Profiling dehydrin gene sequence and physiological parameters in drought tolerant and susceptible spring wheat cultivars.

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PROFILING DEHYDRIN GENE SEQUENCE AND PHYSIOLOGICAL PARAMETERS IN DROUGHT TOLERANT AND SUSCEPTIBLE SPRING WHEAT CULTIVARS

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Abstract

Physiological and yield traits such as stomatal conductance (mmol $m^{-2}s^{-1}$), Leaf relative water content (RWC %) and grain yield per plant were studied in a separate experiment. Results revealed that five out of sixteen cultivars viz. Anmol, Moomal, Sarsabz, Bhitai and Pavan, appeared to be relatively more drought tolerant. Based on morphophysiological results, studies were continued to look at these cultivars for drought tolerance at molecular level. Initially, four well recognized primers for dehydrin genes (DHNs) responsible for drought induction in *T. durum* L., *T. aestivum* L. and *O. sativa* L. were used for profiling gene sequence of sixteen wheat cultivars. The primers amplified the DHN genes variably like Primer WDHN13 (*T. aestivum* L.) amplified the DHN gene in only seven cultivars whereas primer TdDHN15 (*T. durum* L.) amplified all the sixteen cultivars with even different DNA banding patterns some showing second weaker DNA bands. Third primer TdDHN16 (*T. durum* L.) has shown entirely different PCR amplification prototype, specially showing two strong DNA bands while fourth primer RAB16C (*O. sativa* L.) failed to amplify DHN gene in any of the cultivars. Examination of DNA sequences revealed several interesting features. First, it identified the two exon/one intron structure of this gene (complete sequences were not shown), a feature not previously described in the two database cDNA sequences available from *T. aestivum* L. (gi|21850). Secondly, the analysis identified several single nucleotide polymorphisms (SNPs), positions in gene sequence. Although complete gene sequence was not obtained for all the cultivars, yet there were a total of 38 variable positions in exonic (coding region) sequence, from a total gene length of 453 nucleotides. Matrix of SNP shows these 37 positions with individual sequence at positions given for each of the 14 cultivars (sequence of two cultivars was not obtained) included in this analysis. It demonstrated a considerable diversity for this gene with only three cultivars i.e. TJ-83, Marvi and TD-1 being similar to the consensus sequence. All other cultivars showed a unique combination of SNPs. In order to prove a functional link between these polymorphisms and drought tolerance in wheat, it would be necessary to conduct a more detailed study involving directed mutation of this gene and DHN gene expression.

Introduction

Among abiotic stresses, drought provides the most challenging task from an economic point of view and is the focus of many breeders' efforts. Traditionally, the multifaceted nature of drought coupled with an incomplete knowledge of genetic and physiological bases of yield in water-limited conditions have considerably hindered breeding progress (Ludlow and Muchow, 1990; Boyer, 1996; Passioura, 1996, 2002; Khan & Khan, 2010). To overcome the low response to direct selection for yield under drought conditions, substantial efforts have been targeted on manipulation of physiological traits influencing drought resistance through an escape, avoidance and tolerance mechanism (Blum, 1996; Saeed *et al*., 2010). However, indirect selection strategy has only been successful in a limited number of cases notably in wheat cvs. Drysdale and Rees which were selected for adaptation to their unique dryland conditions. Christopher *et al*., (2008) conducted six detailed experiments to compare the growth, development and yield traits of cultivars SeriM82 and Hartog. The yield of SeriM82 was 6-28% greater than Hartog, besides SeriM82 also exhibited a stay-green phenotype by maintaining green leaf area longer during the grain-filling period in both stress and non-stress environments.

As compared to conventional approaches, the advent of molecular markers has enabled scientists to dissect quantitative traits into their single genetic components, i.e. quantitative trait loci (QTLs; Dudley 1993; Tanksley 1993; Prioul *et al*., 1997; Tuberosa *et al*., 2002), and to assist the selection and pyramiding of beneficial QTL alleles through marker-assisted breeding (Ribaut *et al*., 2002). More recently, bioinformatics (Bray, 2002) and the deluge of information generated by the post-genomics platforms (Tuberosa *et al*., 2002; Hazen & Kay, 2003) have added new dimensions for deciphering the role and function of genes governing the response to drought. Despite all these impressive and fascinating technological breakthroughs, the overall impact of marker assisted selection (MAS) and genomics on the release of drought-resilient cultivars has so far been disappointingly negligible.

A large proportion of modern molecular research on drought tolerance also attempts to identify those genes whose expression contributes to differences in drought tolerance. Although by no means universal, a substantial proportion of such studies lack any rigorous measurement of either the environmental stress imposed or the resulting tissue water status. The broad types of water-status measurements such as relative water content (RWC %) and osmotic potential have been reported between 2003 and 2005 where the main objectives have included an analysis of molecular responses to drought (Jones, 2006). In such studies, over half of the research had no measure of water-status whatsoever, which raises serious concerns as there is a lack of critical information on water-status indicated that experiments are likely to be both difficult to

repeat with any certainty and more importantly, almost certainly limits the value of the information collected. Tolerance to abiotic stress is a complex phenomenon, comprising a number of physio-biochemical processes at both cellular and whole organism levels activated at different stages of plant development. Several mechanisms have been adopted by drought-tolerant plants to adapt to water stress. Examples of these mechanisms may be reduction in water loss by increasing stomatal resistance, increasing photosynthetic activity and increase in water uptake by developing larger and deeper root systems, and accumulation of osmolytes (Bartels & Nelson, 1994). One of the main molecular events occurring during water-deficit is extensive modification of gene expression resulting in a strict control of the physiological and biochemical responses to stress. Several genes specifically involved in stress response have been identified. Among these are the genes encoding the so-called late embryogenesis abundant (LEA) proteins. LEA proteins accumulate under stress conditions such as drought, salinity and low temperatures, but they are also present in ABA-treated vegetative plants. To this family belong the dehydrin (*DHN)* genes (Yongchun, 2010), which are up-regulated during the stress (Zhu *et al*., 2000). Association between accumulation of members of the *DHN* family and tolerance to stresses involving dehydration has been shown in several species such as sunflower (Cellier *et al*., 1998), barley (Zhu *et al*., 2000) and wheat (Lopez *et al*., 2003).

Although genotypic differences in dehydrin expression have been reported in response to cold and drought tolerance, it is important to relate the expression of dehydrins in relation to changes in Relative water content (RWC) and stomatal conductance when wheat plants were exposed to drought stress (Yongchun, 2010). Lopez *et al*., (2003) worked with seven winter wheat cultivars with the objective to determine the relationship of dehydrin gene expression with the acquisition of drought tolerance in specific cultivars under drought stress at seedling development stages. The expression of a 24-KDa dehydrin was observed in cultivars Connie, TAM105 and Gene at 4 days' stress while no expression of this gene was detected in non-stress control plants.

Similar results were recorded by Patrizia *et al*., (2006) who identified resistant and sensitive genotypes based on the measurement of relative water content (RWC %). The purpose of this research was to evaluate the genetic diversity of dehydrin gene sequence and also relating it with some physiological and grain yield traits such as relative water content (RWC %) and stomatal conductance under water-stress and non-stress treatments in greenhouse conditions.

Materials and Methods

Seeds of sixteen spring wheat cultivars (*T. aestivum* L.) namely, Anmol, Inqilab, Moomal, TJ-83, Sarsabz, Khirman, SKD-1, TD-1, Kiran, Abadgar, Marvi, Mehran, Bhittai, Z.A. 77, Pavan, and Imdad were grown in Greenhouse at the Department of Plant Sciences, University of Reading, U.K. during January, 2009. For morphophysiological studies, the pots were filled with compost. One day after watering the pots, six seeds at equal distance were sown in each pot. After germination, three seedlings were thinned and the remainder three were allowed for evaluation and data recording. The trial was laid-out in green house with randomized complete blocks in factorial arrangement, the irrigation regimes and cultivars were considered as 2 factors. Four replications and two irrigation regimes i.e. well-watered (frequently irrigated) and drought-stress was arranged for drought tolerance screening via yield physiological parameters. Drought-stress treatment was irrigated when moisture level dropped to 25% of the water field capacity (WFC) and re-watered back to only 35% of the WFC, so this treatment never reached to actual WFC but was put in continuous stress during entire experimentation period. Hereafter, 75% drought-stress treatment will be referred to as just drought-stress (DS).

Morpho-physiological trait measurements

About 24 random measurements were taken for recording the data per cultivar over replications and treatments. Stomatal conductance (mmol $m^{-2}s^{-1}$) was measured on full expanded flag leaves by Porometer AP4, Delta Devices, Cambridge, U.K. Care was taken to take the measurements during 12:00 noon to 3:00 P.M. in light conditions when the stomatal function is believed to be more active and screening of plants for drought tolerance was more reliable. Leaf relative water content (RWC %) was determined on fully expanded flag leaves which were randomly excised at their base, placed in grip plastic bags and transported to the laboratory. Fresh weight was determined within 2 hours of excision. While turgid weights were determined after leaves were soaked in distilled water for 18 hours at room temperature (20 ± 2^{o}C) and 60% relative humidity under low light conditions. The leaves were then taken out of water, blotted on tissue papers and turgid weight was obtained. Turgid weights were obtained after oven drying the leaves at 70^{0}C for 24 hours. Leaf water content was calculated according to Schonfeld *et al*., (1988) i.e. RWC % = (fresh weight-dry weight)/(turgid weight-dry weight) x 100. Grain and biological yield per plant (g) were determined after each plant was harvested and threshed separately.

Gene sequencing

For molecular analysis, the leaf below the flag leaf was excised, put in 15 ml Falcon tubes and very quickly immersed into liquid nitrogen. For longer storage, the plant material was wrapped in plastic bags and placed in -80^{0}C freezer. Primer design was carried-out with **t**en primers (five forward and five reverse) responsive for drought tolerance in wheat and rice obtained from Invitrogen (Illumina products). These primers are part of dehyrin genes (Table 1) and the information about their sequence and target genes was obtained through public database using BLAST (http://www.ncbi.nlm/BLAST) and gene bank accession number. While preparing 100µM primers for PCR reaction, an appropriate quantity

of T x TE buffer (nmoles of primer x 10 µl of T x TE buffer) was added. The designed primers were stored at -20°C for later-on use. For DNA extraction and PCR reaction, about 100 mg of frozen leaf sample weighed and ground with mortar and pestle, and about 20 mg dried sample was used for DNA extraction. The total DNA was extracted using QIAGEN DNeasy Plant Mini-kit (50), Cat. No. 69104 and followed the supplier's instructions. For PCR reaction, 2 µl of DNA (template), 25 µl of Biomix (2x bioline, prepared) containing (Taq Poly., DNTP, Mg^+, and $NH4^+$), 1.75 µl of each forward and reverse primers and 19.5 µl of distilled water were put in 0.2 ml of PCR tubes. Thus, the final volume for PCR reaction was 50 µl. One negative control without DNA but containing Biomix, primer and water was also used in a reaction. The PCR tubes were first mixed with mixer and then placed in PCR machine. Amplification of gene was performed in a thermal cycler (GenAmp, PCR System 9700 PE Applied Biosystems) using one step of 2.0 min at 94^0C and then 30 cycles each of 30s at 94^0C, 30s at 58^0C (annealing temperature optimized), and 60s at 72^0C, followed by a final step of 7.0 min at 72^0C. PCR products were separated in 1% agarose gel (1.0 g agarose dissolved in 100 ml TAE buffer) and stained with 1.0 µl of Ethidium Bromide. Gels were scanned and analyzed under Syngene Gel Documentation system and DHN band intensities were measured with 100 bp ladder as an internal control. PCR product clean-up was performed by using QIAquick PCR Purification kit (QIAGEN). PCR-products were sequenced using the Big Dye Terminator Sequencing Kit (Applied Biosystem). The Thermal Cycler conditions using GenAmp, PCR System 9700, PE Applied Biosystems were; 30 cycles each of 1:00 min. at 96^0C), 20s at 95^0C (denaturing temp. and time), 10s at 50^0C (annealing temp. and time) and 4:00 min. at 60^0C (extension temp. and time). Samples were analyzed by an ABI Prism 3130x Genetic Analyzer (Applied Biosystem). DNA sequences obtained were compared with public databases using BLAST (http://www.ncbi.nlm/BLAST) programmes. MEGA (version 3.0; http:/www.Nmega software.net) programme (Kumar *et al*., 2004) was used to perform a cluster analysis and a phylogenetic tree construction.

Table 1. Primers used in PCR Amplification

Primer sequences (5' to 3')	Target gene	Gene Bank accession number
GCGTCATGGAAAGCATCAC (F) GTCCAGGCAGCTTGTCCTT (R)	WDHN13 (*T. aestivum* L.)	AB076807
ATGGAGTTCCAAGGGCAG (F) TCAGTGCTGTCCCGGCAGCTT (R)	*TdDHN15* (*T. durum* L.)	X78431
ATGGAGTACCAGGGACAGCAG F) GGGCAGCTTCTCCTTGATCTT (R)	*TdDHN16* (*T. durum* L.)	X78429
TCGACGTGTACGGCAACCG (F) GGGAGCTTCTCCTTGATCTT (R)	*RAB16C* (*O. sativa* L.)	CA753127

Note: Target genes and their relative Gene bank accession number are reported.

Results and Discussion

Characterization of wheat genotypes contrasting in their response to water-stress was attempted by conducting experiments in the greenhouse earlier than carrying molecular analysis. Physiological and yield traits were measured and drought resistant and susceptible genotypes were labeled according to their performance in water stress conditions. Among several physiological measurements, stomatal conductance (mmol $m^{-2}s^{-1}$) and leaf relative water content (RWC %) were found as the most reliable traits to be used as selection criteria for screening drought tolerant genotypes. With respect to grain yield exhibited strong positive correlation with physiological traits and supported the same genotypes to be drought tolerant (Fig. 1a, b and c). Results revealed that five out of sixteen cultivars were relatively drought tolerant i.e. Anmol, Moomal, Sarsabz, Bhitai and Pavon (Fig. 1c). Similar results were reported by Patrizia *et al*., (2006) who identified resistant and sensitive genotypes based on RWC %. Further characterization of genotypes differing in their response to water stress, and studied water loss rate (WLR) and free proline content after different periods of dehydration. Under drought stress, the water content of wheat leaves decreased, but membrane permeability increased. Western blot analysis showed that there was a specific protein of 28 ku under drought stress, the expression of dehydrin in weak drought tolerant wheat leaf was earlier than that in strong drought tolerant wheat leaf, and the content of dehydrin in strong drought tolerant wheat was higher than that in weak drought tolerant wheat. After redehydration, the water content of wheat leaves increased, the membrane permeability decreased, and this dehydrin could exist in plant for some time. It showed that harm to wheat under drought stress and expression of this dehydrin is closely related to drought resistance in wheat. For yield traits, Christopher *et al*., (2008) conducted six detailed experiments to compare the growth, development and yield traits of cultivars SeriM82 and Hartog. The yield of SeriM82 was 6-28% greater than Hartog. After these results, studies were continued to look at the genetic diversity of dehydrin genes among the drought tolerant and susceptible wheat genotypes at molecular level.

Details of PCR- products: Fig. 2a and 2b are the PCR-products with Primer 13 where L, 0 lanes represent Ladder and negative, respectively while Nos. 1 to 16 are the cultivar. Likewise with Primer 15 (Fig. 3a & 3b) and Primer 16 (Fig. 4a & 4b) are their ladders, negatives and cultivar numbers. Using the 4 primers listed in Table 1, we noticed that all the four primers amplified the DHN genes variably. Primer WDHN13 (*T. aestivum* L.) amplified the DHN gene in only seven (v1, v5, v6, v11,

v12, v14 and v15) out of sixteen genotypes (Fig. 2a & 2b). Whereas primer TdDHN15 (*T. durum* L.) amplified the DHN gene in all sixteen genotypes with even different DNA banding patterns (some showing second weak DNA bands) (Fig. 3a & 3b). Third primer TdDHN16 (*T. durum* L.) has shown entirely different PCR amplification pattern, specially showing two strong DNA bands and of course worked for all sixteen genotypes (Fig. 4a & 4b). However, the fourth primer RAB16C (*O. sativa* L.) failed to amplify DHN gene in any of the genotypes.

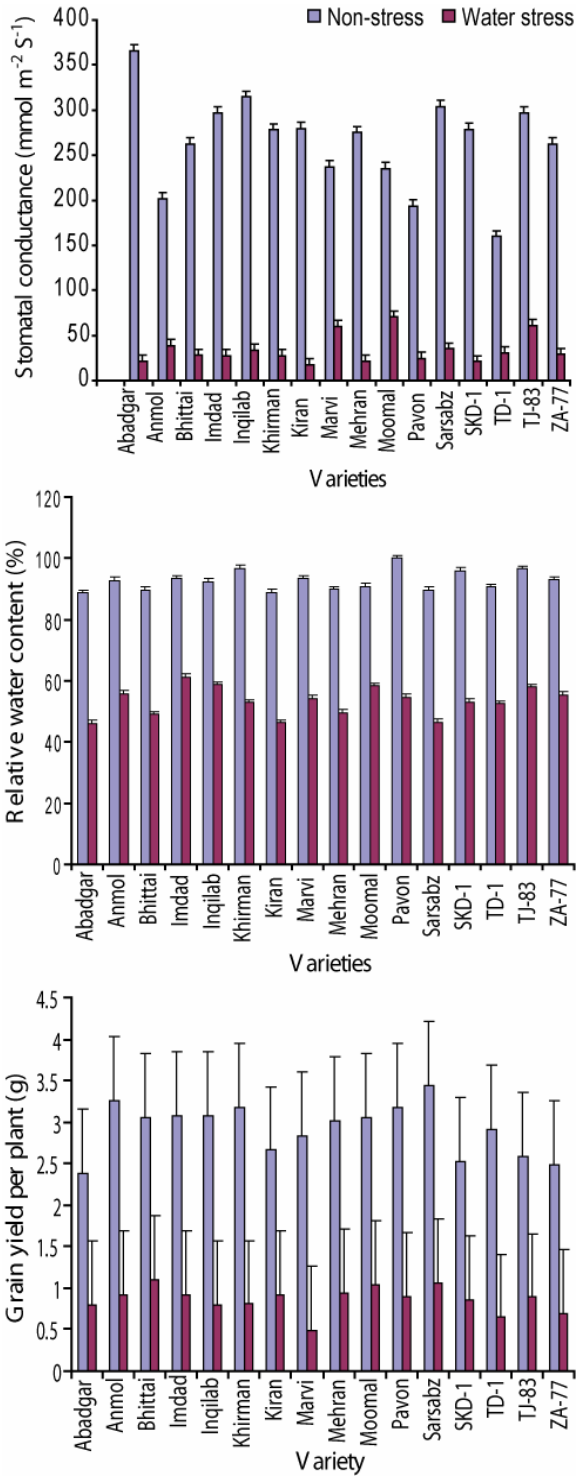

Fig. 1. Graphical presentation of cultivars for (a) stomatal conductance (b) relative water content (c) and grain yield per plant (c) in water stress and non-stress conditions.

The consensus sequence of DHN gene with primer WDHN13 revealed no genetic diversity in all seven cultivars where DHN gene was amplified. This suggests that well adapted cultivars may not differ much in DHN genes sequence. While on other hand, not amplifying the DHN gene in other nine cultivars indicated that these genotypes might possess entirely different DHN genes (different from the primer WDHN13) which were not picked-up by primer 13 which could be that primer and had more conserved coding region. The more reliable DHN gene sequence in that case, may be needed by two other Primers such as TdDHN15 and TdDHN16. This difference in DHN gene band actually shows that 7 cultivars were genetically diverse from other nine cultivars regarding DHN gene.

In contrast, examination of 14 sequences of wheat genotypes shows several interesting features. First it identifies the 2 exon/one intron structures of this gene, a feature not previously described in the two database cDNA sequences available from *T. aestivum* L. (data base number = gi|21850) and *T. turgidum* L. (data base number = gi|1181295). Secondly, the analysis identifies several single nucleotide polymorphisms (SNPs), positions in the gene sequence where there is variation in the specific nucleotides at certain positions (Table 2). Although complete gene sequence was not obtained for all the cultivars yet there is a total of 38 variable positions in the exonic (coding) sequence, from a total gene length of 453 nucleotides. Table 2 provides a list of these 38 positions with individual sequence at these positions given for each of the 14 cultivars included in this analysis (two cultivars viz. Imdad and Pavon's sequences were not clean and were not included in sequencing analysis and alignments).

This demonstrates a considerable diversity for this gene with only three cultivars i.e. TJ-83, Marvi and TD-1 being similar to the consensus sequence. In addition, these cultivars suffered the most in stress conditions based on especially physiological attributes. All other cultivars have a unique combination of SNPs.

Bibi *et al*. (2010) generated induced mutation and assessed genetic diversity through Random Amplified Polymorphic DNA and then investigated drought tolerance through sequence tagged site techniques. Out of 100 alleles amplified with fifteen primers, 78% were polymorphic. Results indicated that eleven genotypes amplified the DREB F1R1 fragments whereas thirteen genotypes amplified DREB F3 R3 sequence. Wheat DNA amplification with DREB genes yielded 190-220bp bands. In present studies, in order to prove a functional link between these polymorphisms and drought tolerance in wheat, it would be necessary to conduct a more detailed study involving directed mutation of this gene. This sort of studies will be very meaningful to other molecular biologists who wish to carry gene expression studies in such type of genotypes.

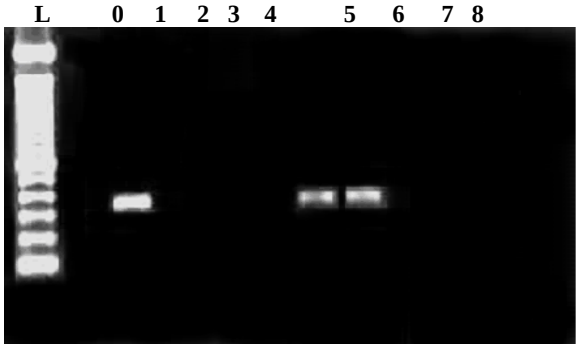

Fig. 2a. Primer WDHN13

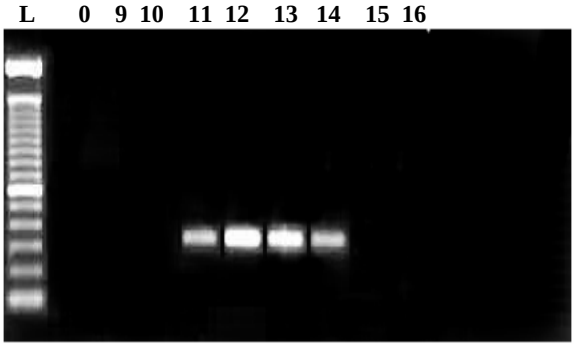

Fig. 2b. Primer WDHN13

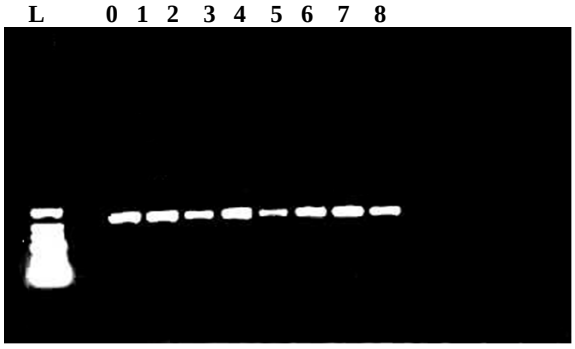

Fig. 3a. Primer TdDHN15.

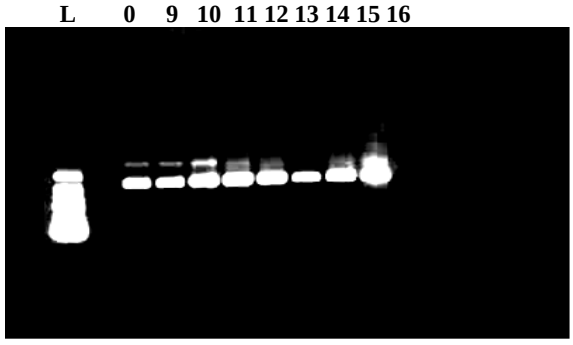

Fig. 3b. Primer TdDHN15

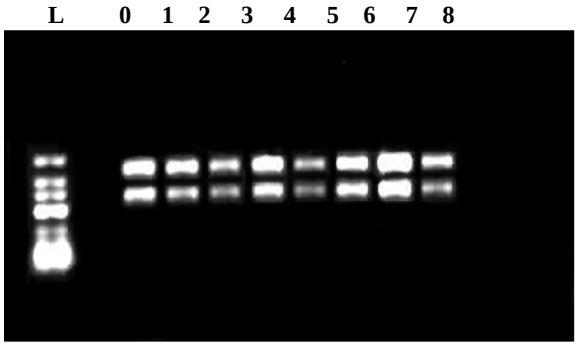

Fig. 4a. Primer TdDHN16

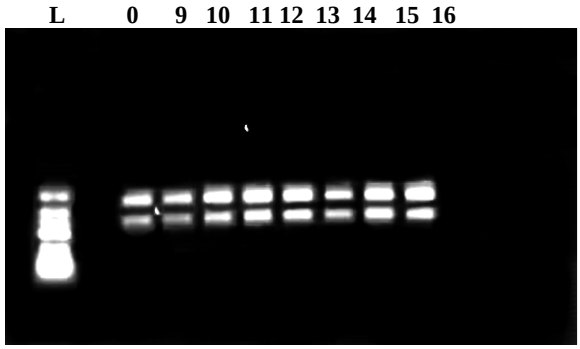

Fig. 4b. Primer TdDHN16

Table 2. Summary of the Single Nucleotide Polymorphisms (SNPs) present in the 38 positions of 14 cultivars tested, together with the reference sequences from *T. aestivum* L. and *T. turgidum* L.

Consensus	**CCGGCCGGGCTAGCGGGTAGGGGGGCGCGGCAACCGAT**
TJ-83	**No SNPs were detected**
Moomal	T A
Anmol	G A
Abadgar	T
Inqilab	AGT
TD-1	**No SNPs were detected**
Mehran	T A G AGT
Marvi	**No SNPs were detected**
ZA-77	A A A AA C TT
Bhitai	AA A A TT
SKD-1	A AAAA AAAAC TT
Sarsabz	A A AAA A AAAAC A TA
Khirman	A A
Kiran	AA
TAM W-101	TTA A A G ACG T T A G C
T.turgidum	ATA A G C AG A

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