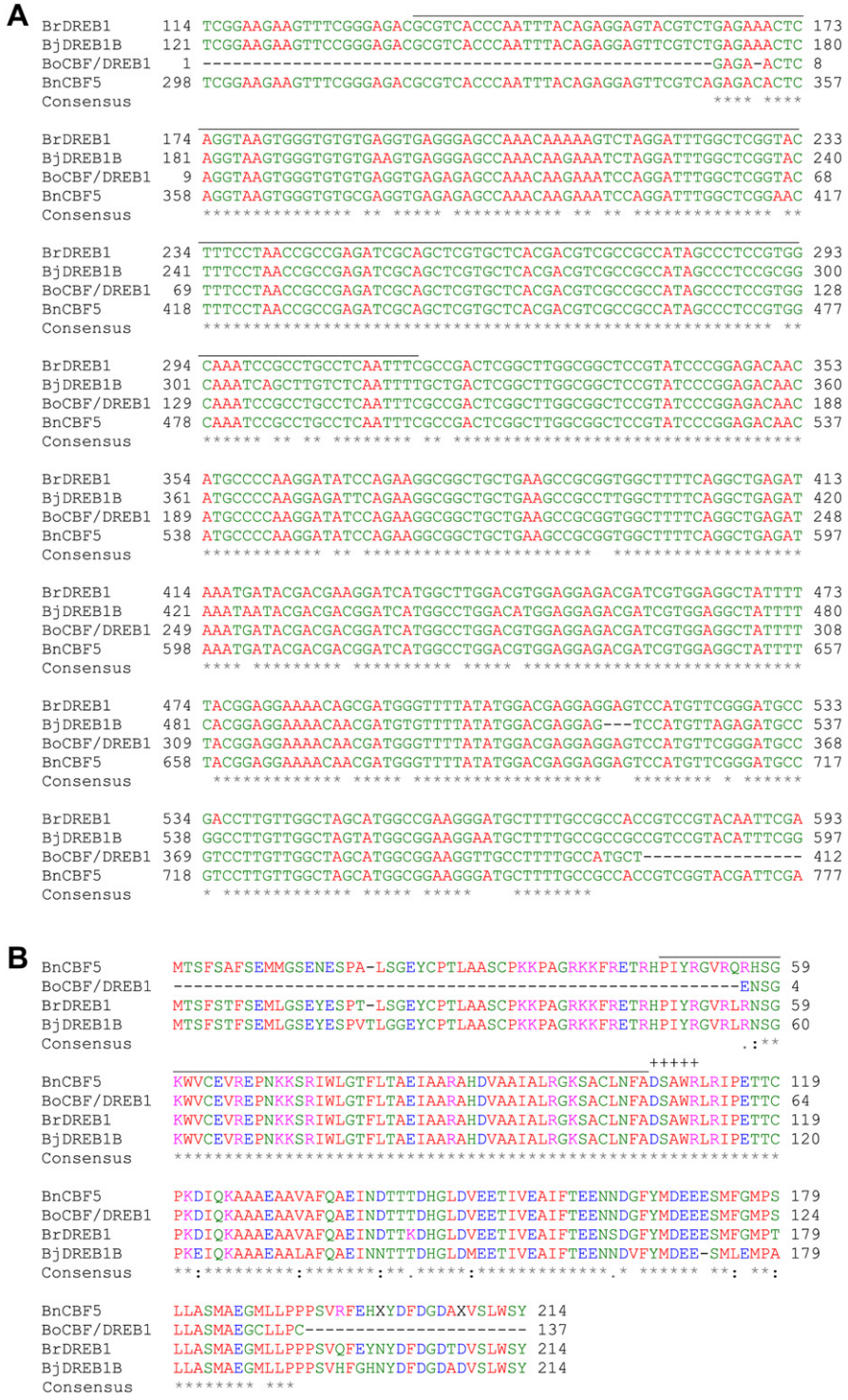

Fig. 3. A. Nucleotide sequence (cDNA) of obtained putative CBF/DREB fraction from cauliflower and multiple sequence alignments with other Brassicas species. Alignments were made using ClustalW2 EMBL-EBI. Consensus symbols denoted as: "*" indicates that the nucleotides in that column are identical in all sequences in the alignment. ":" indicates that conserved substitutions have been observed, "." indicates that semi-conserved substitutions are observed. The AP2 region is indicated by the overline. BrDREB1 from *Brassica rapa* subsp. *pekinensis*, BjDREB1B from *Brassica juncea*, BoCBF/DREB1 sequence isolated from *Brassica oleracea var. botrytis*, BnCBF5 from *Brassica napus*. B. Multiple alignment and comparison of the deduced amino acids sequence of *BoCBF/DREB1* with amino acid sequences of Brassica species. *Brassica napus BnCBF5/DREB1* (GeneBank, AAM18958), *Brassica juncea BjDREB1B* (ABX00639), *Brassica rapa* subsp. *pekinensis DREB1* (ACL12046), *Bo. Brassica oleracea.* The symbol "*" indicates the consensus and the DNA (CRT/DRE) binding AP2 domain is indicated by a thick overline. + indicates the CBF signature motif DSAWR and ":" indicates the conserved substitutions and "." indicates semi-conserved substitutions. The dotted line shows the missing amino acids of the full length gene sequence because of the isolation of only a partial sequence of the gene from *B. oleracea var. botrytis*. Percent consensus among the Brassica species was calculated and found to be 90% homology for 137 amino acids sequence of *BoCBF/DREB1*. The % consensus calculated as the number of "*"/total amino acid sequence which is 123 aa and multiplied by 100 = 123/137 × 100 = 90%. C. Multiple alignment of the *BoCBF/DREB1* deduced amino acids sequence with members of Brassicaceae family other than Brassica species. *BoCBF/DREB1* (*Brassica oleracea*), *Raphanus sativus RsCBF1* (ACX48435), *Thlaspi arvense TaCBF* (ABV82985), *Eutrema salsugineum EsDREB1* (AAS00621) and *Arabidopsis thaliana AtCBF2* (ABV27090). The symbol + indicates the CBF signature motif DSAWR, "*" indicates the identical amino acids in all sequences. ":" indicates conserved substitutions, "." indicates semi-conserved substitutions, the % consensus was calculated as the number of "*"/total amino acid sequence which is 137 aa and multiplied by 100 = 91/137 × 100 = 67%.

C

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TaCBF          MNSFSAFAEMFGSEYESPDTIGADYCPTLATSCPKKPAGRKKFRETRHPIYRGVRRRNSG 60
EsDREB1        MNSFSAFAEMFGSEYESPVTVGGDYCPTLATSCPKKPAGRKKFRETRHPIYRGVRRRNSG 60
BoCBF/DREB1    ------------------------------------------------------ENSG 4
RsCBF1         MTSFSTFSELLGSEHESPVTLGEEYCPKLAASCPKKPAGRKKFRETRHPIYRGVRLRNSG 60
AtCBF2         MNSCSAFSEMFGSDYESPVSSGGDYSPKLATSCPKKPAGRKKFRETRHPIYRGVRQRNSG 60
ApCBF          MSSFSAFSEMFGSDYESMISSVGDYSPTLATSCPKKPAGRKKFRETRHPVYRGVRQRNSG 60
Consensus                                                          .:***

                                                                   +++++
TaCBF          KWVCEVREPNKKSRIWLGTFPTAEMAARAHDVAAIALRGRSACLNFADSAWRLRIPESTC 120
EsDREB1        KWVCEVREPNKKSRIWLGTFPTAEMAARAHDVAAIALRGRSACLNFADSAWRLRIPESTC 120
BoCBF/DREB1    KWVCEVREPNKKSRIWLGTFLTAEIAARAHDVAAIALRGKSACLNFADSAWRLRIPETTC 64
RsCBF1         KWVCEVREPNKKSRIWLGTFLTAEIAARAHDVAAIALRGKSACLNFADSAWRLRIPETTC 120
AtCBF2         KWVCELREPNKKTRIWLGTFQTAEMAARAHDVAAIALRGRSACLNFADSAWRLRIPESTC 120
ApCBF          KWVSELREPNKKTRIWLGTFQTAEMAARAHDVAAIALRGRSACLNFADSVWRLRIPESAC 120
Consensus      ***.*:******:******* ***:**************:*********.*******::*

TaCBF          AKDIQKAAAEAAVAFQAE-MSDTTT-DHGLDMEETIVEAIVNEE-QSGGFYMDEE-AMFG 176
EsDREB1        AKDIQKAAAEAAVAFQAE-MSDTMTSDHGLDMEETTVEVIVTEEEQSEGFYMDEE-AMFG 178
BoCBF/DREB1    PKDIQKAAAEAAVAFQAE-INDTTT-DHGLDVEETIVEAIFTEE-NNDGFYMDEEESMFG 121
RsCBF1         PKDIQKAAAEAAVAFQAE-INDTTT-DHGLDVEETIVEAIFTEE-NSDGFYMDEEESMFG 177
AtCBF2         AKEIQKAAAEAALNFQDE-MCHMTTDAHGLDMEETLVEAIYTPEQSQDAFYMDEE-AMLG 178
ApCBF          AKDIQKAAAEAALAFQNELMSDTATTDHGLDMEETLVEAIVTAEQ-IDTFYIDEE-TMFG 178
Consensus      .*:*********: ** * : .  .  ****:*** **.* . *     **:*** :*:

TaCBF          MPRLLANMAEGMLLPPPSVQWGQNYDCDGDADVSLWSY 214
EsDREB1        MPRLLANMAEGMLLPPPSVQWGHNYDCDGDADVSLWSY 216
BoCBF/DREB1    MPSLLASMAEGCLLPC---------------------- 137
RsCBF1         MPTLLASMAEGMLLPPPSVQFGHTYDFDGDADVSLWSY 215
AtCBF2         MSSLLDNMAEGMLLPSPSVQWNYTFDVEGDDDVSLWSY 216
ApCBF          MPSLMANMAEGMLLPLPSIQWINNYDVEGDADMPLWSY 216
Consensus      *. *: .**** ***
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Fig. 3. (*continued*).

2.6. Correlation between proline production and frost resistance

There was a positive and significant correlation between proline content and frost resistance under cold acclimation at all of the tested freezing temperatures (Fig. 6A). Non-acclimated clones showed a very weak relationship between proline and frost resistance (Fig. 6B).

2.7. Detection of COR15 gene

The results of protein fractionation are presented in Fig. 7A. The Western blot results confirmed the presence of COR15 protein and this was repeated 2–3 times to verify the results (Fig. 7B). The results clearly demonstrated the influence of cold acclimation on

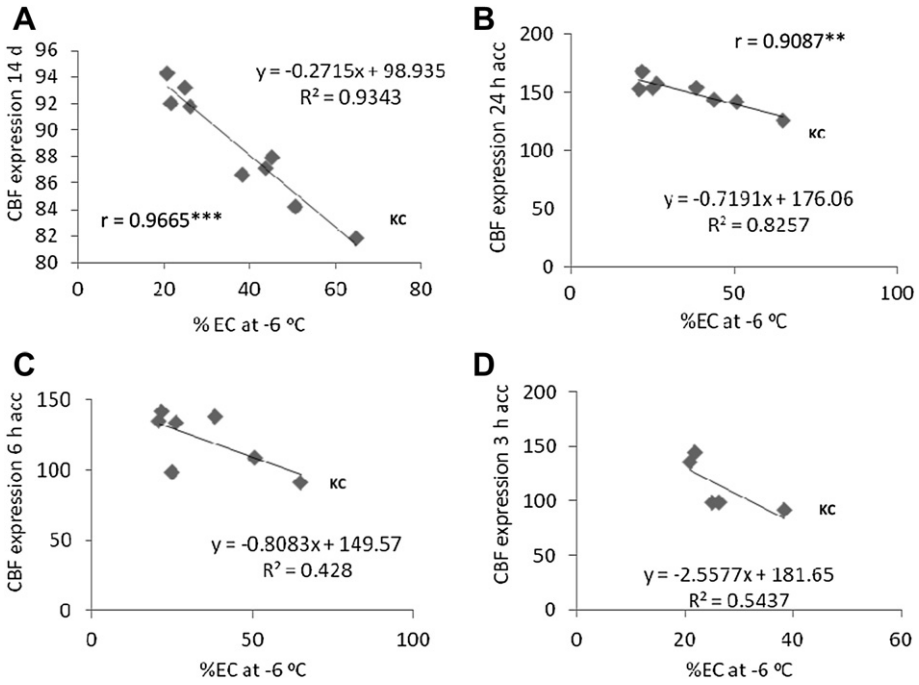

Fig. 4. Correlation between frost resistance (EC% at −6 °C, −4 °C and −2 °C) and CBF expression after 14 d (A), 24 h (B), 6 h (C) and 3 h cold acclimation (D). The location of the control clone is indicated by KC. The data at −6 °C temperature only is presented here because the other data at −4 °C and −2 °C also showed about similar correlation response, so to avoid figures repetition other data not presented.

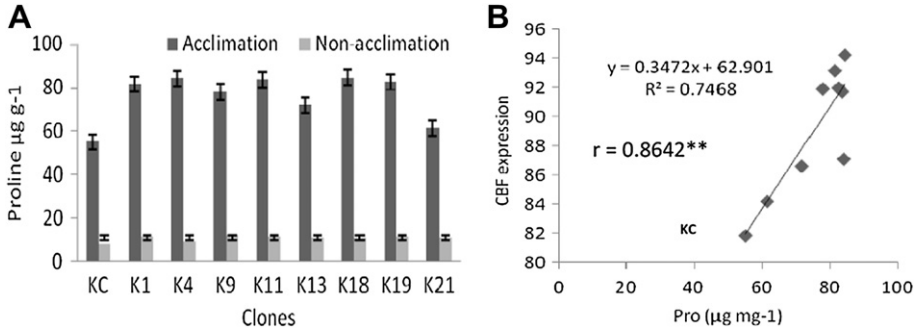

Fig. 5. (A) The effect of cold acclimation on proline production, KC (control) and K1–K21 (Mutants). The bars indicate the standard error of the mean of replicates. (B) Correlation between CBF expression and Proline production under cold acclimation. The location of the control clone is indicated by KC.

the expression of *COR15* and all the genotypes showed the presence of COR15 protein after 14 d acclimation (Fig. 7B). In non-acclimated conditions, mutants K1, K4 and K21 also showed clear blots while for other clones no blots were observed (Fig. 7C). This suggests constitutive expression of COR15 in these clones without cold acclimation. These three mutants also showed slightly higher frost resistance compared to other clones (K9, K11, K19, KC) under non-acclimation (Fig. 7D).

3. Discussion

In all the physiological tests there was no significant leakage of electrolytes from leaf discs treated at −2 °C, while there was a sharp increase in leakage at −4 and −6 °C. This suggests *B. oleracea* is resistant to −2 °C but sensitive to freezing temperatures below this. Increased leakage of electrolytes reflects damage to cell membranes [26]. The physiological screening evidences could suggest that there might be a group of cold responsive genes in cauliflower induced under cold acclimation. The molecular evidences showed the identification and expression of BoCBF/DREB1 and COR15 genes along with higher level of free proline under cold acclimation. The high resemblance (90% homology) of deduced amino acid sequence of BoCBF/DREB1 (Fig. 4A) with CBF from other species of Brassica suggests that the isolated sequence is highly conserved in Brassicas. These findings demonstrate the existence of some part of the CBF regulon pathway in cauliflower, as previously reported that CBF regulon includes COR genes, membrane stabilizing proteins and cryoprotectents such as proline [27]. The RT-PCR technique used in this investigation can be supported by previous scientists who had isolated cDNA sequences of CBF/DREB genes from other plants [28,29]. The cDNA sequences from both mutant and control clones were identical, that shows that in isolated part of gene sequence no mutation has occurred. Since there is evidence for differences in expression pattern then this suggests that the mutation might be in an upstream position or in a regulator gene like ICE (Inducer of CBF expression) a master switch of CBF genes regulation [30]. Further investigation is needed to explore the upstream DNA in this pathway for mutations in these mutants.

Cold acclimation increased frost resistance with expression of the *BoCBF/DREB1* gene and the transcript level in mutants increased up to 24 h cold acclimation and then declined by 14 d. Many scientists have reported a similar pattern of CBF/DREB1 expression [29,31–33]. Cold acclimation also induced frost resistance in control clones in comparison with non-acclimated conditions. This evidence showed that cold acclimation works to increase the frost resistance in cauliflower. The mutants showed highly frost resistance compared to the control clones under cold acclimation and even though the transcript of *BoCBF/DREB1* appeared in control clones but very low when compared to the mutants. This evidence showed that mutagenesis has increased the level of expression of this gene, ultimately enhanced frost resistance in mutants. Similar effect of over-expressed CBF on frost tolerance has been reported [15,34].

Proline was always produced in clones under both situations of either cold acclimation or non-acclimation but cold acclimation increased the proline level by about 8 fold and confirmed the previous findings of many researchers [14,23,35]. One reason here for the significant increase in proline level under cold acclimation might be the expression of *BoCBF/DREB1* gene in mutants under cold acclimation because there was no transcript observed in non-acclimated clones. Gilmour [15] also reported increased proline production with over-expressed CBF gene. The increased proline and *BoCBF/DREB1* transcript in mutants over control clones might

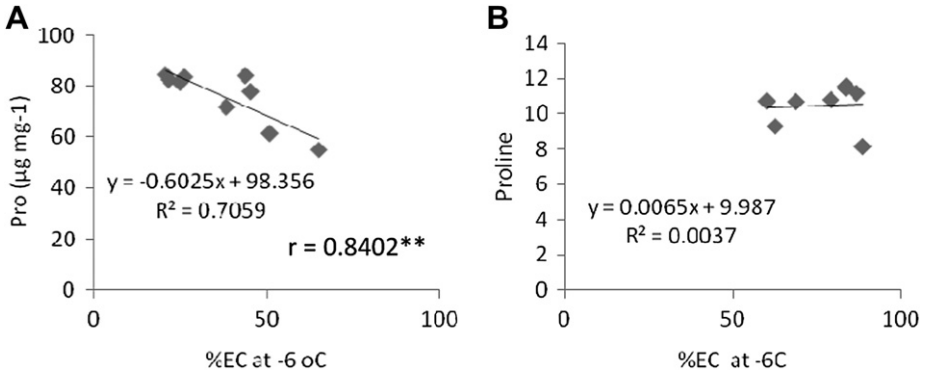

Fig. 6. Correlation between proline level and frost resistance (EC% at −6 °C) under cold acclimation (A) and non-acclimation (B).

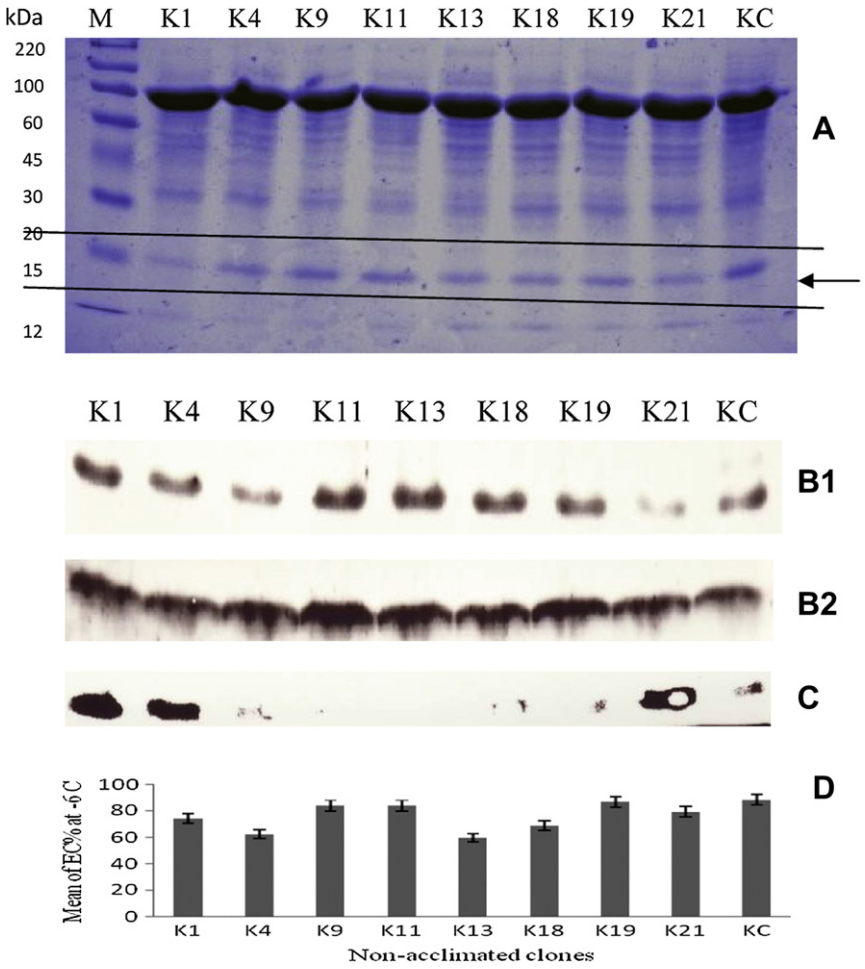

Fig. 7. (A) SDS-PAGE analysis, the arrow flanked by lines shows the band of interest. (B) Western blot of cold-acclimated clones for COR15 (B1 and B2 repeats for confirmation) (C) Western blot of non-acclimated clones for COR15. (D) Frost damage in non-acclimated clones (Electrical conductivity EC%). M is marker proteins, K1–K21 are mutants and KC is control. The bars indicate the standard error of the mean of replicates.

be due to mutagenesis. The cold acclimation induced increase in proline level in correlation with frost tolerance can confirm the previous findings [15,36]. The mutants always showed higher proline level and frost resistance compared to control plants but the difference between mutants and control plants become very clear under cold acclimation. These findings demonstrated that the mutagenesis positively increased the proline level and subsequently enhanced frost resistance. Proline contributes to improved freezing tolerance in mutants over control plants [37].

All of the cold-acclimated clones expressed the COR15 gene, but under non-acclimation only in three mutants (K1, K4, K21) this gene expressed, that might indicate the presence of other transcription factors genes in cauliflower which induced the COR15 gene expression because in these mutants no RT-PCR bands appeared under non-acclimation. The frost resistance in cold-acclimated clones where COR15 strongly expressed was slightly higher than non-acclimated clones. COR15 protein can reduce the susceptibility of membranes to freezing stress [38,39]. Other interesting thing observed here is the frost resistance level of mutants K13 and K18 which is about similar to K1, K4, and K21 under non-acclimation, while COR15 blots were not present for K13 and K18 under non-acclimation, that could suggests the presence of other genes and there might be some upstream mutation which might enhance the frost resistance in these mutants under non-acclimation. These results suggest further investigation to analyze these two mutants (K13 and K18). The appearance of the success of mutagenesis in this project confirmed that this is a promising method of producing mutant lines with better stress resistance in *B. oleracea var. botrytis*. Mutation breeding can have many advantages, such as multiple traits can be identified compared to transgenic approach where a single trait can be introduced in the crop, low cost, rapidity, simplicity and mutated varieties have more chances of survival in the environment [40]. Conclusively, the chemical induced mutagenesis and over expression of the identified BoCBF/DREB1 of cauliflower (*B. oleracea var. botrytis*) is a promising approach to improve frost resistance.

4. Materials and methods

4.1. Plant materials, mutagenesis and selection of stress resistant mutants

Chemically induced mutants along with control (wild type) clones were maintained through sub-culturing under the *in-vitro* conditions in the Plant Physiology Lab, University of Plymouth UK. These mutants were assessed for their expression of stress resistance [23]. In the present investigation the highly frost resistant mutants were selected and transferred to greenhouse. The regeneration of clones was carried out from greenhouse and retransferred to in-vitro condition. Then the in-vivo grown clones in the

greenhouse and the regenerated in-vitro clones were correlated for frost resistance for the confirmation of the induced resistance stability [41]. After regeneration these clones were symbolized as 'K' in this paper and the highly frost resistant mutants (K1, K4, K9, K11, K13, K18, K19, and K21) from the mutated population along with control clone (KC) were subjected to molecular and biochemical investigations.

4.2. Plant growth conditions

The *in-vitro* clones were taken from the growth cabinet (23 °C and 16 h photoperiod) and transferred to *in-vivo* conditions through a weaning process and then grown on in the glasshouse. Plants were then subjected to either cold-acclimating or non-acclimating conditions in Growth Cabinets. Acclimation conditions were 14 d at 4 °C, 8 h photoperiod, 180 μmol m^{-2} s^{-1} light intensity and 75% humidity. Non-acclimation conditions were 22 °C, 16 h photoperiod 180 μmol m^{-2} s^{-1} light intensity and 52% relative humidity.

4.3. Analysis for frost tolerance

Acclimated and non-acclimated plants were assessed for frost resistance using a leaf disc relative conductivity assay [22,23]. Ten leaf discs of 1 cm diameter each were cut from fully expanded photosynthetically active leaves and placed in boiling tubes. Three replicate tubes were used for each treatment for each clone. Total tubes used were = 9 clones × 3 replicates × 2 unacclimated/acclimated × 4 different test temperatures (216 tubes). Three tubes without leaf discs (blanks) were used for monitoring EC contamination. The percent relative electrical conductivity was calculated as REC% = Post freezing EC/Post autoclaving EC × 100. Tubes were held in the frost chamber in the dark at +1 °C for 2 h, ice added to each tube to initiate freezing, a sample taken, and the remainder then exposed to freezing to −2, −4, and −6 °C with a hold of 2 h at each test temperature to ensure equilibration.

4.4. RNA extraction

Non-acclimated leaves were sampled after 14 d and cold-acclimated leaves sampled after 3 h, 6 h, 24 h and 14 d of cold acclimation. All samples were immediately frozen in liquid nitrogen to avoid RNA degradation. RNase cleaning agent (Sigma RNase ZAP cat # R2020) was used for cleaning all surfaces before RNA isolation. The tissues were then ground in liquid nitrogen and 100 mg of frozen tissue powder for each sample was weighed into 1.5 mL RNase free micro-centrifuge tubes (Ambion) and stored at −80 °C prior to RNA isolation. Leaf tissue samples (100 mg) were taken from −80 °C and 500 μl lyses solution (Sigma cat # L8167) added and immediately vigorously vortexed for at least 30 s and then incubated at 56 °C for 5 min. Total RNA was extracted from each clone using Sigma cat # STRN50 and RNAlater® for RNA protection and the purified RNA in flow through eluate was distributed into small aliquots and stored at −20 °C for a short time and at −80 °C for long term storage.

4.5. Identification of BoCBF/DREB1 gene

Total RNA was used as the template for the synthesis of first strand cDNA using an ImProm-II™ Reverse Transcription (RT) System (Promega cat # A3800). Each RNA sample (0.8 μg/reaction) was diluted with nuclease-free water (Sigma w1754) and primer oligos $(dT)_{15}$ (0.5 μg/reaction) were combined for a final volume of 5 μl/RT reaction in a 0.2 mL nuclease-free PCR tube (Ambion). A final volume of 20 μl of RT mixture for each sample was prepared by combining 5 μl of RNA + oligo $(dT)_{15}$ primer mixture with 15 μl RT reaction mixture. The first strand cDNA was synthesized in a thermal cycler (Perkin Elmer 9700) under the thermal cycle (Annealing: 25 °C for 5 min, Extension: 42 °C for 60 min, Inactivation of Reverse Transcriptase: 70 °C for 15 min). The amplification of first strand cDNA was made in a 50 μl PCR reaction of 25 μl PCR master mix 2× (Promega cat # M7502), 5 μl (10 μM) of each forward and backward primers, 5 μl of first strand cDNA, and nuclease-free water (Sigma cat # w1754) added to make 50 μl of final reaction mixture for each sample. The gene specific (degenerate) forward and reverse primers (Forward, 5-AAGAAGTTT CGTGAGACCCGTCA C-3 and Reverse, 5-GGCAAAAGCATA CCTTC CGCCAT-3) were used. Initial denaturation at 94 °C for 3 min once, denaturation at 94 °C for 1 min, annealing at 61 °C for 1 min, extension at 72 °C for 2 min (35 cycles) and then a final extension at 72 °C for 10 min followed by 4 °C ∞. The PCR products were run on a 0.8% high melting agarose (Sigma) gel and then visualized under UV light and photographs taken with a gel documentation system. The PCR products were compared with a PCR marker ladder (Promega cat # G3161) and band intensities were semi-quantitatively measured using Quantity one 4.6.3 Bio-Rad software.

4.6. Sequence analysis and multiple alignments

The cDNA fragment was excised in a minimum volume of agarose gel using a clean sharp scalpel. Each slice was then transferred to a 1.5 mL micro-centrifuge tube (Ambion) and isolation of the DNA fragment was carried out using the ® SV Gel and PCR Clean-Up System (Promega A9281). The weight of the tube was noted before and after addition of the slice. Membrane binding solution was added to the tube at a ratio of 10 μl/10 mg of agarose gel slice, mixed very gently and incubated at 61 °C until the gel slice dissolved completely. This was centrifuged briefly at room temperature and DNA was purified with an SV mini-column and the purified DNA was subjected to sequencing (Eurofins MWG Operon, Germany). Some of the purified DNA was stored at 4 °C for a short time and at −20 °C for longer storage. Multiple sequence alignments of the deduced amino acid sequence of the isolated gene with other cold induced genes sequences were carried out using ClustalW2. EMBL-EBI [24] and BLAST (NCBI).

4.7. Genomic DNA isolation

Leaves from each clone were ground into a fine powder in liquid nitrogen, using a mortar and pestle, and 100 mg of the powder transferred to a 1.5 mL micro-centrifuge tube (Ambion) on ice and 350 μl lyses solution A and 50 μl of lyses solution B were added and isolation of DNA was carried out (Sigma G2N10).

4.8. Proline extraction and estimation

Free proline was extracted from leaf frozen powders stored at −80 °C following the method of Bates [42]. Samples of 100 mg were homogenized in 1.5 mL of 3% sulfosalicylic acid in 2 mL tubes and centrifuged at 13,000 × g for 5 min at room temperature. 300 μl of the supernatant was incubated for 1 h with 2 mL glacial acetic acid and 2 mL acid ninhydrin in test tubes at 100 °C in a water bath. The tubes were then immediately dipped in ice. The reaction mixture was extracted with 1 mL toluene by mixing vigorously for 10–30 s. The chromophore containing toluene was pipetted into a fresh tube, warmed to room temperature and its absorbance noted at 520 nm by spectrophotometer using toluene as a blank. The proline concentration in samples was determined from a predetermined standard curve. The reaction for each sample was performed in triplicate.

4.9. Protein extraction and Western blotting

Leaf tissues from −80 °C were thawed on ice, placed in a mortar and pestle and 2 mL extraction buffer [43] (100 mM potassium phosphate, 1 mM DTT, 1 mM EDTA, 1% Triton X-100, 10% glycerol, pH 7.8) was added to 1 g of tissues and ground. 1 mL of the liquid grindate was transferred to a microfuge tube (1.5 mL) on ice. The supernatant was centrifuged twice at 13,000 × g for 15 and 10 min respectively at 4 °C. The clear supernatant containing proteins was separated using SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis) [44] and visualized using Coomassie blue staining solution. Unstained gels were used for Western blotting and the proteins were transferred to a nitrocellulose membrane by electro-blotting at 15 V for 90 min. One liter of 1× Tris–glycine transfer buffer was prepared by dissolving Tris–HCl 3.05 g, Glycine 14.4 g, adding methanol 200 mL, ddH_2O 800 mL, pH 8.3 adjusted with NaOH [45]. After transferring the proteins onto the nitrocellulose membrane, the membrane was incubated in blocking solution (PBST + 2% BSA) for 1 h at room temperature with mild shaking. After incubation, the membrane was washed with PBST twice for 5 min each, followed by incubation with primary COR15 antibodies obtained from the Lab of the Dr. Michael Thomashow (MSU, USA). Primary antibodies were diluted (1:1000) in a total volume of 20 mL PBST and the membrane was incubated at room temperature overnight with very mild shaking. The membranes were then washed 3 times with PBS while agitating, for 10 min each and the membrane then incubated in goat anti rabbit IgG horseradish peroxidase conjugated secondary antibody (PBS diluted 1:20,000) (Abcam) for 90 min at room temperature with mild shaking to detect primary antibodies attached to the COR15 protein. The membranes were washed 3 times with PBS for 10 min each and then incubated in developmental solution (DAB 0.06 g, $NiCl_2$ 0.03 g in 100 mL PBST) for about 15 min. The membranes were washed repeatedly with PBS, and blots were observed and digital images made.

4.10. Statistical analysis

Analysis of variances (ANOVA) was performed using Minitab 15 and correlation among the different parameters was investigated using Microsoft Excel and values of the correlation coefficient for different levels of significance investigated according to Fisher and Frank [46].

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