Pak. J. Agri. Sci., Vol. 58(2), 571-581; 2021
ISSN (Print) 0552-9034, ISSN (Online) 2076-0906
DOI: 10.21162/PAKJAS/21.1445
http://www.pakjas.com.pk

GENE EXPRESSION ANALYSIS OF SOME GENES ENCODING NF-Y TRANSCRIPTION FACTORS UNDER DROUGHT STRESS IN *Triticum aestivum* L.

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The expression of drought tolerance in wheat is based on a complex genetic mechanism and involves many genes. Gene expression is contingent upon various transcription factors which are proteins in nature, hence themselves being products of specific genes known as Transcription Factor genes. In the present studies, one such gene family; the NF-Y Gene Family has been explored for its role in conferring drought tolerance in wheat. One hundred genotypes of wheat were screened at seedling stage for morphological traits related with drought tolerance. Expression analysis of nine NF-Y genes was carried out to observe their expression patterns and drought responsiveness. The genotypes 29-SAWSN 11-12 (1014) and Chakwal-50 were the highest performers, while Nax-2 5924 was the lowest performer under drought. The reportedly drought responsive gene Traes_3AS_9151583B9.1 expressed differentially in case of drought tolerant and susceptible genotypes. Its ortholog, Traes_2BS_F23D9EA93.1 gave a similar expression pattern, thereby indicating its potential drought responsiveness. The results of expression analysis supported the results of phenotypic evaluation. The information generated might be useful for breeding drought tolerant wheat by harnessing the potential of drought responsive Ta-NFY genes.
Keywords: Drought; Expression analysis; NF-Y, Transcription factor; Wheat

INTRODUCTION

The importance of wheat as the second most produced cereal crop in the world and the leading cereal crop of Pakistan cannot be denied. Wheat is the primary food of one-third of the world population and contributes to one fifth of the total worldwide calory consumption. In view of the rampant increase in world population, the overall yield of wheat will have to be increased from 2.6 to 3.5 tonnes per hectare during the forthcoming 25 years to meet the projected demand (Saharan and Tiwari, 2011). In Pakistan, wheat contributes 1.7% to GDP and 7% to value added in agriculture (Pakistan Economic Survey, 2019-20). Wheat production is encountered by many yield limiting factors. In the semi-arid regions like Pakistan, drought is one of such major factors, and its importance as a limiting factor is destined to magnify due to gradual global warming at the rate of 0.18 ^{o}C temperature increase each decade. Drought is particularly detrimental to wheat yield if it occurs at tillering, anthesis and grain-filling stages of growth (Qadir *et al*., 2019). Its occurrence at the latter stage is assessed to cause a loss of up to 40% in grain-yield (Wollenweber *et al.,* 2003). Breeding wheat for drought tolerance and development of such cultivars that are able to furnish sustainably high yields under water limited conditions is therefore a prime focus of wheat breeders in Pakistan.

Tangible improvements in wheat for its yield and other traits have been achieved through the use of conventional techniques wherein the pedigree and bulk selection remained the breeding methods of choice due to their simplicity and effectiveness (Blum, 2011; Acquaah, 2012). These methods are generally based on classic phenotyping techniques like visual selection and are hence inaccurate to a considerable extent with high probability of erroneous selections, besides being labour intensive, destructive and slow (Moose and Mumm, 2008; Dreher *et al.,* 2009; Ahmar *et al.,* 2020).

Among the non-conventional approaches for breeding drought tolerant wheat is to harness the potential of drought responsive transcription factor genes (Singh and Laxmi, 2015). These genes are responsible for generating such proteins (called transcription factors) that regulate transcription of drought responsive genes and hence their ultimate gene expression. Studies have shown that 6-8% of the plant genomes code for Transcription Factor genes (Franco-Zorrilla *et al.,* 2014). Many of these transcription factors genes (of various families) have been reported to respond to drought and water stress in wheat. Nuclear Factor-Y (NF-Y) is one such transcription factor family that has gained attention due to its significant role in drought tolerance

(Gahlaut *et al.,* 2016). In wheat, 37 NFY TF genes under the sub-families (NF-YA, NF-YB and NF-YC and Dr1) have been identified, out of which 9 NFY and 2 Dr1 genes have been found to respond significantly to drought (Stephenson *et al.,* 2007). Although no drought tolerant cultivar of wheat developed using transcription factor genes has yet been released, nonetheless, the success of utilization of TFs in breeding other cereal crops like rice has further strengthened the idea that TFs can be utilized for developing wheat varieties (Xu *et al.,* 2006).

In the present research, drought tolerant and susceptible genotypes were identified from the material observed. Then these genotypes were characterized for presence of NFY TF genes. Thereafter, expression analysis of NFY TF genes was carried out in drought tolerant and susceptible genotypes under drought conditions. The information generated could be utilized in wheat breeding programs to exploit the potential of NF-Y transcription factors for conferring drought tolerance.

MATERIALS AND METHODS

The experiments were conducted at the Department of Plant Breeding and Genetics, University of Agriculture, Faisalabad, Pakistan. The coordinates of said area are 73.8^0 East Longitude and 31.43^0 North Latitude. The height above sea level is 184 meters. Faisalabad city, Agro-climatically, falls under the semi-arid zone where climate is characterized by extremely hot summers and mild winters.

***Plant Material and Growing Conditions*:** Plant material consisted of hundred wheat genotypes along with Chakwal-50 which was used as control being an established drought tolerant cultivar (Mahmood *et al.,* 2013; Tariq *et al.,* 2013). This material was collected from different sources including CIMMYT and Ayub Agricultural Research Institute (AARI), Faisalabad, Pakistan. The genotypes were evaluated in Hoagland solution (Hoagland and Arnon, 1938) under normal and drought conditions. The drought condition was simulated by applying 20% Polyethylene glycol (Hajihashemi and Sofo, 2018; Hellal *et al.,* 2018; Shivakrishna *et al.,* 2018) and normal condition was no Polyethylene glycol.

***Screening of Germplasm*:** Seeds of 100 genotypes along with Chakwal-50 as control were sown under hydroponic conditions using cigar roll system (Zhu *et al.,* 2005) with three replications and two treatments of normal and drought condition (20% PEG) in a completely randomized factorial design. For germination in cigars, 0.5% solution of NaOCl was used for surface-sterilization of wheat seeds for approximately 60 seconds. They were then rinsed in deionized H_2O. Cigar rolls were made by selecting 3 seeds of similar size for each genotype and were wrapped in filter paper. Two groups of 303 cigar rolls each were soaked in two separate plastic containers of Hoagland solution having 0% PEG and 20% PEG in vertical position. Data were collected on 14 days old seedlings for the following traits:

(i) Root Length, Shoot Length and Coleoptile Length (cm): The seedlings carefully taken out of cigar rolls were placed along a measuring rod. The root length was measured from the crown to the tip of root. Shoot length was measured from the crown up to tip of the longest leaf. Coleoptile length was measured from the base of the seedling up to the tip of coleoptile.

(ii) Root Fresh Weight and Shoot Fresh Weight (g): The seedlings taken out from the cigar sheet (floating on nutrient solution) were gently pressed with blotting paper to remove water from the seedling surface. The root and shoot portions of each seedling were detached from each other and weighed separately with electronic balance.

(iii) Root Dry Weight and Shoot Dry Weight (g): The same roots and shoots that were used for calculating the fresh weight were oven-dried and weighed individually on electronic balance for recording dry weight.

***Statistical Analysis*:** Data were subjected to Analysis of Variance using GenStat 10^{th} edition (Fisher, 1921). Thereafter, biplot analysis was performed using the same software to analyze the performance of genotypes over the two moisture levels (0% and 20% PEG) in order to identify drought tolerant and susceptible genotypes (Gabriel, 1971).

***Bioinformatic analysis and primer designing*:** The complete set of NF-Y transcription factor protein sequences found in wheat was downloaded from the global genomic/DNA and TF resource http://planttfdb.cbi.pku.edu.cn/ (Plant Transcription Factor Database, accessed on 14-09-2015), an online repository maintained by the Center for Bioinformatics, Peking University, China from where 76 protein sequences (22 of NF-YA, 34 of NF-YB and 20 of NF-YC) were reported to be found in bread wheat (*Triticum aestivum L.*). These protein sequences were blasted for obtaining the corresponding cDNA sequences using TBLASTN facility available at https://www.ncbi.nlm.nih.gov (URL for the National Center for Biotechnology Information official website, accessed on 17-12-2015). Besides construction of phylogeny, the relevant genomic and transcript sequences were also obtained from https://phytozome.jgi.doe.gov/, the Plant Comparative Genomics portal of the Department of Energy's Joint Genome Institute, JGI, University of California.

***Gene specific primer amplification*:** The DNA of drought tolerant and susceptible genotypes identified from the initial screening was extracted using CTAB method. After DNA extraction, a confirmatory test was done to check the quality of DNA by running the samples on 1% agarose gel stained with ethidium bromide along with 1 kb DNA ladder, and visualized under UV light. Moreover, quantification of DNA was determined by using Nanodrop 2000 spectrophotometer. After obtaining good quality DNA, the pooled DNA was subjected to gradient PCR to obtain and optimize the annealing temperature for each primer.

The isolated DNA of drought tolerant and susceptible genotypes were then subjected to PCR amplification using specific primers for selected drought responsive NF-Y genes along with those of phylogenetically related genes (but not reported with respect to drought responsiveness). Visual scoring was done through ethidium bromide stained agarose gel electrophoresis and documentation.

Gene expression analysis

(i) Growing of plant material: The most drought tolerant, and the most drought susceptible genotype, along with control (total three genotypes) were carried further for expression analysis of the NF-Y genes detected. The three genotypes were grown in Hoagland solution under hydroponic conditions with two treatments of Polyethylene glycol (0% and 20%) and two replications in a completely randomized factorial design.

(ii) RNA isolation and synthesis of cDNA: RNA was isolated from roots and shoots separately at three leaf stage. The isolations were made by using Trizol method (Chomczyński and Mackey, 1995) at 0, 0.75, 1.5, 3, 6, 12, 24, 48, 96 and 192 hours of the application of polyethylene glycol. The presence of RNA was confirmed through gel electrophoresis. Quantification of RNA was done through the Nanodrop 2000 spectrophotometer. Thereafter, cDNA was prepared using high-quality RNA following the protocol of Thermo Fisher Scientific (catalog number: K1622).

(iii) Quantitative RT-PCR: Before proceeding for Quantitative (Real Time) PCR, the cDNA was subjected to Semi-quantitative PCR (Semi qPCR). The Semi qPCR was performed individually for all primers over all the three genotypes under study. Its protocol is the same as gene specific primer amplification with the only difference that cDNA (instead of directly isolated DNA) is used (Walker *et al.,* 2003). Following the Semi-Q PCR reactions, gel electrophoresis was used to confirm the results. The Ta-NFY genes whose primers visually exhibited expression were carried forward for qPCR Analysis (Real Time PCR) for determining their level of expression (Heid *et al.,* 1996). The qPCR primers were designed from Complementary DNA Sequences (CDS) of each selected gene, keeping the amplicon length within the range of 200 to 250 base pairs to ensure accurate quantification of expression. For Real Time PCR (qPCR) analysis, each reaction was performed in a final volume of 10ul, containing 5ul of SYBR Green PCR Master Mix, 0.5ul each of forward and reverse primers, 1ul of cDNA template and 3ul of nuclease free water. Initial denaturation was carried out at 95 ºC for 10 mins, followed by 40 cycle of 15 seconds at 95 ºC and 60 seconds at 60 ºC. Actin-2 (TC234027) was used as control and qPCR data were calculated/presented in terms of relative expression.

RESULTS

***Screening of Genotypes*:** The 100 wheat genotypes along with Chakwal 50 (cultivar of rainfed areas) were screened over two levels of moisture (0% and 20% PEG) under hydroponic conditions. Significant variation ($p \leq 0.01$) was found among wheat genotypes for all the traits under study. Biplot analysis classified the genotypes into drought tolerant and susceptible genotypes and gave their comparative performances over the normal (0% PEG) and drought (20% PEG) conditions (Fig. 1). The genotypes assessed as drought tolerant and susceptible along with their mean values for each trait are mentioned in Table 1. On an overall basis, 29-SAWSN 11-12-1014 (genotype no. 40 in Fig. 1) and Nax-2-5924 (genotype no. 1 in Fig. 1) exhibited the best and the worst performances under drought conditions respectively.

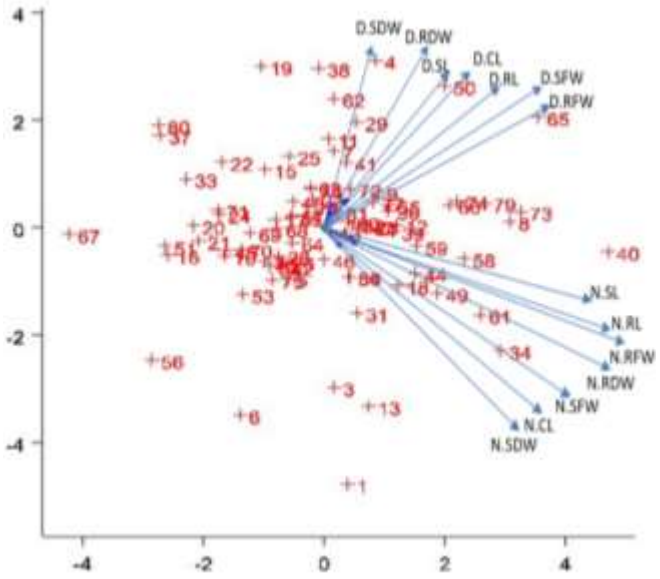

Figure 1. Biplot Analysis for screening of 100 wheat genotypes along with control under normal and drought conditions.

Phylogenetic analysis of Ta-NFY gene family: The phylogeny of NF-Y transcription factor protein sequences was constructed using MEGA software for assessing the evolutionary relationships among the sequences, shown in Fig. 2.

The NF-Y gene family consists of three sub-families, NF-YA, NF-YB and NF-YC, consisting of 22, 34 and 20 genes respectively (total 76 genes). Of these NFY genes, 4DL_F512146B2.2, 2BL_3237AA694.1, 6AL_C0E74F959.2, 6BL_953171851.2, 2DL_DA577AF57.1 and 3AS_9151583B9.1 have been reported to respond to drought in *Triticum aestivum L*. (Stephenson *et al.,* 2007). These drought responsive gene sequences along with such other sequences that are

Table 1. Mean value of traits in respect of selected genotypes screened for drought tolerance and susceptibility.

Performance	Genotype	Condition	Trait						
			Root length	Shoot length	Root fresh weight	Root dry weight	Shoot fresh weight	Shoot dry weight	Coleoptile length
High performers	Galaxy-13	Normal	5.25	5.97	0.52	0.34	0.33	0.24	2.97
		Drought	4.17	4.52	0.34	0.30	0.18	0.17	2.87
	UAF-AL-9801	Normal	4.85	5.79	0.41	0.36	0.28	0.14	3.47
		Drought	4.47	4.03	0.30	0.30	0.17	0.13	3.27
	Suleman-96	Normal	4.54	6.02	0.37	0.35	0.25	0.19	4.70
		Drought	4.39	4.77	0.27	0.29	0.14	0.13	3.23
	Chakwal-86	Normal	4.93	6.20	0.42	0.35	0.28	0.20	3.50
		Drought	4.66	3.43	0.35	0.29	0.13	0.11	2.90
	Chakwal-50	Normal	7.74	8.05	0.65	0.57	0.43	0.28	6.50
		Drought	6.99	6.40	0.56	0.55	0.25	0.19	3.50
	Nax-1 (5907)	Normal	5.32	5.39	0.38	0.36	0.29	0.18	4.27
		Drought	4.35	4.75	0.33	0.29	0.15	0.11	3.23
	29-SAWSN-11-12 (1014)	Normal	6.69	7.84	0.57	0.55	0.39	0.25	6.60
		Drought	5.85	7.27	0.56	0.52	0.27	0.22	5.83
	STW-739	Normal	4.95	5.51	0.40	0.40	0.27	0.15	3.09
		Drought	4.41	4.38	0.31	0.30	0.18	0.16	3.07
	76	Normal	5.27	5.02	0.52	0.34	0.31	0.25	2.77
		Drought	4.22	4.77	0.34	0.32	0.19	0.18	2.62
	1985	Normal	4.91	6.29	0.41	0.37	0.30	0.23	3.57
		Drought	4.61	4.78	0.37	0.30	0.16	0.15	2.95
	10113	Normal	7.14	7.87	0.67	0.52	0.41	0.24	6.42
		Drought	6.31	5.41	0.51	0.45	0.37	0.15	4.53
	1916	Normal	5.34	5.01	0.31	0.35	0.30	0.19	4.65
		Drought	4.71	4.70	0.30	0.31	0.19	0.15	3.83
Low performers	Kharchia-65	Normal	4.69	4.76	0.44	0.35	0.38	0.20	5.20
		Drought	5.46	3.25	0.29	0.30	0.14	0.15	3.27
	Nax-2 (5924)	Normal	3.03	4.05	0.37	0.21	0.27	0.15	2.77
		Drought	2.87	2.63	0.27	0.16	0.11	0.08	2.63
	ZA-2	Normal	5.70	3.56	0.46	0.30	0.29	0.20	3.12
		Drought	5.89	3.40	0.32	0.27	0.14	0.14	2.97
	Rohtas-90	Normal	7.09	6.62	0.45	0.34	0.39	0.20	3.03
		Drought	5.38	6.19	0.40	0.28	0.16	0.16	2.93
	ZA-4	Normal	5.64	3.28	0.52	0.32	0.30	0.27	3.17
		Drought	4.39	3.43	0.41	0.26	0.17	0.18	3.07

phylogenetically related to them (thirty two in total) were selected to design the full-length primers for further use in DNA amplification and expression analysis shown in Table 2.

***Characterization of core set of wheat*:** Ten of the Ta-NFY primers were optimized. Details of optimized primer sequences along with their annealing temperatures are given in Table 3.

***Gene Expression Analysis*:** SemiQ qPCR using the 9 optimized (and amplified) primers was carried out before proceeding for qPCR. The results of Semi- qPCR analysis are presented in Fig. 3.

In Fig. 3, the genotypes V1, V2 and V3 stand for 29-SAWSN 11-12 (1014), Nax-2 5924 and Chakwal-50 which are, the most drought tolerant, the most drought susceptible (as assessed via phenotypic evaluation) and the known drought tolerant check variety respectively. Moreover, the portion/bands at Serial No. 1 to 10 of each gel depict the temporal RNA isolation at 0 hours (normal moisture conditions), 0.75 hrs, 1.5 hrs, 3 hrs, 6 hrs, 12 hrs, 24 hrs, 48 hrs, 96 hrs and 192 hrs after application of drought conditions (20% PEG).

Since, UIK 7 and UIK 32 gave little or no expression they were not analyzed further. The remaining (expression exhibiting) gene primers *i.e*. UIK 1, UIK 8, UIK 9, UIK 10, UIK 26, UIK 28 and UIK 32 were carried forward for the qPCR analysis. For this purpose, primers were designed manually

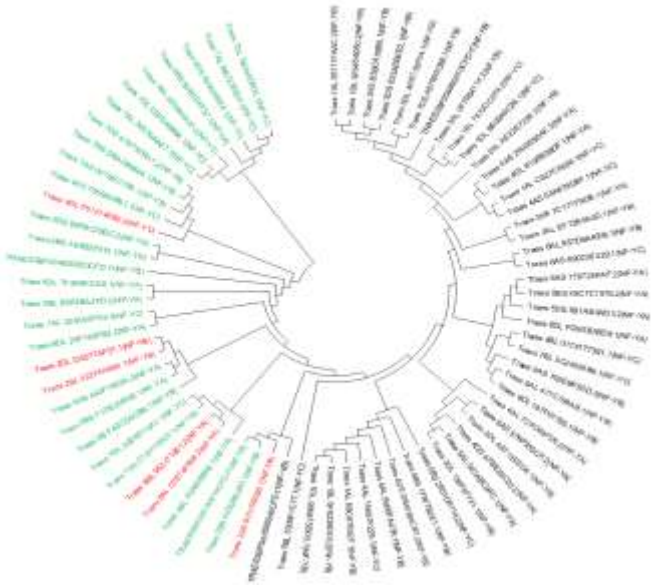

Figure 2. Phylogeny of NF-Y Transcription Factor genes in *Triticum aestivum L.* Genes highlighted in "Red" depict the already reported drought responsive Ta-NFY genes, while the ones highlighted in "Green" depict the Ta-NFY genes for which full length primers were designed (in addition to those designed for reported drought responsive genes).

Table 2. Forward and reverse full-length primers for selected Ta-NF-Y genes.

Sr.	Gene ID	Sub-family	Forward	Reverse
1	Traes_5DS_443F1993A.2	NFYA	CATCATCAGCTGGAGCTGGA	GATATTTCGGCTGTCCGTCA
2	Traes_4BL_7DA93696E.1	NFYA	CCGATGCAATCGCGTCAGTGA	GGATGATTTGCCAGGGGGCAAA
3	Traes_4BS_B7B7EFF37.1	NFYA	CGTGCGTGTCAACCTGTCA	GGTTGCATCAAGGTGCCACT
4	Traes_6DL_29F162FB2.2	NFYA	GTGCTCCTTGGAACAATGGA	CAGAGGACCCCTTGTTACGT
5	Traes_2DS_69FBC78BC.2	NFYA	GTTGCCGCTGCAACTGTCTTT	CAGCGCACCAGAGGATGCTTT
6	Traes_5BS_F1DE20B4B.1	NFYA	GTTGGTGGGCGCTTAGTCA	GATATTTCGGCTGCCCGTCA
7	**Traes_6AL_C0E74F959.2**	**NFYA**	**CGGCGGAGGCAA\GCTAGTTT**	**GGGCTGCAAAGCACTGTGAGA**
8	**Traes_6BL_953171851.2**	**NFYA**	**CGTCGGAGGCAAGCTAGCTT**	**CCATGAGACACACTATCTCTGA**
9	Traes_5DL_7F3ABFDD6.1	NFYA	CTTCCTTGGCAAAGATAGGCA	GATCTGGGTGCCTGCGTATA
10	Traes_5BL_B5F4BA2FD.2	NFYA	CTTCCTTGGCAAAGCTAGGCA	CTTGATCTGGGTGCCTGCGTA
11	Traes_2BS_A95B28510.1	NFYA	GTGGCTTCTTGGTTCTTGATT	GCGCACCAGAGGGTGCTTT
12	Traes_7DS_A1673EBA1.2	NFYB	CCTTGGCTAGCTCTAGCCA	CAGTACGCAGGACGCAGAA
13	Traes_3B_03BE0E869.2	NFYB	CGCATCATGAGGAAGGCCAT	CAGCTGCACCTGTTACAGCT
14	Traes_7BS_DBAD99848.1	NFYB	GACCGTCAACGCCGAAGACAT	CGCTGCACCTGATTCTGAACCT
15	Traes_3B_FA3CD3CB6.1	NFYB	CAAACACATGTACAGCAAACT	GTTCCGCTTGCAGTTGTGTTA
16	**Traes_2BL_3237AA694.1**	**NFYB**	**CCTCCTCTCACTCACCTAGTA**	**GCCACATCGCTCGAATATTTCA**
17	**Traes_2DL_DA577AF57.1**	**NFYB**	**CTGCTGCTAGCTAGCTAGCTA**	**CTCGTGGGCACATCGCTCGA**
18	Traes_2BS_F23D9EA93.1	NFYB	GAGTTGCTCTGTTTCTTGGCT	GTCATGCCCTGTCTTGCCT
19	Traes_7AS_067BEC7B5.1	NFYB	GCCATGGAAAACGACGGCGT	GTAGTATTGCTGGCCCATGACA
20	Traes_3B_4D37853E1.1	NFYB	GCGGATCATGAAGCAGGTCCT	CTGCCTGATCTGATGATGGAGT
21	**Traes_3AS_9151583B9.1**	**NFYB**	**GTTCACAGCTCCATCCCTTCTT**	**CACTCACTTTGGTCAGCTCAAA**
22	Traes_7DL_52D5D13A1.1	NFYC	CAGTGCTCAGGAGCAGCTTA	CTGAAACGAGGGCTCTGAAT
23	Traes_1DS_C1D7139D7.1	NFYC	CGCCCATTTCTCGGTTGGTT	CACCACCTTGTCTGTCCATT
24	Traes_7DL_D97D4666A.1	NFYC	CTTGGAAACCTCAGCTTGCA	GGCTCACATGTTGTCAGCCA
25	Traes_7AL_BB7235524.1	NFYC	GCAAGATCAAGCAGTCGACA	CCACGACCTCAGTGTCAACT
26	**Traes_4DL_F512146B2.2**	**NFYC**	**GCAAGCTAGATCGCAATGGA**	**GCATACAGCTCTGGCATCGA**
27	Traes_6DS_8436285E5.1	NFYC	CCGTCTCCTACGTCCTCCTT	CATACCAGCATCGAAGCTACCA
28	Traes_4AS_73658DB91.1	NFYC	CTTGCAGATCGCAAGCTAGATA	CATGCAGCTCTGGCATCGAT
29	Traes_7AL_34519AA27.1	NFYC	CTTTGGCAACGTCAGCTTGCA	GGAACTTTGCATGTCCTGGCTA
30	Traes_1AL_323AE5FA3.4	NFYC	GAGAAGATTAGGTAGCTAGCTA	GCATGCATTGCACCGACAAGT
31	Traes_7BL_0258A9A33.2	NFYC	GATCGAGAGATTCCAGTTCCA	GGTGGTTGCTCACAATGTTGT
32	Traes_7DL_3B340D60A.1	NFYC	GGACAAGCAAGACCAAGCAGT	CTAAACTGAGGGTACTGAATCT

Note:- Serial No. 7, 8, 16, 17, 21 and 26 are already reported to be drought responsive (Stephenson *et al*. 2007)

Table 3. Optimized Ta-NF-Y gene primers with their annealing temperatures.

Sr.	Gene ID	Sub-family	Primer ID	Amplicon length	Forward primer	Reverse primer	Annealing temp. (^{O}C)
1	Traes_7BS_DBAD99848.1	NFYB	UIK09	309	GACCGTCAACGCCGAAGACAT	CGCTGCACCTGATTCTGAACCT	60.8
2	Traes_7AL_BB7235524.1	NFYC	UIK10	460	GCAAGATCAAGCAGTCGACA	CCACGACCTCAGTGTCAACT	55.6
3	Traes_7DL_52D5D13A1.1	NFYC	UIK01	645	CAGTGCTCAGGAGCAGCTTA	CTGAAACGAGGGCTCTGAAT	57.4
4	Traes_2BS_F23D9EA93.1	NFYB	UIK26	713	GAGTTGCTCTGTTTCTTGGCT	GTCATGCCCTGTCTTGCCT	55.4
5	Traes_4DL_F512146B2.2	NFYC	UIK11	764	GCAAGCTAGATCGCAATGGA	GCATACAGCTCTGGCATCGA	55.4
6	Traes_7DL_D97D4666A.1	NFYC	UIK08	774	CTTGGAAACCTCAGCTTGCA	GGCTCACATGTTGTCAGCCA	55.4
7	Traes_4BS_B7B7EFF37.1	NFYA	UIK07	1877	CGTGCGTGTCAACCTGTCA	GGTTGCATCAAGGTGCCACT	56.3
8	Traes_7AS_067BEC7B5.1	NFYB	UIK28	672	GCCATGGAAAACGACGGCGT	GTAGTATTGCTGGCCCATGACA	60.2
9	Traes_7DL_3B340D60A.1	NFYC	UIK30	841	GGACAAGCAAGACCAAGCAGT	CTAAACTGAGGGTACTGAATCT	56.6
10	Traes_3AS_9151583B9.1	NFYB	UIK32	899	GTTCACAGCTCCATCCCTTCTT	CACTCACTTTGGTCAGCTCAAA	57.8

Each optimized Ta-NFY gene primer was then amplified for the isolated DNA of 17 genotypes screened for drought tolerance and susceptibility. Of the 10 optimized primer, the amplification of UIK 11 was negligible.

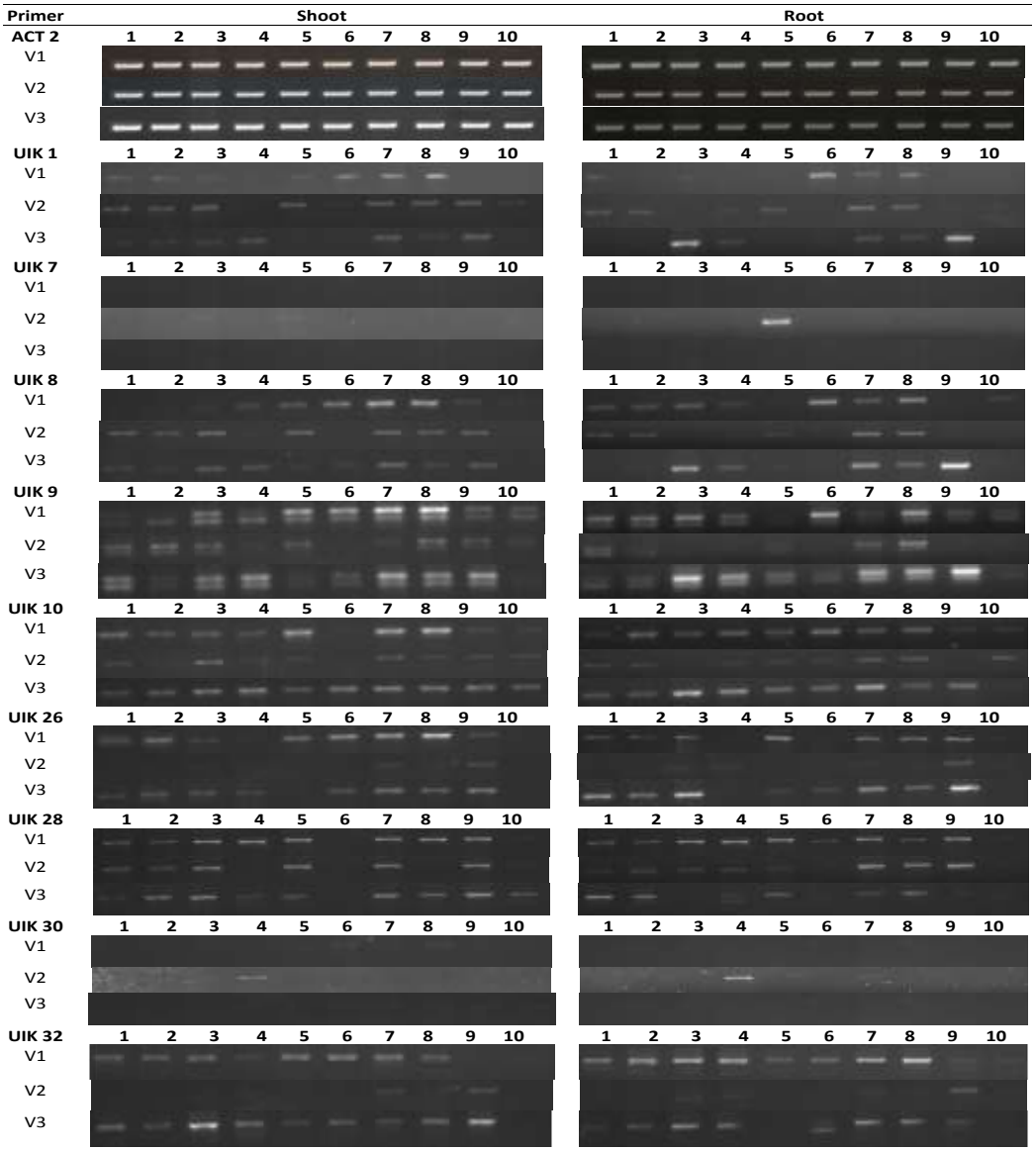

Figure 3. Spatial and Temporal Semi-Quantitative PCR based expression in three wheat genotypes: 1014 (V1), Nax-2 5924 (V2) and Ch-50 (V3). The numbers 1 to 10 represent the ten RNA isolation times *i.e.* 0, 0.75, 1.5, 3, 6, 12, 24, 48, 96 and 192 hrs after drought induction.

Table 4. Ta-NFY gene primer sequences designed for Quantitative (Real Time) PCR.

Sr.	Gene ID	Primer ID	Sub-family	Amplicon Length	Annealing Temp.	Forward	Reverse
1	Traes_2BS_F23D9EA93.1	UIK 26	NFYB	211	58.5	GCGAGAAGCGCAAGACCATCAA	CCATTGTTCCTTGGCGTCGACT
2	Traes_7AS_067BEC7B5.1	UIK 28	NFYB	228	59.5	CTGATGCCGATCGCAAACGTGA	GGGCACGACGTAGTCGTCGAA
3	Traes_7BS_DBAD99848.1	UIK 9	NFYB	216	59.5	CTGATGCCCATCGCGAATGTGA	GTCGTCGAAACCGAGGCGGTT
4	Traes_7AL_BB7235524.1	UIK 10	NFYC	227	56.6	GGAACCGTCTTCACAACCTCA	GCTGGAGCTGCTGGTGAAATT
5	Traes_7DL_52D5D13A1.1	UIK 1	NFYC	232	59.7	GTACGAGCAGTCGCAGGAGTA	CTCCAGTGTGAACATCTCGCAT
6	Traes_7DL_D97D4666A.1	UIK 8	NFYC	251	59.7	GTGATGTTGCTGGTGGATCACA	CTGCTCGATCTCTGACCGTTGT
7	Traes_3AS_9151583B9.1	UIK 32	NFYB	254	58.5	CTTCCTGCCCATCGCCAACGT	GGAACTTGTGGAGGTAGTGCTT

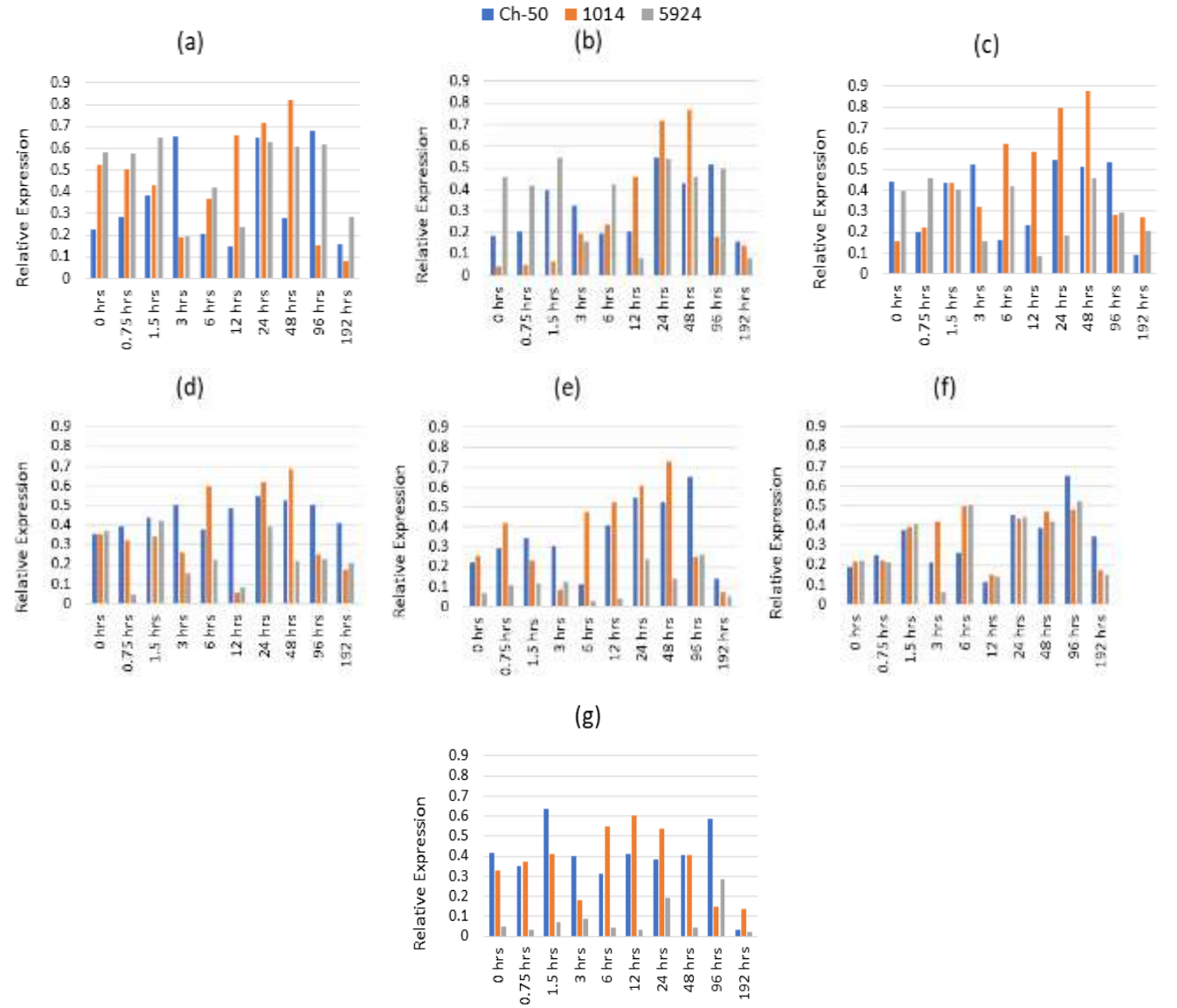

Figure 4. Relative Gene expression of selected Ta-NFY genes: The charts (a), (b), (c), (d), (e), (f) and (g) depict relative expression among shoots of three genotypes Ch-50 (Chakwal-50), 1014 (29-SAWSN-134-11-12) and 5924 (Nax-2) for the genes Traes_7DL_52D5D13A1.1 (TaNFYC-HAP5), Traes_7DL_D97D4666A.1 (TaNFYC-CCAAT related), Traes_7BS_DBAD99848.1 (TaNFYB-B6 related), Traes_7AL_BB7235524.1 (TaNFYC-CCAAT related), Traes_2BS_F23D9EA93.1 (TaNFYB-unknown), Traes_7AS_067BEC7B5.1 (TaNFYB-B6 related) and Traes_3AS_9151583B9.1 (TaNFYB-unknown). Actin 2 (TC234027) was used as a housekeeping gene.

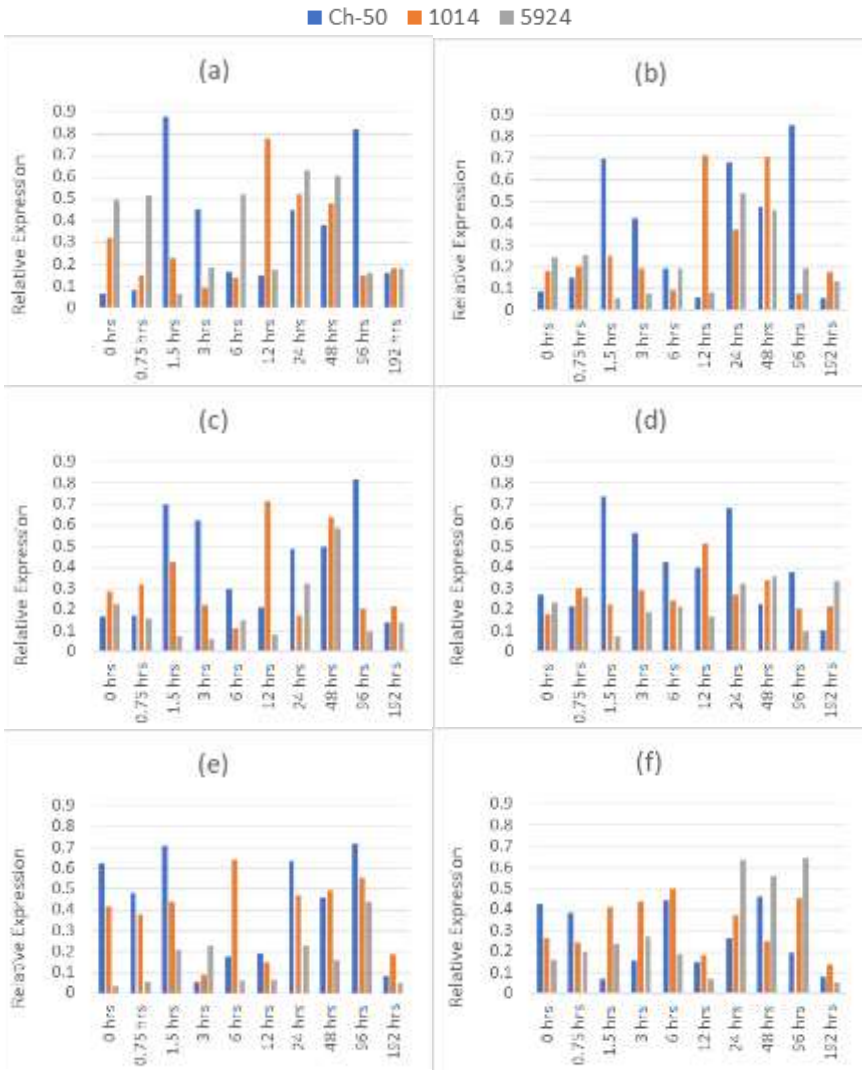

Figure 5. Relative Gene expression of selected Ta-NFY genes: The charts (a), (b), (c), (d), (e), (f) and (g) depict relative expression among roots of three genotypes Ch-50 (Chakwal-50), 1014 (29-SAWSN-134-11-12) and 5924 (Nax-2) for the genes Traes_7DL_52D5D13A1.1 (TaNFYC-HAP5), Traes_7DL_D97D4666A.1 (TaNFYC-CCAAT related), Traes_7BS_DBAD99848.1 (TaNFYB-B6 related), Traes_7AL_BB7235524.1 (TaNFYC-CCAAT related), Traes_2BS_F23D9EA93.1 (TaNFYB-unknown), Traes_7AS_067BEC7B5.1 (TaNFYB-B6 related) and Traes_3AS_9151583B9.1 (TaNFYB-unknown). Actin 2 (TC234027) was used as a housekeeping gene.

from CDS keeping in view the primer designing rules/requirements. These are mentioned in Table 4 and the qPCR results in terms of relative expression are presented in Fig. 4 and Fig. 5.

DISCUSSION

The basic objective of this study was to carry out screening, genotyping and expression analysis of selected Ta-NFY genes on the germplasm. Phenotypic screening was carried out to identify drought tolerant and susceptible genotypes manifested in the form of high performers and low performers under drought conditions. Following screening, genotyping of the selected material was done through gene specific primer amplification using selected NFY genes. Finally, spatial and temporal expression analysis of selected NFY genes was carried out under drought to determine which NFY genes are drought responsive and hence might have a role in conferring drought tolerance in wheat.

Root length, shoot length, root fresh weight, root dry weight, shoot fresh weight, shoot dry weight and coleoptile length were the traits used for screening of germplasm at seedling stage. Vigorous seedlings are very important to determine the overall drought tolerance of the plant and ultimately translate

into better yields (Misra, 1990; Chowdhry *et al.*, 1999). All these traits were positively corelated with drought tolerance. Seedling traits generally give medium to high variability across environments with additive gene action, and can proved to be effective selection criteria at early stage of the plant (Rauf *et al.*, 2008; Hameed *et al.*, 2010; Ahmed *et al.*, 2019). Water stress affects the roots first, hence longer roots with more weight provide the seedling with more access to soil moisture (Ahmad *et al.*, 2014). Ability to develop healthy shoots with greater length and weight under water stressed conditions is also an indication of drought tolerance of the plant (Faisal *et al.*, 2017; Basal and Szabo, 2020). Coleoptile is a sheath that protects the young shoot tip. Its length is one of the major indices that reflect the ability of a genotype to resist desiccation and water deficits at early stages of growth (Dhanda *et al.*, 2004; Rebetzke *et al.*, 2005; Mohan *et al.*, 2013). Seventeen genotypes (out of 100) were identified through Biplot analysis, that included the best (tolerant) and the worst (susceptible) performers under drought. These were namely Suleman-96, UAF AL-9801, Galaxy-13, 29SAWSN 11-12 (1014), Nax-1 5907, Chakwal-50, Chakwal-86, 1916, STW 739, 76, 1895, 10113, Rohtas-90, K-65, ZA-2, Nax-25924 and ZA-4. The genotypes on the extremes were 29-SAWSN 11-12 (1014) which was the highest overall performer and Nax-2 5924 the lowest performer under drought. Whereas, Chakwal-50, a known drought tolerant check variety, also performed well under drought in this study. From these screening and biplot analyses, two genotypes namely 29-SAWSN 11-12 (1014) and Nax-2 5924 were identified to be the most tolerant and the most susceptible genotypes.

The best PCR amplification was observed for UIK 9, 8, 10, 1 and 32. Whereas, the primer UIK 11 (despite being optimized) could not be properly amplified. The amplicon length of each primer was observed to have profound effect on amplification results and the quality of PCR reaction. Generally, primers having amplicon lengths within 1000 bp yielded good amplification, while those having amplicon lengths beyond 1000 bp did not yield good quality results. This implies that the quality of PCR reaction deteriorates with the increase in amplicon length of primer applied and the concurrent increase in extension time of the reaction. Based on PCR amplification, the primers/genes UIK 8, 9, 10, 1, 26, 28, 32, 7 and 30 were carried forward for expression analysis of these genes. Amplification of a gene does not necessarily imply that it is actually expressing in the individual. Such validation is only possible through qPCR or at least Semi-Q-PCR expression analysis.

The Semi-Q PCR results are shown in Fig. 3. As it appears from the figure, UIK 7 and UIK 30 did not exhibit any mentionable expression in any of the three genotypes. In case of other primers, it can be generally observed that the most prominent expression of genes occurred at stages 7 and 8 (*i.e.* 24 hours and 48 hours after application of PEG). Also, it can be easily observed in the figure that the expression was silenced at stage 10 *i.e.* after 192 hours of the application of PEG. Moreover, in most cases (except for UIK 9, 10 and 32), the expression generally commences at the initial stages (1, 2, and 3 etc.), then diminishes at stages 4, 5 and 6, only to reappear at stages 7, 8 and 9. This shows that the expression of these genes generally proceeds in rhythmic fashion at the initial stages of the application of drought. The reduction in gene expression might be due to the role of certain genes as negative regulators in oxidative stress response (Zhang *et al.*, 2020). The appearance of rhythmic expression patterns is also supported by previous studies which show that plant gene expression is often exhibited in an oscillating manner in response to abiotic stress (Grundy *et al.*, 2015; Kuintzle *et al.*, 2017). Furthermore, in these studies, the expression was overall more profound in shoots relative to roots at the same stage of the application of drought.

The Ta-NFY gene Traes_3AS_9151583B9.1 (represented by primer UIK 32) is a reportedly drought responsive gene and it can be observed from electrophoresis (Fig. 3) that the genotypes that were phenotypically evaluated/labelled as drought tolerant and drought susceptible gave differential expression for the said gene. Specifically, the expression pattern for the said gene in case of two drought tolerant genotypes 29-SAWSN 11-12 1014 (V1) and Chakwal-50 (V3) was similar to each other, while it was distinctively different for Nax-2 5924 (V2) wherein the gene expression appears to be negligible. In the light of the already reported fact that Traes_3AS_9151583B9.1 is a drought responsive gene, these differential expression patterns (between drought tolerant and susceptible genotypes) thus validate the results of the initial phenotypic screening experiment. A similar expression pattern was observed for the gene Traes_2BS_F23D9EA93.1 (represented by primer UIK 26) wherein the gene expression for 29-SAWSN 11-12 1014 (V1) and Chakwal-50 (V3) is similar, and collectively different from that of Nax-2 5924 which does not exhibit the same levels of expression at all stages drought conditions. It is also to be noted here that these two genes namely Traes_3AS_9151583B9.1 (UIK 32) and Traes_2BS_F23D9EA93.1 (UIK 26) are phylogenetically related to each other as evident in the Fig. 2. Also, both of them belong to Sub-family NFY-B. Therefore, it is concluded that Traes_2BS_F23D9EA93.1 (UIK 26) is also a potentially drought responsive gene, that has not been reported earlier in the literature. However, nine other NF-Y genes have already been reported as drought responsive in previous studies, indicating that the potential of NF-Y gene family can be harnessed for breeding drought tolerant wheat (Stephenson *et al.* 2007). The real-time expression of these two genes under discussion assessed via qPCR analysis also gave similar patterns in terms of relative expression as presented in Fig. 4 and Fig. 5. Since, the expression levels of these two genes are significantly higher among drought tolerant genotypes as

compared to the susceptible ones, it can therefore be inferred from these results that up-regulation of both these genes enhances the ability of a plant to endure drought conditions and to exhibit drought tolerance. The remaining five genes (coded UIK 9, 10, 1, 8 and 28), the expression patterns in both Semi-q and qPCR analyses were similar for all of three genotypes and did not sufficiently differentiate between drought tolerant genotypes and the susceptible ones. Therefore, it may be stated that possibly these genes are not drought responsive.

For the sake of validity of these conclusions, the Semi-q PCR analysis for all the seven genes selected for expression analysis and qPCR analysis for the genes Traes_3AS_9151583B9.1 (UIK 32) and Traes_2BS_F23D9EA93.1 (UIK 26) were repeated three times, each time using a new set of reagents.

***Conclusions*:** Based on the analysis of phenotypic screening data, the genotypes 29-SAWSN 11-12 1014 and Nax-2 5924 were assessed as drought tolerant and drought susceptible genotypes respectively. The gene expression analyses carried out via Semi-q and qPCR protocols conformed with the results of phenotypic evaluation, as the drought responsive genes namely Traes_3AS_9151583B9.1 (already reported) and Traes_2BS_F23D9EA93.1 (potential) gave differential expression among the genotypes that had been assessed as drought tolerant and susceptible based on screening data. On an overall basis, the genotypes that performed well under normal conditions also generally performed well under drought conditions. The expression level of any Ta-NFY gene at any stage of drought was greater in the shoot than in the root of the same plant in all genotypes, tolerant or sensitive. Expression of Ta-NFY genes proceeded in a wave-like rhythmic fashion. At seedling stage (three leaf stage), the greatest level of gene expression was exhibited between 24 hours and 96 hours after application of drought. The reportedly drought responsive gene namely Traes_3AS_9151583B9.1 (UIK 32) behaved as expected, hence supporting the previous studies regarding its drought responsiveness. The Ta-NFY gene Traes_2BS_F23D9EA93.1 (UIK 26) which is phylogenetically related and evolutionarily close to Traes_3AS_9151583B9.1 (UIK 32) was also assessed as drought responsive according to these studies. It is further concluded that up-regulation of both these genes may confer drought tolerance in wheat.

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[Received 12 Dec 2020; Accepted 26 March 2021; Published (online) 18 April 2021]