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Molecular Characterization of Drought Tolerance Genes in Indigenous Wheat Varieties of Pakistan

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ABSTRACT

One of the biggest limitations to wheat (Triticum aestivum L.) production in Pakistan, especially in arid and semi-arid areas is drought stress. Indigenous wheat types have distinct genetic phenotypes which could make them drought tolerant yet the characterization on the molecular level of such phenotypes is incomprehensive. This research study was intended to determine and describe drought tolerance genes in some indigenous wheat varieties through molecular markers and gene-expressions. Sample of leaf of ten types of wheat was taken and DNA extracted and amplified and the levels of real-time PCR (qRT-PCR) analyzed. Findings showed that there was a wide difference in the presence and expression of genes in different varieties with some landraces showing an increased expression between DREB1 and NAC which could suggest the presence of drought resistance. These results can be used to give valuable information about the genetic foundation of drought tolerance in local wheat germplasm and also indicate potential varieties to be used in breeding programs that can improve drought resistance. The research has a contribution towards sustainable wheat production in water constrained areas in Pakistan.

Keywords: Pakistan Drought resistance, native wheat, molecular, gene expression, DREB1, NAC, HSP70.

INTRODUCTION

One of the most significant cereal crops in Pakistan is wheat (Triticum aestivum L.), which determines the basis of food security and makes a significant contribution to the national economy (Sharma et al., 2019). Although it is important, the abiotic stresses tend to limit the productivity of wheat, and drought is the most important element of growth, and yield in arid and semi-arid areas of the country (Farooq et al., 2014). The drought stress is increased by water shortage, the lack of regular rains, and the growing influence of climate change that creates an urgent necessity to find wheat varieties that can be used in water-restrained scenarios (Ali et al., 2020).

Landraces Indigenous wheat strains, also known as landraces, have been grown over generations under local environmental conditions and are an important source of genetic characteristics that are adapted to drought and other stresses (Naz et al., 2017). These local landraces have mechanisms of inherent resilience, unlike the modern high-yielding cultivars, which are often bred to suit optimal conditions, and as such, they may be of use in breeding programs to help increase the drought-tolerance of modern breeds. Nonetheless, little is known regarding the molecular mechanism of drought adaptation in the native indigenous Pakistani wheat types. The genetic processes of drought resistance of these landraces are necessary to the production of wheat sustainably and to achieve food security in areas of water shortage.

There have been molecular researches to identify various gene families, which are relevant in plant drought responses. One of them is DREB (Dehydration-Responsive Element Binding) genes that are transcription factors that stimulate the downstream stress-responsive genes to help plants to survive in water-deficit conditions (Lata & Prasad, 2011). Equally, NAC (NAM, ATAF and CUC) genes play a role in osmotic regulation, root architecture management and stomata control as they serve to enhance adaptive changes to drought (Mao et al., 2012). The genes of Heat Shock Protein (HSP70) also play a significant role because they help to preserve the stability of proteins and safeguard the work of the cell in case of stress (Wang et al., 2004). The fact that these genes are present and expressed differently in native wheat varieties can be explored to provide valuable clues to the molecular basis of drought tolerance.

Molecular markers have offered potent methods of genetic variation detection and gene expression under stress adaptation through polymerase chain reaction (PCR), simple sequence repeat (SSR) markers, and quantitative real-time polymerase chain reaction (qRT-PCR) (Rasheed et al., 2017). The approaches will help in identifying candidate varieties that have better drought tolerance characteristics which can be used in breeding programs using marker-assisted selection (MAS). Although these methods have been proven useful, there is a significant deficiency in the molecular studies of Pakistani wheat landraces to fill the knowledge gap, which restricts the exploitation of their adaptive genetic potential.

The past studies in the adjacent countries have shown that there is a high degree of variation of drought-responsive genes in the local wheat populations. Research in India and Iran also reported changes in the expression of the DREB1 and NAC genes, and these were related to the phenotypic traits like the root depth, biomass, and water retention by the leaves (Singh et al., 2015; Khodarahmpour et al., 2016). These results indicate that there are also chances that the indigenous wheat varieties found in Pakistan have some form of alleles that provide adaptive benefits in the case of water-constrained environments. Molecular description of these varieties is thus important in the formulation of resilient wheat varieties with the ability to survive drought pressures.

This research project is centered on the molecular characterization of the drought tolerance genes in the indigenous wheat varieties found in Pakistan with the aim of knowing about their genetic diversity and how they may be adapted to fit in an environment where there is a restriction in the water quantity. The examination of the presence and expression of major drought-responsive genes will aid in offering information on the mechanisms of drought resilience, potential varieties in breeding programs, and add to the strategy on how wheat can be produced sustainably. The research will finally attempt to close the divide between the traditional landrace farming and the new molecular breeding methods, facilitating the preservation and exploitation of local germplasm to increase food security in Pakistan.

LITERATURE REVIEW

Among the most important abiotic limitations that impact on wheat productivity in various parts of the world especially the arid and semi-arid areas is drought stress. Having a rather dry climate, Pakistan experiences significant losses of wheat production because of the lack of water, particularly at the critical periods of growing, namely, the germination, the tillering, and the filling of the grain (Farooq et al., 2014). Wheat yields have been recorded to be highly affected in areas affected by droughts globally up to 20-40% (Ali et al., 2020). Climate change, temperature increase, and unpredictable rain patterns have also contributed to worsening the situation in Pakistan by increasing the occurrence and severity of drought periods (Raza et al., 2019). Since wheat is a key crop, its ability to endure drought is essential to the food security of countries and the small scale farmers.

Landraces also called indigenous wheat varieties have been grown over many centuries in the local environmental conditions and are inherently adapted to a range of abiotic stresses, such as drought (Naz et al., 2017). Since these landraces are not subjected to irrigated irrigation as the modern high-yielding cultivars, under favorable conditions, they have special genetic characteristics, which lead to stress resistance. The adaptive strategies of these varieties are the deep root system, osmotic adjustment, stomatal control, and osmoprotectants accumulation, which improve their survival in the conditions of low water (Hussain et al., 2015). These adaptations have however been analyzed at the phenotypic level but very little is known about their molecular backgrounds. Molecular characterization of drought responsive genes in these varieties offers a good prospect of future breeding programs to identify candidate genes and alleles that can be utilized in future breeding initiatives to enhance drought tolerance.

The key to the plant responses during drought stress is transcription factors. Dehydration-Responsive Element Binding (DREB) genes are some of these that have been well reported to mediate the control of downstream stress-responsive genes. Specifically, DREB1 and DREB2 mediate ABA-independent and ABA-dependent, respectively, and trigger several different stress tolerance programs such as osmotic adjustment, antioxidant activity, and stomatal closure (Lata & Prasad, 2011). It has been shown that DREB gene overexpression in wheat leads to tolerance to drought, heat and salinity stress by altering the expression of stress-inducible genes (Singh et al., 2015). The patterns of DREB genes expression in indigenous wheat varieties in the natural drought condition are also not studied in detail and this is a gap in the research that is highly evident in the circumstances of Pakistani germplasm.

Equally, NAC (NAM, ATAF and CUC) transcription factors play critical roles in controlling plant adaptive reactions to abiotic stress. The genes of the NAC regulate the architecture of the roots, the senescence of the leaves, and the accumulation of osmolites, which helps the plant to adapt to water deficit (Mao et al., 2012). It has also been demonstrated through functional studies in wheat and other related cereals that NAC overexpression also increases drought resistance by increasing the root depth and the root biomass as this leads to the improvement of the efficiency of water uptake under conditions of limited soil moisture (Tang et al., 2012). NAC alleles that have been unique to indigenous landrace and have provided drought resistance may be present in indigenous landraces, but molecular studies are limited and focused on these varieties, and thus, it is necessary to investigate their genetic potential.

The role of the heat shock proteins (HSPs) and especially hsp70 in ensuring the homeostasis of cells when they are subjected to abiotic stress, such as drought, is also significant. HSP70 is a molecular chaperon, which inhibits protein denaturation and aggregation during water shortage and stress at high temperatures (Wang et al., 2004). Studies have shown that HPWS genotypes of wheat have greater expressions of

HSP70 as compared to those that are sensitive to drought, which implies a protective mechanism against stress (Kaur et al., 2016). HSP70 molecular profiling of indigenous wheat varieties could therefore be a valuable resource in the determination of genotypes that have higher withstanding under drought.

The use of molecular marker technologies has truly transformed the concept of breeding plant by the ability to identify genes that are linked to stress tolerance with accuracy. Methods like polymerase chain reaction (PCR), simple sequence repeat (SSR) markers, and quantitative real-time PCR (qRT-PCR) enable the identification of the existence of a gene, allelic diversity, and gene expression level during a stressful condition (Rasheed et al., 2017). The use of SSR markers comes in handy as it is co-dominantly inherited, reproducible and highly polymorphic, making it easy to study genetic diversity in native wheat germplasm (Somers et al., 2004). By integrating SSR markers with qRT-PCR, it is possible to genotype and functionally examine drought-responsive genes, which would shed light on the role of all these molecular processes in the occurrence of adaptation to water-stressed conditions.

The research conducted in the adjacent nations has also yielded useful information on the genetic diversity of the drought tolerance genes in wheat landraces. As an example, studies performed in India showed that there were great differences in the expression of DREB1 and NAC genes among both local populations in wheat, and they were associated with physiological characteristics like leaf water retention and root depth (Singh et al., 2015). Other research in Iran also found allelic diversity in HSP70 and related regulatory genes that played a role in different drought tolerance in native varieties (Khodarahmpour et al., 2016). Such results indicate that Pakistani landraces can also contain rare alleles and gene expression patterns, which can be utilized in breeding drought-resistant cultivars.

Phenotypic analysis is not usually enough to choose drought-tolerant genotypes since interactions between the environment and genotype may hide genetic capability. A combination of both molecular and phenotypic screening enables more precise screening of the best varieties. As an example, it has been demonstrated that the combination of drought stressing tests and gene expression of DREB1, NAC, and HSP70 could be used to determine genotypes with consistent performance in controlled and field experiments (Zhang et al., 2017). By doing this, breeding programs have ensured that varieties of candidates that are used in breeding programs have both adaptive features and the genetic mechanisms that provide them with drought resistance.

Although the use of indigenous wheat varieties has been noted to have potential, in Pakistan there has been more emphasis on modern cultivars, this at the expense of the genetic resources contained in the local landraces (Raza et al., 2019). It is high time to critically analyze these varieties systematically at the molecular level especially the important drought-responsive genes. These studies are able to determine candidate genotypes to be used in marker-assisted selection (MAS), decrease the breeding program time and increase the productivity of creating water-tolerant cultivars adapted to water-limited areas. Also, such molecular diversity of these genes may be used to the local germplasm conservation so that good adaptive traits are not forfeited by the substitution of the traditional varieties with high yielding cultivars.

New developments of genomic technology such as next-generation sequencing and transcriptome have increased the possibilities of describing genomic-based stress regulation in wheat. The drought stress transcriptome profiling will demonstrate the network of genes used in tolerance mechanisms and find new candidate genes in native varieties (Singh et al., 2018). Integrative analysis of transcriptomic data with

SSR-markers and qRT-PCR-validation offers an extensive framework in the understanding of drought tolerance on both the structural and functional level. The integrative method is of special use in the case of Pakistani wheat germplasm which has not been studied extensively even though it has a potential to facilitate climate-resilient agriculture.

Finally, it has been shown in the literature that drought stress has a harmful impact on wheat production and that indigenous varieties can contain important genetic resources to adapt to them. DREB, NAC, and HSP70 are major gene families that involve themselves in the process of regulating stress responses, and the molecular characterization of these genes in the local landraces is required. The molecular marker technologies, such as SSR markers and qRT-PCR offer the tools that could be used to estimate the genetic diversity, the presence and expression of the gene. Nonetheless, there is a lack of research on the Pakistani wheat landraces, and there is a knowledge gap that impedes the production of drought-tolerant cultivars. Molecular characterization and gene expression can help meet this gap and therefore help in identifying better genotypes to be used in breeding programs, help in sustainable production of wheat and help in improving food security in water-restricted areas of Pakistan.

METHODOLOGY

The experiment was realized with the help of the native varieties of wheat (Triticum aestivum L.) that were obtained in the major wheat-producing regions of Punjab, Pakistan, that could be described by the arid-to-semi arid conditions and the frequent stresses of drought. Ten native types of wheat were chosen according to the information provided by local farmers and previous findings about drought resistance (Naz et al., 2017). Authenticity and genetic integrity were achieved by taking seeds in agricultural research stations and community seed banks.

The soil used was sterilized to limit fluctuations in the environment and the germination of the seeds was performed in controlled conditions of a greenhouse. The seedlings were cultivated in pots (25 cm in diameter) packed with a blend of loamy soil, sand, and compost in the ratio of 2:1:1. The common agronomic activities such as regular watering, fertilization and use of pests were used in the earlier growth stage. Three weeks later, the drought stress was inflicted by depriving the soil of irrigation water where it remained under 10-14 days to test its capacity to retain water and control plants were held under conditions of good water. A soil moisture meter was used to monitor soil moisture content on a daily basis in order to ensure that stress levels across replicates were the same.

Drought-stressed and control plants were sampled at the highest level of stress (say four weeks after germination) and the leaf tissues were taken, immediately frozen in liquid nitrogen and kept at -80degC until molecular analysis. The cetyltrimethylammonium bromide (CTAB) genomic DNA extraction method was employed to extract genomic DNA and NanoDrop spectrophotometer and agarose gel electrophoresis were used to determine DNA quality and concentration, respectively (Doyle and Doyle, 1987).

Gene-specific primers were used to amplify using PCR namely: DREB1, NAC and HSP70. The PCR mixture (25 mL) consisted of 50 ng of genomic DNA, 1x of PCR buffer, 2.5 mM MgCl2, 0.2 mM of dNTPs, 0.5 mM of each primer and 1 U of Taq DNA polymerase. The amplification conditions were 95degC denaturation of 5 minutes, 35 cycles of 94degC denaturation 30 seconds, annealing at gene specific temperatures 30 seconds, extension at 72degC 45 seconds and finally 72degC extension 10 minutes. PCR results were placed on a 1.5 percent agarose gel that was stained using ethidium bromide, and photographed using UV light.

In order to analyze the expression of the genes, the entire RNA of the leaf tissues was extracted with the help of TRIzol reagent (Invitrogen, USA). The RNA integrity was checked by using agarose gel electrophoresis and concentrations were determined by using a NanoDrop spectrophotometer. cDNA was produced using a reverse transcription kit (Thermo Scientific, USA). qRT-PCR with SYBR Green chemistry was used to measure the levels of expression of DREB1, NAC, and HSP70 genes on a StepOnePlus Real-Time PCR System (Applied Biosystems, USA). The internal reference gene was actin, and the 2 -DDCt method was used in computing the relative expression (Livak and Schmittgen, 2001).

The PCR score was given on the presence/absence of the genes and the results of the qRT-PCR were analyzed on the basis of ANOVA to determine the variations in gene expression across the varieties. Comparison was performed at p = 0.05 using Tukey HSD test to make post hoc comparisons. The correlation coefficient tool was applied to test the relationships between the phenotypic traits and gene expression, including the score of the leaf wilting, the relative water content, and the reduction of biomass. All the analyses were done in the SPSS version 25.

The high expression varieties were also tested in terms of physiological characteristics such as relative water content, chlorophyll content, stomatal conductance, root length and biomass traits in order to confirm the candidate phenotypes of the molecular observation and use them in breeding to improve the drought tolerance (Blum, 2011).

DATA ANALYSIS & FINDINGS

Occurrence of Drought-Responsive Genes

The presence of three of the most important drought-responsive genes, DREB1, NAC and HSP70, were identified by PCR amplification in ten native wheat varieties. The gel electrophoresis showed presence of gene specific fragments that depicted each target gene. Variety W3, W6 and W9 were those that were uniformly positive in all three genes whereas the varieties W1 and W2 were negative in HSP70. The other varieties showed a partial occurrence, which meant that there was genetic variation in the distribution of genes related to drought.

Table 1: Presence/Absence of Drought-Responsive Genes in Indigenous Wheat Varieties

Variety	DREB1	NAC	HSP70
W1	+	+	–
W2	+	+	–
W3	+	+	+
W4	+	+	+
W5	+	+	–
W6	+	+	+
W7	+	+	+
W8	+	+	–
W9	+	+	+

W10	+	+	–

The findings show that all varieties had DREB1 and NAC genes, which implies that the gene is universal in drought resistance in these landraces. But the HSP70 existence is variable which suggests that varieties have different strategies in terms of their molecules in response to water stress. These results are consistent with previous works demonstrating that landraces can have different compositions of drought-responsive genes, which causes the differences in stress adaptation (Singh et al., 2015; Khodarahmpour et al., 2016).

Analysis of Gene Expression when exposed to Drought

Quantitative real time PCR (qRT-PCR) was done to determine the relative expression of DREB1, NAC and HSP70 gene under drought stress relative to well-watered controls. Normalization of expression was done using actin as a reference gene and relative quantification of expression was done by 2 -DDCt (Livak and Schmittgen, 2001).

Table 2: Relative Expression Levels of Drought-Responsive Genes in Wheat Varieties under Drought Stress

Variety	**DREB1 Expression**	**NAC Expression**	**HSP70 Expression**
W1	1.8	2.1	0.7
W2	2.0	2.3	0.6
W3	3.5	3.8	2.9
W4	2.8	3.0	1.5
W5	2.1	2.5	0.9
W6	3.2	3.5	2.7
W7	2.9	3.2	1.8
W8	2.0	2.4	0.8
W9	3.6	3.9	3.0
W10	1.9	2.2	0.7

All three genes were upregulated in varieties W3, W6 and W9 with 3.5-3.9 fold upregulation of DREB1 and 2.7-3.0 fold upregulation of HSP70. This shows that such varieties will have a high likelihood of having an improved drought tolerance on a molecular level. Contrary to this, W1, W2, W5, W8 and W10 exhibited reduced levels of HSP70 expression (<1), which indicates that protein stabilization mechanisms are less important in these genotypes during water deficit. The findings of ANOVA proved that there were significant differences in seed varieties in terms of gene expression ($p < 0.01$) and the post hoc Tukey test revealed that the best varieties in gene activation related to stress were W3, W6, and W9.

Association of Gene Expression With Physiological Characteristics

The physiological parameters, such as relative water content (RWC), score of the wilting of the leaf, chlorophyll material, root length and biomass reduction were measured to confirm the practical importance of the gene expression data. The correlation analysis of Pearson showed that there was a strong positive correlation between DREB1 and NAC activity and RWC ($r = 0.82$ and 0.79, respectively; $p < 0.01$), and these varieties that had better transcriptional factor activity were able to maintain water status despite being affected by drought. HSP70 expression was also positively correlated with root length ($r = 0.68$, $p < 0.05$) and biomass retention ($r = 0.72$, $p < 0.05$), a fact that proves the protective effect of HSP70 in cellular functioning and protein stability.

Table 3: Correlation Between Gene Expression and Physiological Traits under Drought Stress

Trait	DREB1	NAC	HSP70
Relative Water Content	0.82*	0.79*	0.55
Leaf Wilting Score	–0.75*	–0.72*	–0.50
Chlorophyll Content	0.69*	0.66*	0.60
Root Length	0.58	0.61	0.68*
Biomass Reduction	–0.70*	–0.68*	–0.72*

*Significant at $p < 0.05$

The correlations show that transcription factors DREB1 and NAC are strongly related with water retention and stress tolerance, whereas HSP70 plays a bigger role in protection at structural and protein levels. The findings reveal that drought tolerance of native types of wheat is a complex response of transcriptional regulation and molecular chaperone action.

Ranking Variety and Interpretation Statistically

Integrating molecular and physiological evidence, W3, W6 and W9 were revealed to be better drought-tolerant varieties. They were always highly expressed with respect to genes, water retention, decreased wilting of leaves and also biomass and root development during stress. On the other hand, the varieties W1, W2, W5, W8 and W10 had moderate and low molecular responses and the respective physiological decreases when subjected to drought. The result of the ANOVA showed that there was a very significant difference ($p < 0.01$) between the varieties in terms of gene expression and physiological parameters, which supports that the traits of drought tolerance are genetically diverse between indigenous wheat landraces. These results confirm other research works in India and Iran, where other types of gene-physiology correlations were also found in landrace wheat populations (Singh et al., 2015; Khodarahmpour et al., 2016).

Integrated Findings

The combined test proposes that Pakistani native wheat varieties in drought tolerance are regulated by the expression level and presence of important genes. DREB1 and NAC transcription factors mainly mediate the regulation of osmotic adjustment/water retention, whereas HSP70 aids in protein stabilization/biomass retention. The best varieties that shed light at both molecular and physiological systems, namely W3, W6 and W9, may be used as the potential breeding programs to be used in improving drought resilience. The findings have some useful implications on the genetic basis of the adaptation of drought to the local germplasm and the highlight of the significance of molecular characterization in the selection of a better genotype to be used in sustainable production of wheat.

CONCLUSION AND RECOMMENDATIONS

The results of this paper show that there is a high level of genetic variation of the significant drought-responsive genes such as DREB1, NAC, and HSP70 in indigenous wheat varieties of Pakistan, which make them adapt to water-restricted conditions. Molecular studies indicated that DREB1 and NAC were generally prevalent in all the varieties studied, and thus played a central role in the drought tolerance process, especially during osmotic adjustment, stomatic regulation and water retention. Conversely, HSP70 was found to vary in presence and expression whereas this could imply that the different varieties have different protein stabilization mechanisms. The quantitative expression analysis has shown that the varieties W3, W6 and W9 are characterized by much better expression of all three genes, which were strongly correlated with the better physiological performance of these plants in drought conditions, which is denoted by higher relative water content, less wilt of leaves, increased chlorophyll content, and the preserved biomass and root growth.

These findings verify that the drought tolerance of native wheat genotypes is an intricate characteristic comprising of transcriptional regulation and defensive molecular characteristics. Combining molecular data and physiological characteristics has led to a detailed explanation of the genetic context of drought adaptation, and this demonstrates the importance of native landraces in breeding programmes as genetic resources. The research also points out that phenotypic measurements can possibly not give a complete picture of the genetic potential of drought tolerance and that molecular characterization is required when it comes to specifically choosing better genotypes.

It is possible to make a number of recommendations based on the findings. To begin with, wheat such varieties as W3, W6, and W9 should be given precedence in breeding programs aimed at breeding successful wheat varieties in drought-prone areas of Pakistan since these varieties have been shown to respond highly to the molecular aspect in addition to their physiological capabilities. Second, the incorporation of the DREB1, NAC, and HSP70 gene-based marker-assisted selection (MAS) can fast-track the generation of drought-tolerant cultivars since it would be possible to select them at the molecular scale. Third, the promotion of conservation and utilization of the indigenous wheat germplasm should be adopted to preserve the genetic diversity which is important in the event of long-term adaptation to climatic changes. Lastly, future studies need to concentrate on the identification of more genes in response to drought including transcription factors and stress-signaling pathways and the use of sophisticated genomic technologies like transcriptome sequencing to reveal new alleles and regulatory pathways that increase stress tolerance.

To sum up, the paper gives a good indication that molecular characterization of the native wheat varieties can be used to determine the genotype that has a high drought tolerance feature. Integration of molecular understanding and conventional breeding can play a great role in ensuring sustainable wheat production in Pakistan, hence food security in those regions where water scarcity is a serious issue among others saving on precious genetic resource.

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