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Presence of seventeen genes potentially involved in cold tolerance in sugarcane and *Saccharum spontaneum* genotypes

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Abstract

Cold is one of the most important stress which effect the growth and productivity of plants. Various pathways and mechanisms are affected by cold stress in plants. In the present work the Genomic DNA of three sugarcane genotypes and another from the wild species *Saccharum spontaneum* were tested for the presence of 17 candidate's genes/miRNA involved in cold stress tolerance. Among these genotypes two sugarcane cultivars are cold tolerant, namely CP 85_1491 and SPSG 394, while the CP 77_400 is cold susceptible. Presence of these 17 gene/miRNA was confirmed by PCR and gel electrophoresis. In the genomic DNA of cultivar SPSG 394 all genes were amplified while the CP 85_1491 and *S. spontaneum* showed amplification for 96% of the genes/miRNAs and low results of these genes were studied in Genotype CP77_400. Therefore we concluded that cultivar SPSG 394 are more cold tolerant.

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Introduction

Low temperature or cold stress, chilling (low temperature above 0C) and freezing (below 0C) is one of the major abiotic stress which reduce and limits the growth, productivity and geographical distribution of agriculture crops and huge loss of crops (Xin and Browse, 2000). Although the freezing and chilling temperature have differ from each other chilling temperature effect the cell while freezing temperature stop the cellular activity by dehydration (Pearce, 1999). Formation of ice crystal in the intracellular spaces can Couse the disruption of physical structure of cell and tissue while dehydration can Couse the injury which effect the membrane structure and function (Uemura *et al.,* 1995).Cold stress or low temperature change the phytohormone content and activate the cold acclimation signal pathways although the cross-talk between signaling pathways proceeds is still unclear (Xia *et al.*, 2009). In the senescence of plant organs cytokinins (CKs) have profound effect (Mikkelsen *et al.,* 2004) in response to environmental stimuli the CKs participate in the morphogenesis and metabolism modulation (Sakakibara, 2006) at very extreme cold temperature 0C to-13C some herbaceous plants from temperate region can survive while plants from tropical and subtropical regions have little or no capacity to survive in cold stress, plants have very significantly ability to survive in freezing and chilling stress (Thomashow, 2001).

Modern sugarcane cultivars (Saccharum hybrid L) is economically important commercial crops and provide 40% of renewable source of energy biofuel ethanol (Lam *et al.,* 2009). The cultivars sugarcane are widely growing in all parts of globe approximately 120 countries. And are mostly consider as a cold sensitive plants (Tai and Lentini, 1998). Cold stress (freezing and chilling) temperature damaged the plants, the magnitude of damaging on duration and severity of cold stress, the cultivars resistance to post freezing and the time gap and temperature variation between the harvesting and freeze events (Tai and Lentini, 1998). Although some field experiments shown that sensitivity to cold stress of same cultivars of sugarcane have varies among verities Du *et al.* (1999) some of the subtropical sugarcane cultivars are more cold tolerant then tropical hybrids, therefore in cold stress a view of transcriptome dynamics and identification of cold responsive genes family and pathways could be important aspect in breeding program to identifies cultivars which are more cold tolerant and the related stresses (Que *et al.,* 2014). Yung plants and ratoons re-growth can be killed by cold damage of mature plants about 35^{o}C temperature are optimum for sugarcane growth while the temperature below 20^{o}C largely limit the yield and growth (Moore, 1987).

Significant studies have been directed to identify the nature of cold responsive genes and the pathway of these cold responsive genes in cold stress. Cold regulated miRNA in different plants species were reported like sugarcane miR319, miR393 (Zhang *et al.*, 2009; Thiebaut *et al.*, 2012; Wang *et al.*, 2014). Moreover some previous reported studies directed that a set of genes is more reliable than a single reference gene used for normalization (Vandesompele *et al.,* 2002; Janská *et al.,* 2013). On the base of above analysis gene set of miR171/miR5059 and miR171/18s rRNA were shown as the best miRNA candidates genes express in normalization during cold stress in buds of sugarcane cultivars ROC22 and FN39, followed by miR171 and 18s rRNA. Gene miR171 belong to family of conserved miRNA, miR171 gene belong to family of conserved miRNA, which regulating the members of the transcription factor family SCARECROW-LIKE (SCL). These SCL genes are the members of GRAS family these genes take part Gibberellic Acid in response of stimulating and regulate development of flowering and apical meristem (Bolle, 2004; Lee *et al.,* 2008). Recently Research has demonstrated that miR171 is play a key role in the meristem differentiation in many plants (Engstrom *et al.*, 2011; Curaba *et al.,* 2013). As the buds are one of the most important axillary meristems in sugarcane, it's reasonable to deduce that the expression of miR171 should be relatively continuous and stable to promote the differentiation of the buds, miRNA expression analysis in sugarcane

under cold stress, miR171/18S rRNA, and miR171/miR5059 were the best reference genes for multiple use, and the miR171 and 18S rRNA were the best single reference genes.

The aim of present research work are to identify the cold resistant sugarcane cultivars of KP region of Pakistan, by studying the miRNAs Cold Responsive genes in the genomic DNA of selected sugarcane cultivars. These research will be useful for studies on miRNA function in response to cold stress, and other abiotic stresses in sugarcane.

Material and methods

Sample collection

For present study three sugarcane genotypes and *Saccharum spontaneum* were selected. The sugarcane genotypes were collected during maturing stage from Sugarcane Crop Research institute Mardan KP Pakistan. Then the cultivars were harvested in Research field of genetic Department Hazara University Mansehra KP Pakistan. The name of varieties are shown in table 1. Before DNA extraction disease free green and fresh leaves were collected. The leaves were quickly stored at -80C.

DNA Extraction

Genomic DNA were extracted from the fresh leaves of sugarcane and *Saccharum spontaneum* by modified CTAB method (Dolye & Dolye 1990).

Take O.5 gm. Leaves tissue and was crushed in liquid nitrogen with help of mortar and festal. Then the grinded tissue were transfer into fresh Eppendrof tube. Added 900µl 2% CTAB Buffer (CTAB 2gm, EDTA 1.6 gm., PVP 2.4 gm. Tris 2.4gm. Nacl 16.4 gm. dH2O 100 ml. 200ul of beta marcepto ethanol) to each tube containing tissue. The samples were then incubate for 24 hours at 56°C in incubator after incubation 200 µl of phenol Chloroform: Iso Amyl Alcohol (24:1) were added to each sample. The samples were centrifuge at 10,000rpm for 20 mints. After centrifugation the supernatant was transfer to fresh Eppendorf tube and added 500 µl of cold isopropanol to each tube. Then centrifuge the samples for 15 mints at 8000 rpm. After centrifugation the supernatant were discorded and the pellet was washed with 70% ethanol. Centrifuge the samples at 8000 rpm for 5 mints. After centrifugation the ethanol were discorded and the samples were kept at room temperature for overnight. After drying 60 µl of dd H2O were added to each tube.

Gel electrophoresis of Extracted DNA

The quality of extracted DNA were checked by 1% agarose gel. The preparation of agarose gel are given below.

98ml of DH_2O was taken in conical flask and 2ml of 50xTAE were added and 1gm of agarose powder were added to the solution, the solution were shacked properly and heated for several minutes in oven, after heating the solution , the flask were kept at room temperature for few minutes, when the solution was slightly cold 25ul of ethidium bromide were added to the solution and transfer into gel plate where combs were fixed, the gel plate were kept at room temperature for solidification, the combs were removed and the gel were transfer into gel tank and 7ul of extracted DNA with 3ul DNA loading dye were added in each well and 1kb DNA marker was also loaded where 80Volt current were passing from electrolytic solution cantoning in gel tank, after 35 minutes the gel were examined in UV apparatus and electronic gel picture was captured as shown follow.

Primer Designing and selection

Literature were studied and Primer were design and selected from a review article Yang *et al.,* (2016) the primer with sequence and name are shown in table. 2.

Polymerase Chain Reaction of Cold tolerance genes

The amplification of seventeen reference genes in the genomic DNA of Saccharum genotypes, thermo scientific PCR kit Catalog #EP0402 were used.

The PCR mixture consist of 3ul of 10xbuffer, 3ul of $MgCl_2$, 3ul of DNTPS, 2ul of primers 1ul of each primer forward and reverse respectively, *Taq*-polymerase 0.5ul, template DNA 2ul and PCR water

11.5ul while PCR condition are initial denaturation 94°C for 5mints, cycling denaturation 94°C for 40sec annealing 55°C for 40sec and termination 72°C for 40sec and final elongation 72°C for 10mints while 40 cycle were given.

Agarose Gel electrophoresis of PCR amplified product

The PCR amplified product were further confirmed by 1.5% gel and the band size was compared with 1Kb DNA marker. The procedure of Agarose gel preparation are as follow.

Take 1.5gram of Agarose and dissolved in 98ml of dH_2O and added 2ml of 50xTAE buffer and the solution was heated for few minutes and then kept at room temperature, when the solution become cold then 25ul of Ethidium bromide was added and solution were transfer into gel plate. Then all PCR amplified product were loaded with 4ul loading dye after 30 mints the gel was checked in UV apparatus and photograph was taken. As shown in fig. 2.

Data analysis

The reference genes were further confirmed in Genomic DNA of Sugarcane and Saccharum spontaneum genotypes by Gel electrophoresis. Name of genes and product size were shown in the following tables 3. The table.4. Shown the total number of miRNAs in each genotype.

Result

Confirmation of genes by Agarose gel

The PCR amplified product of 17 reference genes were confirm on 1.5% Agarose gel. The fig. 2. Shown, from sample 1 to sample 4 are miR159 gene, sample 5 to sample 8 are miR160 gene, sample 9 to sample 12 are miR167 gene, sample 13 to 16 are miR171 gene, sample 17 to sample 20 miR398 gene, sample 21 to sample 24 are miR1520 gene, sample 25 to sample 28 are miR5059 gene, sample 29 to sample 32 are miR5072 gene, sample 33 to sample 36 are mir5655 gene, sample 37 to sample 40 are eEf_1a gene, sample 41 to sample 44 are miR156 gene while from sample 45 to sample 48 are miR396 gene. The Gene miR159 optimize in CP85_1491, CP77_400 and SPSG 394 while no band are optimize in *S. Spontaneum,* while the Band size is 150bp, miR160 optimize in all samples and produced band of 500bp size, miR167 gene optimize in CP 85_1491 and SPSG 394 and formed 500bp size band, while no band are optimize in CP 77_400 and S. Spontaneum, miR171 gene optimize in all genotypes and produced 150bp size of band, miR398 gene are optimize in all genotypes and produced 150bp size of band, miR1520 gene are optimize in all samples and produced 150 bp size of band, miR5059 gene are optimize CP 85_1491, CP77_400 and SPSG 394 band size is 200bp.

Table 1. Shown the sugarcane genotypes which were studied in this work.

Serial no	Genotype Name
1	CP 85_1491
2	CP77_400
3	SPSG 394
4	*Saccharum spontaneum*

While in *Saccharum spontaneum* the band size is 150bp, miR5072 optimize only in CP85_1491 and band size is 200bp while in *Saccharum spontaneum* shown band of 150bp, miR5655 optimize in CP85_1491, CP77_400 and SPSG394 while no band are shown in *S. Spontaneum* band size are 300bp.

The eEF_1a gene optimize in CP85_1491, CP77_400 and SPSG394 while no band are shown in S. Spontaneum band size are 300bp, miR156 are optimize in optimize in CP85_1491, CP77_400 and SPSG394 while no band are shown in S. Spontaneum band size are 150bp, miR 396 gene are optimize in all genotypes and band size are 150bp.

The 5s_rRNA gene are optimize in CP 77_400, SPSG 394 and *S. Spontaneum* while no band are shown in CP85_1491. Similarly the gene 18s_rRNA are optimize in SPSG 394 and Saccharum Spontaneum while no bands are shown in CP 85_1491 and CP77_400. The bands size is 200bp. The U6_snRNA shown band in CP 77_400, SPSG 394 and *Saccharum spontaneum*. While no band are optimize in CP

85_1491, and 25s_rRNA shown band in all sample. The band size of U6_snRNA are 200bp and 25s_rRNA are also 200bp. The GAPDH gene are optimize in all genotypes and band size are 230bp. As shown in fig. 3 and 4. In table. 3. The product size and optimization of reference genes in each sugarcane genotypes and Saccharum spontaneum were shown.

Table 2. Primer sequences and amplicon characteristics for each of the 17 candidate reference genes. (Yang *et al.,* 2016).

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
miR159	AGCGGTTTGGATTGAAGGGA	GTGCAGGGTCCGAGGT
miR160	ATTATGCGTGCAAGGAGCCA	GTGCAGGGTCCGAGGT
miR167	ATCGTGAAGCTGCCAGCATG	GTGCAGGGTCCGAGGT
miR171	ATACGTGATTGAGCCGTGCC	GTGCAGGGTCCGAGGT
miR398	ATTCGCCGCAGGTGATGAGA	GTGCAGGGTCCGAGGT
miR1520	CGGCGGATCAGAACTGGTAC	GTGCAGGGTCCGAGGT
miR5059	ATCATCGTTCCTGGGCAGCA	GTGCAGGGTCCGAGGT
miR5072	ATATGAGTTCCCCAGCGGAG	GTGCAGGGTCCGAGGT
miR5655	CGGCAACACATGTGGATTGAGA	GTGCAGGGTCCGAGGT
5S rRNA	GCGTAGAGGAACCACACCAATC	CGAGCTATTTTGCCGCAGG
18S rRNA	CTACGTCCCTGCCCTTTGTACA	ACACTTCACCGGACCATTCAA
GAPDH	CACGGCCACTGGAAGCA	TCCTCAGGGTTCCTGATGCC
eEF-1α	TTTCACACTTGGAGTGAAGCAGAT	GACTTCCTTCACAATCTCATCATAA
miR156	AGGCGCCTGACAGAAGAGAGT	GTGCAGGGTCCGAGGT
miR396	ACCTGCGGTCAAGAAAGCTGT	GTGCAGGGTCCGAGGT
U6 snRNA	ACAGAGAAGATTAGCATGGCCC	GACCATTTCTCGATTTATGCGTG
25S rRNA	GCAGCCAAGCGTTCATAGC	CCTATGGTGGGTGAACAATCC

Analysis

The total number of seventeen reference gene in the Genomic DNA of selected sugarcane and Saccharum spontaneum Genotypes are shown in the following table 4. While table. 3. Shown the optimization and product size of each gene in each Saccharum genotypes.

The cultivar SPSG 394 shown high optimization rate of 17 reference genes. These genes were confirmed by PCR and Agarose gel electrophoresis. Total of 17 reference genes (miRNAs) were optimized in SPSG 394 shown 100% result. While the remaining genotypes have low optimization of reference genes, like CP 85_1491 have 15 genes optimize, CP 77_400 have 11 and Saccharum spontaneum have 15 genes optimized as shown in fig.5, so therefore the cultivar SPSG 394 are more cold tolerant among these selected sugarcane and Saccharum spontaneum genotypes, the value in table 4, for cold susceptible "0" were used while value"1" shown cold tolerant. As described above and also shown in the fig. 5.

Table 3. Table Show the result of 17 candidates Reference miRNA genes in selected genotypes. 0 for absence and 1 for present of genes.

Gene	CP 85/1491	CP 77/400	SPSG 394	Saccharum spontaneum	Band size
miR159	1	1	1	1	200bp
miR160	1	0	1	1	200bp
miR167	1	1	1	1	150bp
miR171	1	0	1	1	200bp
miR398	1	0	1	0	200bp
miR1520	1	0	1	1	200bp
miR5059	1	1	1	0	200bp
miR5072	1	1	1	1	200bp
miR5655	1	1	1	1	200bp
5S rRNA	1	0	1	1	200bp
18S rRNA	1	1	1	1	200bp
GAPDH	1	1	1	1	496bp
eEF-1α	1	1	1	1	150bp
miR156	1	1	1	1	150bp
miR396	0	1	1	1	220bp
U6 snRNA	0	1	1	1	200bp
25S rRNA	1	0	1	1	150bp

The cultivar SPSG 394 shown high optimization rate while the remaining genotypes have low optimization rate of 17 reference genes. This results will confirm by PCR and gel electrophoresis. While further confirmation will be done by expression study, sequencing of desired fragment and transcriptome sequencing.

Table 4. Shown the number of 17 reference genes in selected Saccharum spp. Genotypes.

Verities name	total genes present	gene absent	Cold susceptible	cold tolerant
CP 85_1491	15	2	1	0
CP 77_400	11	6	1	0
SPSG 394	17	0	0	1
Saccharum spontaneum	15	2	1	0

Discussion

In present work three sugarcane genotypes and a wild species *Saccharum spontaneum* were selected. In These genotypes CP85_1491 and SPSG 394 are cold tolerant while CP 77_400 are cold susceptible. These cultivars were grown in research field of genetics Department Hazara University. Fresh leaves were collected from the sugarcane and *Saccharum spontaneum* genotypes. Total genomic DNA was extracted by CTAB method (Dolye & Dolye 1990). Modern sugarcane cultivars (Saccharum hybrid L) are mostly consider as a cold sensitive plants (Tai and Lentini, 1998). Cold stress (freezing and chilling) temperature damaged the plants, the magnitude of damaging on duration and severity of cold stress, the cultivars resistance to post freezing and the time gap and temperature variation between the harvesting and freeze events (Tai and Lentini, 1998).

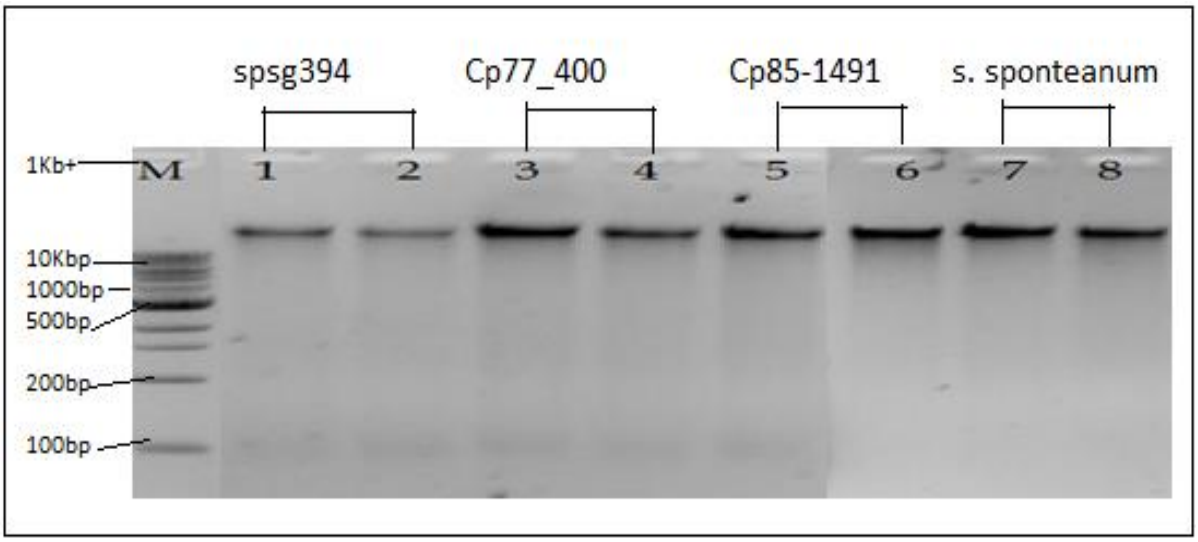

Fig. 1. Gel photograph of extracted DNA from fresh leaves of sugarcane and *Saccharum spontaneum*. Sample 1, 2 are SPSG 394, sample 3, 4, are CP77_400, sample 5, and 6 are CP85_1491 and sample 7 and 8 are *Saccharum spontaneum*.

The reference genes or cold regulated miRNA in different plants species were reported like sugarcane miR319, miR393 (Zhang *et al.,* 2009; Thiebaut *et al.,* 2012; Wang *et al.,* 2014). Were study and confirm in genomic DNA of Sugarcane and Saccharum spontaneum by PCR and gel electrophoresis. Reference genes miR171/miR5059 and miR171/18s rRNA were shown as the best miRNA candidates genes express in normalization during cold stress in buds of sugarcane cultivars ROC22 and FN39, followed by miR171 and 18s rRNA. Gene miR171 belong to family of conserved miRNA, miR171 gene

belong to family of conserved miRNA, which regulating the members of the transcription factor family SCARECROW-LIKE (SCL). These SCL genes are the members of GRAS family these genes take part Gibberellic Acid in response of stimulating and regulate development of flowering and apical meristem (Bolle, 2004; Lee *et al.,* 2008).

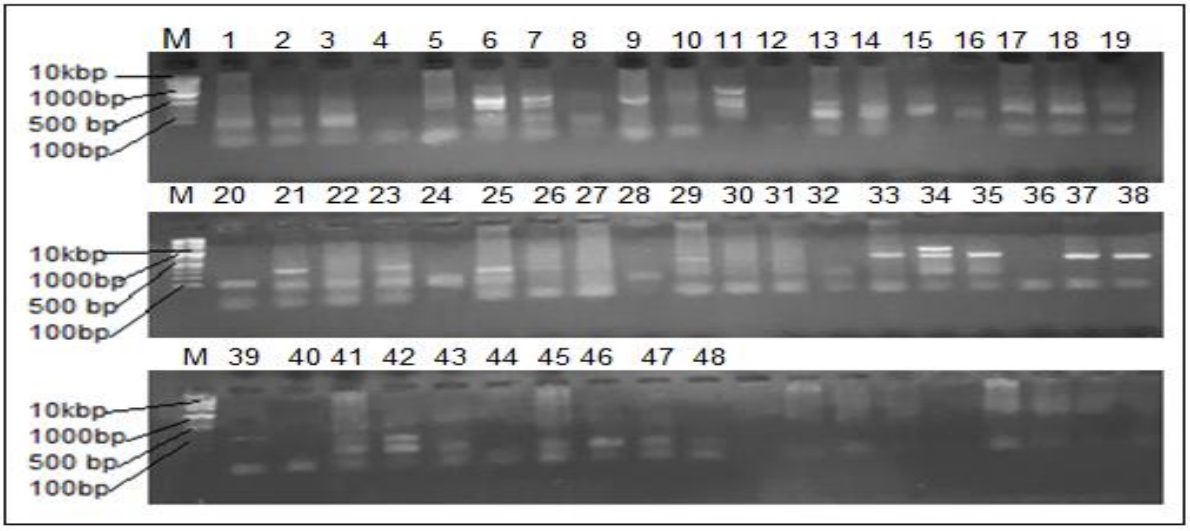

Fig. 2. Gel Photograph of PCR amplified product of 12 reference genes. the sample1, 5, 9, 13, 17, 21, 25, 29, 33, 37, 41 and 45 are CP85/1491 sample 3, 6, 10, 14, 18, 22, 26, 30, 34, 38, 42, 46 are CP77/400 sample 3, 7, 11, 15, 19, 23, 31, 35, 39, 43, 47 are SPSG 394 while sample, 4, 8, 12, 16, 20, 24, 32, 36, 40, 44, 48 are *saccharum spontaneum*.

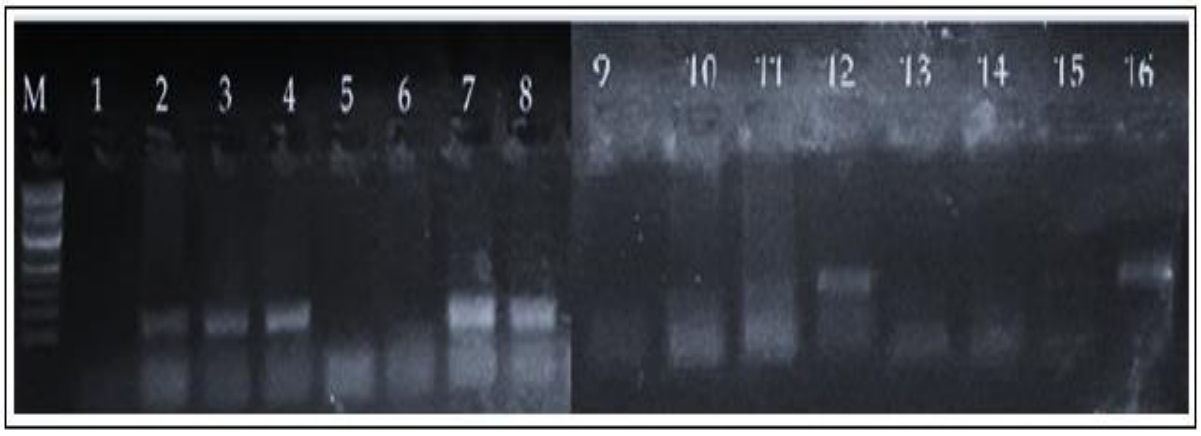

Fig. 3. The Agarose gel photographs shown PCR amplified product of 5s_rRNA, 18s_rRNA, U6_snRNA and 25s_rRNA, From sample 1 to sample 4 are 5s_rRNA and from sample 5 to sample 8 are 18s_rRNA From sample 9 to 12 are U6_snRNA and from sample 13 to 16 are 25s_rRNA Sample 1, 5, 9, 13 are CP85_1491, sample 2, 6, 10, 14 are CP 77_400, sample 3, 7, 11, 15 are SPSG 394 while sample 4, 8, 12, 16 are *S. Spontaneum*.

The genes optimization were confirm from gel photograph of the PCR amplified product. In Cultivar SPSG 394 all seventeen reference genes were optimize which shown that these sugarcane cultivars are cold tolerant and shown 100%result. The CP 85/1491 PCR result shown 98% and two genes namely miR5655 and 18s_RNA are not optimize. While in CP 77/400 12 reference genes were optimized In PCR study five genes namely miR160, miR1520, miR5059, miR5072 and 5s_rRNA are not optimize and shown 85% result. In Saccharum spontaneum 15 genes were optimized during PCR study although two gene were not optimize in the genomic DNA of *S. Spontaneum* namely miR398 and

miR5059. *Saccharum spontaneum* shown 98 % result of optimization of reference. Saccharum are wild species and they have the ability to resist cold stress. Moreover some previous reported studies directed that a set of genes is more reliable than a single reference gene used for normalization (Vandesompele *et al.,* 2002; Janská *et al.,* 2013). Although various studies has been shown that these reference genes are working in normalization during cold temperature. In selected sugarcane genotypes the SPSG 394 shown 100% result. All genes are optimized in this cultivars.

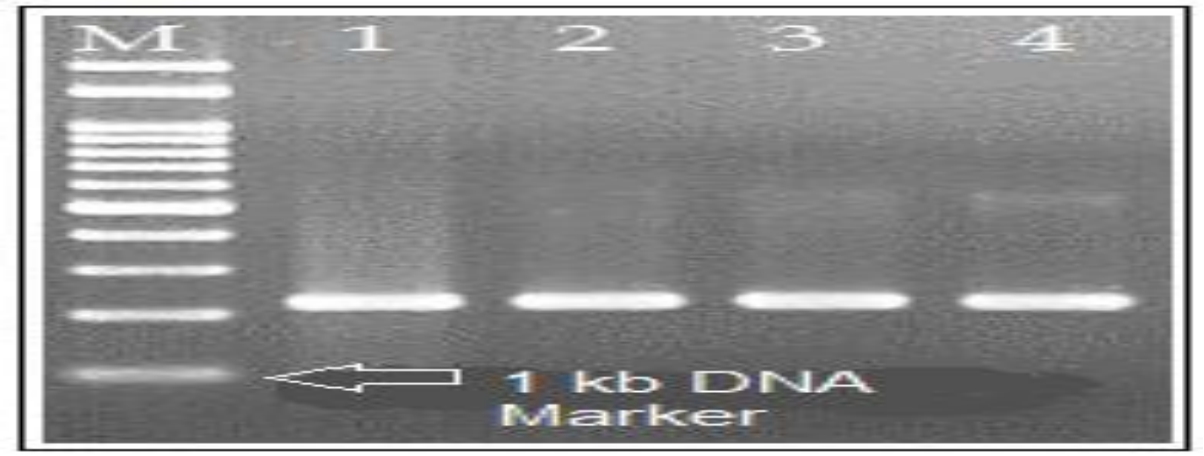

Fig. 4. Agarose gel photograph of PCR amplified product of GAPDH Sample 1 are CP85_1491, sample 2 are CP77_400, sample 3 are SPSG 394 and sample 4 are *Saccharum spontaneum*.

Identification of cold responsive genes and the cold signaling pathways are the most important resources for identification of cold tolerance cultivars of sugarcane in breeding program and to identify the genetic difference among cultivars and to obtain transgenic genotype with cold tolerance and its related stress.

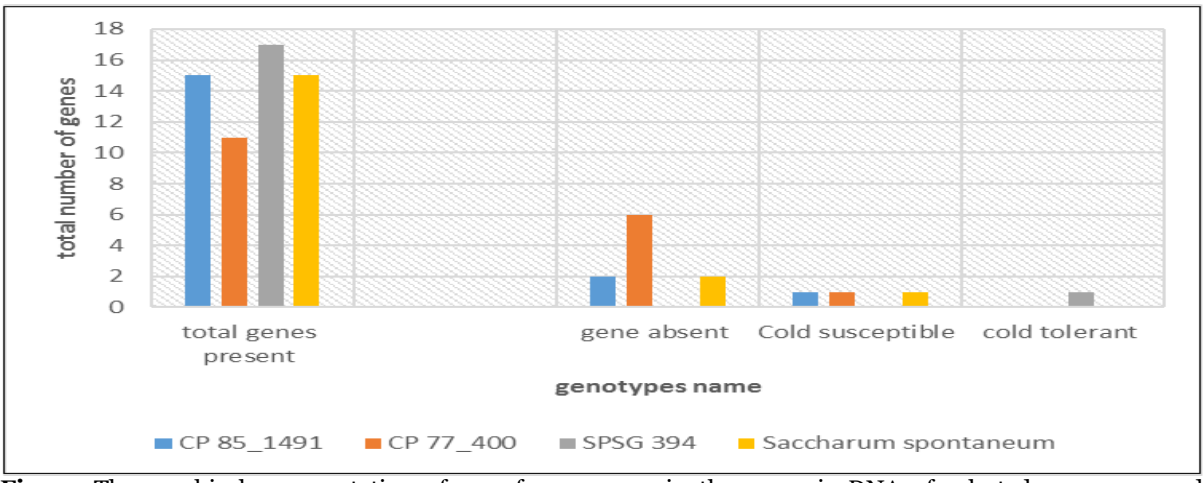

Fig. 5. The graphical representation of 17 reference gens in the genomic DNA of selected sugarcane and Saccharum spontaneum genotypes.

Conclusion

In this study we only study the miRNAs in the genomic DNA of selected sugarcane and *Saccharum spontaneum*. Although this is PCR based study and shown that these reference genes are working in cold stress. We concluded from this experiment that SPSG 394 are more cold tolerant then the other two selected sugarcane genotypes. This cultivar has the ability to resistant to cold stress.

The cultivar CP 77_400 are most harvested cultivars in KP region of Pakistan, but susceptible to cold stress. If modern breeding techniques were used to produce a good cultivar from these two sugarcane cultivars SPSG 394 and CP 77_400. Furthermore work are needed like transcriptome sequencing and expression study of these genes in the selected sugarcane genotypes. It can lead a better understand of tolerant cultivars.

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