EVALUATING GENETIC DIVERSITY OF CHILLING STRESS IN COTTON GENOTYPES

ABSTRACT

In order to study genetic diversity and some physiological features related to chilling stress using molecular markers, an experiment was conducted at University of Mohaghegh Ardabili. Treatments were set in a factorial experiment based on randomized complete block design with 3 replications and 3 stress levels (25, 15 and 5°C) between 20 cotton genotypes. The results showed that chilling stress influenced on some physiological features such as the activity of catalase, proline content, soluble carbohydrates and proteins. Cluster analysis carried out using WARD method in physiological features showed that genotypes located in three groups in the acclimation level and after acclimation, respectively. Nazilli, Ciakra, Avangard and B-557 were in the better group in studied levels. Also based on the results Avangard, Chegurava, Tashkand and Shirpan 603 were the most tolerant genotypes. In the ISSR marker analysis using of 12 primers produced 96 polymorphic bands. The mean of PIC, MI and EMR were 0.283, 1.065 and 3 respectively, for all primers. Some of markers had promising results that confirmed ISSR markers as powerful tool in any marker assisted program for plant breeders.

Key words: chilling, cotton, ISSR marker

INTRODUCTION

Cotton cultivars (diploid and tetraploid cultivars) have sown in 17 out of 30 provinces in Iran. Based on report published by Agriculture ministry (Iran cotton report to 68th plenary meeting of ICAO, Cape Town, South Africa, 2009). Cotton (*Gossypium hirsutum* L.) is sensitive to chilling condi-
tions during germination and establishment (Lauterbach et al., 1999; Bolek, 2010). The chilling effect is exhibited by physiological agitation, a phenomenon known as chilling injury (Yan et al., 2010). Following this process many biochemical and physiological changes occurred including: increase levels of carbohydrates, antioxidant enzymes, proline, gene expression and membrane lipid composition changes (Xin and browse, 2000), the appearance of new isoforms of protein (Heidarvand and Maali amiri, 2010) that all of which lead to cold acclimation. Inter Simple Sequence Repeat or ISSR (Zietkiewicz et al. 1994) markers are more and more in demand, because they are known to be abundant, very reproducible, highly polymorphic, highly informative and quick to use (Zietkiewicz et al. 1994, Bornet and Branchard 2001, Sofalian et al. 2009). ISSR uses the presence throughout the genome of Simple Sequence Repeats (SSR) which is ubiquitous, abundant and highly polymorphic tandem repeat motifs composed of 2 to 5 nucleotides (Sofalian et al., 2009). Liu and Wendel (2001) declared ISSR as an easy and efficient genetic marker system in cotton for display both inter and intraspecific variations.

The present study focused on the characterization of genetic diversity among cotton genotypes, belonging to G. hirsutum using ISSR DNA markers and its relationship to physiological features influenced chilling tolerance.

MATERIALS AND METHODS

Experiment was conducted during the summer season of 2012 at the research greenhouse of the University of Mohaghegh Ardabili. Treatment were set in a factorial experiment based on randomize complete block design (RCBD) with 3 replication per treatment. The seeds of 20 genotypes (Table 1) belonging to the Gossypium hirsutum species were used in this study. Uniformly germinated seeds were conveyed to plastic pot in the greenhouse under a controlled environment at 25±1°C and 16 h light/8 h dark photoperiod. Then, plants at 4 and 5 leaf stages of each genotype were divided into two groups. One group of each species was maintained in the same chamber with 16 h light / 8 h dark photoperiod and at 25±1°C as the control. The other group of each genotype was transferred in a chamber for cold acclimation with: 16 h light/8 h dark photoperiod and 15°C for 1 day as well as 5°C for 1 day. 48 and 72 h after treatment, seedlings were randomly taken from chilling-treated seedlings of each genotype (Rapacz, 2002). Leaves were ground with mortar and pestle using liquid nitrogen and kept at -70°C for further analyses.
Proline content was measured using the acid ninhydrin by method of (Bates et al., 1973). In order to measure the total soluble proteins from leaves utilized Guy et al. (1992) procedure. Leaves were homogenized in 50 mM Tris-HCL, pH 7.5; 0.04 % (v/v) 2-mercaptoethanol and 2 mM EDTA. Then centrifuged at 11500 rpm and 4°C for 21 min. supernatant stored at -20°C for analysis. Protein concentrations were assayed with bovine serum albumin (BSA) as standard protein according to the Bradford (1976) method. Soluble sugar content was analyzed using the enthrone method described by Irigoyen et al. (1992). Absorbency of the resulting solution was read at 625 nm and a calibration curve with D-glucose was performing as a standard. The activity of catalase as well as poly phenol oxidase enzymes was determined using the Tris (50 mM; pH=7) and hydrogen peroxide (3%) (Kar and Mishra, 1976). Electrolyte leakage was estimated as reported by Fry et al., (1991).

**DNA extraction, PCR methods and gel electrophoresis**

Genomic DNA was extracted from young leaf tissue using the CTAB extraction method according to Saghai-maroof et al., (1984) with some minor modifications. Quantity and quality of the isolated DNA was measured by spectrophotometer and visualized using 0.8% agarose gel electrophoresis. Amplification reaction was carried out in a total volume 20 μl with Tris-HCl, MgCl₂, dNTPs, Taq DNA polymerase, corresponding primer and

| Cultivar Number | Cultivar Name | Origin | Cultivar Number | Cultivar Name | Origin |
|-----------------|---------------|--------|-----------------|---------------|--------|
| 1               | Chegurava     | Turkey | 11              | Nazil‘i       | Turkey |
| 2               | Sahel         | Iran Trade | 12              | Bakhtegan     | Iran Trade |
| 3               | Belisoovar    | Turkey | 13              | Varamin 349   | Iran Trade |
| 4               | 4.S.4         | Greece | 14              | 43347         | Greece |
| 5               | 4325          | Greece | 15              | Khordad       | Iran Trade |
| 6               | Tashkand      | Uzbekistan | 16              | Opal          | America |
| 7               | No-228        | Greece | 17              | Ciakra        | Iran Trade |
| 8               | Syndose       | Greece | 18              | Avangard      | Bulgaria |
| 9               | No-200        | Greece | 19              | Oltan         | Iran Trade |
| 10              | Shirpan 603   | Bulgaria | 20              | B557          | Pakistan |
30 ng DNA templates using thermal cycler. After initial denaturation at 94°C for 5 min, 40 cycles of PCR were conducted where each cycle consisted of 30 sec denaturation at 92°C, 40 sec annealing (annealing temperature optimized for each primer according to theoretical Tm°C for hybridization, 45-54) and 1 min extension at 94°C with a final extension five min at 72°C. 12 ISSR primers were selected based on polymorphism and robustness of the bands obtained. All the generated patterns were repeated twice to verify reproducibility. Products of PCR reaction were mixed with 4 μl of 6X loading dye, separated on 1.5% agarose gel in 1X TBE buffer (Tris 0.89 M, EDTA 2 mM, Boric Acid 0.89 M, pH= 8.3), stained with ethidium bromide and visualized under U.V light.

**ISSR and physiological data analysis**

ISSR products were scored as the presence (1) or absence (0) of bands and a binary matrix was constructed. Only ISSR fragments that could be scored unambiguously were included in the analysis. Cluster analysis was performed for the molecular data based on Unweighted Pair Group Method Analysis UPGMA and using NTSYS-pc. GenALEx6.3 was used to calculate Shannon Diversity Index, Marker Index (MI) and Polymorphism Information Content (PIC) and to perform Mantel’s test. ISSR data were also subjected to genetic analysis using POPGENE 1.32 software. Physiological Data analysis was done by SPSS and MSTATC.

**RESULTS AND DISCUSSION**

Cluster analysis based on Physiological features was carried out using WARD method according to the Euclidean distance on standardized data. The genotypes were divided into three clusters at acclimation level (Figs 1, 2). Group one includes seven genotypes, with higher mean for soluble protein, catalase and poly phenol oxidase activity. Considering the features, this group can be considered as tolerant genotypes based on the investigated features under chilling stress. Second group, which includes two genotypes with higher mean for electrolyte leakage and average proline concentration. Group three includes Removed genotypes. This group has lower mean of carbohydrate, electrolyte leakage, and catalase and poly phenol oxidase activity. Also three clusters identified after acclimation level (Figs 3, 4) which among them group 1 and 3 demonstrated the highest and lowest the deviations from the total mean respectively. Group one comprised eleven genotypes with higher mean for soluble protein and middle mean for catalase and poly phenol oxidase activity. Tertiary Group includes six genotypes. This Group has higher mean of carbohydrate.
sis, Nazilli, Ciakra, Avangard and B-557 were been in the better group in studied levels.

Fig.1. Cluster analysis dendrogram of 20 cotton commercial cultivars based on physiological features at acclimation level

Fig.2. The average deviation of the mean cluster at acclimation level
Fig. 3. Cluster analysis dendrogram of 20 cotton commercial cultivars based on physiological features after acclimation level.

Fig. 4. The average deviation of the mean cluster after acclimation.

**ISSR Data Analysis**

At the beginning 34 ISSR primers were screened, of these, 12 primers produced 69 reliable bands (Fig. 5), out of which 48 were polymorphic (69.57%). The average number of polymorphic loci amplified per primer was 4. Primers ISSR-1 and ISSR-14 were polymorphic in all loci (100% polymorphic). The mean PIC value of the polymorphic loci was 0.283. These values can range from 0.0 for monomorphic markers to 0.5 for markers that are present in 50% of accessions and absent in the
other 50% (Gomes et al., 2009). The MI values ranged between 0.27 for primer ISSR-2 and 2.72 for primer ISSR-14 with average 1.065. The primers that showed higher polymorphism had higher EMR values. This trait was observed from 0.33 to 8 with a mean value of 3 per primer (Table 2).

**Table 2**

|   | Primers                 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|-------------------------|---|---|---|---|---|---|---|---|---|----|
| 1 | ISSR-1 5/ AGAC AGACGC 3/ | 3 | 3 | 100 | 0.220 | 0.66 | 3 | 0.226 | 0.383 |
|   | ISSR-2 5/ GACAGACAGACA GACA 3/ | 5 | 3 | 60 | 0.095 | 0.27 | 1.8 | 0.125 | 0.119 |
|   | ISSR-3 5/ AGAGAGAGAGAGAGC 3/ | 8 | 4 | 50 | 0.116 | 0.44 | 2 | 0.158 | 0.115 |
|   | ISSR-9 5/ TCTCTCTCTCTCTCC 3/ | 6 | 4 | 66 | 0.362 | 1.44 | 2.64 | 0.242 | 0.361 |
|   | ISSR-14 5/ CACACACACAGT 3/ | 8 | 8 | 100 | 0.349 | 2.72 | 8 | 0.353 | 0.527 |
|   | ISSR-15 5/ AGAGAGCGAGCGAGC 3/ | 6 | 3 | 50 | 0.326 | 0.96 | 1.5 | 0.165 | 0.253 |
|   | ISSR-16 5/ CACACACACAGG 3/ | 5 | 4 | 80 | 0.245 | 0.96 | 3.2 | 0.297 | 0.314 |
|   | ISSR-19 5/ AGAGAGAGAGAGAGT 3/ | 8 | 6 | 75 | 0.361 | 2.16 | 4.5 | 0.274 | 0.412 |
|   | ISSR-22 5/ ATGATGATGATGATGATG 3/ | 3 | 1 | 33 | 0.355 | 0.37 | 0.33 | 0.2578 | 0.187 |
|   | ISSR-24 5/ GACAGACAGACAGACA 3/ | 6 | 5 | 83 | 0.339 | 1.65 | 4.15 | 0.284 | 0.426 |
|   | ISSR-31 5/ GAGGAGGAGGC 3/ | 6 | 5 | 83 | 0.294 | 1.45 | 4.15 | 0.247 | 0.387 |
|   | ISSR-32 5/ AGAGAGAGAGAGAGAC 3/ | 5 | 2 | 40 | 0.331 | 0.66 | 0.8 | 0.132 | 0.119 |
| Mean | | 6 | 4 | 69.57 | 0.283 | 1.065 | 3 | 0.231 | 0.306 |

1 - Primers
2 - Primer sequences
3 - Number of amplified bands
4 - Number of polymorphic bands
5 - Polymorphic / amplified bands [%]
6 - PIC
7 - MI
8 - EMR
9 - Nei’s gene diversity
10 - Shannon information index
Estimation of genetic diversity

Nei’s gene diversity index (Nei, 1973) ranged from 0.125 for primer 2 to 0.353 for primer 14, with an average 0.25 and the Shannon information index observed between 0.115 for primer 3 to 0.527 for primer 14 with an average 0.30. In this regards, Sheidai et al., (2010) reported the average 0.22 and 0.34 by the two indices, respectively among thirteen cultivars of G. hirsutum using RAPD markers.

Cluster analysis based on molecular data was performed to develop a dendrogram by unweighted pair group method with arithmetic mean
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(UPGMA) according to Jaccard’s similarity coefficients (Fig. 6). The co-
phenetic correlation was high ($r=0.94$, $\alpha=1\%$) indicating a very good fit of
the cluster analysis performed. Therefore, the 20 genotypes were divided
into four groups. Group one includes two genotypes (4: S: 4, Varamin 349,
No-200, Avangard, Shirpan 603, Cindose, Beliisovar, Khordad, Opal, 4325,
Sahel, 43347). Second Group includes (‘Chegurava 15:18, Ciaca, ‘No-
223). Group three includes (Nazil’, Oltan) and Bakhtegan, B557, Tashkand
placed in Group four. Rana and Bhat (2005) studied genetic similarity
among 41G. hirsutum cultivars of cotton using RAPD marker and the UP-
GMA cluster analysis placed all the tetraploid cultivars within their respec-
tive known taxonomic groups.

Identification of informative markers

Table 3

| Protein | PPO | CAT | Carbo | Proline | EL |
|---------|-----|-----|-------|---------|----|
| Intercep | 12699.472 | -41.501 | 34.109 | 4.299 | 2.592 | 32.842 |
| P5n8 | | | | 0.119 | |
| P9n1 | | | | 0.425 | |
| P9n4 | | | | 0.714 | |
| P9n6 | 0.757 | | | -0.932 | |
| P14n2 | | | | | 0.514 |
| P14n8 | | | | -0.498 | -0.743 |
| P15n4 | 0.313 | | | | |
| P15n5 | | -0.461 | -0.946 | | |
| P15n6 | 0.542 | | | | 0.348 |
| P16n5 | | | | -0.563 | |
| P16n1 | | | | | 0.704 |
| P19n3 | | | | -0.605 | |
| P28n1 | | | 0.262 | -0.565 | |
| P30n4 | | | | -0.450 | |
| R2 | 0.691 | 0.500 | 0.168 | 0.566 | 0.818 | 0.929 |

Phenotypic selection has limitations especially when interest is focused
on more complex physiological features. A more accurate way of selection
would be at the genetic level where markers linked to the gene(s) or quanti-
tative trait loci (QTLs) underlying the trait can be screened for.
A prerequisite for genotypic selection is the establishment of associations
between features of interest and genetic markers. QTL mapping of physio-
logical features will provide crop breeders with a better understanding of
the basis for the genetic correlation between economically important traits. This has potential to facilitate a more efficient incremental improvement of specific individual target traits (Graham et al, 2008). Information on QTL analysis has accumulated quickly, and will eventually help the manipulation of complex features in cotton breeding (Tanksley, 1993; Preetha and Ravendredren, 2008). The results of stepwise regression analysis revealed a significant correlation/association between the physiological features and some of the studied ISSR loci. One or more informative markers were identified for almost all of the studied features. The important informative markers in acclimation level are listed below. Markers P9n6, P15n4 and P15n6 accounted for 69% of total variability of protein feature. P14n8, P15n5 and P19n3, were responsible for carbohydrate feature with 52%, P5n8, P9n1, P9n4P9n6, and P28n1 for proline feature with 81% of the total variability. Markers which responsible for electrolyte leakage explained 92% of variation (Table 3). After cold acclimation three markers were associated with soluble protein, two markers for electrolyte leakage and one marker with catalase, carbohydrate and proline concentration features. Markers in association with soluble protein explained 67% of variation whereas amount of explained variance by electrolyte leakage were 75% (Table 4).

Table 4

| Protein PPO CAT Carbo Proline EL |
|-------------------------------|
| Intercept 21501.457 52.327 33.518 3.651 3.844 31.354 |
| P9n1 | |
| P9n6 0.624 0.268 |
| P15n3 |
| P15n4 0.427 |
| P15n5 0.410 -0.451 -0.678 |
| P16n2 0.432 |
| P19n5 -0.499 |
| R2 0.677 0.185 0.164 0.430 0.163 0.758 |

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