Transcriptional repressor gene, *TaDr1*:

Expression analysis under drought conditions in bread wheat from different countries

By

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ABBREVIATION

ALFP	Amplified fragment length polymorphism
Dr1	Down-regulator Transcription 1
DREB	Dehydration-responsive element-binding
DW	Dry Weight
FW	Fresh Weight
GWAS	Genome Wide Association Studies
MAS	Markers Assisted Selection
NFY	Nuclear Factor Y
PCR	Polymerase chain reaction
QTL	Quantitative Trait Loci mapping
RAPD	Random amplified polymorphic DNA
RBR	Relative Biomass Ratio
RFLP	Restriction Fragment Length Polymorphism
RT-qPCR	Reverse Transcription- quantitative Polymerase Chain Reaction
SHWs	Synthetic Hexaploid Wheats
SNP	Single Nucleotides Polymorphism
TFs	Transcriptional factors

ABSTRACT

Wheat is one crucial staple cereal crop of great significance to the human population. It has, and continues to, contribute to the livelihoods of many human beings including benefits of their economy and culture. Wheat production is threatened by drought stress. The impact of drought stress in wheat production calls for development of drought tolerant genotypes. Breeding for drought tolerance is a challenging process as its genetic control is complex and is influenced by environmental conditions. Researchers have identified some of the genes which can be influenced by environmental conditions and involved in the regulation of gene expression which are called Transcription factors. Among other transcription regulators, one regulatory protein encoded by the *TaDr1* gene has been reported to be upregulated under drought conditions. *Dr1* is a transcriptional repressor and couples with *DrAp1* to execute repressive transcriptional activity. In this study, the expression of *TaDr1* under different levels of drought stress was evaluated and compared across six wheat accessions originating from different countries. Results indicated that VK-1 from Kazakhstan was more drought tolerant and SST-398 was more drought sensitive compared to other accessions under greenhouse conditions. However, the trends of drought tolerance in wheat accessions from Australia under conditions were slightly different from field trails results observed by seed providers but both greenhouse and field trails findings were similar in Kazakhstan and South- African accessions. Further analysis of expression profile revealed that two homeologs of *TaDr1* were upregulated under drought conditions in most of the accessions. Moreover, results suggested that *TaDr1a* had strong influenced on *TaDr1* consensus expression profiles. This indicates differences between greenhouse and field trials experiments. It was concluded that *TaDr1* was drought responsive and strongly genotype-dependent which confirmed by other researchers.

DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

KATSO LETHOLA

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CHAPTER 1.0: LITERATURE REVIEW

General Introduction

Wheat is one of the leading cereal crops that feed the world's population, however its yield is threatened by drought stress which is only exacerbated by climate change (Farooq et al. 2014). It can be expected that under the current rate of change in global temperatures, there will be a significant reduction of wheat production, which poses a threat to meeting the growing world population food demand estimated by 2050 (Lobell et al. 2011; Ray et al. 2013). Prolonged drought stress reduces wheat yield, and this has been reported in major wheat growing countries such as Australia (Stephenson et al. 2007). For example, in 2006, a 46% reduction in Australian wheat production was recorded that could be attributed to drought stress (van Dijk et al. 2013). Reduction in crop productivity associated with drought can also lead to a shortage of other major staple crops such as maize and rice (Karim & Rahman 2015). Impacts of drought stress in wheat demonstrate the urgent need to develop drought tolerant wheat cultivars which can meet the demands of the expected 2050 population. Development of drought tolerant wheat genotypes is a challenging process as drought tolerance is a complex trait which involves multiple responses at levels ranging from genetic, biochemical, morphological, and physiological (Nezhadahmadi et al. 2013). In addition, wheat has a hexaploid genome making identification of genetic control and subsequent breeding a complicated process. Both of these factors will be discussed in greater detail below.

This study will be looking at how molecular responses, with an emphasis on gene expression patterns, change under drought conditions, and how this information may be used to improve drought tolerance. Studying gene expression patterns may provide insightful information about the regulation of genes that are drought responsive, and this can be useful in the development molecular markers linked to morphological and

phenological traits important in drought tolerance enhancement. Recent studies have shown that overexpression of a gene, which was identified as drought responsive in wheat, *Dehydration-responsive element-binding* (*DREB*), was associated with yield increase under drought conditions (Shavrukov et al. 2016). These authors conducted a study which demonstrated that wheat cultivars transformed with the drought responsive gene, *TaDREB3*, had improved drought tolerance. Another drought responsive gene, *TaDr1* has been reported to be upregulated under drought conditions in wheat (Stephenson et al. 2007).

1.1 History of wheat cultivation and its significance

1.1.1 Wheat cultivation

The cultivation of wheat can be traced back about 10,000 years. It started by the domestication of *Triticum* wild grasses through selection processes which led to higher yield yet a reduced ability to survive in wild competition (Shewry 2009). The commonly grown wheat, *T. aestivum*, is a hexaploid species composed of three genomes, A, B and D, developed by spontaneous hybridization, during its domestication. It is believed to result from the hybridization of a diploid wild grass species, *Aegilops tauschii* (progenitor of the D genome), and cultivated emmer wheat, *T. dicoccoides,* as illustrated in Figure 1 (Feldman et al. 2001). *T. dicoccoides* is a tetraploid originating from the hybridisation of *T. urartu* (A genome) and a progenitor of B genome, which was lost during evolution, with the current species, *Aegilops speltoides* remaining as the closest relative to the progenitor. *T. aestivum* has a somatic chromosome number of 42 (2n = 6x = 42) and can be classified by either a spring or winter wheat, depending on the planting season (Ma 2005).

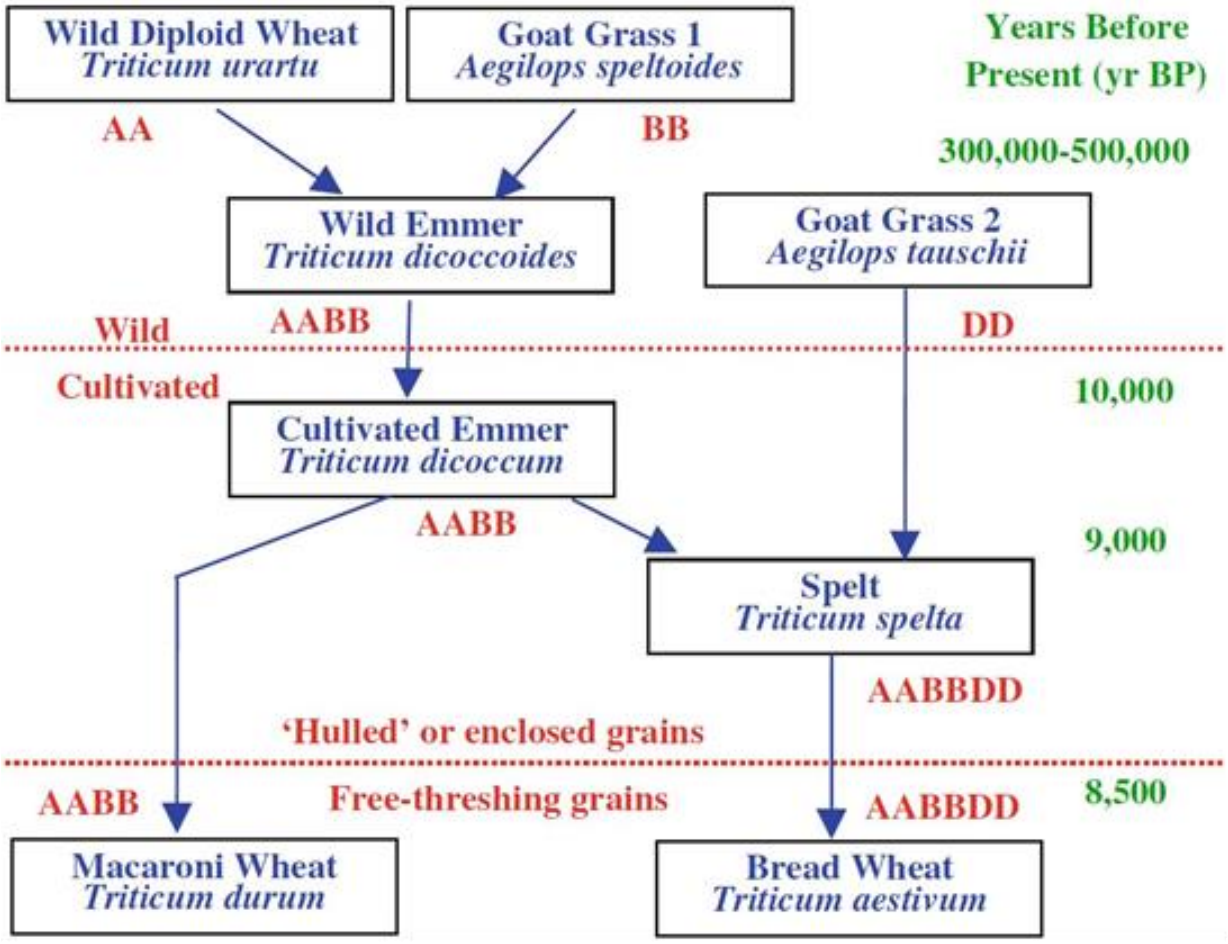

Figure 1: The ancestral lineage of *T. aestivum*. The lineage demonstrates ancient wild parents of wheat grass together with their diploid level until development *T. aestivum*. Adapted from (http://www.newhallmill.org.uk/wht-evol.htm).

The ability of hexaploid wheat to grow in a wide range of environmental conditions has contributed to its high uptake as compared to grasses of other ploidy and species, and it currently constitutes 95% of all cultivated wheat (Feldman et al.1995; Shewry 2009).The selection for breeding of wheat cultivars is influenced by both the environment and the use, as these factors differs from region to region. This could also have contributed to genetic diversity of wheat cultivars and the development of modern wheat genotypes.

1.1.2 Wheat significance

Wheat is grown for human and animal consumption (Sallam et al. 2019). According to Becker et al. (2016), 20% of the human food supply in 2014 was derived from wheat. For many people, wheat is an important source of dietary protein, carbohydrates and other minerals such as selenium, iron and zinc (Shewry et al. 2015). Moreover, wheat accessions have been explored as a mode of iron and zinc enhancement to address mineral deficiency in developing countries where wheat is a staple diet (Palmer et al. 2014). However, one issue with wheat consumption is the emerging sensitivity to gluten, the major component of bread wheat flour that is responsible for the elastic nature of the bread making dough. Diseases such as coeliac and, non-coeliac gluten sensitivity have been reported in a few human populations (Gasbarrini & Mangiola 2014). To alleviate this issue, non-allergic wheat has been reported in the Bobwhite cultivar by suppression of coeliac disease gluten epitopes using RNAi plasmids (Barro et al. 2016; Borisjuk et al. 2019).

On balance however, wheat is still a critical grain in the human food supply. It is an important source of revenue in wheat exporting countries such as Australia. It has been reported that A$2-3 billion is generated yearly in Australia through wheat exports, with 50% of this by Western Australia alone (Department of Primary Industries and Regional Development 2019). Therefore, the successful growth and production of wheat under a range of conditions, including drought is crucial for human survival.

1.2 Drought tolerance

Drought stress has negative impacts on plant growth and development. Reynolds et al. (2006) stated that drought stress can cause 50% of yield loss which, and given the discussion above, can lead to significant food shortages. Furthermore, drought stress effects on yield differ depending on wheat ploidy level. Wang et al. (2017) observed that

drought had a more severe effect on diploid (2n) as compare to tetraploid (4n) and hexaploid (6n) wheat. In general, plants overcome the adverse effects of drought through the development of different mechanisms and respond by undergoing changes at a morphological, physiological and molecular level. Plants respond by producing hormones, accumulating osmo-protectants as well as changing signal transduction pathways (Bohnert et al 1995). Morpho-physiological changes associated with drought stress adaptation include development of thick and deep root systems, leaf rolling and early flowering, while also altering gene expressions levels (Hu & Xiong 2014). Early flowering is identified as a drought escaping strategy to avoid terminal drought stress, and leaf rolling as a water conservation strategy used by many crop plants through reduction of leaf surface area (Basu et al. 2016; Shavrukov et al. 2017).

As mentioned earlier, breeding for drought tolerance in wheat is a more complicated and challenging process than for many other crop plants, due to its hexaploid genome. Also, the growth and development of wheat is affected by drought stress at different growth stages, and there is limited genetic diversity in modern cultivars due to its self-fertilisation and long history of domestication (Nezhadahmadi et al. 2013; Becker et al. 2016). The reproductive stage of wheat has been identified as highly sensitive to drought and can lead to lower yield as grain development will be negatively affected (Zhang et al. 2018). The effects imposed by drought stress on different stages of plant development and growth complicates enhancement of drought tolerance through breeding, and furthermore it is regulated by a wide array of factors which includes interactions between multiple genes and pathways (Mwadzingeni et al. 2016). According to Tardieu and Tuberosa (2010), the identification of genes with immense impact on drought tolerance mechanisms is a commonly used approach to address challenges presented by drought tolerance improvement. Such genes with large impacts on drought tolerance in wheat include Transcriptional factors such as *TaNFYC- A7* and *TaDREB5* (Zotova et al. 2018).

1.2.1 Increase of genetic diversity for drought tolerance

A long history of wheat domestication has narrowed the phenotypic and genetic diversity of genotypes for the selection of drought tolerance in bread wheat (Becker et al. 2016). The domestication of wheat cultivars during the 'Green Revolution' of 1950-80s has contributed to a loss of important drought tolerance traits as more focus was given to the improvement of yield (Budak et al. 2013; Hussain 2015). The genetic diversity in wheat can however be improved through the development of synthetic hexaploid wheats (SHWs) using a wide range of *Ae. tauschii* accessions. Genetic variation for abiotic stress has been observed in SHWs which indicates their potential application in breeding for drought tolerance (Bhatta et al. 2018; Dreisigacker et al. 2008). Another approach which can be used for enhancement of genetic diversity for drought tolerance is molecular breeding through genetic transformation of modern wheat cultivars with genes associated with drought tolerance (Shavrukov et al. 2016).

1.2.2 Strategies used for breeding for drought tolerance

The development of improved drought tolerance involves crossing often drought tolerant wild cultivars with drought sensitive commercial wheat genotypes in order to transfer the advantageous traits to their progeny (Sallam et al. 2019). Breeding for drought tolerance can be done using morphological and physiological trait selection such as yield, chlorophyll content, root system development, stomata conductance, cell stability and proline content (Iqbal 2019). The widely used trait for drought tolerance is yield under drought conditions, and it has generated some notable drought tolerant genotypes. Nevertheless, yield production rates cannot meet the current demand of food production (Tester & Langridge 2010).

An alternative to selection for yield is morphological traits selection for drought tolerance improvement. Becker et al. (2016) conducted a study on root morphology trait selection to improve drought tolerance in wheat. This report indicated drought survival improvement of SHW lines with long roots as compare to with shallow roots due to effective extraction of water. Evidence from research results reported by Xu et al. (2013) suggest that plants had reduced rate of shoot growth during stress which led to increase of root length density and root biomass. The application of physiological or morphological trait selection to improve drought tolerance in wheat has yielded some successful stories. However, these approaches also appear to be slow to meet the required rates of wheat yield under drought conditions for predicted 2050 population demand (FAO 2006).

Another method of breeding for drought tolerance is to use molecular technologies. Markers Assisted Selection (MAS) is a technique employed for selection of genes with drought tolerant traits and to combine these into a pyramid of genes in a single genotype (Randhawa et al. 2013). Application of molecular markers has sped-up the process of breeding. However, it can be more expensive and require skilled labour as compare to traditional breeding based on selection of phenotypical traits only. Molecular plant breeding allows breeders to assess the relationship between drought tolerant morpho-physiological traits and molecular markers, which can be determined by Quantitative Trait Loci (QTL) mapping, or Genome Wide Association Studies (GWAS) (Sallam et al. 2019). The application of GWAS and QTL mapping involves deployment of specific analyses of sequences such as Single Nucleotide Polymorphisms (SNPs), Random Amplified Polymorphism DNAs (RAPDs) and Amplified Fragment Length Polymorphism (AFLP) are known as molecular markers. They can also be used to link important phenotypic traits with genes, as well as to trace genes of interest or mapping genes encoding drought tolerant traits in wheat genotypes (Gupta et al. 1999). The expression patterns exhibited by molecular markers allow breeders to detect and follow the presence of drought tolerant

genes. The presence or absence of drought associated genes can be investigated by genotyping, as exemplified by *NFY* and *DREB* gene expression during molecular wheat breeding (Sallam et al. 2019).

Genomic markers such as SNPs can be used in QTL analysis to genotype transcription factors. In a recent study, one of the genomic tools, Amplifluor-like SNP markers, was successfully used for the genotyping of *TaDREB5* and *TaNFYC-A7* genes associated with drought tolerance (Zotova et al. 2018). Another such application was demonstrated in rice improvement for drought tolerance, by mapping a QTL for the *Deeper Rooting 1* (*DRO1*) gene which controls root growth. It was reported that overexpression of *DRO1* was positively correlated with increased yield in rice (Uga et al. 2013). After identification of the QTLs associated with drought tolerance, further analysis of gene expression assessment is required.

1.3 Gene expression

Plants alter their gene expression patterns by regulating transcription rate, thus plant scientists/physiologists use gene expression patterns to understand crop adaptation strategies toward drought stress at a molecular level (Sukumaran et al. 2018). Analysis of gene expression patterns under drought conditions can be evaluated using molecular techniques such as Northern blotting, Reverse Transcription quantitative PCR (RT-qPCR), Serial analysis of gene expression (SAGE) and Microarrays. Each of these will be discussed in more detail.

1.3.1 Methods used to determine gene expression

SAGE is defined as a molecular technique employed for quantification of multiple genes expressed at a particular time and it measures by sequencing constructed cDNA after separation of unique sequence tags (Yamamoto et al. 2001). Wang et al. (2011) denoted that the application of SAGE allows breeders to determine expression of multiple genes at the same time. These techniques however are very expensive. Fleury et al. (2010) reported that microarrays were successfully used in the assessment of glutathione-related genes expression and that they were down-regulated under drought conditions in a drought tolerant synthetic wheat breeding line. On the other hand, Northern blotting and RT-qPCR are widely used for observation of particular genes (Taniguchi et al. 2001). Northern blotting analysis is highly specific although it requires a lot of mRNA for analysis (Chelly & Kahn 1994). Application of Northern blotting analysis in gene expression often requires the use of radioactively labelled probes that can be more difficult, expensive, and potentially hazardous (Streit et al. 2008). RT-qPCR is a highly-sensitive technique which measures amplicons per cycle by CYBR fluorescence detection and it has real time quantification capability (Smith & Osborn 2009). The application of RT-qPCR in gene expression analysis is usually accompanied by the use of one or two Reference (or Housekeeping) genes such as *Alpha-tubulin* or *Actin* in various plant species. This avoids bias during relative expression assessment of a gene of interest (Evers et al. 2005; Paolacci et al. 2009). RT-qPCR has superseded the use of the Northern blot in gene expression studies for a single gene due to its simplicity and speed. Therefore, RT-qPCR was selected as a technique for determination of expression profiles since it was reasonable price and accurate and it have been used in previous research of *Dr1* expression studies. An example of the use of RT-qPCR was successful evaluation of *TaDr1* expression in wheat under drought conditions (Zotova et al. 2019).

1.4 Identification of genes associated with drought tolerance through profiling of gene expression

Zhang et al. (2017) stated that crops respond to drought stress by regulating expression of Transcription factors (TFs). Transcription factors are regulatory proteins which control expression of genes by activating or suppressing their expression through binding to specific DNA sequences (Latchman 1993). Researchers employ molecular tools such as genome wide study of gene expression under drought conditions to understand and identify TFs that regulate drought responsive pathways. Some of the TFs that have been reported in plants under drought stress are: *Myeloblastosis* (*MYB*), *Myelocytomatosis* (*MYC*), *Tryptophan-arginine-lysine-tyrosine* (*WRKY*), *Basic leucine zipper* (*bZIP*), *No Apical Meristem* (*NAM*), *Arabidopsis Transcription Activation Factor-* (*ATAF*), *Cup-shaped Cotyledons* (*CUC*) and *Dehydration-responsive element-binding* (*DREB*). Okay et al. (2014) observed that genes from family members of *TaWRKY* proteins can be up-regulated in wheat under drought conditions and identified them as drought-responsive genes. In addition, Nakashima and Yamaguchi-Shinozaki (2006) reported that transgenic rice with *DREB1A* had enhanced drought tolerance under greenhouse conditions. Therefore, the up-regulation of TFs by transgenesis in crop plants could play a vital role in the improvement of drought stress tolerance.

Another example of TF transgenesis-induced drought tolerance was reported by Zhang et al. (2015) who overexpressed *Nuclear Factor Y* (*NFY*) gene which showed enhanced drought tolerance in transformed *Arabidopsis*. TFs are not the only regulatory proteins implicated in regulation of pathways under drought conditions. Other regulatory proteins have been identified to be involved in regulation of pathways and they are called transcriptional repressors. Rojo (2001) described a transcriptional repressor as a protein that inhibits binding of RNA polymerase by interfering with the function of the promoters. In *Arabidopsis*, the ethylene responsive factor, AtERF4, was identified to be overexpressed

and implicated in the repression of reporter gene basal transcription level by binding with a GCC box under drought conditions (Fujimoto et al 2000). Yuan et al. (2018) reported that overexpression of *Oryza sativa* drought-responsive zinc finger protein 1 (*OsDRZ1*) was associated with improved seedling drought tolerance in rice by inhibiting transactivation activity of Ethylene-responsive element binding factors (ERFs) with a DLN-box/EAR-motif. In high yielding wheat cultivars, a nuclear-localised transcriptional repressor gene, *TaDr1* was reported to be up-regulated under drought conditions which suggests that this gene expression patterns can be beneficial for yield improvement or drought tolerance (Zotova et al. 2019).

According to Willy et al. (2000), *Dr1* and *DrAp1* genes are classified as general transcriptional repressors in the fruit fly, *Drosophila melanogaster*. However, their functions differ in plants and animals. For example in plants, *DrAp1* is a repressor and *Dr1* is a co-repressor, but in animals, *DrAp1* may have 'swapped' functions (Zotova et al. 2019). It has been observed that multiple-proteins in a hetero-tetramer Dr1/DrAp1 complex may have repression activity of transcription which involves RNA Polymerase II and RNA Polymerase III but not RNA Polymerase I, as it does not have a TATA motif (Inostroza et al. 1992; Purrello et al.1996). Previous studies demonstrated that RNA Polymerase II transcription factors, TFIIA and TFIIB, required for the facilitation of TBP binding to the promoter DNA, failed to interact with the TFIID-TATA Binding Protein (TBP) in the presence of Dr1/DrAp1 complex. This suggested that they were inhibited to bind with TBP as illustrated in Figure 2 (Kim et al. 1995; Orphanides et al. 1996). In *Drosophila*, Dr1/DrAp1 complex was identified to have two roles which were down-stream promoter element (DPE) activation, and repression of TATA-containing promoters' activities (Willy et al. 2000).

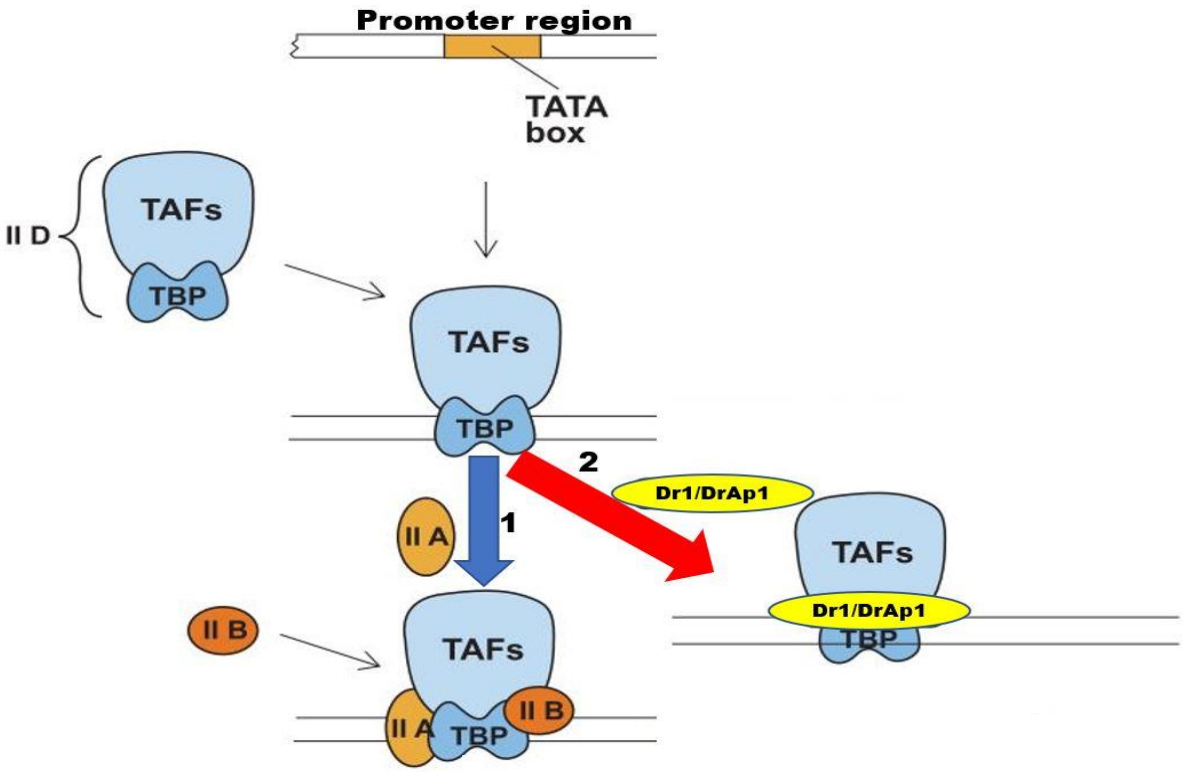

Figure 2: Illustration of the inhibitory effect of Dr1/DrAp1 complex in the TATA-containing promoter region in animal cells. During transcription in the absence of Dr1/DrAp1 complex, Transcription Factor IID containing TATA Binding Protein (TBP) and TBP-Associated Factors (TAFs) will attach to TATA Box, followed by binding of Transcription Factors IIA and IIB to TBP as demonstrated by blue arrow pointing downwards (pathway 1). The red arrow displays prevention of Transcription Factors IIA and IIB binding to TBP by Dr1/DrAp1 complex (pathway 2). The figure was modified from (Stolk et al. 2006).

Previous studies about *Dr1* expression in wheat have displayed its upregulation at Day 10 and there is lack of information about expression of *Dr1* at different time-points in accessions development from different geographical locations. Our study will be assessing the *TaDr1* expression under different levels of drought stress using wheat germplasms originating from different continents. The information obtained from this study will be useful for practical application in developing SNP markers used for enhancement of breeding efforts to improve drought tolerance.

Hypothesis and Aims

Hypothesis:

• *TaDr1* gene is up-regulated in leaves of wheat plants under drought conditions compared to controls.

Aims:

• To assess the expression of the transcriptional repressor gene, *TaDr1*, under different levels of drought stress.

• To identify how expression of *TaDr1* is similar or different in wheat germplasms originated from three different countries, Australia, Kazakhstan and South Africa.

CHAPTER 2.0: MATERIALS AND METHODS

2.1 Plant materials

The study involved comparison of wheat accessions from different geographical origins and therefore the following wheat accessions were selected as Flinders University of South Australia have a collaborative wheat project with LongReach Plant Breeders (Australia), Kazakh Agro-Technical University (Kazakhstan) and Sensako (PTY) LTD (South Africa). In addition, drought conditions of the three countries are different. Australia has a terminal drought and Kazakhstan has a specific - short drought while South Africa has a moderate terminal drought (Y. Shavrukov, personal communication). Seeds of six spring wheat accessions from three origins were used: cultivars Lancer and Trojan were obtained from LongReach Plant Breeders; two accessions, Akmola-2 and VK-1, were provided by Kazakh Agro-Technical University; and two breeding lines, SST-398 and SST-843, were supplied by Sensako (PTY) LTD.

2.2. Wheat plants cultivation

Sixty seeds of each accession were pre-germinated at room temperature for six days in 9 cm diameter Petri dishes containing two layers of water-soaked paper towels. Then seedlings were transplanted into pots (6 inches in diameter) containing 1.2 kg of BioGro soil (six seedlings per pot). Seedlings were grown under green-house conditions for one month, after which four control pots of each accession were well-watered, while seedlings from treatment pots were withdrawn water for 13 days. The samples were collected in four different time-points as follows; Day 0, Day 5, Day 10 and Day 13. In each sampling time-point, five different leaves were randomly sampled from different seedlings in control and drought-treated pots. The leaves were individually placed into 10 ml tubes and frozen

immediately in liquid nitrogen. Samples were then stored in a -80^{o}C freezer until RNA extraction.

2.3. Relative biomass ratio and level of drought

The rate of drought development was determined by recording the weight of pots per time-points. The weight of control and treatment pots was measured twenty-four hours after watering using accurate kitchen scale. The drought tolerance was determine using Relative Biomass Ratio (RBR) which was calculated based on fresh weight and dry weight of shoots and roots of control and drought-treated young plants. The fresh weight of shoots and roots from 15 control and treated plants per accession at Day 13 was measured. The dry weight was measured after leaf samples were placed in 60^{o}C oven for two weeks. Average weight of shoots and roots was used in RBR calculations based on both fresh and dry weight as percentage of treated biomass /control biomass × 100%, as described earlier (Shavrukov et al. 2009).

2.4. RNA extraction and cDNA synthesis

Five samples of frozen leaves (~500 mg) collected from treated and control plants were crushed to powder using 9-mm stainless steel balls, by vortexing (Appendix A). RNA was extracted using TRIzol-like reagent protocol adopted from Shavrukov et al. (2013). The quality of RNA was assessed by electrophoresis in 1.5% agarose gel (Appendix B). Prior to cDNA synthesis, the concentration of RNA was measured using a ThermoScience NanoDrop 1000 spectrophotometer and adjusted to 2.0 µg for cDNA synthesis (Shavrukov et al. 2013). The samples were treated with 2 µl of 2 U/ul DNase (Qiagen kit) per reaction to remove traces of DNA, following the protocol of Zotova et al. (2018). The cDNA was synthesised using Protoscript II Reverse Transcriptase kit (NEBiolab) containing 2 µg of RNA, dNTPs and oligo-dT($_{20}$) primers in BioRad MyCycler Thermal Cycler PCR machine

(Appendix C). The quality of cDNA was assessed by electrophoresis in 1.2% agarose gel. Optimization of the protocol was performed in order to ensure that Threshold cycles (Ct) values generated by qPCR machine were from bulks product of interest after observing multiplied bands in 1.2% agarose gel. Single bands were observed after electrophoresis in 1.2% agarose gel after optimizing the protocol using a temperature gradient approach at annealing steps of cycles (Appendix D).

2.5. qPCR analysis

Three sets of forward and reverse primers used were developed based on *TaDr1* sequences Stephenson et al. (2007) (Appendix E). The first set of primers was consensus and, therefore, it amplifies fragments of three *TaDr1* homeolog in chromosomes 3A, 3B and 3D. The forward primer of *TaDr1* consensus was 5'-GTATTGTGCGTGTCGTGTCAGA -3' and the reverse primer was 5'-CCAGACAACTCGCAACTTAG-3'. The second specific set, *TaDr1a*, was identified by Stephenson et al. (2007) and it amplified chromosome 3A *TaDr1a* homeolog. *TaDr1a* forward primer was 5'- GGCCTGGACTGGGACAGTT -3' and reverse primer was 5'-TGGCTGAAATCACACACGATTTA -3'. *TaDr1B* primer set designed by Zotova et al. (2019) targeted amplification of *TaDr1B* homeolog at chromosome 3D and the forward primer set used was 5'- GCAAGCGGAACATGGCCTGA -3' and reverse primer was 5'-ACTACAGCAGTCATAAACACAGTAT -3'. They were used to assess gene expression patterns after synthesising cDNA from non-degraded RNA. The qPCR analysis of the diluted cDNA (1:10) was performed using the protocol reported by Zotova et al. (2018) in a total reaction volume of 10 µl, containing 5 µl KAPA SYBR FAST qPCR Master Mix (2X), 1 µl of forward and reverse specific primers (3 µM each) and 4 ul of cDNA sample in 96 well plate. Ta54825, *Actin* and Ta22845, *ATP-dependent 26S proteasome*, were used as reference genes during the qPCR experiments (Zotova et al. 2019). The expression of

cDNA samples was analysed using a procedure suggested by the manufacturer (KAPA-Biosystem in Bio-Rad CFX9TM Real-Time system C1000TM Thermal Cycler) as follows: initial denaturing temperature 95^{o}C for 3 mins, denaturing temperature 95^{o}C for 3 secs, annealing temperature 56^{o}C for 30 sec (cycle 40 times), followed by 95^{o}C extension (10 sec), then 65^{o}C melting curve photo capturing (5 sec) with a final extension at 95^{o}C for 50 sec. The mean of gene expression at day 0 was compared to other means from Day 5, Day 10 and Day 13 in each accession. The mean significant difference was calculated with Student's *t*-test in Excel spread sheet obtained from Handbook of Biological statistics (http://www.biostathandbook.com/twosamplettest.html).

CHAPTER 3.0: RESULTS

Breeding for drought tolerance using molecular approaches to assess transcription regulators has become increasingly important to global food security to meet the growing world population demand. In this study, drought tolerance of wheat seedlings was determined using Relative Biomass Ratio based on the fresh and dry weight of shoots and roots. The assessment of drought tolerance led to the identification of drought tolerant and drought sensitive genotypes. Further analysis of qPCR was carried out with synthesised cDNA from RNA which was extracted from leaves of treated and control seedlings, to determine *TaDr1* expression profiles on both drought tolerant and sensitive genotypes of different origins at several time-points. The expressions profiles of *Dr1* homeolog genes were normalised with the average of reference genes (*Actin* and *ATP-dependent 26S proteasome*) expression profiles. The expression profiles of *TaDr1* were determined using three sets of forward and reverse primers designed based on sequences of *Dr1* homeolog genes.

3.1. Plant Biomass

The measurement of fresh and dry weight of roots and shoots were used in the Relative Biomass Ratio (RBR) calculations to determine drought tolerance. The results revealed that the RBR of dry weight was higher than of the fresh weigh in studied plants (Figure 3). The large difference between fresh weight biomass and dry weigh biomass could be attributed to their lower values of Relative fresh weight biomass ratio (FWR) as compared to Relative dry weight biomass (DWR) values. The results revealed that FWR of SST-398 was significantly smaller than Lancer, Akmola-2, VK-1 and SST-843 but not differ from Trojan. The wheat accessions of Lancer, Trojan, Akmola-2, VK-1 and SST-843 did not show significant difference in FWR. DWR in VK-1 was significantly larger than those in Lancer, Trojan, Akmola-2 and SST-398 but not differ from SST-843. No significant

difference of DWR was found between Trojan, Akmola-2, SST-398 and SST-843. However, the DWR in SST-398 was significantly smaller than in Lancer and VK-1.

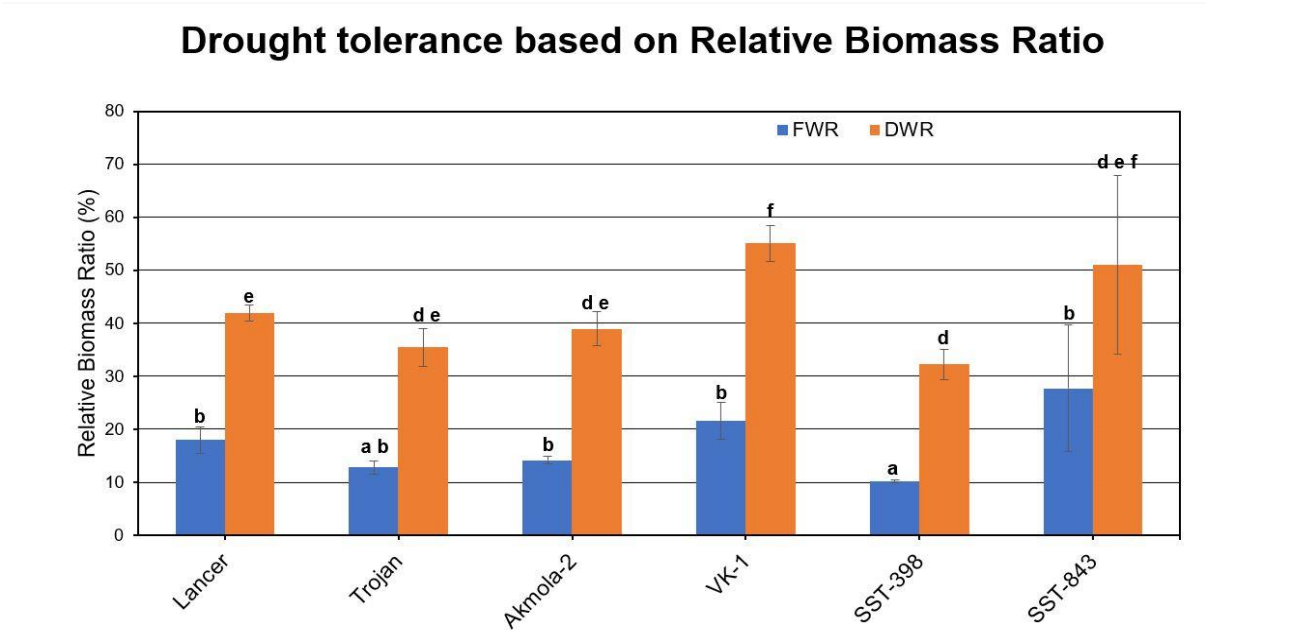

Figure 3: The Relative Biomass Ratio of fresh and dry weight of wheat accessions. The average data of 15 seedlings per accession were used in Relative Biomass Ratio calculation. FW, Fresh weigh; DW, Dry weight. Different letters above the error bars indicated significant differences ($p<0.05$). Comparison of FWR column bars between six cultivars represented by letters 'a – b' while letters 'd – f' represented comparison between DWR column bars.

The results showed that SST-843 had the highest average value of FWR ratio at 27.7% while SST-398 displayed lowest value of FWR ratio at 10.1%. Both accessions have South African origin. VK-1 had the highest value of DWR ratio at 55.0%, while amongst six wheat accessions, SST-398 had the lowest FWR ratio with value of 32.2%. Figure 4 showed average pot weight from six accessions per sampling time-points, which was rapidly declining during drought treatment while the average pot weight in controls in six accessions was relative constant over 13 days.

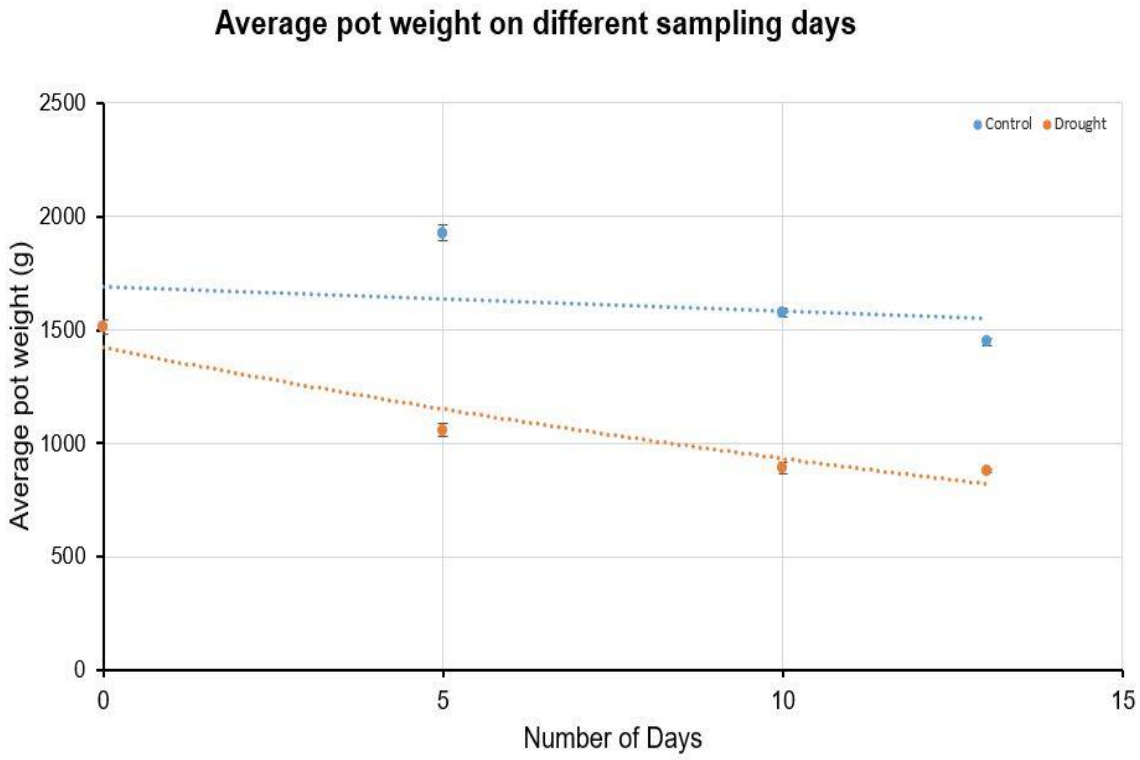

Figure 4: The average weight of pots in control (blue dotted line) and drought-treatment (orange dotted line) in six accessions per sampling time-points for 13 days.

3.2 *TaDr1* gene expression

The evaluation of *TaDr1* gene expression profiles was carried-out by qPCR analysis using three sets of primers, after imposing drought stress on six wheat accessions for 13 days. The primer set which amplify common *TaDr1* fragments (Consensus) were the first to be assessed, and results showed varied but consistent up-regulation of *TaDr1* gene expression across six wheat accessions. The *TaDr1* expression patterns of were classified into three groups. The first group comprised of accessions with constitutively up-regulated expression in Day 5, Day 10 and Day 13 which were VK-1 and SST-398 (Figure 5).

Trojan and Akmola 2 constituted a second group which showed up-regulation in Day 10 but decreased after. The third group contained Lancer and SST-843 which displayed highest expression in latest Day 13. However, Lancer had only late expression in Day 13

while SST-843 had consistent up-regulation of *TaDr1* gene expression. Lancer exhibited highest value of normalised expression in Day 10 and lowest value was observed in Day 13 of Trojan. Based on the results, *TaDr1* was upregulated in all six wheat accessions.

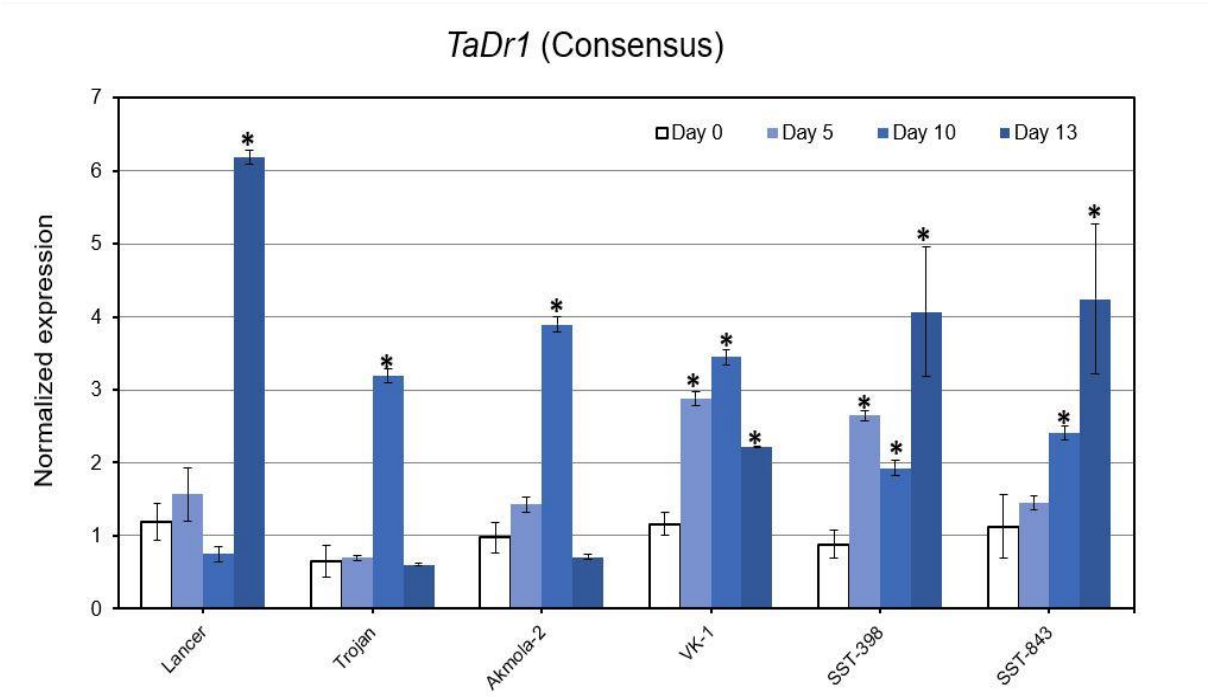

Figure 5: *TaDr1* normalised expression in six wheat accessions. Within each accession, Day 5, Day 10 and Day 13 were compared with Day 0. The asterisk (*) denotes the differences with significance level ($p<0.05$).

The second evaluation was for specific primers of *TaDr1a*, which targeted expression of isoform located at chromosome 3A. The specific primers were able to amplify 130 bp fragment and qPCR analysis revealed that Trojan and Akmola 2 had earlier expression of *TaDr1a* (3A) in Day 10 while Lancer had late expression in Day 13 (Figure 6).

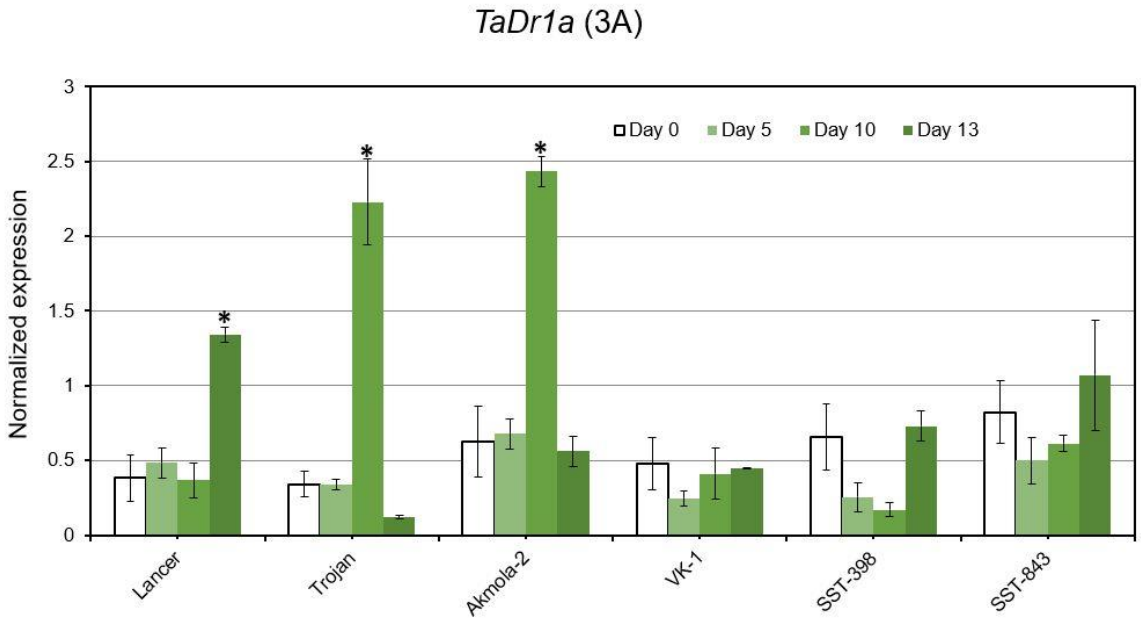

Figure 6: Normalised expression of *TaDr1a* (3A) in six wheat accessions. Within each accession, Day 5, Day 10 and Day 13 were compared with Day 0. The asterisk (*) denotes the differences with significance level ($p<0.05$).

A decreasing trend was registered after Day 10 in Trojan and Akmola-2. There were no significant differences found across time-points in VK-1, SST-398 and SST-843. At Day 5 and Day 10 of SST-398 and SST-843 showed down-regulation. Day 10 of Akmola-2 had highest normalised expression value and the lowest value was recorded in Day 13 in Trojan. Therefore, isoform of *TaDr1a* was only up-regulated in three accessions, Lancer, Trojan and Akmola-2.

The third specific primers set for isoform mapped in chromosome 3D were used to assess expression of *TaDr1b*. The *TaDr1b* expression analysis also exhibited variability across wheat accession. The results of *TaDr1b* outlined in Figure 7 shows that there was no up-regulation in Lancer and Akmola-2 as no significant differences were observed across different time-points.

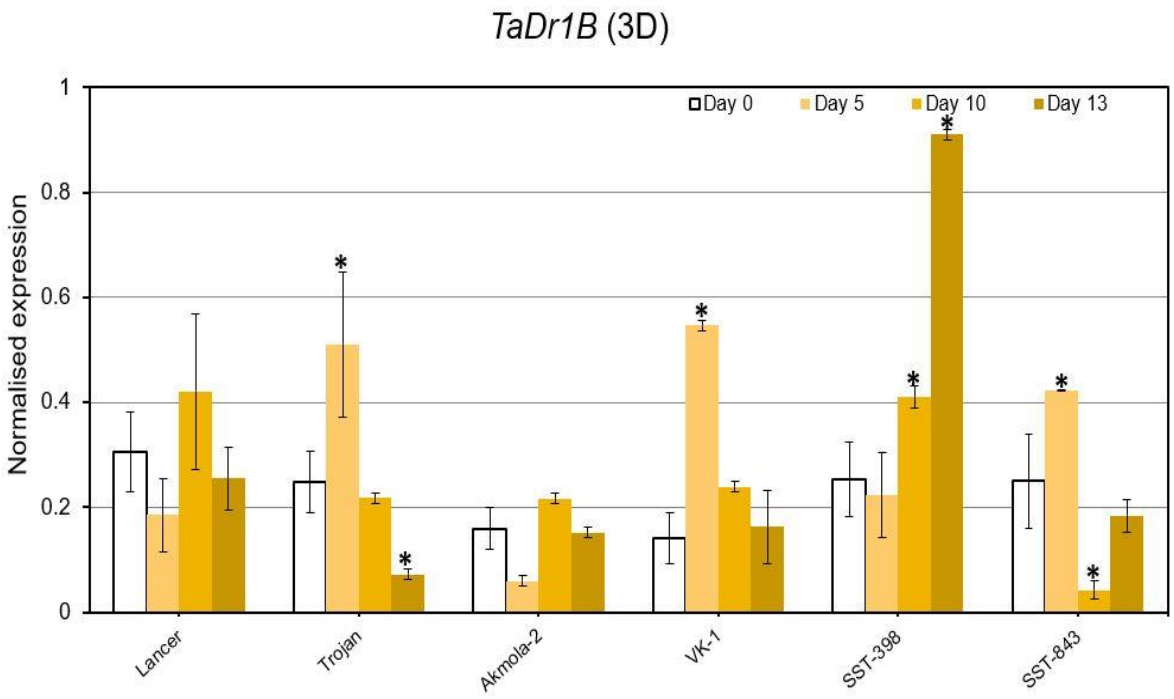

Figure 7: Normalised expression of *TaDr1B* gene on chromosome 3D in six wheat accessions. Within each accession, Day 5, Day 10 and Day 13 were compared with Day 0. The asterisk (*) denotes the differences with significance level (p<0.05).

Based on the presented results, Trojan, VK-1 and SST-843 had an earlier up-regulation at Day 5 of *TaDr1b* expression. The results displayed that SST-398 had medium to late expression at Day 10 and had the strongest expression at Day 13. A decreasing trend after Day 5 up-regulation was observed in both Trojan and SST-843. SST-398 had highest value of the gene expression at Day 13 while lowest normalised expression was found in Day 10 in SST-843.This reveals that up-regulation of *TaDr1b* isoform occurred in four accessions, Trojan, VK-1, SST-398 and SST-843. The results further depicted that *TaDr1* homeolog differed in patterns of expression between the wheat accessions from different countries as *TaDr1a* was responsive in Akmola, Lancer and Trojan while *TaDr1b* was drought responsive in SST-398, VK-1, Trojan and SST-843. Similar expression patterns were observed between *TaDr1a* homolog and *TaDr1* consensus while differ with *TaDr1B* homolog

CHAPTER 4.0: DISCUSSION

Drought tolerance at the vegetative stage in wheat accessions originating from different countries was assessed using RBR followed by evaluation of *TaDr1* expression patterns. Further analyses were carried out using two specific primer sets for *TaDr1* homeologs (*TaDr1a* and *TaDr1B*) together with one consensus primer set to understand the expression profile of these genes at four time-points of drought (Appendix F).

In a study by Sallam et al (2019), it was reported that drought tolerant wheat accessions exhibited higher RBR as compare to drought sensitive, and it was attributed to less water loss. Research presented here revealed that Australian wheat accessions, Lancer had better drought tolerance compare to Trojan in greenhouse conditions, whereas in field trials (B. Jacobs, personal communication) Trojan was more drought tolerant than Lancer. This suggests different conditions in our experiments compared to that of commercial field trails. The results of drought tolerance trends of Kazakh accessions presented here indicated that VK-1 had a better drought tolerance compare to Akmola-2 and this information was consistent with that provided earlier (Y. Shavrukov, personal communication). The findings of drought tolerance trends presented here of South African breeding lines demonstrated that SST-843 had better tolerance to drought compare to SST-398 and was corresponding to drought tolerance in field trials (F. Koekemoer, personal communication).

The qPCR results suggest that there was significant influence of drought on the expression of *TaDr1* homeologs, and therefore *TaDr1* homeologs' expression could be dependent on the environment. Furthermore, qPCR analysis also supported observations made by Stephenson et al. (2007) and Zotova et al. (2019) whereby *TaDr1* was strongly up-regulated under drought conditions. Expression profiles were genotype-dependent, for example earlier or later expression profiles were found in different genotypes. The results

also suggest that *TaDr1a* homeolog had a strong influence in the expression of *TaDr1* consensus as both had similar expression patterns. Therefore, the ancestors of Australian accessions may differ with ancestors of South African accessions and VK-1 may be have a closer pedigree relationship with South African accessions. In addition, the results suggest that *TaDr1* homeologs were differential expressed in spring wheat accessions and were congruent with observations made by Zotova et al. 2019 which demonstrated that differential expression of *TaDr1* homeologs associated with *TaVrn* co-expression. This phenomenon could also support a suggestion made by Zotova et al. 2019 that *TaDr1* expression may influence or be influenced by *TaVrn* expression. *Vrn* gene is responsible for modulation of vernalisation and the transition of plants from vegetative to reproduction stages of development and has wide genetic polymorphism for gene structure and function (Deng et al. 2015). The expression patterns are similar and therefore possibly correlated through a master control pathway(s). The occurrence of differential expression patterns displayed by *TaDr1* homeolog can also be seen in other genes of wheat with homeolog such as an ammonium transporter (AMT) and a nitrate transporter (NRT). A similar phenomenon was reported in studies of *TaAMT* expression whereby homeolog located on chromosome 5 were differential expressed in roots of five wheat lines (Bajgain et al. 2018). However, the relationship between RBR and *TaDr1* gene expression demonstrated absence of any significant correlations. This can indicate more complicated genetic control of drought tolerance where other genes are also involved in plant growth and RBR.

It can be concluded that *TaDr1* homeolog were responsive under drought conditions and their expressions were strongly genotype-dependent. The study was limited to *TaDr1* expression and did not give complete representation of repression caused by a *Dr1/DrAp1* complex. The results therefore provided initial information about the role played by *TaDr1* in drought tolerance. Further gene expression analysis requires inclusion of the *TaDr1 (3B)* homeolog which is part of *TaDr1* expression under drought stress. The study reflects

that other biochemical and physiological parameters known to be affected by drought stress, such as photosynthesis, stomatal conductance and proline production, should be considered in the future for correlation between biomass and *TaDr1* expression. It has been reported that drought affects the rate of photosynthesis, stomatal conductance and proline accumulation (Pandey 2015). Therefore, investigation of interaction between *TaDr1* expression profiles and these parameters could provide of crucial information useful in the breeding for drought tolerance.

Successful application of *TaDr1* primers in drought studies may produce information for the development of potential Transcriptomic biomarkers in the molecular breeding for drought tolerance. For example, application of biomarkers in breeding will enable breeders to distinguish drought tolerant from drought sensitive genotypes and can also assist in the identification of plant development stage most suitable for drought tolerance evaluation. Additionally, it will speed-up the characterisation of parental genotypes used in breeding for drought tolerance and the identification of functional genes which will be beneficial in the application of Marker assisted selection (Rasheed et al. 2015). However, to improve breeding efficiency, Marker assisted selection must be complemented with other advanced technologies such as Next-generation sequencing since drought tolerance is complex trait which involves many factors.

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APPENDICES

Appendix A: protocol of isolation of total RNA using TRIZOL

Materials:

TRIzol – like reagent – lab stock in fridge in fridge (1 mL per tube) (In fridge)

Chloroform (200 uL per tube) (In fridge)

Iso-propanol (500 uL per tube) (In fridge) (own stock)

75% Ethanol (in autoclaved MQW) (1 mL per tube) (own stock)

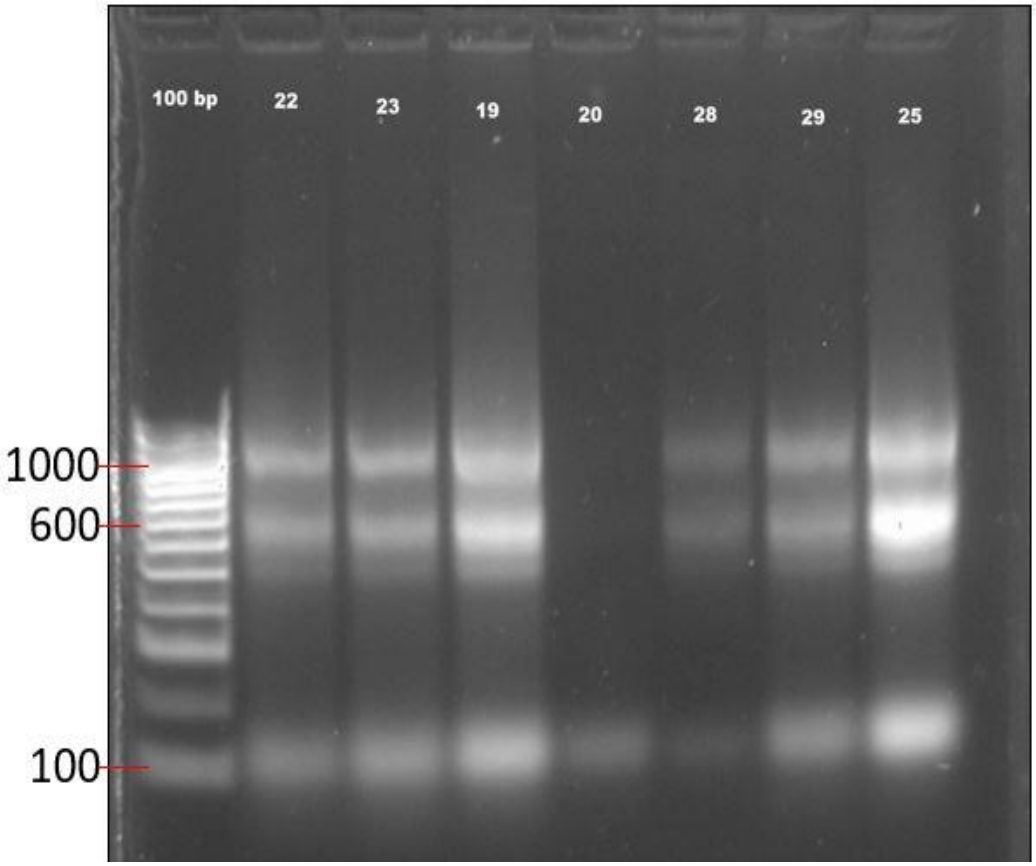
Autoclaved MQW, tips and tubes

Protocol: Always pre-wet tips with the relevant reagent before dispensing.

1. Transfer frozen tissue powder into 2 mL tube (no more than half of the volume).
2. Immediately add 1 mL of **TRIzol-**like reagent Note: Add TRIzol-like reagent under fume hood and let the samples to thaw with open lids before vortexing.
3. Close lids, vortex the samples briefly (or overhead).
4. Put samples in orbital mixer for at least 5 min (could be done longer).
5. Centrifuge at 13,200 rpm (max speed) for 10 min in cold room (if this is available).
6. Transfer supernatant into a new 1.5 mL tube.
7. Add 200 uL of **chloroform** to each tube using the SAME tip, but not touching the walls.
8. Vortex tubes vigorously for 10 sec.
9. Incubate for 15-20 min at room temperature.
10. Centrifuge at 13,200 rpm (max speed) for 20 min in cold room.
11. RNA remains in colorless upper aqueous phase. Carefully transfer the aqueous phase into fresh tube using 200 uL pipette with “wide tip tips “. **Note**: Do not try to remove the very last bit. If the bottom phase got disturbed –spin again.
12. Add 500 uL of **Iso-propanol** to each tube using the SAME tip, but not touching the walls.

13. Mix by vortexing or manually and leave at room temperature for 15-20 min.
14. Centrifuge at 13,200 rpm (max speed) for 10 min in cold room.
15. RNA forms gel-like pellet. Discard supernatant over-heading the tubes.
16. Add 1 mL **of 75% ethanol** to each tube using the SAME tip, but not touching the walls.
17. Mix manually first then by vortexing
18. Centrifuge at 10,000 rpm for 5 min in cold room.
19. Remove ethanol without disturbing the pellet over-heading the tubes. Leave tubes drying up side down for 10-15 min in paper-towel to air-dry RNA pellet.
20. When RNA pellet starts turning from white to clear (very small drops of water could still be seen on tubes wall) - re-suspend RNA in 50 uL of fresh **autoclaved milli-Q water**.
21. Incubate for 10 min at 60C.
22. Measure concentration using Nano-Drop.
23. Store at -80C.

Appendix B: A picture of RNA samples from Day 5 in 1.5 % agarose gel with 100bp Bioline DNA ladder. The samples ID were as follows; 22- Trojan Control Sample 1, 23- Trojan Control Sample 2, 19- Lancer Control Sample 1, 20- Lancer Control Sample 2, 28- VK-1 Control Sample 1, 29-VK-1 Control Sample 2 and 25- Akmola-2 Control Sample 1.



Appendix C: Adapted protocol for cDNA library construction using NEB Biolab ProtoScript.

(1) Measure RNA concentration and calculated how much RNA needs making **2 µg (2000 ng)**. Calculate how much water (volume, ul) has to be added making to volume of diluted RNA **9 ul.** Prepare the calculated RNA samples and dilute with calculated volume of water in strips with eight tubes with individual lids (2-snap tubes, Chromoplas, Australia).

(2) Calculate and prepare the following mix for each RNA sample. Add **3 ul** of the mix in each microtube in strips with diluted RNA (**9 ul**) making the total volume for **12 ul**:

			× 100 samples (for example)
(A)	50 uM Oligo $d(T)_{20}$	= 2.0 ul	200.0
(B)	10 mM dNTP	= 1.0 ul	100.0

(3) Mix and heat for 65^0C for 5 min.

(4) Transfer to ice or in cold-block for 1 min.

(5) Remove ice, add **2 ul** of DNase to each sample mix (No buffer). The total volume becomes **13 ul.** Incubate for 15 min at room temperature.

(6) During incubation, prepare next component mix, **7 µl** in total for each sample:

			× 100 samples (for example)
(C)	5 × ProtoScriptII Reaction Buffer	= 4.0 ul	400.0
(D)	0.1M DTT	= 2.0 ul	200.0
(E)	Murine RNase Inhibitor	= 0.3 ul	30.0
(F)	ProtoScriptII RT	= 0.25 ul	25.0
(G)	H_2O	= 0.45 ul	45.0

[This information for the ordering only: ProtoScriptII-L = 50 ul. For 200 samples, split for two equal parts. ProtoScriptII-X = 200 ul. ProtoScriptII-S = 20 ul].

(7) Add and mix two reactions for each sample making total volume for **20 µl** in each PCR tube.

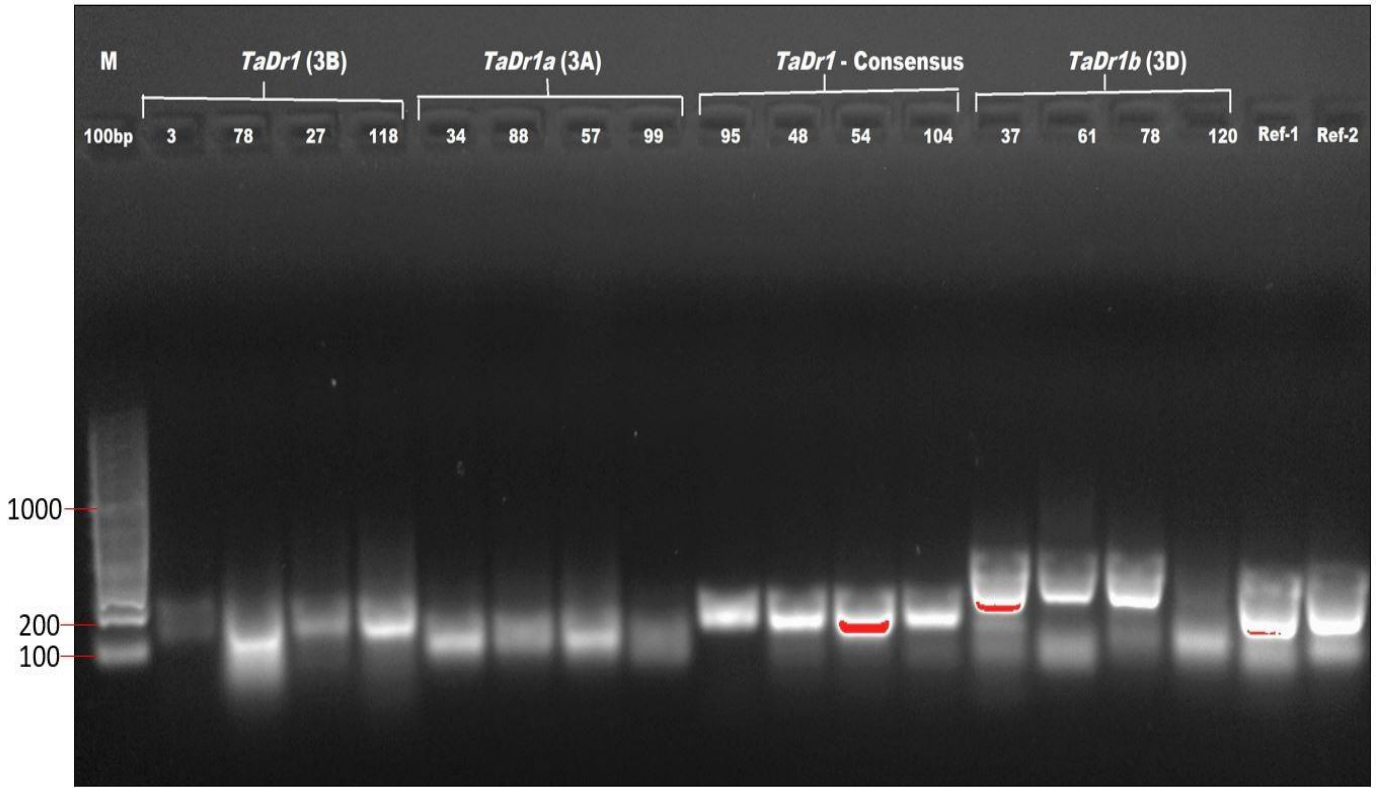
(8) Set PCR machine for program:

(8a) Incubate for 1 hour at 42^0C for Reverse Transcriptase reaction;

(8b) Incubate for 10 min at 65^0C to inactivate the enzyme;

(9) Dilute for 1:10 prior using in qPCR.

Appendix D: qPCR products of four primers mix and reference genes in 1.2% agarose gel with 100bp Bioline DNA ladder. Molecular sizes were as follows; *TaDr1* (3B) -113 bp, *TaDr1a* (3A)-130 bp, *TaDr1* (Consensus)-86 bp, *TaDr1B* (3D)-162 bp, Ref-1; Ta22845 - 202 bp and Ref-2; Ta54825 - 215 bp.



Appendix E: The alignment between two sequence of *TaDr1* during primer set development.

BLAST analysis of sequences of *TaDr1* three isoforms

```
AF464903        AACCTCCGCCCCAGCAATCCCCGCAATCTCAACTGCAGCTCCATCAGCAACCCCAGCCGA
BT009234        AACCTCCGCCCCAGCAATCCCCGCAATCTCAACTGCAGCTCCATCAGCAACCCCAGCCGA
                ************************************************************

AF464903        CGCTAGTGCCGCCGCCGC---------AACCTCAACCCCAGCCACTTGAACTGCAGCAGC
BT009234        TGCAAGTGCCGCTGCCGCCGCTGCCGCAACCTCAACCCCAGCCACCTGAACTGCAGCAGC
                 ** ******** *****         ****************** **************

AF464903        CCCAGCCGCTAACACAACTGCAAGCGGAACATGGCCTGGACTGGGACAGTTAGTGGTTCG
BT009234        CCCAGCCGCTAACACAACTGCAAGCGGAACATGGCCTGAACTGG-ACAGTTAGTGGTTCG
                ************************************ ***** ***************

AF464903        GAACATGTAGCGTCACTATAAGTTAAGACTCTGCCTCCTTTAAAATTGTGCGTTAGGTTT
BT009234        GAACATGTAGCGTCACTATAAGTTAAGACTCTGCCTCCTTTAAAATTGTGCGTTAGGTTT
                ************************************************************

AF464903        GCCTGCATCTTGTACAATGTAAATCGTGTGTGATTTCAGCCACCGTGTC--TAATAATCT
BT009234        GCCTGCATCTTGTACAATGTAAATCGTGTGTGATTTCAGCCACCGTGTCTCTAATAATCT
                ************************************************  **********

AF464903        GAAGCTCTCTAGTAAGCGATGTACTTACTGCGCTGGGTACTGTGTTTATGACTGCTGTAG
BT009234        GAAGCTCTCTAGTAAGCGATGTACTTACTGCGCTGGATACTGTGTTTATGACTGTTGTAG
                ************************************ ***************** *****


AF464903        TCTCATGGTATTGTGTGTGACGTGTCAGAAGCTACTCCATTACCAGTGTAATCAATTGCC
BT009234        TCTCATGGTATTGTGTGTGACGTGTCAGAAGCTACTCCATTACCAGTGTAATCAATTGCC
                ************************************************************

AF464903        TAACTTAATGTTCACCCGTGATGAT-----------------------
BT009234        TGACTTAATGTTCACCCGTGATGATAGTAATTGATTTCAGTGTGCTAA
                * ***********************
```

Primers:

```
TaDr1-F-conc: GTATTGTGTGTGACGTGTCAGA
TaDr1-R-conc: CAATTATTATCATCACAGGCGAACA
TaDr1-R-conc (RevCom): TGTTCACCCGTGATGATAGTAATTG

TaDr1a-F: GGCCTGGACTGGGACAGTT
TaDr1a-R: TGGCTGAAATCACACACGATTTA
TaDr1a-R (RevCom): TAAATCGTGTGTGATTTCAGCCA

TaDr1B-F: GCAAGCGGAACATGGCCTGA
TaDr1B-R: ACTACAGCAGTCATAAACACAGTAT
TaDr1B-R (RevCom): ATACTGTGTTTATGACTGTTGTAGT
```

Appendix F: Primers sequence of Reference and *TaDr1* genes used in the experiment.

Name	Amplicon size (bp)	Tm (°C)	GC content (%)	Primer sequence
Ta54825	215	51.1	53	Forward (F-1); 5'-TGACCGTATGAGCAAGGAG-3'
		51.8	50	Reverse (R); 5'-CCAGACAACTCGCAACTTAG-3'
Ta22845	202	53.8	55	Forward (F-1); 5'-GCTGGCTCGTTCAACTGATG-3'
		54.8	50	Reverse (R); 5'-GGACCAAGCGTTCTGATTACTC-3'
TaDr1	86	50	54.8	Forward (Fc); 5'-GTATTGTGCGTGTCGTGTCAGA -3'
		36	52.8	Reverse (Rc); 5'-CAATTATTATCATCACAGGCGAACA -3'
TaDr1a	130	63	55.4	Forward (Fd); 5'- GGCCTGGACTGGGACAGTT -3'
		39	51.7	Reverse (Rd); 5'-TGGCTGAAATCACACACGATTTA -3'
TaDr1B	162	60	55.9	Forward (BT-F); 5'-GCAAGCGGAACATGGCCTGA -3'
		36	52.8	Reverse (BT-R); 5'-ACTACAGCAGTCATAAACACAGTAT -3'

Appendix G*:* The *p*-values of normalised expressions of Day 5, Day 10 and Day 13 compared with Day 0 for three homologous genes in six wheat accessions across different time points.

Gene	Accessions	*p*-value		
		Day0/Day5	Day0/Day 10	Day0/Day13
TaDr1a (3A)	Lancer	0.8182261	0.971644	0.040994
	Trojan	0.98648	0.6.36E-7	0.251459
	Akmola-2	0.941929	0.024366	0.932469
	VK-1	0.537232	0.875129	0.95531
	SST-398	0.538196	0.364993	0.913727
	SST-843	0.552924	0.751222	0.657969
TaDr1b (Consensus)	Lancer	0.526435	0.469882	0.003831
	Trojan	0.909715	0.0057129	0.922611
	Akmola-2	0.349514	0.0091397	0.503262
	VK-1	0.041156	0.00667096	0.008758
	SST-398	0.001809	0.0064767	5.07E-5
	SST-843	0.702294	0.0131324	0.009281
TaDr1B(3D)	Lancer	0.518772	0.553065	0.825468
	Trojan	0.045589	0.858308	0.0304509
	Akmola-2	0.397811	0.641699	0.951548
	VK-1	0.010923	0.50623	0.880361
	SST-398	0.88859	0.0465494	0.008269
	SST-843	0.0253656	0.0183974	0.644942