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# Molecular Analysis of Cold Responsive (COR) Genes in Selected Sugarcane and *Saccharum spontaneum* L.

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## Abstract

**Background:** Sugarcane (Saccharum derived) is an important commercially harvested crop in all parts of the world including tropical and subtropical areas. Saccharum hybrid is the tall perennial true grasses with sweet stalk rich in sucrose and it is the main source of sugar.

**Methods:** Initially, 23 genes differentially expressed during cold stress in other Andropogoneae tribe members were retrieved from NCBI GenBank and were investigated in the genome of selected sugarcane and *Saccharum spontaneum* L. Samples. Their presence in our samples was analyzed and confirmed through PCR and Agarose Gel Electrophoresis (AGE).

**Results:** Most of these (COR) genes (21/23) were confirmed in cold tolerant cultivars namely, SPSG-394, CP-851491 and *Saccharum spontaneum* L. while the least number of genes was observed in cold sensitive cultivar namely, CP-77400. Moreover 10 cold responsive genes, namely CBF1, CBF2, CBF3, COR 6.6, COR 78, COR 47, WCOR 80, WCOR14, C17 and 85KDA were sent for sequencing. Nucleotide sequences analysis of selected genes revealed the homology to stress responsive protein. Furthermore, during a conserved domain search, three conserved domains had been detected, namely gypsy transposon, zinc binding for reverse transcriptase and pepsin like aspartate proteases.

**Conclusion:** The analysis of cold responsive genes in sugarcane could help breeders to select cold tolerant sugarcane cultivars through PCR amplification.

## Introduction

Sugarcane (Saccharum derived) is an important commercially harvested crop in all parts of the world including tropical and subtropical areas. Saccharum hybrid is the tall perennial true grasses with sweet stalk rich in sucrose and it is the main source of sugar [1]. The modern complex hybrid Saccharum is derived by the interbreeding of Saccharum species [2]. The plant growth and productivity are affected by several biotic and abiotic factors. Low temperature or freezing is one of the major abiotic factors which influences the growth and development of the plant. The plant has shown various mechanisms to cold stress, and this phenomenon is called cold acclimation. The normal temperature required for growth of sugarcane plants is about 35°C; the temperature bellow 20°C can reduce the plant growth and yield [3]. Although some field experiments have shown the variation in the sensitivity to cold stress of some sugarcane cultivars [4]. It is noted that some of the subtropical sugarcane cultivars are colder tolerant than tropical hybrids [5]. Therefore, a view of transcriptome dynamics and identification of cold responsive genes family and pathways could be an important aspect in breeding programs to identify cultivars which are more tolerant to cold and the related stresses [5]. But it depends on the cultivar tolerance to post freezing and the time gap and temperature variation between the harvesting and freeze events [6]. The cold responsive genes, including COR15a, [7], alfalfa Cas15 [8], and wheat WCOR 14, WCS 120 [9] have been reported. The expression of cold responsive genes has shown to be critical for both cold acclimation and chilling tolerance in plants [10]. The cold responsive genes of Arabidopsis include COR 15a, COR 6.6, COR 47 and COR 78/RD29 are expressed during cold, dehydration or ABA stress. The COR 15a polypeptide is targeted to the chloroplast [11]. The CBF1, CBF2 and CBF3 (transcription factors) are not induced during exogenous ABA pathway; therefore it suggested that these transcription factors participate during induced ABA pathway [12, 13]. During abiotic stresses like cold, salinity and drought, these transcription factors bind to the dehydration elements/C-repeat (DRE/CRT) of the stress resistance genes and enhance the expression of these stress resistance genes [14]. In non-acclimated transgenic Arabidopsis plant, the constitutive overexpression of the CBF1 and CBF3 induces the expression of other cold inducible genes which increases the plant tolerance to chilling and freezing stress [15].

Sugarcane is the 2nd major crop in Pakistan and plays a vital role in the agro-economy. The production and yield of the crop is affected by several biotic and abiotic factors among this cold is also one of the major factors which causes significant losses to Pakistan Agro industry. In this study we investigate the cold tolerance genes (COR), transcription factors (CBF1, CBF2 and CBF3) and WCOR14 gene in selected commercially grown sugarcane cultivars and wild relative *Saccharum spontaneum* L.

## Methods

### Sample collection

The plant materials for this research were collected from Sugarcane Crop Research Institute Mardan, Khyber Pakhtunkhwa Pakistan. Three commercially grown sugarcane cultivar namely SPSG 394, CP77_400, CP85-1491 based on resistance to cold stress and wild type *S. spontaneum* L. was selected as control from cold region Murree, Khyber Pakhtunkhwa Pakistan. The sugarcane buds were collected during maturing stage and harvested in the research field of Genetics Department Hazara University, Mansehra (Table 1).

| Cultivars | Year of Release | Cane Yield (t/ha) | CCS% | Maturity | Cold Responses |
|---|---|---|---|---|---|
| SPSG-394 | 2003 | 50.00 | 11.00 | Early | Cold tolerant |
| CP-851491 | 1994 | 60.00 | 11.00 | Early | Cold tolerant |
| CP-77400 | 1996 | 74.00 | 12.18 | Mid | Cold susceptible |
| *Saccharum spontaneum* L. | N/A | N/A | N/A | N/A | Cold tolerant |

**Table 1:** Detail of selected sugarcane cultivars collected from Sugarcane Crop Research Institute Mardan and *Saccharum spontaneum* L. collected from Murree, Pakistan (Source: Sugar Crops Research Institute, Mardan, Khyber Pakhtunkhwa)

### DNA Extraction

The genomic DNA was extracted from the fresh leaves of sugarcane cultivars and wild relative *S. spontaneum* L. by using modified CTAB methods [16].

The fresh leaves of sugarcane cultivars and *S. spontaneum* L. were crushed in liquid nitrogen with the help of pestle and mortar, and crushed samples (0.25 mg) was taken in 2 ml Eppendorf tube, and the pre-heated CTAB buffer (900µl) was added to each tube, and then incubated at 56°C for 24 hours. After incubation, 500µl mixture (composition) of Phenol Chloroform (PCI) was added to each tube and the samples were centrifuged at 8000xg for 20 minutes. After centrifugation, the clear supernatants were transferred into fresh Eppendorf tubes and 500ul of cold isopropanol were added to the supernatants. The samples were kept in -20°C for 20 minutes and then centrifuged at 8000xg for 20 minutes. After centrifugation, the supernatant was discarded, and the pellet was washed by adding 500µl of 70% ethanol. After washing, the samples were left at room temperature and 60µl of TE buffer were added to each tube and vertex well. The DNA quality and quantity was checked on 1 % Agarose gel.

### Gel Electrophoresis

The extracted genomic DNA was checked on 1% agarose gel. One gram agarose powder was dissolved in (98 ml dH2O and 2 ml 50x TAE (Tris-acetate-EDTA), the mixture was boiled in a conical flask. Till the agarose dissolved completely, 25µl ethidium bromide was added and then gel was cast in a gel tray with a comb. After solidifying, gel was placed in the gel tank containing 50x TAE. Five µl of DNA from each sample was taken, mixed with 2µl loading dye and loaded in the wells. The gel was then run at constant voltage of 75 volts for approximately one hour and 30 minutes. The gel was observed under UV light using "Uvitech" gel documentation system.

### Selection of Genes and Primer designing

Several cold responsive genes were selected from public database and reported literature of Andropogoneae tribe. Further, primers were designed according to the conserved regions of the genes (Table 2) and these primers were synthesized from Macrogen Korea.

### PCR amplification

For the amplification of twenty three cold responsive (COR) genes in the genome of sugarcane cultivars and *S. spontaneum* L. Thermo scientific PCR kit (Catalog #EP0402) was used and followed the instruction of the manufacturer. The PCR amplified products were further confirmed by 1.5% gel and the amplicon size was compared with 1Kb DNA marker. The 23 cold tolerance genes (COR) were further confirmed in genomic DNA of sugarcane cultivars and *Saccharum spontaneum* L. by gel electrophoresis. Although for further confirmation the samples were sent to the Beijing Genomic, Institute China for sequencing. The optimized sequences of sugarcane cultivars and *S. spontaneum* L. were analysed by using NCBI protein and nucleotide BLAST. The sequences of cold tolerance genes (COR) of sugarcane cultivars and *S. spontaneum* L. were also analysed to study the conserved domains in the conserved domain (CDS) search NCBI.

## Results

### DNA Confirmation

Whole genomic DNA was extracted from fresh leaves of three commercially grown sugarcane cultivars and a wild relative *S. spontaneum* L. from Pakistan either resistance or sensitive to cold stress. DNA was analysed by electrophoresis prior to use as a template in PCR reactions. The high qualities DNA free from impurities i.e. RNAs, protein which could hinder PCR amplification, was purified (Fig. 1).

### Confirmation of targeted genes

For PCR optimization of target DNA using cold tolerance genes (COR) various condition were used. The conditions were also modified by changing the annealing temperature and annealing time. The amplified PCR product of cold tolerance gene (COR) was confirmed by 1.5% Agarose gel. The cold responsive genes were further analysed in the genome of selected sugarcane cultivars and wild relative *S. spontaneum* L. through PCR and recorded on Agarose gel electrophoresis (AGE). The analysis of selected cold responsive genes (COR) showed 100% amplification, among 23 genes 16 were successfully amplified in all selected sugarcane cultivars and *S. spontaneum* L. while 7 genes were found unique (Table 3).

| Gene name | TM (°C) | Forward primer (5'-3') | Reverse primer (5'-3') |
|---|---|---|---|
| cor6.6 | 60 | CTGGCAAAGCTGAGGAGAAG | CGGATCGCTACTTGTTCAGG |
| kin2 gene | 59 | GCTGGCAAAGCTGAGGTAGT | GCCTTGTCCAGCAGAACATT |
| COR15 | 60 | GAAAAGAAGGCGCAAAGAAG | AATGTGACGGTGACTGTGGA |
| cor15b | 50 | GTCCTCATGGCCATGTCTTT | GAGGATGTTGCCGTCACTTT |
| C17 | 60 | GGATCCATGAATGGAGATGG | CGCTGTACTCTTGACGGTGA |
| 85 kDa | 59 | CCTACAGCTGCAAAGCATCA | CCAAGAACCCCTTCTTTTCC |
| WCS120 | 59 | GGTCGTTGGAGGAGAGAATG | GCTGCGTCTGTCTCTTGGAT |
| Wcs66 | 59 | ACACGGGAACTACTGGCACT | ATTCTCTCCTCCAACGACCA |
| Wcor726 | 60 | CCCGCTACCTTTGGAGAATA | ACACGGTTTGAACCAAGAGG |
| Wcor80 | 59 | GAAGAGCCTCATGGACAAGG | ACATTCGCTCCTCCAATGAC |
| COR410 | 59 | CGTGACTGGGAGTGAGTGTT | TAGCCCAGCCGAATTACAAC |
| WCOR615 | 59 | CAAAGTGGCTCGAGGAGAAC | ACGAAACGAAAAGTGGGCTA |
| cor47 | 59 | AGCGATGAAGAAGGTGAGGA | ACACTGGTACCGGGATGGTA |
| cor78 | 59 | GAACACTCCGGTCTCTCTGC | CAATCTCCGGTACTCCTCCA |
| Cor78 | 59 | GGAAGAGTCGGCTGTTTCAG | CAATCTCCGGTACTCCTCCA |
| WCS120 | 59 | TTACATGCCGACACTTTGGA | CTACGTGTGGTCGCATCAGT |
| WCOR410 | 50 | GAGAAGGAGGAGGAGCTGGT | CTTTTCCTTGAGCCCCTTCT |
| WCOR14 | 59 | TGCTGGTGTTTGTTCTTTGC | CTACCGCCTCCTGTACCTTG |
| WCoR14 | 60 | CTTCTTCTTCCGTGCTGCTC | TCGCAAAGAACAAAACACCA |
| CBF1 | 60 | CAGCCTTAACAAGACGCACA | CCGTTTGCTAGCTTTTGAGC |
| CBF2 | 60 | GTGTGGCCAGAGGAGGAGTA | CGAGCCAGATCCTGGAGTAA |
| CBF3 | 55 | CGCGCTCTGGAGCTACTACT | GGGAAGACGACAAGAACAGC |
| CBF3 | 59 | CCTGGAGTTGGATGCGTACT | GGGTTGGCTGTCAAGCTTAT |

**Table 2:** The list of primers for cold tolerance genes used in this study.

| Gene name | CP 85/1491 (cold tolerant) | CP 77/400 (Cold susceptible) | SPSG 394 (Cold tolerant) | *Saccharum spontaneum* L. | Band size |
|---|---|---|---|---|---|
| *CBF1* | 1 | 0 | 0 | 1 | 1100bp |
| *CBF2* | 1 | 0 | 1 | 1 | 1100bp |
| *CBF3 (DNA)* | 1 | 1 | 0 | 0 | 1100bp |
| *CBF3 (RNA)* | 1 | 0 | 1 | 1 | 200bp |
| *C17* | 1 | 1 | 1 | 1 | 350bp |
| *85 KDA* | 1 | 1 | 1 | 1 | 200bp |
| *COR 47* | 1 | 1 | 1 | 1 | 150bp |
| *WCS 120* | 1 | 1 | 1 | 1 | 150bp |
| *WCS 66* | 1 | 1 | 1 | 1 | 130bp |
| *WCOR 726* | 1 | 1 | 1 | 1 | 130bp |
| *COR 6.6* | 1 | 1 | 1 | 1 | 900bp |
| *KIN 2* | 1 | 0 | 1 | 1 | 500bp |
| *COR 15a* | 1 | 1 | 1 | 1 | 400bp |
| *COR 15B* | 1 | 1 | 1 | 1 | 1000bp |
| *WCOR 410* | 1 | 1 | 1 | 1 | 400bp |
| *COR 78 (DNA)* | 0 | 1 | 1 | 0 | 2350bp |
| *WCOR 615* | 1 | 1 | 1 | 1 | 450bp |
| *COR 410* | 1 | 1 | 1 | 1 | 400bp |
| *WCS 120* | 1 | 1 | 1 | 1 | 200bp |
| *WCOR 80* | 1 | 1 | 1 | 1 | 480bp |
| *WCOR 14(DNA)* | 1 | 1 | 1 | 1 | 597bp |
| *WCOR14 (RNA)* | 1 | 1 | 1 | 1 | 520bp |
| *COR 78 (RNA)* | 0 | 1 | 1 | 0 | 2350bp |
| Total Genes | 21 | 19 | 21 | 20 | - |

**Table 3:** The detail of selected 23 cold tolerance genes (COR) in selected sugarcane cultivars and wild relative *Saccharum spontaneum* L value 1 shown presence while 0 shown absences

For further investigation, the amplified COR genes were selected for sequencing based on presence of COR genes in the genome of investigated sugarcane cultivars and wild relative *S. spontaneum* L. Furthermore, the successful sequences of COR genes in selected sugarcane cultivars and *S. spontaneum* L. were further analysed by using NCBI Protein BLAST (Basic Local Alignment Sequence tool) for comparison of already available data. Further, analysis of the successful sequences of COR genes showed that these COR genes encode cold and other abiotic stress responsive protein (Table 4).

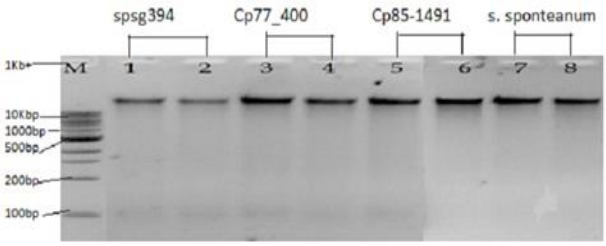


**Figure 1:** Genomic DNA extracted from sugarcane cultivars and *Saccharum spontaneum* L. lanes. 1 and SPSG-394, lanes: 3 and 4 CP-851491, lanes: 5 and 6 CP-77400 and lanes: 7 and 8 *S. spontaneum* L. M represents DNA ladder mix marker (Fermentas; cat# SM0331), respectively.

| Gene name | Sample name | Protein name | Function of protein |
|---|---|---|---|
| *CBF1* | SPSG-394 | DRE | This protein involved in different abiotic stresses |
| | CP-851491 | | |
| | *Saccharum spontaneum* L | | |
| *CBF2* | SPSG-394 | RIRE2 | This protein helps in cell function |
| | CP-851491 | | |
| | *Saccharum spontaneum* L | | |
| *CBF3* | SPSG-394 | DRE | This protein involved in different abiotic stresses |
| | CP-851491 | | |
| | *Saccharum spontaneum* L | | |
| *WCOR14* | SPSG-394 | COR 14 | This protein involved in cold stress |
| | CP-77400 | | |
| | CP-851491 | | |
| | *Saccharum spontaneum* L | | |
| *COR78* | SPSG-394 | GTPs | Small GTP binding protein regulate the function of other protein |
| | CP-851491 | | |
| | *Saccharum spontaneum* L | | |
| *COR6.6* | SPSG-394 | KIN2 | Cold responsive protein |
| | CP-851491 | | |
| | *Saccharum spontaneum* L | | |
| *WCS66* | SPSG-394 | Aspartate Proteases | These are large class of plant enzyme help in hydrolysis of peptide bond |
| | CP-851491 | | |
| | *Saccharum spontaneum* L | | |
| *COR47* | SPSG-394 | hypothetical protein | This protein help in Calvin cycle catalyzing the carbon fixation and also help in plant metabolism |
| | CP-851491 | | |
| | *Saccharum spontaneum* L | | |
| *85KDA* | SPSG-394 | Cold shock protein CS66-like | This protein is involved in cold stress |
| | CP-851491 | | |
| | *Saccharum spontaneum* L | | |
| *C17* | SPSG-394 | Phosphoprotein ECPP44-like | This protein help in Phosphorylation activity |
| | CP-851491 | | |
| | *Saccharum spontaneum* L | | |

**Table 4:** The detail of analysed sequences of COR genes in selected sugarcane cultivars and *Saccharum spontaneum* L.

### Sequence Analysis

The sequences of the selected cold tolerance genes (COR) were also analysed using NCBI Conserved Domain search (CDS). Three significant conserved domains were detected, during conserved domain analysis of CBF2, WCOR14 and WCS66 genes retrieved from SPSG-394, CP-851491 and *S. spontaneum* L. Conserved domain (Gypsy type transposon) was detected during the analysis of CBF2 gene sequence. These families of plants genes revolved close association with gypsy type transposon and have an important role in the regulation of genes. The conserved domain (Zinc binding region) was detected in COR47 gene sequences, and this domain also called zinc finger acts as small protein motif and characterized as binding region for various molecules and also helps in order to stabilize the protein folding. Furthermore, conserved domain (aspartate proteases) detected by WCS66 gene sequence proteases (peptidases or proteinases) are a large category of enzymes that catalyze the hydrolysis of peptide bond. The current research work was conducted for the first time in the Khyber Pakhtunkhwa region of Pakistan for the identification of cold tolerant commercially grown sugarcane cultivars. The PCR based analysis of cold tolerance genes in selected sugarcane cultivars and wild relative *S. spontaneum* L. showed positive and significant conclusion and results. Sugarcane plant during cold stress reprogram their gene expression through transcriptional, translational and posttranslational mechanism, CBF transcriptional factors and other cold responsive genes plays crucial role in cold stress. Based on results of cold tolerance genes in selected sugarcane cultivars and wild relative *S. spontaneum* L. these cold responsive genes showed 100% amplification, while among the investigated sugarcane cultivars SPSG-394 and CP-851491 showed significant results most of the cold responsive genes were amplified in their genome.

## Discussion

The response of plants to cold stress is a complex process involved many physiological and biochemical modifications. The expression of genes and protein metabolites in the response to cold stress has been reported [16]. In this study transcription factors, namely CBF1, CBF2 and CBF3 were investigated in selected sugarcane cultivars and wild relative *S. spontaneum* L. through PCR and further confirmed by sequencing. Moreover, CBF1 gene was observed in cold tolerant cultivar CP-851491 and wild relative *S. spontaneum* L. further, CBF2 gene was optimized in sugarcane cultivars CP-851491, SPSG-394 and wild relative *S. spontaneum* L. while the CBF3 gene was optimized in all sugarcane cultivars and wild relative *S. spontaneum* L (Table 3). In this study, WCOR14 gene was optimized in all selected sugarcane cultivars and wild relative *S. spontaneum* L. while the sequence result of WCOR14 showed that this gene encodes COR14 protein (Table 4).

Further, cold responsive gens example includes COR15a and COR15b, are expressed during cold [8]. In this study COR15a and COR15b were amplified in all sugarcane cultivars and *S. spontaneum* L. Moreover, wheat cold tolerance genes WCOR 726, WCOR80,

WCOR410, WCS 120, and WCS66 that contributes to freezing tolerance [11]. In this study, WCS66, WCS120, WCOR726, WCOR 615 and WCOR80 were amplified in all sugarcane cultivars and wild relative *S. spontaneum* L. while the WCS66 gene sequence analysis showed conserved domain (aspartate proteases) during NCBI CDS search. The cold responsive genes C17, 85KDA, COR410, COR 6.6, COR 47 and COR 78/RD29 are expressed during cold, dehydration or ABA stress. The expression of these genes has shown to be critical for both cold acclimation and chilling tolerance in plants [10]. In this study the C17 and 85KDA genes were amplified in all sugarcane cultivars and wild relative *S. spontaneum* L. and encode proteins like cold shock protein CS66-like and Phosphoprotein ECPP44-like, these protein help in cold temperature. While COR47 encodes protein, hypothetical protein, which help in Calvin cycle catalyzing the carbon fixation and also help in plant metabolism, and COR78 encode small GTPs protein which helps in regulating the function of other protein, while COR6.6 encode KIN2 which helps in cold temperature and other abiotic stresses.

The current study was conducted to investigate the cold responsive genes in Pakistani sugarcane commercially grown cultivars. Our results revolved that many genes are involved in sugarcane during cold stress. Although in this study the genes were confirmed by PCR and DNA sequencing, moreover conserved motifs were also studied. We recommend more study on large scale using transcriptomic approaches could be more significant for the sugarcane industry of Pakistan.

## Competing Interest

The authors declare that there is no conflict of interest.

## Author Contributions

All authors contributed equally to this study and manuscript.

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