Identification, Classification of AOX genes in *T. aestivum* and Their Expression Patterns to Salt Stress

by

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

ABARES	Australian Bureau of Agricultural and Resource Economics and Sciences
ADP	Adenosine Diphosphate
AGI	Arabidopsis Genome Initiative
Aox	Alternative Oxidase (nuclear gene)
AOX	Alternative Oxidase (protein)
At	Arabidopsis thaliana
ATP	Adenoside Triphosphate
BGI	Beijing Genomics Institute
BLAST	Basic Local Alignment Search Tool
bp	Base Pair
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium
CAC	Citric Acid Cycle
cDNA	Complementary DNA
CREs	*cis*-Regulatory Elements
CS	Chinese Spring
CSS	Chromosome Survey Sequences
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EDTA	Ethylenediamine Tetraacetic Acid
ENA	European Nucleotide Archive
EST	Expressed Sequence Tags
ETC	Electron Transport Chain
FAOSTAT	Food and Agriculture Organization Corporate Statistical Database
FW	Fresh Weight
g/mg	grams/milligrams
Gb	Gigabases
gDNA	Genomic DNA
IBGSC	International Barley Genome Sequencing Consortium
IBI	International Brachypodium Initiative
IMM	Inner Mitochondrial Membrane

IMS	Intermembrane Space
InDels	Insertion and Deletions
IRGSP	International Rice Genome Sequencing Project (IRGSP)
IWGSC	International Wheat Genome Sequencing Consortium
Kb	Kilobase
kDa	kilodalton
L/ml/µl	litre/millilitres/microliters
LTR	Low-temperature responsiveness
M/mM	molar/millimolar
MDA	Malondialdehyde
mETC	mitochondrial Electron Transport Chain
MMT	Metric Million Tonnes
mRNA	Messenger RNA
mROS	mitochondrial reactive oxygen species
Mw	Molecular Weight
MYA	Million Years Ago
NAD(P)H	NADH and NADPH
NADH	Nicotinamide Adenine Dinucleotide [reduced]
NADPH	Nicotinamide Adenine Dinucleotide Phosphate [reduced]
NCBI	National Center for Biotechnology Information
NEB	New England Biolabs
nt	nucleotide
ORF	Open Reading Frame
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with Tween® detergent
PCR	Polymerase Chain Reaction
Pfam	Protein family database
PGSC	Potato Genome Sequencing Consortium
PMF	Proton Motive Force
PVPP	Polyvinyl-polypyrrolidone
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RDW	Relative Dry Weight
RNA	Ribonucleic Acid

ROS	Reactive Oxygen Species
SDS	Sodium Dodecyl Sulphate
SDW	Shoot Dry Weight
SEM	Standard Error of the Mean
SHAM	Salicylhydroxamic acid
SNP	Single-Nucleotide Polymorphism
STI	Salinity Tolerance Index
T_a	PCR cycle annealing temperature (°C)
TAE	Tris-Acetate-EDTA
TAO	*Trypanosomal* alternative oxidase
TBA	Thiobarbituric Acid
TBARS	Thiobarbituric acid reactive substances
TCA	Tricarboxylic Acid
TES	N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid
TGAC	The Genome Analysis Centre, UK
TGC	Tomato Genome Consortium
T_m	PCR cycle melting temperature (°C)
TSA	Transcriptome Shotgun Assembly
URGI	Unité de Recherche Génomique Info, France
w/v	weight/volume
WGS	Whole-Genome Shotgun
µg/ng	micrograms/nanograms
µM/nM	micromolar/nanomolar

Abstract

Crop yields are subject to environmental stresses. Increasing the knowledge of stress-tolerant plant genotypes could increase crop yields. *AOX* genes have been shown to play a significant role in stress tolerance of several plant species. For crop species to date, research has focused on AOX in rice and barley and its stress responses, and it has been observed that AOX expression responds to low temperatures and salinity stress, as environmental stressors.

At the start of this project, publications on AOX and the alternative pathway of respiration in wheat were scarce. The primary objective of this research was to identify and characterise the *AOX* gene family in *T. aestivum*. Specifically, the aims were to (1) identify the gene candidates in commercial bread wheat; (2) assess which of these genes were stress responsive when exposed to chemical and a biological stress, such as salinity; and (3) assess *AOX* gene families' members and expression to determine differences, if any, between *T. aestivum* and its wild ancestors during its evolution.

The research characterised the structure, phylogeny and expression profile of the *AOX* gene family. In *T. aestivum* and its related species, 89 *AOX1* genes were identified, of which, 58 were confirmed as complete genes. However, owing to the quality of genome assemblies of *Ae. sharonensis*, it was not possible to determine the full length of *AeshAox1a*. From the phylogenetic analyses, those identified AOX proteins were classified as AOX1a, AOX1c, AOX1d and AOX1e type. The *in silico* analyses showed that the *AOX* genes in *T. aestivum* were expressed at precise developmental stages as well as when subjected to both abiotic and biotic stresses.

Four commercially significant *T. aestivum* cultivars, Chinese Spring (CS), Opata M85 (Op.), Gladius (Gl.) and Drysdale (Dr.), showed different salinity tolerances in response to salt stress. A comparative study between tolerant versus sensitive cultivars revealed tissue-dependent variations in *AOX* gene expression, physiological and biochemical responses. Thirteen *Aox1* genes were studied at the transcript level using qRT-PCR. Under chemical and salinity stress, qRT-PCR analyses showed that *Aox1a* clades and *Aox1d* clades were the most responsive isoforms in *T. aestivum*.

Similarly, *Aox1a* clades and *Aox1d* clades genes were the most responsive *Aox1* gene expression in four direct ancestors of *T. aestivum*. Consistent with the transcript findings,

immunoblot analysis revealed that AOX protein abundance was induced by KCN and salinity stress. This study's findings showed that the AOX protein abundance was higher in the seedling under KCN treatment than in the seedling under salinity stress. The findings suggest that the elevated *AOX* expression in both sensitive species reflects the role of AOX attempting to minimise ROS when subjected to salinity stress. In contrast, tolerant species exhibited higher pre-existing AOX protein levels than salt-sensitive species, which gives salt-tolerant species an advantage in coping with oxidative stress.

Declaration

I certify that this thesis does not incorporate without acknowledgement 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.

Signed 

Date 05/12/2019

Conference

Certificate of Participation, Poster title: 'Identification and characterization of homologues *Aox1* genes encoding alternative oxidase in bread wheat (*Triticum aestivum L.*)', at the 7th International Crop Science Congress, held on 14–19 August 2016 in Beijing, China.

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Chapter 1: Introduction and Literature Review

1.1 Introduction

Wheat, maize and rice are the three main cereal crops that account for more than 50% of the world's production of cereal grains in 2019 (Table 1.1). Globally, humans consume over 50% of their daily caloric intake from cereal grains (Daryanto et al. 2016; Tilman et al. 2002). Wheat production occupies the largest hectare area at 220.2 Mha, and yet, the yield as a percentage of production area is only 34% (Table 1.1). Comparatively, maize has the highest productive yield as a percentage of land area at 5.63 times, with rice yielding 4.57 times its productive area. Moreover, wheat is the leading grain that is traded globally both in terms of productive metric million tonnes (MMT) and percentage entering trade markets. When comparing wheat with maize, wheat occupies 12.7% greater land area (ha) and yet yields 31.9% less.

Barley is the most salt-tolerant of the cereal crops (Munns & Gilliham 2015), and when comparing with wheat's productive area, which is 4.6 times greater, its productive area would yield only 674 MMT of barley. When comparing rice with wheat, rice's productive area (ha) when increased by 33% (for similar comparison) would yield 1005.6 MMT. Thus, rice would produce 25.5% more grain yield for the same productive area as wheat. Hence, the implication is that despite wheat's greater acreage (ha), factors such as drought, climate change and salt tolerance affect its productive capacity. Similarly, other factors, such as an expanding global population, increase the demand for wheat (Figure 1.1).

Table 1.1: World production of cereal grain in 2019

Grain	Area (Mha)	Production (MMT)	International trade (MMT)	International trade (%)
Maize	195.3	1100.2	147	13.36
Wheat	220.2	749	183	24.43
Rice (paddy)	165.2	756.1	40	5.29
Barley	47.6	145.7	31	21.27
Sorghum	45.3	63.3	8.6	13.58

Sources: FAOSTAT database. 2019 (http://www.fao.org/faostat/en/#data)

Wheat production faces significant challenges because of this escalating demand and needs to be increased by more than 35% to meet the projected demand of the global population by 2050 (Ray et al. 2013, 2015). The impact of changing weather conditions and expanding global

demand for wheat necessitates increasing the production yield (Challinor et al. 2014). Changing climatic conditions, particularly variations in temperature, affect the crop and are expected to affect yields negatively. Challinor et al. (2014) found that adapting the wheat and rice crops could result in 7–15% increase in productive yield capacity.

Changing weather patterns in countries such as Australia has affected wheat production. The productive yield of wheat decreased by 46.7% from 2016–2017 to 2018-2019 (Table 1.2; ABARES 2019). This represents the lowest volume since 2007–2008, and this decrease is due to extreme drought occurring in New South Wales and Queensland. Wheat exports are likely to significantly decrease year-on-year owing to lower production and increased domestic demand. This demand for wheat is also attributed to the increasing use as animal feed for livestock because of the dry weather conditions, which have reduced grazing areas. The average productive yield over three years (2016–2019) was 23.33 MMT, which reflects a decrease of 26.66% from 2016–2017. With productive yield capacities decreasing year-on-year, this negative effect will similarly affect the export of wheat despite continuing demand both domestically and internationally.

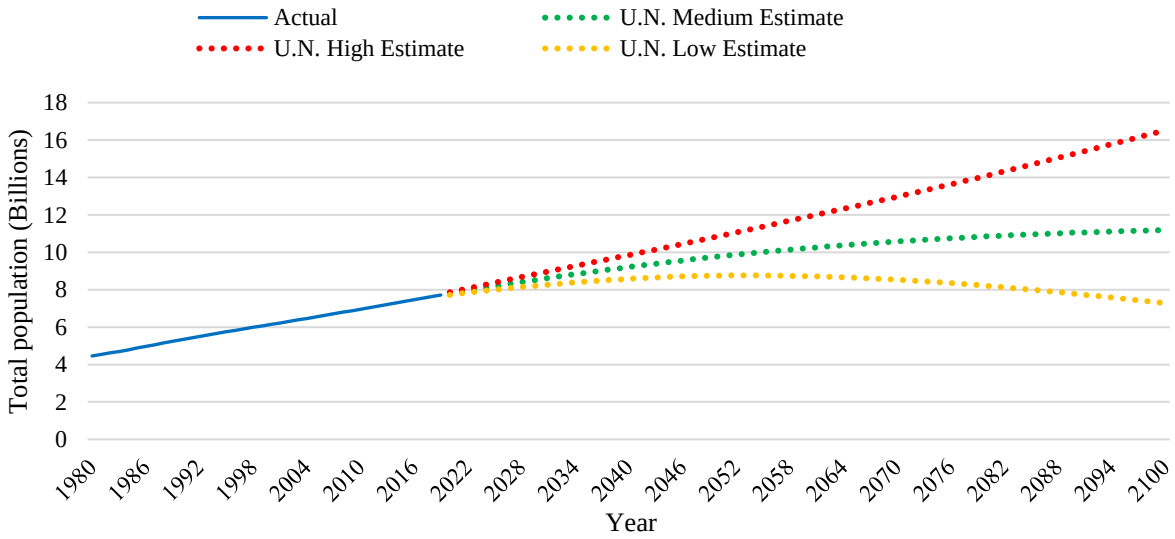

Figure 1.1: United Nations 2017 world population projection

Table 1.2: Australian wheat production in MMT from 2016-2017 to 2018-2019 (ABARES 2019)

States	2016-2017 MMT	2017-2018[s] MMT	2018-2019[f] MMT	Three-year average 2016–2019
New South Wales	9.81	4.49	1.98	5.42
Victoria	4.66	4	1.95	3.53
Queensland	1.50	0.68	0.43	0.87
South Australia	6.13	4.09	2.86	4.36
Western Australia	9.64	7.94	9.68	9.08
Tasmania	0.05	0.03	0.047	0.04
Australia (Total)	31.81	21.24	16.95	23.33

f = forecast, s = estimation (Source: ABARES 2019, Australian Crop Report: February 2019, No. 189).

The identification of new types of stress-tolerant genotypes and genes controlling such tolerance is vital for improving the cultivation and yield of crops. Identifying and creating new types of stress-tolerant cultivars is especially important for increasing crop yields for those crops susceptible to environmental stressors. Alternative Oxidase (*AOX*) genes play a significant role across several crop species in increasing stress tolerance. *AOX* genes have been shown to be responsive to abiotic stresses such as cold in hexaploid wheat (Mizuno et al. 2008; Takumi et al. 2002). The fact that *AOX* genes have been shown to be of vital importance in stress tolerance in wheat provides a basis for extended research. The focus of this thesis research is in identifying the genetic responses of *AOX*, to improve crop yield, by evaluating salinity stress associated with *AOX* expression in bread wheat, *T. aestivum*.

1.2 *Poaceae* Genome Organisation

The *Poaceae* family has fewer species and genera than other families but is considered of greater ecological and economic significance (Gaut 2002). The grass family phylogeny for the subfamily *Pooideae* is determined by its genome diversity based on the genome size (Gaut 2002). The *Pooideae* subfamily has 3,300 known species, with a genome size of 2.25–17.9 Gb (Gaut 2002). There was a divergence at the time of *Bambusoideae*, *Ehrhartoideae* and the *Pooideae* clade, between *Erhartoideae* (rice) and the *Pooideae* subfamily, which resulted in the *Triticeae* having diverged from oats 25 million years ago (MYA) (Gaut 2002). The origin of wheat occurred in this clade some 13 MYA (Figure 1.2).

Figure 1.2: Evolutionary dynamics of *Poaceae* genomes (Gaut 2002)

Restriction fragment length polymorphism genetic map markers, which illustrate the differences between deoxyribonucleic acid (DNA) sequences, became prevalent in the 1980s, and by the 1990s, genetic maps of grass genomes were then comparable (Gaut 2002). Wheat genomes A, B and D were compared, and it was determined that, typically, markers maintain their order throughout the grass genomes (Gaut 2002). This colinearity of marker and gene order has resulted in the comparative study of DNA sequences, including those of diploid wheat (Feuillet & Keller 2002). It has been determined that genes are not randomly organised and that those species with the largest genomes have high numbers of gene clusters along the chromosomes (Feuillet & Keller 2002). With large wheat and barley genomes, microcolinearity has been found at the orthologous gene receptor kinases (Feuillet & Keller 2002).

Paux et al. (2008) approximated that the wheat genome was 17 Gb. Hence, this has posed challenges to sequencing because >80% are repetitive sequences and the wheat chromosome 3B is more than double the size of domesticated and wild rice genomes, which ranged from 261 Mb to 389 Mb (Paux et al. 2008; Stein et al. 2018). Rice was selected as the closest model plant as a monocot to wheat (Rensink & Buell 2004). The maize genome is approximately equivalent to three wheat chromosomes: 2.5 Gb (Li et al. 2004; Paux et al. 2008). Bread wheat is considered a recent hexaploid and has three homoeologous genomes (A, B and D) that have related progenitor species (Paux et al. 2008).

1.3 Orthology Relationships Between *Poaceae*

Making numerous comparisons between genomes enables the clarification of evolutionary species (Wang et al. 2015). Thus far, there has been research on the most economically viable *Poaceae* representing the genome sequencing of eight grass clades (Wang et al. 2015). When comparing orthologous gene pairs, the genomes' evolutionary rates vary between them when duplicated simultaneously (Wang et al. 2015). The nucleotide substitution was found to be 48% quicker in all grasses except rice, with diploid wheat genomes showing that about ~50% of the genes are anchored to chromosomes, and thus, do not have a slow evolutionary rate (Wang et al. 2015). *Ae. tauschii* and *T. urartu* as two diploid wheat genomes have the least colinear genes and regions inferred because approximately half the genes are attached to chromosomes (Wang et al. 2015). Differentiation between crop species can also vary significantly with regard to genome size (Table 1.3). *T. aestivum* (bread wheat) differs from rice by a factor of 40, with rice being 4.3×10^8 bp and bread wheat being 1.7×10^{10} bp (Keller & Feuillet 2000).

The orthology, the common evolutionary conservation of loci of genomes amongst *Poaceae,* has identified colinearity between genomes of wheat, maize and rice (Keller & Feuillet 2000). More recent mapping has shown some exceptions to the known orthology and colinearity. The leaf rust resistance genes in wheat and rice have been shown to lack colinearity at the locus *Lr1* on the chromosome 5DL (Keller & Feuillet 2000). Sorrells et al. (2003) found many chromosome rearrangements when conducting a comparative sequence analysis of the locations of rice genome with the homologous wheat genomes. This finding shows that there are flaws in transferring cross-species information when using rice as a base model (Sorrells et al. 2003).

Table 1.3: Examples of genome size variation in monocot and dicot plants

Arabidopsis Genome Initiative (AGI), International Brachypodium Initiative (IBI), International Rice Genome Sequencing Project (IRGSP), Potato Genome Sequencing Consortium (PGSC), Tomato Genome Consortium (TGC), International Barley Genome Sequencing Consortium (IBGSC), International Wheat Genome Sequencing Consortium (IWGSC).

Species	Common name	Estimated size (Gb)	Reference
Arabidopsis thaliana	Arabidopsis	0.125	AGI 2000
Brachypodium distachyon	Brachypodium	0.272	IBI 2010
Oryza sativa	Rice	0.389	IRGSP 2005
Phoenix dactylifera	Date palm	0.658	Al-Dous et al. 2011
Sorghum bicolor	Sorghum	0.73	Paterson et al. 2009
Solanum tuberosum	Potato	0.844	PGSC 2011
Solanum lycopersicum	Tomato	0.9	TGC 2012
Glycine max	Soybean	1.1	Schmutz et al. 2010
Zea mays	Maize	2.5	Li et al. 2004
Aegilops tauschii	Goat grass	4.3	Luo et al. 2017
Triticum urartu	Red wild einkorn	4.94	Ling et al. 2018
Hordeum vulgare	Barely	5.1	IBGSC 2012
Triticum monococcum	Einkorn wheat	5.6	Fox et al. 2014
Triticum turgidum ssp. dicoccoides	Wild emmer	10.1	Avni et al. 2017
Triticum turgidum L. ssp. *durum* (Desf.)	Durum wheat	10.45	Maccaferri et al. 2019
Triticum aestivum	Bread wheat	17	IWGSC 2014

1.4 Bread Wheat Genome Evolution

Diploid einkorn wheat, *T. monococcum* spp. *monococcum*, is a domesticated spring wheat. It was domesticated from the wild winter wheat form, *T. monococcum* spp. *aegilopoides* (Fox et al. 2014). Approximately 0.5 to 0.36 MYA (Figure 1.3), wild tetraploid emmer wheat *T. dicoccoides* was derived from the natural hybridisation between *T. urartu* ($2n = 2x = 14$ A^uA^u) and an unknown species with B genome associated to the progenitor *Aegilops speltoides* ($2n = 2x = 14$, SS). The newly evolved species *T. dicoccoides* created a cultivated tetraploid emmer wheat *T. dicoccon* ($2n = 2x = 28$, A^uA^u BB) (Dvorák et al. 2012; Feldman & Levy 2005).

Further natural hybridisation occurred between *T. dicoccoides* (the newly formed tetraploid) and *Ae. tauschii* (a third diploid species) as the D genome donor, forming the hexaploid wheat *T. aestivum* ($2n = 6x = 42$, A^uA^uBBDD) (Feldman & Levy 2012; Jia et al. 2013; Li et al. 2014;

McFadden & Sears 1946). Another hybridisation along with a chromosome doubling between *T. timopheevii* (2*n* = 4*x* = 28, A^uA^u GG) and *T. monococcum* (2*n* = 2*x* = 14, A^mA^m) gave rise to the hexaploid species *T zhukovskyi* (2*n* = 6*x* = 42, $A^mA^mA^uA^u$GG) (Huang et al. 2002). *T. zhukovskyi*'s genomic constitution was the first hexaploid wheat that was different from the common bread wheat *T. aestivum* (2*n* = 6*x* = 42, A^uA^u BBDD) (Upadhya & Swaminathan 1963).

In the past few years, the whole genomes of several *Triticum* and *Aegilops* species have been sequenced, such as *T. aestivum*, *Ae. tauschii* and *T. urartu*. Initial whole-genome sequencing of *T. aestivum* was successfully achieved by Brenchley et al. (2012) using the whole-genome shotgun sequencing approach. Soon after, genome sequences of wheat-related species were produced along with bread wheat updated versions (Avni et al. 2017; Clavijo et al. 2017; IWGSC 2014; 2018; Ling et al. 2013; 2018; Luo et al. 2017; Maccaferri et al. 2019; Zhao et al. 2017b).

Advancement in plant genome sequencing has enabled research to study hexaploid *T. aestivum* and was made possible through the Next Generation Sequencing (NGS) (Bierman & Botha 2017). Bread wheat *T. aestivum* consists of a large hybrid combination of A, B and D subgenomes and has been highly debated over the past several decades. The IWGSC (2018) has conducted research for 13 years, to identify an annotated reference of the *T. aestivum* genome sequence. Researchers have been able to annotate reference the sequencing of 21 chromosome assemblies resulting in 107,891 high-confidence genes and their genomic regulatory sequences (IWGSC 2018). In doing so, the IWGSC (2018) has identified the dynamics of change brought about by environmental conditions to complex gene families, which affects the end-use quality at the subgenome level. By understanding the annotated reference assembly, it resolved the genetic basis and identified the quantitative trait locus for determining abiotic stress resistance (IWGSC 2018).

The result of this research has increased the understanding of the high-quality chromosome genome structure, thereby enabling greater insight into gene networks that regulate the expression traits that can result in higher crop yield (IWGSC 2018). The ability to access sequence-level information will enhance the ability to locate genomes that need to be changed for breeding due to the annotated reference of the genomic sequence (IWGSC 2018). This breakthrough requires the establishment of DNA marker platforms being newly identified (IWGSC 2018).

Figure 1.3: Evolutionary ancestry of *Triticeae aestivum*

The black arrows indicate the evolutionary hybridisation steps, green arrows indicate the domestication steps and red arrows indicate the selection steps that have resulted in rye, barley and modern wheat cultivars. The scale indicates the time in million years (MY). Genome constitutions are given beside the species names (Feuillet et al. 2007).

1.4.1 The A Genome

In hexaploid wheat *T. aestivum*, there is one A genome as compared with *T. zhukovskyi* that has two A genomes (Dvorák et al. 1993). The source of the A genome in *T. aestivum* has been traced to the progenitor *T. urartu* (diploid AA) (Dvorák et al. 1993; Ling et al. 2018). The genomic study of *T. urartu* has clarified the evolutionary structure of tetraploid and hexaploid wheat (Ling et al. 2018). There are similarities in the nucleotide sequences in A genomes between species *T. zhukovskyi* and *T. urartu* (Dvorák et al. 1993; Upadhya & Swaminathan 1963). The other A genome found in *T. zhukovskyi* was inherited from *T. monococcum* (Dvorák et al. 1993; Upadhya & Swaminathan 1963).

1.4.2 The B Genome

It is believed that the initial B genome donor found in wheat cannot be found in the wild anymore because it was lost during evolution (Feuillet et al. 2007). Studies have shown that the diploid ancestor of the B genome in tetraploid and hexaploid wheat is probably a member of the *Sitopsis* section directly associated to *Aegilops speltoides* ($2n = 2x = 14$, SS) (Blake et al. 1999; Feldman et al. 1995; Feuillet et al. 2007). Salse et al. (2008) suggested that *Ae. speltoides* seemed more likely to have a direct evolutionary association to the B genome found in *T. aestivum* rather than both A and D genomes. Wheat genotypes can potentially be improved

for stress tolerance through the use of the *Sitopsis* group species due to their unique genes (Zhang et al. 2001).

1.4.3 The D Genome

The D genome that is found in *T. aestivum* originated from the *Ae. tauschii*, and this genome carries the alleles and genes that enable the species *Ae. tauschii* to adapt to changes in climatic conditions found in Central Asia (Feuillet et al. 2007). As a result, the presence of the D genome in wheat *T. aestivum* enables plants of hexaploid wheat to be grown more widely across the world, more than other *Triticum* species (Feuillet et al. 2007). It is the D genome that provides the soft grain endosperm from the encoded proteins that are essential for bread, whereas the hard endosperm of tetraploid durum wheat without the D genome is suitable for pasta (Chantret et al. 2005).

More recent research has shown that the *Ae. tauschii* genome is directly associated with the D genome of hexaploid wheat (Luo et al. 2013). The D genome found in wheat is directly inherited from *Ae. tauschii* (Luo et al. 2017). Researchers have found that a group of accessions in Iran near the Caspian Sea, *Ae. tauschii* ssp. *tauschii* var. *meyeri*, have a direct relationship with the D genome of bread wheat, which is more than the *Ae. tauschii* ssp. *strangulata* accession AL8/78 (Luo et al. 2017). *Ae. tauschii* has been shown to play a vital role in wheat breeding, and its genome is a vital reference in wheat genomics sequencing (Brenchley et al. 2012; Luo et al. 2013).

1.5 Effect of Salinity in Bread Wheat and its Relatives

1.5.1 Salinity

Salinity stress is abiotic stress brought about by environmental factors that have a harmful effect on photosynthetic systems. Salinity stress affects the growth of crops and thereby inhibits productive yield by affecting soil structure (Munns & Gilliham 2015). The soil stress caused by salinity results in ion toxicity, particularly chlorine (Cl^-) and sodium (Na^+) ions, and is brought about by poor drainage, low-quality water, waterlogging and poor irrigation (Fayrap & Koç 2012). Soil salinity causes considerable wheat yield loss (Oyiga et al. 2016). The survival of higher plant species is constantly under threat from numerous abiotic stresses, such as extreme weather conditions. Severe temperatures, drought, UV-radiation, waterlogging and irrigation with saline water can result in a salt imbalance, whereby plants are unable to extract water and receive nutrients (Najeeb et al. 2015).

1.5.2 Mechanisms of Salinity Tolerance

Salinity tolerance is a response to salinity stress and varies significantly between and within plant species and varieties (Gupta & Huang 2014). In the case of wheat (*Triticum aestivum*), it is less salt-tolerant than barley (*Hordeum vulgare*) (Gupta & Huang 2014). In cereal crops, such as wheat, salinity tolerance is where a productive crop yield occurs despite having grown in salinised soils. Salinity tolerance includes three main components: first osmotic tolerance, second, tissue tolerance to Na^+ or Cl^-, and lastly, the exclusion of Na^+ or Cl^- (Figure 1.4) (Munns & Tester 2008). Through these types of saline tolerance, plants such as wheat are able to manage the saline environment.

Figure 1.4: Three mechanisms of salinity tolerance (Roy et al. 2014)

1.5.2.1 Osmotic Tolerance

Osmotic tolerance is a plant's ability to tolerate stresses brought about by salt stress (Eynard et al. 2005). This results in reductions of crop yields owing to alterations to metabolic processes, creating an increase in osmotic pressure that thereby restricts water uptake, a form of physiological drought (Eynard et al. 2005). Further, it brings about abnormal pH levels and ionic competition, which results in limited nutritional uptake (Eynard et al. 2005). Salt stress is ion-specific, due to changes to the ionic composition, such as sodium toxicity (Na^+), which has been shown in herbaceous crops, such as wheat (Eynard et al. 2005; Läuchli & Epstein, 1990). Osmotic tolerance can result in 10–90% failure of wheat crop yield (Eynard et al. 2005).

Osmotic tolerance is associated with reduced shoot growth, as well as possibly linked to signalling and sensing mechanisms (Roy et al. 2014). Ion exclusion occurs mainly in the roots, where the transport process of Na^+ and Cl^- restricts the toxic accumulation of ions in the leaves (Roy et al. 2014). Osmotic tolerance is controlled by long-distance signals that result in reduced shoot growth prior to shoot Na^+ increasing (Roy et al. 2014). The understanding of the osmotic tolerance phase is limited. One aspect that is known is that the process involves fast long-distance signalling, which is understood to occur from ROS waves (Mittler et al. 2011). Variances in osmotic tolerance could be attributed to variations in the long-distance signalling or to how the salt is initially perceived or could be due to variations in signal response (Roy et al. 2014).

The lack of a clear understanding of the specific mechanism of salt tolerance allows for increased speculation. The function of abscisic acid (ABA) is where ABA increases as a result

of high salinity levels (Addicott & Van Steveninck 1983; Guy 1990; Sánchez-Serrano et al. 1991; Zeevaart & Creelman 1988). It is the function of ABA that controls plant growth, particularly in saline environments, by altering the ratio of shoot and root. When wheat is in an environment that lacks water, it is ABA that is partly responsible for the transpiration stream stomata closure (Munns 1992). Plant antioxidant systems have also played a significant function in increasing wheat osmotic tolerance (Sairam et al. 1997; Sairam et al. 2000).

When wheat is subjected to osmotic stress, photosynthesis reduces, which results in ROS, for example, hydrogen peroxide, hydroxyl radical ($^{\bullet}OH$) or superoxide (O_2^- or O_2) found in the plant cells. Since ROS is toxic, it has been shown to damage proteins, RNA and DNA and destroy plant cells through oxidative stress; in response, plant cells increase their antioxidant enzymes (Jiang & Zhang 2002; Mittler 2002). The defensive nature of the enzymes acts as a detoxification process of the ROS, thereby activating stress-responsive signalling pathways (Garratt et al. 2002). When wheat cultivars are subjected to deficient water conditions, a stress response activates an increase in antioxidant enzymes and metabolites, such as α-tocopherol, ascorbic acid, carotenoids and glutathione, in some tolerant wheat species versus those that are more sensitive to drought conditions (Sairam et al. 1997; Sairam et al. 2000).

1.5.2.2 Exclusion of Na^+ or Cl^-

Salt tolerance in plants has been associated with increased efficiency of the selective uptake of K^+ instead of Na^+ (Tester & Davenport 2003). Na^+ exclusion results in a low level of accumulation, with an increased K^+, in the shoot and is a primary salinity tolerance mechanism for *Triticum aestivum* (Cuin et al. 2008; Gorham et al. 1987). *T. aestivum* (bread wheat) is more saline tolerant than *T. turgidum* ssp. *durum* (durum wheat) (Colmer et al. 2006; Francois et al. 1986; Gorham et al. 1987; Joshi et al. 1982; Maas & Greive 1990; Rawson et al. 1988). Durum wheat cultivars have been shown to have a greater sensitivity to salinity due to increased accumulation of Na^+ in the plant shoot (Francois et al. 1986; Maas & Greive 1990). However, Genc et al. (2007) more recently analysed 51 wheat genotypes and found no apparent relationships between salinity tolerance and leaf Na^+.

T. durum and *T. aestivum* have been shown to vary in salt tolerance when grown with different concentrations of NaCl (1–150 mM) and Ca^{2+} (10 mM) solutions, which results in Na^+ of 15–50 mmol in their roots (Husain et al. 2004). The Na^+ permeates through cation channels in the

root (Tester & Davenport 2003). However, plant roots typically (>95%) prevent salt accumulation, which is the primary mechanism for salinity tolerance (Munns 2005). Moreover, a strong relationship exists for numerous plant species between salt exclusion and salt tolerance (Munns & James 2003; Tester & Davenport 2003). In wheat, there are distinctions in salt sensitivity between varieties, for instance, durum wheat *T. turgidum* ssp. *durum* has increased salt sensitivity as compared with bread wheat (*T. aestivum)*, which limits productive farming capacity where soils are heavily salinised or sodic (Munns 2005).

In the case of bread wheat, when subjected to Na^+ and Cl^- of a solution of 50 mM NaCl concentration to the xylem, the percentage of exclusion varied between bread genotypes. Janz had a concentration of 0.3 mM Na^+ as compared with Chinese Spring at 1.1 mM Na^+, Kharchia-65 at 1.4 mM Na^+ and Punjab-85 at 1.6 mM Na^+ (Munns 2005). The exclusion percentages were for Janz at 99%, Chinese Spring 98%, and both Kharachia-65 and Punjab-85 at 97% (Munns 2005). Bread wheat typically has a low rate of Na^+ transport to the plant shoots as compared with other genotypes, such as barley, which have a high rate of Na^+ (Munns 2005).

Husain et al. (2003) researched six durum wheat genotypes to measure the effects of Na^+ exclusion on the transport of Na^+ in leaves as a way to improve crop yield. They found that those leaves with high concentrations of Na^+ had rapid chlorophyll loss and died sooner than those with lower Na^+ (Husain et al. 2003). Differences only occurred between genotypes at the low level, 75 mM NaCl, whereas at higher levels, 150 mM NaCl, the effects of osmotic stress had the same effect on both genotypes (Husain et al. 2003). The yield on the low Na^+ increased by 20% when subjected to moderate salinity, whereas there was no improvement in yield when subjected to a high salinity concentration. They concluded that in genotypes, where there is a high salinity concentration, other traits are essential since the 'osmotic effect of the NaCl outweighs its salt-specific effect on growth and yield' (Munns 2005, p. 649).

1.5.2.3 Na^+ Tissue Tolerance

Salinity tolerance and controlling the accumulation of Na^+ in cereal crops, particularly in the leaf blade, is essential (Munns & Tester 2008). However, frequently, there is minor or no correlation between the leaf content and Na^+ tolerance in wheat (Genc et al. 2007). Several studies have shown a weak correlation between salinity tolerance and the level of exclusion (Ashraf & McNeilly 1988; Genc et al. 2007; Hollington 2000). Following the transportation of

Na^+ to the leaves, plants can tolerate ion-specific stress through vacuole compartmentalisation (Munns & Tester 2008).

Vacuoles are storage organelles found in plant cells and function to store inorganic ions that accumulate when plants are subjected to saline and adverse climatic conditions (Fan et al. 2015). The sequestering of Na^+ is the primary mechanism by which salinity tolerance occurs for some halophytes (Munns & Tester 2008). This is necessary because of enzyme intolerance to high levels of Na^+ despite the high-level accumulation that occurs in the plants' leaves (Munns & Tester 2008). It has been shown that the enzymes in leaf cells are functional despite high concentrations of Na^+ and Cl^- (in excess of 200 mM), which is the result of the ions being compartmentalised into the cell's vacuoles, thereby reducing toxicity (Fan et al. 2015; Tester & Davenport 2003).

There are many compatible solutes, which vary within plant species and between the genotypes. Compatible solutes include nitrogen-based compounds and carbohydrates (Mansour 2000; Munns 2002). Crops subjected to salinity stress have high concentrations of nitrogen-based compounds, such as glycine-betaine (GB) and proline. Halophytes are protected from osmotic cell damage by accumulating higher levels of GB and proline in the leaf. In wheat, salinity tolerance has been associated with high accumulations of GB (Sairam et al. 2002). Compatible solutes not only provide osmotic adjustment but are also ROS scavengers (Wang et al. 2003a). ROS scavengers are a cohort of antioxidant substances that provide cells protection from oxidative stress. Heat, drought, salt and oxidative stress are associated with the creation of ROS, such as OH, O^{2-} and H_2O_2 (Mittler 2002).

1.6 Plant Mitochondrial Metabolism

1.6.1 Mitochondria

Mitochondria in plant cells are energy-producing organelles along with the chloroplast. The DNA of mitochondria are semi-autonomous organelles and are made from protein encoded by both nuclear and mitochondrial genes. Mitochondria multiply by fission as opposed to *de novo* synthesis. Mitochondria produce >90% of adenosine triphosphate (ATP) in a cell when subject to aerobic conditions through oxidative phosphorylation. Organic molecules release energy through the process of cellular respiration, which occurs partly in the mitochondria, whereby

ATP is synthesised from adenosine diphosphate (ADP) and inorganic phosphate (P_i) (Taiz & Zeiger 2002). Mitochondria are composed of a double membrane, with the inner invaginated referred to as the cristae, surrounded by a thick outer membrane that surrounds the whole organelle (Figure 1.5). The region between the membranes is known as the intermembrane space, or perimitochondrial space, and the region enclosed by the inner membrane is the matrix, where the citric acid cycle (CAC) is found (Finnegan et al. 2004). Plant productivity is understood to be directly correlated to mitochondrial respiration. Other roles that mitochondria play in the cell include components of metabolism of nitrogen, phosphorus, carbon and sulphur. In addition, mitochondria participate in cell signalling and programming cell death, thereby determining a cell's fate (Fernie et al. 2004; Sweetlove et al. 2007).

Figure 1.5: Mitochondria

Three compartments of the mitochondria are shown: the outer membrane, inner membrane and matrix (Nelson & Cox 2000).

1.6.2 Mitochondrial Electron Transport Chain (mETC)

Photosynthesis in plants is a primary pathway for fixing carbon, which is then used in growth and respiration, and providing energy. The photosynthetically produced carbohydrates ($C_6H_{12}O_6$) are used to generate energy for growth and produce CO_2 and H_2O by oxidation. Oxidation of carbon compounds is coupled with nicotinamide adenine dinucleotide phosphate ($NADP^+$) reduction to nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) at the start of the mitochondrial Electron Transport Chain (mETC) in the TCA pathway. Both $NADP^+$ and NADPH are required for biosynthetic reactions. However, NADH and succinate undergo oxidative phosphorylation, where electrons from NADH are accepted ultimately by O_2 in the inner mitochondrial membrane (IMM), with a subsequent release of energy that powers ATP synthase and produces ATP from ADP (Figure 1.6).

Mapping of mETC and the TCA in mammalian mitochondria has shown eight sites that have the capability to release both H_2O_2 and $O_2^{-\bullet}$, but as yet, these sites are not fully characterised (Brand 2010; Perevoshchikova et al. 2013; Quinlan et al. 2012). In plants, the mETC has

distinctive non-phosphorylating respiratory pathways, which include AOX, uncoupling proteins 5 (UCP) and type II NAD(P)H dehydrogenases (Finnegan et al. 2004; Picault et al. 2004; Rasmusson et al. 2004). The mETC starts with oxidation of the NADH to NAD^+ and involves Complex I (NADH CoQ reductase or NADH dehydrogenase Type I). Complex I functions as the link between glycolysis, the CAC and fatty acid oxidation to the mETC. Then, Complex II (succinate dehydrogenase or succinate-CoQ reductase) combines the CAC to the mETC. Complex III (CoQ reductase) requires $CoQH_2$ for the next step, and Complexes I and II both yield it. Electrons from the ubiquinol-10 are rechannelled from the CoQ reductase and reduce cytochrome *c* and form a substrate for the terminating Complex IV (CoQ reductase). Complexes I, III and IV use the energy released during the electron transfer to pump protons and produce a proton gradient between the matrix and IMM space. This is the proton motive force (pmf) and is dissipated by the Complex V (ATP synthase) by which ADP and inorganic phosphate (P_i) are combined to generate ATP (Figure 1.6). This is known as the 'cytochrome pathway'; electron flow through this pathway results in the maximal ATP synthesis and hence is known as the phosphorylating pathway. NAD(P)H oxidation can also occur via the internally orientated NADH dehydrogenases (NDin), along with external-orientated NDex, as well as the AOX, which are not coupled to proton transport. This defines the 'alternative pathway', and when it is operational, electrons channelled from the $CoQH_2$ are divided between AOX and Complex III, cytochrome c and Complex IV (CP).

Figure 1.6: The plant mitochondrial electron transport chain

Complex I; the NADH dehydrogenase, CII; Succinate dehydrogenase, CIII; Cytochrome c reductase, IV; cytochrome c oxidase and V; ATP synthase. Q is the ubiquinones (CoQ) pool, internal- and external-oriented alternate NADPH dehydrogenases (NDin and NDex) and AOX are the alternative non-proton-pumping pathways that catalyse the NADPH and NADH oxidation. IMM denotes the inner mitochondrial membrane; MnSOD denotes manganese superoxide dismutase enzyme (Vanlerberghe, 2013).

Since the ND to AOX bypasses proton translocation, it does not contribute to the pmf, and as a result, the free energy released because of electron transfer down the reduction potential gradient is dissipated as heat. Thus, the alternative pathway is considered a non-energy conserving, non-phosphorylating pathway. If electron transport occurs via Complex I to AOX, one-third of ATP can by synthesised compared with the CP, owing to the proton-pumping Complex I to the AOX pathway. The presence of AOX, NDin and NDex pathways found in the mETC enables ATP turnover to be modulated, and this is dependent on the components in the mETC, which are active between the oxidation of NAD(P)H to O_2. The AOX, NDin and NDex pathways play vital roles in plant physiology as modulators of ATP synthesis and hence metabolic flux, which is regulated by the demand for ATP. Hence, these alternative respiratory pathways play a significant role in plants undergoing physiological changes brought about by abiotic stresses, and therefore, understanding the presence of the alternative pathway and the way it is regulated is essential (Vanlerberghe 2013).

1.6.3 Respiration

The cytosolic oxidative pentose phosphate, mitochondrial tricarboxylic acid cycle and oxidative phosphorylation are three primary metabolic pathways that plants use for respiratory metabolism (Van Dongen et al. 2011). Typically, eukaryotic organisms have some form of cellular respiration. The cells release energy in the form of metabolites that contain carbon, which then undergoes complete oxidation to form CO_2 and H_2O. For plants, various reduced

carbon compounds are used in cellular respiration, such as amino, organic and fatty acids, as well as carbohydrates.

Cellular respiration comprises three pathways, namely, glycolysis in the cytosol, pentose phosphate pathway and a side pathway. Small amounts of ATP are generated, when glycolysis converts the glucose and fructose (as carbohydrate compounds with reduced carbon) to pyruvate, an organic acid. Pyridine nucleotides, including NADH and NADPH, are produced from the glycolysis and pentose phosphate pathways. The pyruvate is the end product from the glycolysis process and is transported through the IMM (McCommis & Finck 2015; Siedow & Day 2000). The pyruvate is decarboxylated by pyruvate dehydrogenase, thereby generating CO_2, NADH and acetyl-CoA. Acetyl-CoA then enters the Krebs cycle or tricarboxylic acid (TCA) cycle, which undertakes a series of chemical reactions to release stored energy in NADH and $FADH_2$, and CO_2.

During oxidative phosphorylation, several sites found in the mETC suffer electron leakage due to the inefficiency of mETC (Jastroch et al. 2010). Each electron leakage can combine with molecular oxygen, which forms O_2^- (superoxide anion) (Vanlerberghe 2013). As a result of electron leakage, the primary mETC sites experience high levels of mitochondrial reactive oxygen species (mROS), which are found in the IMM at Complexes I and III (Brand 2010; Vanlerberghe 2013).

These sites, Complex I (IQ) and III (IIIQo), are both ubiquinol-redox sites and the Complex I site includes flavin of Complex I (site IF) (Brand 2010; Quinlan et al. 2012). In addition, G3PDH (glycerol 3-phosphate dehydrogenase), pyruvate and 2-oxoglutarate dehydrogenases, and the electron transferring flavoprotein, ETFQOR (Q oxidoreductase) of fatty acid beta-oxidation, have been defined as other mitochondrial ROS-producing sites (Brand 2010; Quinlan et al. 2012). Another contributing factor to significant mROS accumulation is Complex II, where high levels of reactive oxygen species (ROS) are generated (Quinlan et al. 2012). Despite this, only two sites can release $O_2^{-\bullet}$, sites IIIQo and G3PDH, whereby the $O_2^{-\bullet}$ is released straight into the intermembrane space (IMS) (Sena & Chandel 2012). Further, significant amounts of $O_2^{-\bullet}$ are released at sites IQ and IIIQo toward the mitochondrial matrix. Here, the $O_2^{-\bullet}$ is converted to H_2O_2 by way of the SOD2 enzyme (superoxide dismutase 2). From here, the H_2O_2 is dispersed into the cytosol through both mitochondrial membranes. Some of H_2O_2 is oxidised by peroxiredoxins (PRX) or by glutathione peroxidases (GPX) to form H_2O (Sena & Chandel 2012). This can be noted in Figure 1.7.

Figure 1.7: Mitochondria produce reactive oxygen species (ROS) for signalling

Superoxide $O2^{-\bullet}$ in the mitochondrial matrix undergoing a reduction process to H_2O_2 (Sena & Chandel 2012).

The moderate production of mROS mediated by the mETC takes place when the mitochondrial respiratory poise is optimal. With that, mROS is essential for ROS-dependent genes induced by both up-regulation and down-regulation and important for regular cellular signalling (Sena & Chandel 2012). This is vital for regulating critical physiological plant processes, such as growth and development, as well as plant response to biotic and abiotic stresses. The effect of the accumulation of mROS is that the mitochondrial photorespiration, when subjected to abiotic stress, becomes inefficient. Higher plants have complex molecular mechanisms to minimise the effects of abiotic stress and increase stress tolerance. Alterations in plant mETC can cause changes in the level of mROS at a cellular level and result in cell death (Amirsadeghi et al. 2006; Atkin & Macherel 2009; Rhoads et al. 2006).

1.6.3.1.1 Roles of ROS

In plant biology, ROS is believed to have two roles, one as a toxic by-product and the other related to its involvement in signalling (Mittler 2017). Initially, it was proposed that the toxic by-products created by aerobic metabolism were ROS, which antioxidative enzymes and antioxidants counteract to remove (Bailey-Serres & Mittler 2006). More recent research has led to the belief that ROS has a role in signalling in plants and in plant development, growth and in responding to abiotic and biotic stimuli (Bailey-Serres & Mittler 2006). ROS (H_2O_2, O^{2-} and OH.) are by-products and are also produced by the NADPH oxidase system (Gamaley & Klyubin 1999). NADPH oxidase, found on the plasma-membrane, uses NADPH produced by anabolic reactions and can initiate and amplify ROS production for plant signalling (Bailey-Serres & Mittler 2006).

During oxidative stress, ROS targets cellular components, such as proteins, lipids, Ribonucleic acid (RNA) and DNA, and for each component, ROS reacts differently (Mittler 2017). Cellular ROS levels have been exploited by necrotrophic plant pathogens, where plant necrosis benefits the pathogen (Mittler 2017). ROS has various roles in cellular systems, including development and growth, and in regulating cell death, signifying its importance in plant life. Since environmental factors can vary significantly, acclimation to these changing conditions is essential for plants. The ability to adapt and acclimate requires plants to have sensing mechanisms and the ability to regulate and communicate intra-cellularly. ROS provides a fundamental role in both responding and acclimating to the environment (Liebthal & Dietz 2017). The AP's alternative role allows acclimation to changing conditions by enabling electrons to pass through the respiratory pathways, for example, in leaves that are subject to varying light conditions (Del-Saz et al. 2018).

Exposing plants to oxidative stress results in an increase in lipid peroxidation and malondialdehyde (MDA) content (Flagella et al. 2006; Zou et al. 2016). The amount of MDA content is an indication of the amount of lipid peroxidation (Del Buono et al. 2011). The thiobarbituric acid reactive substances (TBARs) assay is used to measure MDA content levels to determine a plant's abiotic stress response (Moller et al. 2007). Increase in MDA content is reflected in a corresponding increase in TBARs, particularly when a plant is exposed to salinity or chemical stress since it signifies sensitivity to such stresses (Del Buono et al. 2011). A low level of lipid peroxidation causes lower MDA content, which indicates salt tolerance (Borzouei et al. 2012). A low lipid peroxidation level indicates a plant's capacity to reduce oxidative damage when subjected to salinity, thus making it salt-tolerant, whereby it is able to maintain growth (Borzouei et al. 2012). Taylor et al. (2002) noted that a correlation between lipid peroxidation damage, an increase in MDA content and the induction of AOX when plants were exposed to low temperature and drought. Further, it has been suggested that a plant's induction of AOX in response to environmental stress may be too slow, such that it may not save highly susceptible mitochondrial sites (Taylor et al. 2002).

However, in the case of less susceptible mitochondrial membrane, AOX induction could be effective in slowing oxidative damage (Taylor et al. 2002). The effects of salinity stress and the levels of lipid peroxidation can vary between cultivars based on whether they are salt-tolerant or salt-sensitive (Khan & Panda 2008). Although the mETC-specific sites affected by salinity stress have not yet been characterised, Smith et al. (2009) found that the activities of

Complex I and Complex III are affected by salinity stress. Given that the electron leakage at both Complex I and III accounts for most of the mROS, it can be deduced that they are salt stress-sensitive. It can be further suggested that if the Complexes were protected, then there would be a reduction in mROS when subjected to salt stress (Jacoby et al. 2011).

1.7 Alternative Oxidase (AOX)

Alternative Oxidase (AOX) is an important component of plant respiration and is a cyanide-insensitive terminal oxidase of the plant mitochondria, which is located towards the matrix side of the IMM (Umbach et al. 2005). AOX does not allow energy transfer when electrons pass through it. Subsequently, this energy is lost in the form of heat, and in the case of plants with high concentrations of AOX thermogenesis occurs (Elthon & McIntosh 1987). Elthon and McIntosh's study (1987) is the third after those of Huq and Palmer (1978) and Rich (1978) to isolate AOX. The result of their research was to purify AOX from mitochondria in the thermogenic spadix of *Sauromatum guttatum* (Elthon & McIntosh 1987). Polyclonal antibodies were used to recognise the expression of a protein of 35 kDa – 37 kDa (Elthon & McIntosh 1987). In the past three decades, the evolution and comprehensive knowledge of the structure of AOX protein has been determined (Millar et al. 2011; Shiba et al. 2013; Umbach et al. 2006). To detect AOX protein, the monoclonal antibody (AOA) was successfully used in plant species, including wheat (Jacoby et al. 2010; Sugie et al. 2006; Takumi et al. 2002; Wanniarachchi et al. 2018). AOX is found in mETC the non-energy-conserving pathway and is disengaged from the proton translocation during mitochondrial ATP synthesis (Fiorani et al. 2005; Van Aken et al. 2009). AOX in plants has been studied widely, and it has been shown that AOX redirects electrons from the ATP synthesis through the four-electron reduction process turning oxygen to water.

1.7.1 Classification of AOXs

The AOXs have been characterised in different plant species, and their respective encoding genes have been classified into different families and subfamilies. In general, the *AOX* genes are categorised into two families, the *AOX1* and *AOX2*, where the *AOX1* is present in both monocots and eudicots (Considine et al. 2002). *Arabidopsis thaliana*, a eudicot plant, has two distinct *AOX* subfamilies, *AtAOX1* with four isoforms (*1a*, *1b*, *1c*, and *1d*) and *AtAOX2*, and *AtAOX1* is the most predominant gene (Costa et al. 2014). In monocots, only *AOX1* isoforms are present (Costa et al. 2014).

The monocot rice (*Oryza sativa*) has four *AOX1* isoforms (*OsAOX1a, OsAOX1c, OsAOX1d* and *OsAOX1e),* whereas barley (*Hordeum vulgare* L.) has four (*HvAOX1a, HvAOX1c, HvAOX1d1* and *HvAOX1d2*) (Wanniarachchi et al. 2018). In bread wheat, *Triticum aestivum*, Takumi et al. (2002) identified the first two *Aox1* isoforms, *WaAox1a* and *Waox1c*, by obtaining the full genomic sequence from Chinese Spring. With both monocots and eudicots having *AOX1* genes, this means that an essential group of AOX, which is found in almost every higher plant species, is needed to respond to abiotic stress (Abu-Romman et al. 2012).

1.7.2 AOX Genomic Structure (intron–exon structure)

The *AOX* genomic structure is predominantly composed of four exons interrupted by three introns at well-conserved positions (Polidoros et al. 2009; Pu et al. 2015). Further, it has been observed that the size of the last three exons are conserved 129 bp, 489 bp and 57 bp in a wide variety of plant species (Polidoros et al. 2005; Polidoros et al. 2009). A loss or gain of introns has been shown to result in variations in intron–exon structures, where the *AOX* genes in some species (*A. thaliana*) have two to four introns and three to five exons (Figure 1.8). Some *AOX* members have variations of intron numbers as a result of evolutionary loss/gain of introns (Polidoros et al. 2009). Thus, it is plausible that the genomic structures of plant *AOXs* are changeable, providing a molecular basis for breeding stress-tolerant plant varieties (Campos et al. 2016). However, this requires the presence of adaptable polymorphic *AOX* allelic sequences that can produce transgenic plants with significant phenotypic characteristics with respect to stress adaptation and tolerance (Polidoros et al. 2009).

The intron–exon genomic structure of *Aox1a* in Z. *mays* contains a promoter that contains stress-responsive regulatory motifs for stress adaptation and tolerance (Polidoros et al. 2005). Although *Daucus carota* L. expresses both *AOX1* and *AOX2* gene families, only its *DcAOX1* gene family was demonstrated to exhibit significant allelic variations, indicating *AOX1* is the most variable in plant varieties and cultivars. Indeed, of the eight domesticated cultivars of *D. carota* L. ssp. *sativus*, cv. Rotin, Nevis, Senta, Nantes Normu, Lange Rote Stumpfe, Nairobi, Nikki and Norwich, the first two cultivars (Rotin and Nevis) exhibited a total of three genotypes each, while the rest exhibited only a single genotype (Nogales et al. 2016).

Figure 1.8: Intron–exon plant *AOX* genes organisation

Typically, *AOX* genes exhibit a conserved structure that shows four exons interrupted by three introns. Variations occur with intron gain and loss where *AOX* genes have five or three exons in selected species (Polidoros et al. 2009).

1.7.3 Structure of Plant AOX Protein

Being a resident protein of the IMM, located on the matrix side, a key structural feature of AOX protein includes *N*- and *C*-terminal hydrophilic regions exposed to the mitochondrial matrix as the catalytic core (Berthold et al. 2000). Multiple sequence alignment of the AOX family of proteins from all the six kingdoms of life (Plants, Animals, Protists, Fungi, Archaebacteria and Eubacteria) revealed that catalytic cores of AOXs are highly conserved and exist essentially as either covalently or non-covalently linked dimers (Pennisi et al. 2016).

However, plant AOXs exhibit atypical covalent dimerisation to allow tight conservation in the *N*-terminal region of cysteine (Cys) residue forming the inter-monomer disulfide bond (Pennisi et al. 2016; Umbach et al. 2006).

A typical plant AOX has two highly conserved Cys residues, CysI and CysII, located within the structurally undefined *N*-terminus and at the *N*-terminal end of the hydrophilic portion, respectively. Umbach et al. (2006) demonstrated that both CysI and CysII are the tight biochemical regulators of the entire AOX function. CysI activates the entire activity of AOX when the protein interacts with ά-ketoacids; upon loss of the residue via substitution or oxidative eradication, the enzyme is inactivated. However, the activator CysI residue is not conserved in all plant AOX protein sequences. In AOXs in maize and tomato, a serine residue (SerI) replaces CysI at the same position and, therefore, they are not activated by α-keto acids but by succinic acid. By contrast, site-directed mutagenesis of both CysI and CysII residues in *A. thaliana* has demonstrated that CysII is an alternative activating site for AtAOX1a (Berthold et al. 2000; Holtzapffel et al. 2003; Huh & Kang 2001; Umbach et al. 2006).

To date, three attempts have been made to model AOX. The first model proposed that the active site was centrally located between four small helixes, whereby the molecule was anchored to the membrane by two transmembrane helixes (Moore et al. 1995). Andersson and Nordlund (1999) produced the second model, which Berthold et al. (2002) revised; the transmembrane AOX model was a di-iron carboxylate protein containing four-α-helix bundle that hosts the di-iron catalytic site and two additional α-helices skirting for anchoring the protein to the IMM (Pennisi et al. 2016) (Figure 1.9).

Figure 1.9: Suggested structural models of AOX

(A) The transmembrane model (the SUM model). (B) The Andersson–Nordlund model. The residue numbers represent the *S. guttatum* amino acid sequence. The iron atoms are shown as filled spheres and represent specific iron-binding motifs (EXXH) (Albury et al. 2002).

May et al. (2017) explained the crystal structure of Trypanosomal alternative oxidase (TAO) as identified in the previous models that Moore et al. (2013) and Shiba et al. (2013) proposed. It is widely accepted that the sequence homology of AOX belongs to the di-iron carboxylate superfamily (May et al. 2017). This superfamily is characterised by a di-iron core that is bounded by a four-helix bundle, ligated by four (E123, E162, E213 and E266) conserved glutamate and two histidine residues (H165 and H269) (May et al. 2017). Shiba et al.'s (2013) AOX model showed the homodimer form consisting of each monomer having six long and four short α helices, with the four-helix bundle comprising α2, α3, α5 and α6, along with a long N-terminal arm, six long α-helices and four short α-helices (Figure 1.10) with the bundle acting as a support two iron atoms.

Figure 1.10: Trypanosomal alternative oxidase (TAO) crystal structure

(A) Dimeric structure of TAO; (B) the hydrophobic dimer surface representation on the left and the hydrophilic surface on the right; and (C) the suggested TAO dimer binding model membranes of the surface on the left and cartoon on the right (Shiba et al. 2013).

1.7.4 The Role of Alternative mETC in Abiotic Stress Tolerance

It is important to reassert that it is not possible to select plant genotypes that are tolerant to multiple abiotic stresses. This is because plant responses to abiotic stresses vary based on the type of environmental stress, such as high salinity, drought and extreme temperature. Plant responses are elicited by the oxidative stress events followed by mROS generation at Complexes I and III of the mETC, known to be the major sites of mROS production. As mentioned, the alternative mETC circumvents electron channelling through Complexes I and III, thereby reducing mROS production and plant response to abiotic stress.

The physiological effects of abiotic stress are controlled by energy-dissipating systems found in the alternative mETC. AOX and ND components of the alternative respiratory pathways are both associated with the physiological response to a broad number of abiotic stresses. Specifically, they are associated with high temperature, drought and salinity stresses (Vanlerberghe 2013). The role of the ND component of the alternative respiratory pathway in abiotic stress tolerance has been extensively tested in eudicots such as *Arabidopsis* (Smith et al. 2011), and flowering tobacco (*Nicotiana sylvestris*) (Liu et al. 2008; Michalecka et al. 2004),

but not in monocots (cereals) such as wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), maize (*Zea mays*) and rice (*Oryza sativa*). *AOX1* is present in both monocots and eudicots, which suggests that *AOX1* could be the basic stress-responsive component in nearly all plants. Therefore, up-regulating *AOX1* in monocots should enhance alternative respiratory capacity and abiotic stress tolerance.

Research on wheat has indicated that alterations in the mETC due to salinity stress are detrimental to the plant (Jacoby et al. 2010). Reduced mitochondrial respiration rates have resulted in salinity stress in durum wheat seedlings (Flagella et al. 2006). The effect of this is that an accumulation of mROS initiates mROS scavenging systems and lipid peroxidation (Flagella et al. 2006). Jacoby et al. (2011) observed a positive correlation between plants with salinity tolerance and a positive increase in antioxidant enzyme response, which interacts in scavenging of ROS, for example, in *Arabidopsis*, durum wheat and barley.

Although the mETC-specific sites affected by salinity stress have not yet been characterised, Smith et al. (2009) found that the activities of Complex I and Complex III are affected by salinity stress. Given that the electron leakage at both Complex I and III accounts for most of the mROS, it can be deduced that they are salt stress-sensitive. It can be further suggested that if the Complexes were protected, then there would be a reduction in mROS when subjected to salt stress (Jacoby et al. 2011).

The role of AOXs is well characterised in thermogenic plants and during anthesis (flowering period) in titan arum (*Amorphophallus titanum*) (Figure 1.11), where the dissipated energy in the form of heat functions to vaporise floral scents that allow for pollination by insects (Meeuse 1975; Meeuse & Raskin 1988; Polidoros et al. 2005). AOX also plays a vital role in regulating enzymes for adaptive cellular reprogramming against biotic and abiotic stress factors (Campos et al. 2016; Costa et al. 2010). It has been suggested that AOXs minimise endogenous production of cytotoxic ROS (Fung et al. 2006; Nogales et al. 2016; Polidoros et al. 2005).

This image has been removed due to copyright restrictions. Available online from [https://www.environment.sa.gov.au/goodliving/posts/2016/01/corpse-flower]

Figure 1.11: Image of the titan arum (*A. titanum*) at the Mount Lofty Botanic Garden nursery, Adelaide, Australia

The expression profile of the *AOX* genes has been observed as being up-regulated in response to exogenous chemicals, indicating that up-regulation of *AOX* genes confers protection against injury from abiotic factors. Previous research has noted that AOX expression in plants increases when subjected to KCN (Abu-Romman et al. 2012; Li et al. 2008; Polidoros et al. 2005; Takumi et al. 2002). The protective effect of *AOX*s was previously demonstrated in freshly harvested pink tomato (*Lycopersicon esculentum*) pre-treated with methyl salicylate vapour prior to a chilling challenge (Fung et al. 2006). This vapour increased the expression levels of *AOX* gene in *L. esculentum*, which protected the tomato from chilling injury. Using four tomato clones of *AOX* designated as *LeAOX1a, 1b, 1c* and *LeAOX2*, gene expression analysis using Reverse Transcription Polymerase Chain Reaction (RT-PCR) found marked expression levels of *LeAOX1a* and *1b* genes in leaf, root and fruit tissues, whereas *1c* was expressed preferentially in root tissue (Fung et al. 2006). A similar expression profile of the *LeAOX1a* and *1b* was observed when green tomatoes were kept at 4°C (Holtzapffel et al. 2003).

Differential expression of *VuAox2a* and *2b* in leaves, hypocotyls and roots was also observed in cowpea (*Vigna unguiculata*) (Costa et al. 2010). This finding strongly indicates that *AOX* subfamilies are moderately tissue-specific, where they could be playing collective regulatory functions. The putative regulatory function of AOXs was also demonstrated in *Candida albicans*, where the fungal cultures of *aox1a/aox1a* mutants were markedly retarded, whereas *aox1b/aox1b* mutants and the *aox1a/aox1a, aox1b/aox1b* double mutants were almost entirely

inhibited in the same culture media, following treatment with 1 mM potassium cyanide (KCN) (Huh & Kang 2001). This finding strongly supports that AOX is an essential regulatory component of cyanide-resistant respiration, and thus, abiotic stress should induce mitochondrial accumulation of the protective AOXs. Unlike the cytochrome pathway, which is highly responsive to inhibitors of the electron transport chain, particularly KCN and antimycin A, the alternative respiratory pathway is tolerant to these agents (Campos et al. 2009; Fu et al. 2010). Surprisingly, the resistance of tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum* L.) and cucumber (*Cucumis sativus*) against infection by tobacco mosaic virus, potato virus X and cucumber mosaic virus, respectively, can be achieved by treating with inhibitors of the cytochrome pathway, particularly antimycin A and KCN (Fu et al. 2010).

The protective benefit of cytochrome inhibitors against tobacco mosaic virus was validated, which demonstrated enhanced viral replication and systemic movement in tobacco treated with salicylhydroxamic acid (SHAM), an AOX inhibitor (Berthold & Stenmark 2003; Liao et al. 2012). This finding strongly indicated that cytochrome inhibitors could induce accumulation of AOXs. Indeed, similar to most AOXs from other sources, treatment of *C. albicans* with 1 mM KCN or 10 µM antimycin A significantly up-regulated the expression of *AOX1b* (renamed as *AOX1d* by Costa et al. 2014) characterised by increased *β*-galactosidase activity (Huh & Kang 2001). This is useful for protecting cellular damage by cyanide/antimycin A-induced ROS generation from adverse abiotic and biotic factors (Liao et al. 2012).

It is important to note that despite their similar biochemical properties and functions in the electron transport chain, *AOX1* and *AOX2* gene families exhibit dissimilar expression patterns (Polidoros et al. 2009). For instance, while the *AOX1* gene family is highly responsive to induction by biotic or abiotic stress including pathogens infection, chilling stress and chemical treatment, its *AOX2* gene counterpart is usually not significantly affected by such factors.

Thus far, only four studies have investigated AOX in *T. aestivum* at the transcript level. Takumi et al. (2002) investigated *WaAox1a* and *Waox1c* under cold stress and KCN. They found that both *WaAox1a* and *Waox1c* increased under cold stress, but only *WaAox1a* under KCN. Mizuno et al. (2008) researched two wheat cultivars, one tolerant and the other sensitive, to determine the *WaAox1a* and *Waox1c* response to cold stress. Both varieties *WaAox1a* and *Waox1c* increased at the transcript level until day 5; thereafter, the sensitive variety cv. Chinese Spring exhibited a reduction in *WaAox1a* (Mizuno et al. 2008). Garmash et al. (2105; 2017) studied *WaAox1a* and *Waox1c* under de-etiolation in wheat and found *WaAox1a* was more

highly expressed than *Waox1c* in both studies. In addition, Garmash et al. (2017) found that the NDin genes, NDA1 and NDA2, were highly expressed under de-etiolation stress but that NDex, NDB1 and NDB2 showed varied expression. NDB1 had very low expression, whereas NDB2 had a significant expression after 12h of de-etiolation (Garmash et al. 2017). To date, no studies have investigated AOX in *T. aestivum* and its ancestors when exposed to salinity at the transcript level. This current study is believed to be the first to undertake this research.

In response to salinity stress, AOX has played an important role by increasing its capacity (Hilal et al. 1998; Jolivet et al. 1990). To date, several plant species, including *Arabidopsis*, when exposed to salinity stress have induced AOX transcripts (Kreps et al. 2002; Seki et al. 2002). Salinity stress-induced AOX transcript response in *Arabidopsis* is a defensive mechanism that increases both AOX activity and protein levels (Smith et al. 2009). The plants undergoing salinity stress induce AOX activity to minimise oxidative damage. AOX activity decreases ROS, which thereby reduces oxidative damage in salt-stressed *Arabidopsis* (Smith et al. 2009; Umbach et al. 2005). Jacoby et al. (2013) noted that in *T. aestivum,* the cytochrome pathway respiration was inhibited by high concentration levels of salt, which also induced AOX protein as a responsive measure. Vanlerberghe (2013) noted that ethylene played a role in salt-stressed *Arabidopsis* in inducing AOX activity.

However, in the case of the expression profile of *AOX2* gene, it appears up-regulated in specific plant tissues, appears to change through developmental stages of plants and is affected by most stresses (Saisho et al. 1997). Differential expression of *DcAOX2a* and *DcAOX2b* in various tissues or organs was also reported in *D. carota* L during different development and growth stages (Campos et al. 2009). Nevertheless, Costa et al. (2010) emphasised that the *AOX2* gene also plays an important role in moderating stress related to plastid-dependent signalling *A. thaliana.*

AOX proteins are encoded in the nuclear genome; these are largely involved in plant response and adaptation to a wide range of environmental stresses and are essential for plant growth and development. The *AOX1* and *AOX2* genes play key regulatory functions in both adaptive- and tolerant stress response (Velada et al. 2016). However, to date, the *Araceae* family is the only monocot family where *AOX2* identified in three of its subfamilies, *Lemnoideae, Pothoideae* and *Monsteroideae in silico* (Costa et al. 2017a). Therefore, the occurrence of the *AOX1* subfamily in both monocot and eudicot plants suggests that it is the basic alternative oxidase for abiotic stress tolerance in nearly all plant species (Abu-Romman et al. 2012). The

responsiveness of AOX to a variety of exogenous treatments that induce oxidative stress is the reason that AOX is frequently used to study abiotic stress response in plants (Ho et al. 2007).

1.8 Research Project Aims

1.8.1 Major Aim

Previous findings and existing literature concur that the alternative pathway is associated with the improvement of a plant's tolerance to abiotic stress (Mhadhbi et al. 2013; Miller et al. 2010; Smith et al. 2009; 2011). The effects of various stressors, such as salinity and drought, have varying effects on the mETC, and thereby, can lead to an increased reduction in particular sites within the mETC. This then manifests in increased ROS production. Higher salt tolerance has been found in *Arabidopsis* plants where the *AtAox1a* has been over-expressed when there is an increase in AOX (Smith et al. 2009). From this, it can be deduced that the role of AOX is vital in how plants cope with environmental stressors, such as salt (Smith et al. 2009).

To date, there has been limited research into the alternative respiratory pathway in monocots, particularly cereals and especially for food crops, such as wheat (Wanniarachchi et al. 2018). Takumi et al. (2002) undertook research of AOX in *T. aestivum* and identified the first two *Aox1* isoforms, *WaAox1a* and *Waox1c*. In 2008, a study of two wheat cultivars, one tolerant and one sensitive was conducted to assess AOX response to cold stress (Mizuno et al. 2008). In addition, Garmash et al. (2105; 2017) studied *WaAox1a* and *Waox1c* under de-etiolation in wheat. Feng et al. (2013) stated that no studies had examined AOX in *T. aestivum* and its ancestors when exposed to salinity, which has been conducted in this thesis. Research into *AOX* genes could provide insight into their relationship in improving stress tolerance in wheat, thereby providing a greater understanding of AOX and how it acts to minimise ROS and increase crop yield production. This research will use *T. aestivum* (bread wheat) to test this hypothesis.

1.8.2 Specific Aims

There are three specific aims in this research study.

- The first objective is to identify the genes encoding for AOX in wheat and analyse their expression profiles under unstressed conditions using real-time polymerase chain reaction (RT-PCR) assay.

- The second objective is to assess how the identified genes respond during oxidative stress. This will be achieved by inhibiting the cytochrome pathway using inhibitors such as Cyanide, a Complex IV inhibitor, and Antimycin A, a Complex III inhibitor.
- The third objective is to assess the expression under salinity of *AOX1* in several bread wheat cultivars and the progenitors of wheat.

When commencing this research, very few publications were available about the alternative pathway and specifically AOX in wheat. In fact, only *WAox1a* and *WAox1c* genes had been partly identified. This lack of information led to this project. In the past year, a significant publication identifying some of the AOX gene family members and part characterisation was published in the scientific literature prior to the submission of this thesis for assessment (Brew-Appiah et al. 2018). In this thesis, first, the researcher's work will be presented, and then a review of the study by Brew-Appiah et al. (2018) will be conducted and compared in the context of the thesis findings, which has significantly expanded that study.

Chapter 2: Material and Methods

2.1 Plant Materials and Experimental Design

The plant material used in this study to investigate *AOX* expression profiles during abiotic stress are presented in Table 2.1. Seeds were sterilised in 5% (v/v) bleach and 0.1% (v/v) Tween-20 for 2 min and then were germinated on wet filter paper (Whatman) in Petri dishes for a week at room temperature. In the chemical experiment, 5 mM of KCN or 20 µM of antimycin A were applied to seedlings of *Triticum aestivum* cv. Chinese Spring by spraying and pouring. Samples were collected at four different time points (0, 3, 12 and 24 h). For the salinity experiments in Chapters 4 and 5, the seedlings were transferred into a hydroponics system aerated using an SR7500 Air pump at low speed. A hydroponics system was arranged according to the study by Shavrukov, Genc and Hayes (2012) using 12-litre plastic boxes that were randomly arranged (Figure 2.1). The nutrients were supplied in growth solutions (Appendix A.1). Seedlings were grown hydroponically for 10 days without stress application. Then, growth solutions were replaced 24 h before the start of the salt application. Throughout the experiment, the total volume of the growth solution was maintained by adding reverse osmosis water. The seedlings were grown in greenhouse conditions at 25°C with a 16-hour photoperiod (warm white).

In the salinity experiment Chapter 4 presents, 10-day old seedlings of four bread wheat cultivars, *T. aestivum* cv. Chinese Spring, Opata M85, Gladius and Drysdale, were exposed to salinity with 25 mM NaCl increment twice daily for three days. The final concentration of 150 mM of NaCl was used for two days more for a total of five days from the start of the salinity stress. Plant samples were collected at four different time points after first NaCl application (0, 12, 24 and 72 h). In the second salinity experiment described in Chapter 5, the following 'ancestor' species were used: *T. urartu* (IG45626), *Aegilops speltoides* (AUS-21650), *Ae. tauschii* (AUS-24119) and *T. turgidum* ssp. *dicoccoides* cv. Daliyya. For controls, two bread wheat cultivars were used, *T. aestivum* cv. Chinese Spring and Opata M85. In this experiment, the ancestor species were grown until the stem elongation stage before being exposed to 150 mM of NaCl for three days. In addition, samples were collected at the same four time points as above (0, 12, 24 and 72 h).

Table 2.1: Plant materials used in each experiment

Experiments	Species	Chapter
The impact of chemical stress	*T. aestivum* cv. Chinese Spring (CS)	Chapter 4
	Four bread wheat cultivars: Chinese Spring (CS), Opata M85 (Op.), Gladius (Gl.) and Drysdale (Dr.)	Chapter 4
The impact of salinity on bread wheat cultivars and its progenitors	Bread wheat cv. Chinese Spring and Opata M85; *T. urartu* (IG45626); *Ae. speltoides* (AUS-21650); *Ae. tauschii* (AUS-24119); and *T. dicoccoides* cv. Daliyya	Chapter 5

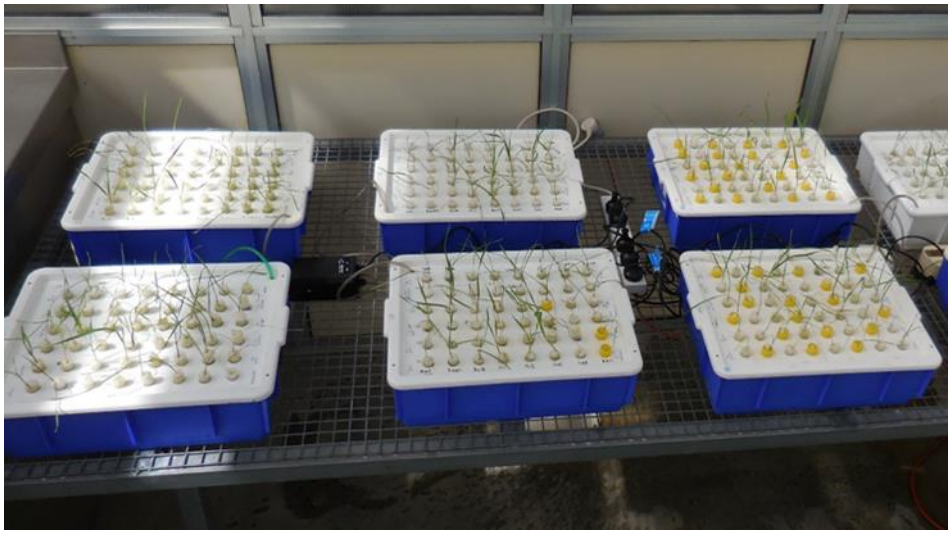

Figure 2.1: Aeration hydroponics system (10 ml open-ended plastic tube) used to grow bread wheat cultivars and progenitors

2.2 Physiological and Biochemical Analysis

2.2.1 Biomass Measurement

To measure the effect of abiotic stress on biomass, fresh and dry weight were recorded for each experiment. For sampling, 3–5 seedlings of each species were taken at each harvest and rinsed with double-distilled water before measurements. The seedlings were cut for shoots and roots and gently blotted with paper towels. Fresh weight, shoot and root tissue were weighed immediately, and the measurements were recorded for each sample. For dry weight measurements, plant samples were dried in an oven for three days at 70°C and weighed.

Relative dry weight (RDW) was calculated using the formula: shoot dry weight (SDW) in treatment/SDW in control, × 100%.

2.2.2 Ion Concentration Measurement

Leaf Na^+ and K^+ concentrations were measured in the third leaf harvested from each plant replicate. The leaf was collected into a 10 ml screw-cap centrifuge tube. A 10 mL of 1% (v/v) nitric acid (HNO_3) was added and incubated in an oven at 70°C for 24 h for leaf tissue digestion. Leaf samples were gently mixed several times to ensure complete digestion. The concentrations of Na^+ and K^+ were measured using a GBC 933 Atomic Absorption Spectrophotometer (GBC Scientific Equipment Pty. Ltd., VIC, Australia). The ion concentrations were calculated as sap based (the tissue water content).

2.2.3 Measurement of Lipid Peroxidation

Frozen leaf tissue (100 mg) was homogenised with a mortar and pestle in 1 mL of 5% trichloroacetic acid (TCA) and vortexed for 10 seconds. The homogenate was centrifuged at 12,000 *g* for 15 min at 4 C. The supernatant was transferred into two new centrifuge microtubes (400 µL each). Both microtubes were used for quantification of lipid peroxidation in terms of MDA content, according to HS Li (2000). In the first centrifuge microtube, 400 µL of 20% (w/v) TCA containing 0.5% (w/v) thiobarbituric acid (TBA) was added. In the second centrifuge microtube, 400 µL of 20% (w/v) TCA was added without the 0.5% thiobarbituric acid (TBA). Both microtubes were heated to 96 °C for 30 min in a water bath and then quickly cooled down using ice. Both reactions were centrifuged at 9,500 *g* for 10 min at 4°C. Then, 100 µL of each supernatant was transferred into a 96-well plate in triplicate. The absorbance of the supernatant was measured at 440, 532 and 600 nm by using a Microplate reader (CLARIOstar, BMG Labtech). MDA content was calculated and expressed as nmol/g FW, based on the following formula: MDA content = $6.45(A_{532} - A_{600}) - 0.56\ A_{440}$.

2.2.4 Measurement of Oxygen Uptake in Leaf Slice

Leaf samples were collected on day 3 from both the treated and control plants. Then, using a sharp razor blade, leaf segments (0.02 g FW) were chopped perpendicularly to the veins into small slices (~1 mm) under respiration medium (Appendix A.2.1). The small slices of leaves were incubated in 1.5 mL respiration medium in darkness for 30 min. The oxygen uptake rate of the sliced leaf was measured using a Clark-type Oxygraph Plus oxygen electrode system

(Hansatech Instruments Ltd) for 10 min in darkness, then 1.5 µL of 1 mM FCCP was added and it was left to stabilise for 10 min. The respiration rates in the presence of 2 mM KCN and, subsequently, 3 mM SHAM, were taken to determine the cyanide resistance and the residual oxygen consumption, respectively. To determine the AOX capacity, the oxygen rate in the presence of KCN was subtracted from the oxygen rate in the presence of SHAM. Respiration rates were calculated accordingly to the leaf area (cm^2).

2.3 Nucleic Acid Analysis

2.3.1 DNA Extraction

The DNA extraction was performed using Phenol/chloroform extractions method described by Weining and Langridge (1991), which was modified as follows: approximately 300 mg of leaf tissue was ground into a fine powder in a 10 mL screw-cap tube with two 9-mm stainless steel ball bearings frozen in liquid nitrogen using Vortex Mixer (Ratex Instruments, Australia). Tissue material remained frozen during grinding and was kept in liquid nitrogen and transferred to −80°C until the start of DNA extraction. DNA Extraction Buffer (1.4 mL) (Appendix A.2.2) was added to each tube and thoroughly vortexed. The homogenate was then carefully mixed with 1.4 mL of Phenol/Chloroform/Isoamyl alcohol (25:24:1), and the tube was inverted for 15 min using a Rotary tube mixer with a disc (Ratek Laboratory Equipment, Australia). Then, the mixture was centrifuged at 1,300 *g* (4,000 rpm) for 10 min in a cold room using Eppendorf minicentrifuge 5415C (Eppendorf AG, Hamburg, Germany). The aqueous phase, 600 mL, was transferred into a new sterile 2.0 mL tube and mixed with 100 µL 3M Na-acetate (pH = 4.8) and 800 µL isopropanol, inverted properly several times, and kept on the bench for 15 min. The solution was centrifuged at 10,000 rpm for 10 min using the Eppendorf minicentrifuge. The DNA pellet was washed twice by using 1 mL 70% ethanol, dried on a paper towel for 20 min and then resuspended in 100 µL of 1/10 diluted TE buffer with RNase (Appendix A.2.3). DNA quantity and quality were checked using a NanoDrop 1000 spectrophotometer (ThermoFisher Scientific Pty. Ltd. Waltham, USA), and DNA samples were stored at −20°C until used.

2.3.2 RNA Extraction

A modified extraction protocol from the Chomczynski and Sacchi (1987) study was used, as described by Shavrukov et al. (2013), using TRIzol-like reagent (Appendix A.2.4). During

sampling, both shoot and root tissues from the seedlings were collected and frozen separately in liquid nitrogen. Total RNA was extracted from the tissue as follows. Approximately 200 mg of sample tissue was ground into a fine powder under liquid nitrogen using cooled mortar and pestle. All samples were transferred into 2 mL tubes, and 1 mL of TRIZOL-like reagent was added immediately and vortexed for 10 seconds. The sample was incubated at room temperature for 5 min and then centrifuged at 11,000 *g* (13,200 rpm) for 10 min in a cold room. The supernatants were transferred to sterile 1.5 mL tubes, and 200 µL of chloroform was added. Each tube was shaken vigorously by hand for 15 seconds and then incubated for 2–3 min at room temperature. The sample was centrifuged at 12,000 *g* for 15 min at 4°C. The aqueous phase contained RNA and was transferred into a new tube. Then, 0.5 mL of 100% isopropanol at room temperature was added to the aqueous phase, followed by 10 min incubation at room temperature. The last centrifugation step before the RNA wash step was performed with the centrifuge at 12,000 *g* for 10 min at 4°C. After the supernatant was removed from the tube, the RNA pellet was washed twice with 1 mL of ice-cold 75% ethanol. Next, the tubes were centrifuged at 8000 *g* for 10 min at 4°C, and then the RNA was air-dried for 5–10 min. Further, 25µL DEPC-treated water was added to resuspend the RNA, and the tubes were stored at −80°C until use.

Two µL RNA of the sample was used to determine the RNA quality and quantity using a NanoDrop 1000 spectrophotometer (Thermo Scientific Pty. Ltd., Waltham, USA). The ratio of absorbance at 260 nM to 280 nM was used to assess the purity of the RNA, and the quality of RNA was examined routinely by agarose gel electrophoresis.

2.3.3 First-strand Complementary DNA (cDNA) Synthesis and DNase Treatment

The ProtoScript II First Strand cDNA Synthesis Kit (NEB Biolab) was used to reverse transcribe mRNA into cDNA. The manufacturer's instructions were modified as follows. 2 µg of extracted RNA was mixed with 12 µL of prepared reaction-mix, which contained 50 µM Oligo $d(T)_{20}$, 10 mM dNTP and water. The mixture was heated at 65°C for 5 min and then transferred to the ice for 1 min. One µL of DNaseI (Zymo Research, USA) was added to each sample and incubated for 15 min at room temperature. Each sample was mixed with another 7 µL of prepared reaction-mix, which contained 5 × ProtoScriptII Reaction Buffer, 0.1 M DTT, 0.5 µL Murine RNaseOut and 0.5 µl ProtoScriptII RT. A total of 20 µL of the reaction-mix was incubated for 45 min at 42°C (Digital Dry Block Heater, Ratek Instruments, Australia) and

then incubated for 5 min at 80°C in the same block to inactivate the enzyme. All cDNA samples were diluted for 1:10 prior to using in qRT-PCR.

2.3.4 Polymerase Chain Reaction (PCR)

For PCR, standard amplifications were performed using GoTaq Flexi DNA Polymerase (Promega, Australia) in a MyCycler Thermal Cycler (Bio-Rad Laboratories, USA). Additionally, Phusion High-Fidelity DNA Polymerase (Finnzymes, USA) was used for high-performance PCR, including sequencing. Volumes of PCR varied from 20 to 50 µL in 0.2 mL PCR tubes (Scientific Specialities Inc., USA). When using PCR reagents from Promega (USA), a standard PCR (50 µL) contained 1 × Green GoTaq Flexi PCR buffer, 0.2 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 mM primers (GeneWorks, Australia) and 0.25 µL of Taq DNA Polymerase (5u/µl), with variable concentrations of template DNA and sterile-autoclaved water. Water was used as a negative control to ensure the accuracy of amplification. The standard PCR cycles when using GoTaq Flexi DNA Polymerase from Promega (USA) were 95°C for 5 min, 40 cycles of 95°C for 30 sec. Annealing temperature was varied depending on primers set (Section 2.3.4.1) for 30 sec and 72°C for 30 sec, with following final extension at 72°C for 7 min and 4°C held infinitely. When using PCR reagents from Phusion High-Fidelity DNA Polymerase, the PCRs and PCR cycles were performed as recommended by the manufacturer.

All primers used in this project were manufactured by Sigma-Aldrich Pty Ltd (Australia). As a starting point for performing PCR, the annealing temperature (T_a) of each primer, shown in Tables 2.2 and 2.3, were determined from the basic melting temperature (T_m) given in the primer data sheet, which is $T_a = T_m$-5°C. The qRT-PCR primers were manually designed to span a 98–158 bp product at the 3'-end of the gene where possible. The self-complementarity of each primer was checked with Oligonucleotide Properties Calculator (Kibbe 2007). The details of the primers used in this study, including sequences for the reference genes, are in Tables 2.2 and 2.3.

Table 2.2. Primer list for amplification of the open reading frame (ORF) of *AOX1* in bread wheat and ancestor species.

Sequence ID	Sequence	Ta (°C)	Product Size (bp)
TaAox1a-2AL-FW	GATTGTGATTCGCGGAGGCGTTC	64.7	1985
TaAox1a-2AL-REV	GTGGTAGTAGCAGTAGTAGCGT	59.1	
TaAox1a-2BL-FW	CCACAGCAACCAACGCAGGCGA	68.5	2110
TaAox1a-2BL-REV	TTTGCTTGGTGGTAGTAGTA	53.6	
TaAox1a-2DL-FW	TTTCCCGGCGCCCAGATGAGCTCA	70.4	2038
TaAox1a-2DL-REV	CATTTGCTTGGTAGTAGTAGTAA	53.9	
TaAox1d1-2AL-FW	ATCGGTTTATTACTTGTCCAAT	53.5	1127
TaAox1d1-2AL-REV	AACAATCCATCTTCTCAAACT	53	
TaAox1d1-2BL-FW	CCTCAAGCTTTTCTACCAGA	54.7	1100
TaAox1d1-2BL-REV	TAGGCGACAGTGGTAGTAAGT	57.9	
TaAox1d1-2DL-FW	ATCAGTCAGCTTACCAGATA	52.8	1216
TaAox1d1-2DL-REV	TAACGGGACAAACGTCGGCGG	65.5	
TaAox1d2-2AL-FW	TCACAAGCTTTTCGATCGC	56.7	1106
TaAox1d2-2AL-REV	GTAGTGTAGTACGCACCAACGGT	62.5	
TaAox1d2-2BL-FW	TTCCCATTCGATCCGCCAC	60.2	1140
TaAox1d2-2BL-REV	AAACATCGGCGACAGTGGTAGT	62.2	
TaAox1d2-2DL-FW	ATCCGCTCACAAGCTTTTCGATCGG	65.8	1160
TaAox1d2-2DL-REV	TACACACACATCGTTTACACAG	56.7	
TaAox1d3-2AL-FW	CTTTTCGACCGGCGTATTTTAGTA	59.4	1136
TaAox1d3-2AL-REV	AACAATCCATCTTCTCAAACT	53	
TaAox1c-6AL-FW	TTGCTCCCACGTCTCGCGTCA	66.6	1587
TaAox1c-6AL-REV	TACCTATCCGACACATATACG	53.9	
TaAxo1c-6BL-FW	AGCCAAGCAGAGCCGCCGTTCA	69.4	1551
TaAxo1c-6BL-REV	TTCTTTACTGACAATGGCAGAGT	58	
TaAxo1c-6DL-FW	CTCTCGGAGCTTGGCACGTCCA	66.7	1496
TaAxo1c-6DL-REV	TACCTATCCGACACACATA	51.9	

Table 2.3: Primer list for qRT-PCR

Sequence ID	Sequence	Ta (°C)	Product Size (bp)
TaAox1a-2AL	GTGTACTACCAGGGTATGC	58	107
TaAox1a-2AL	GTGGTAGTAGCAGTAGTAGCGT		
TaAox1a-2BL	TATGCAGCTGAAGGCCACCCCA	56	98
TaAox1a-2BL	TTTGCTTGGTGGTAGTAGTA		
TaAox1a-2DL	GTGTACTACCAGGGTATGC	58	117
TaAox1a-2DL	CATTTGCTTGGTAGTAGTAGTAA		
TaAox1d1-2AL	ACAACAAGAAGGCCGTGGT	59	129
TaAox1d1-2AL	TGTCGATGGACGTGTCCGGGC		
TaAox1d1-2BL	CGACGCCAACCACTACGCAT	58	150
TaAox1d1-2BL	TAGGCGACAGTGGTAGTAAGT		
TaAox1d1-2DL	CTACCACTGATCAGTTCAAT	57.5	108
TaAox1d1-2DL	TAACGGGACAAACGTCGGCGG		
TaAox1d2-2AL	AGGGAATGACGCTGAATCAAT	58	110
TaAox1d2-2AL	GTAGTGTAGTACGCACCAACGGT		
TaAox1d2-2BL	CGACGCCAACCACTACGCAT	58	158
TaAox1d2-2BL	AAACATCGGCGACAGTGGTAGT		
TaAox1d2-2DL	GCGTACTTCGTTGTCCGA	60.5	140
TaAox1d2-2DL	AGCGAGCGCAGGTGAAGT		
TaAox1d3-2AL	TCAGGCCGTGGGACACGTACA	59	134
TaAox1d3-2AL	CTGGAAGAAGAGGTCGCTGCCCTT		
TaAox1c-6AL	ACATCCATTTCCAGGGGC	59	133
TaAox1c-6AL	TACCTATCCGACACATATACG		
TaAxo1c-6BL	ACATCCATTTCCAGGGGC	60	107
TaAxo1c-6BL	TTCTTTACTGACAATGGCAGAGT		
TaAxo1c-6DL	ACATCCATTTCCAGGGGC	57.5	146
TaAxo1c-6DL	TACCTATCCGACACACATA		
Ta2291-FW	GCTCTCCAACAACATTGCCAAC	58	165
Ta2291-REV	GCTTCTGCCTGTCACATACGC		
Ta54227-FW	CAAATACGCCATCAGGGAGAACATC	59	227
Ta54227-REV	CGCTGCCGAAACCACGAGAC		
Ta2776-FW	CGATTCAGAGCAGCGTATTGTTG	60	242
Ta2776-REV	AGTTGGTCGGGTCTCTTCTAAATG		
Ta54825-FW	TGACCGTATGAGCAAGGAG	61	215
Ta54825-REV	CCAGACAACTCGCAACTTAG		
Ta53908-FW	TTGCTCTGAACGACCATTTC	62	175
Ta53908-REV	GACACCATCCACATTTATTCTTC		

2.3.5 Purification of PCR Products

All primer pairs were tested by standard qRT-PCR using GoTaq Flexi DNA Polymerase, and the presence of a single amplification product of the expected size for each gene was verified by electrophoresis. PCR products were purified using the Wizard SV Gel and PCR Clean-up System (Promega, USA) as per the manufacture's recommendations.

2.3.6 Agarose Gel Electrophoresis

For visualisation of nucleic acids, DNA fragments, RNA or PCR products were separated by agarose gel electrophoresis as proposed by Sambrook and Russell (2001). Gel concentration was 1–2% of analytical grade agarose powder (Promega, Madison, USA) depending on the product or fragment size. During agarose gel preparation, the agarose powder was dissolved in 1 × TAE Buffer (Appendix A.2.5) to 1-2% (w/v) as per the volume of the gel run. The solution was heated in a microwave to dissolve the agarose powder. Then, 1 μL/mL of GelRed (Biotium, USA) was added to stain DNA, RNA or PCR products, and the solution was positioned in a suitable gel tray and left to set. Samples were mixed with 6 × Blue Loading Dye (Promega, USA) if needed at a ratio of 6:1, before loading the samples into the gel wells. The electrophoresis run was set at 90 volts for approximately 45 min. Electrophoresis was performed using systems from Bio-Rad Laboratories (USA). An EPS-300 electrophoresis Power Supply (Pharmacia Biotech, Sweden) was used as the power pack. Gel imaging was performed on a Bio-Rad GelDoc^{TM} EZ imager (USA).

2.3.7 Gene Expression Analysis

In silico, wheat *AOX1* expression was analysed in five different tissues (grain, spike, stem, leaf and root) by using RNA-seq data, which is available at ExpVip (http://www.wheat-expression.com). RNA-seq data were obtained by analysing *T. aestivum* cv. Chinese Spring at different developmental stages (Borrill et al. 2016). In addition, ExpVip was used to study *AOX1* expression in relation to biotic and abiotic stresses for *T. aestivum* (Borrill et al. 2016; Pearce et al. 2015). *In vitro*, the *AOX1* responses to chemical and salinity stresses were investigated using qRT-PCR, to study the change in *AOX1* transcript during oxidative stress.

2.3.7.1 Preparation of qRT-PCR Standards

qRT-PCR standards were prepared for *AOX1* isoforms and five selected reference genes *GAPDH* (Ta30768), *Actin* (Ta54825), *ADP-ribosylation factor* (Ta2291), *Cell division control protein AAA-superfamily of ATPases* (Ta54227) and *RNase L inhibitor-like protein* (Ta2776). Melting curves analysis confirmed all amplicons produced a single-peak at the expected temperature, implying specific amplification. For further confirmation, the presence of a single PCR product at the expected size was verified for each gene by electrophoresis using 2% agarose gel. The PCR products were purified as described in Section 2.3.5 and quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific Pty. Ltd., Waltham, USA). Standards for qRT-PCR were prepared with purified products at concentrations of 10^{-2}, 10^{-4}, 10^{-6} and 10^{-8} fmol/µL for subsequent qRT-PCR analysis.

2.3.7.2 Sample Collection

In the chemical experiment, three biological replicates from individual seedlings were collected at different time points: 0, 3, 12 and 24 h. From these, an assessment was conducted for *AOX1* response in wheat seedlings under chemical exposure and compared with the control (non-stressed). In the salinity experiment, three biological replicates from individual seedling were collected at the following time points: 0, 12 and 72 h. In the experiment described in Chapter 4, leaf tissues were collected individually, while the shoot tissue was collected for the experiment described in Chapter 5. The change in plant material collected was due to the nature of the ancestor's growth habits.

2.3.7.3 Transcript Analyses

Aox1 transcript abundances were determined by qRT-PCR using the Bio-Rad CFX 96 Real-Time Detection System (Bio-Rad, USA). For total RNA extraction and the reverse transcript, see Sections 2.3.2 and 2.3.3, respectively. All qRT-PCR analyses were performed by using the PowerUp™ SYBR® Green Master Mix (Life Technologies Australia Pty. Ltd., VIC, Australia).

To perform the expression analysis, a 10 µL volume of the qRT-PCR mix was used, which consisted of 5 µL of PowerUp SYBR Green Master Mix (2×), 4 µL of 1/10 diluted cDNA and 1 µL of a 3 µM mix of specific primers (Section 2.3.4.1). The qRT-PCR reaction conditions were carried out using the standard cycling mode, as stated in the manufacturer's protocol. The expression level of *AOX* cDNA was normalised against the geometric mean of three selected

reference genes: *ADP-ribosylation factor* (Ta2291), *Cell division control protein AAA-superfamily of ATPases* (Ta54227) and *RNase L inhibitor-like protein* (Ta2776). The $2^{-\Delta CT}$ methodology (Livak & Schmittgen 2001) was used to normalise expression data. The primers used for these analyses are discussed in Section 2.3.4.1. *AOX1* gene expression between time points was statistically analysed using a two-way ANOVA. The statistical analyses and the heat map were derived using GraphPad Prism version 8.1.2 (GraphPad Software Inc., San Diego, USA).

2.3.8 Protein Expression Analysis in Shoot Tissue

All buffers, reagents and solutions used in the protein analysis are described in Appendix A.3

2.3.8.1 Extraction of Total Protein

Total protein was extracted from frozen shoot tissue using a mortar and pestle in the presence of a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, USA) using 2 × Laemmli sample buffer (Appendix A.3.1). Extracted proteins were incubated at room temperature for 5 min and boiled for 5 min at 95°C.

2.3.8.2 Determination of Protein Concentration

Total protein content was determined using an EZQ Protein Quantitation Kit (Invitrogen, Carlsbad, USA) as per manufacturer's guidelines. Bovine serum albumin (BSA) was used as a standard protein.

2.3.8.3 Immunoblot Analysis

Total proteins (20–25 μg) were separated by SDS-PAGE using Mini-Protean TGX Stain-Free Precast Gels (4–20% Tris-HCl, Bio-Rad, USA) following the manufacturer's instructions; running conditions were 100 V constant voltage and 400 mA maximum current for 30 min. Stain-Free gels were activated for 45 seconds using UV light in a ChemiDoc Touch Imaging System (Bio-Rad, USA). Total proteins were transferred from the precast gel to a PVDF membrane using a Trans-Blot Turbo Transfer System (Bio-Rad, USA). The electrophoretic transfer was performed using a pre-programmed protocol, Mixed Mw for 7 min. Then, membranes were blocked for 1 hour in the blocking buffer (Appendix A.3.2), followed by incubation overnight at 4°C in primary antibody, diluted 1:5000 in 15 mL blocking buffer with

gentle agitation. Membranes were washed three times for 10 min each in PBST (Appendix A.3.3) at room temperature with gentle agitation, followed by incubation in secondary antibody (HRP conjugated anti-Mouse IgG 1:20000) at room temperature for 1 hour. Membranes were washed three times for 10 mins each in PBST, before the final step. Clarity Western ECL Substrate Kit (Bio-Rad, USA) were used in 1:1 ratio, where the membrane was incubated in the dark for 5 min with ECL substrate and then visualised using the ChemiDoc Touch Imaging System (Bio-Rad, USA). Protein bands intensity was quantified using ImageLab software version 6.0 (Bio-Rad, USA).

2.3.8.4 Membrane Stripping and Re-probing

The membrane was stripped as described by Legocki and Verma (1981), in 15 mL acidic glycine stripping buffer (Appendix A.3.4) for 30 min at room temperature with gentle agitation. Then, the membrane was washed three times with PBST for 10 min each. Successful primary antibody removal was tested by re-probing the membrane with the secondary antibody for redeveloping the signal using an ECL substrate. Next, the membrane was blocked with a blocking buffer for 1 hour and the same procedure as described in the previous section was followed.

2.4 Bioinformatics Analysis

2.4.1 Identification and Characterisation of the AOX Gene Family in Wheat and its Relatives

The full-length CDS of wheat *TaAOX1* (BAB88645.1 and BAB88646.1) and rice *OsAOX1* (Os04g0600200, Os02g0700400 and Os04g0600300 in rice) were obtained from the National Center for Biotechnology Information (NCBI) database. To predict other *AOX* genes in wheat, wheat *TaAOX1* and rice *OsAOX1* sequences were first used in a BLASTN program on the URGI wheat genome database (http://urgi.versailles.inra.fr/Platform) with an E-value cut-off $<10^{-5}$ (Table 2.4). Homologues sequences of *AOX1* with 80–100% similarity were obtained from the database with an additional 4000 bp up- and down-stream for further analysis. In addition, *in silico* analysis was performed using the BLAST tool to search for Transcriptome Shotgun Assembly (TSA) and Expressed Sequence Tags (EST) in the NCBI databases as resources for gene discovery. Then, a manual examination of TSA and EST sequences was conducted to evaluate all *AOX* genes.

Further investigation was carried out to examine the AOX candidate protein sequences of wheat. The sequences were analysed using the Pfam (http://pfam.xfam.org), and InterProScan (https://www.ebi.ac.uk/interpro/search/sequence-search) databases. The obtained protein sequences were designated as AOX if they had complete AOX domains, whereas the remaining sequences were considered partial AOX (Section 2.4.2). To calculate the biochemical parameters of AOX, the theoretical isoelectric point (pI) and molecular weight (Mw) were calculated using the pI/Mw tool in ExPASy database (www.expasy.org). The subcellular localisation of AOX proteins and the location of the signal cleavage sites were predicted by using MitoProt II version 1.101 (Claros & Vincens 1996), and TargetP version 2.0 (Armenteros et al. 2019) web tools. To access MitoProt II version 1.101 software, (https://ihg.gsf.de/ihg/mitoprot.html) was used, and TargetP version 2.0 server was accessed via (http://www.cbs.dtu.dk/services/TargetP).

To obtain the *AOX1* candidate genes from the wheat relative species, wheat *AOX1* was used in the BLAST tool against genome assemblies publicly available at URGI, Ensembl Plants, NCBI and GrainGenes (Table 2.4). For diploid ancestors, the genome assemblies of *T. urartu* and *Ae. Tauschii,* hosted on NCBI under BioProject PRJNA337888 and PRJNA341983, were used, respectively, while the genome assemblies of *T. monococcum, Ae. speltoides* and *Ae. sharonesis* hosted on URGI platform (Table 2.4) were used. For tetraploid ancestors, the genome assemblies of *T. turgidum* ssp. *dicoccoides, T. turgidum* ssp. *durum* cv. Kronos and cv. Svevo, hosted on GrainGenes were used.

Table 2.4: List of sequence resources of *Triticum* and *Aegilops* species

Databases highlighted in light orange represent the latest resources used for *AOX1* genes identified in this study.

Organism name	Database/GenBank assembly accession	Host	Year
T. urartu	TGAC_WGS_urartu_v1	URGI	2014
	Tu2.0	NCBI	2018
T. monococcum	TGAC_WGS_monococcum_v1	URGI	2014
Ae. speltoides	TGAC_WGS_speltoides_v1	URGI	2014
Ae. sharonensis	TSL_WGS_sharonensis_v1	URGI	2014
Ae. tauschii	TGAC_WGS_tauschii_v1	URGI	2014
	ASM34733v2	Ensembl Plants/ NCBI	2014
T. turgidum ssp. *dicoccoides*	Aet v4.0	NCBI	2017
	Zavitan WEWSeq v.1.0	Ensembl Plants/ GrainGenes	2017
T. turgidum L. ssp. *durum* cv. Kronos	Triticum_turgidum_Kronos_EI v1.1	GrainGenes	2017
T. turgidum L. ssp. *durum* cv. Svevo	Durum Wheat (cv. Svevo) RefSeq Release 1.0	Ensembl Plants/ GrainGenes	2019
T. aestivum L.	IWGSC-CSSv2	URGI	2014
	Wheat TGACv1	URGI	2015
	IWGSC-CSSv3	URGI	2016
	IWGSC-WGA v0.4	URGI	2016
	IWGSC_Refseq v1.0	URGI	2018
	IWGSC_Refseq v1.1	URGI	2018

2.4.2 AOX Gene Nomenclature

This study has adopted nomenclature standards for genes of wheat and its relatives, according to Raupp et al. (1995) and McIntosh et al. (2017). In the diploid ancestors, the identified genes were designated as follows: each gene name begins with prefixes to indicate the species, and then the *AOX* gene family isoforms based on the *AOX* classification scheme proposed by Costa et al. (2014). To avoid the complexity of the nomenclature in both hexaploid and tetraploid wheat, the identified genes were followed by a suffix indicating the gene location, which consists of an Arabic numeral, the homologues genome and the chromosome arm. For example, *TaAOX1a-2AL* indicated that *Triticum aestivum* is the species and *AOX1a* is an isoform according to the *AOX* classification scheme; 2AL indicates the location in the long arm of chromosome 2 of genome A (Figure 2.2B). An additional Arabic numeral indicates a

duplication in the gene isoform. In some cases, a gene was given the suffix '-like' if it had a premature stop codon and it was given the suffix '-partial' if it was partially obtained from the database.

For the purpose of this research, it was necessary to modify the classification scheme published by Costa et al. (2014) to be suitable for the newly identified AOX proteins in wheat and its related species, which are shown in Figure 2.2A. An example of *AOX1* gene nomenclature in wheat is shown in Figure 2.2B.

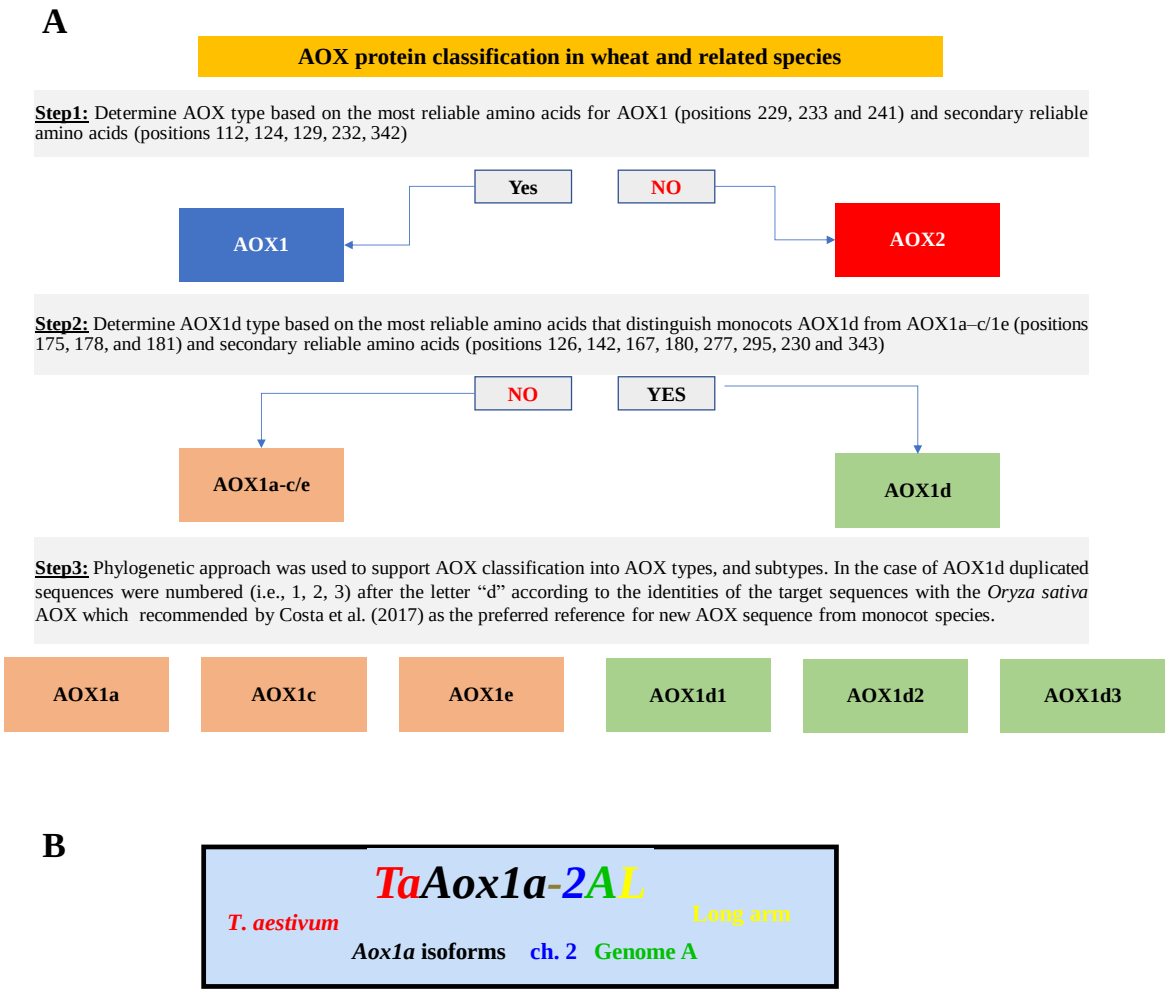

Figure 2.2: AOX protein classification (A) and the nomenclature of *TaAOX1* gene (B) in wheat and related species

Figure 2.2A shows the three steps that were followed to classify AOX proteins. In the first step, the AOX type was determined based on the reliability of the AOX1 amino acids. The most reliable amino acids were at positions 229, 233 and 241, and the secondary reliable amino acids were at positions 112, 124, 129, 232 and 342, which were used to distinguish AOX1 from

AOX2. For the second step, the same process was employed. For determining the AOX1d type, the most reliable amino acids that differentiate monocots AOX1d from AOX1a–c/1e were at positions 175, 178 and 181, while the secondary reliable amino acids were at positions 126, 142, 167, 180, 277, 295, 230 and 343. The final third step was the phylogenetic approach, which was used to support AOX classification into AOX types and subtypes. Where there were AOX1d duplicated sequences, they were numbered (e.g. 1, 2 or 3) following the letter 'd'. This was according to the identities of the target sequences with *H. vulgare* and *O. sativa* AOX, which was suggested by Costa et al. (2017b) as the preferred reference for new AOX protein sequences from the monocot species.

2.4.3 Phylogenetic Analysis

Eighteen AOX amino acid sequences from monocot and dicot plant species were used to construct the phylogenetic tree. These included AOX sequences from *H. vulgare*, *B. distachyon*, *S. bicolor*, *O. sativa* and *P. virgatum*, as monocot species, and dicot species such as *Arabidopsis* (Appendix C9). AOX proteins were obtained from the NCBI. The 20 amino acid sequences from monocot and dicot species were aligned with the TaAOX1 identified in this study using ClustalW2.1 plugin in Geneious Prime software version 2019.1.3 (Biomatters Ltd., Auckland, New Zealand). To investigate the evolutionary relationship among AOX proteins, the phylogenetic tree was constructed by employing the neighbour-joining method using the bootstrap value of 1,000 replicates wrapped in Geneious Prime software, version 2019.1.3 (Biomatters Ltd., Auckland, New Zealand).

2.4.4 Genomic Architecture and Protein Analysis

To investigate the genomic architecture of each AOX1 gene, the coding sequence was aligned with its corresponding genomic DNA sequence. The structure models were illustrated using the gene structure display server (Hu et al. 2015). The NetPhos 3.1 server (http://www.cbs.dtu.dk/services/NetPhos/) was used to predict serine, threonine or tyrosine phosphorylation sites in the AOX protein (Blom et al. 1999), while the Musite prediction program (http://musite.net) was used to predict putative acetylation sites (Gao et al. 2010). The NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) was used to predict putative N-glycosylation sites.

2.4.5 The Chromosomal Localisation of AOX1 and Promoter Region Analysis

The chromosomal localisation of *AOX1* was obtained from genome assemblies of wheat and its relatives. Namely, 2000 bp of *AOX* sequence upstream of the start codon were analysed to predict the *cis*-acting regulatory elements using the PlantCARE database (Lescot et al. 2002).

Chapter 3: Identification and Classification of Alternative Oxidase in Wheat and its Related Species

3.1 Introduction

The world's population is highly reliant on the global supply of bread wheat (*T. aestivum*), and demand for the supply of bread wheat has increased substantially (Longin & Reif 2014; Ray et al. 2013; 2015; Reynolds et al. 2011). Several factors affect the productive yield of bread wheat, such as climate change, decreasing arable farmland and environmental stresses (Godfray et al. 2010; Zhao et al. 2017a). The effects of climate change and the increased demand for wheat require an improvement in productive yield capacity (Challinor et al. 2014; Ray et al. 2013, 2015). Crops exposed to environmental stress cause metabolic responses and gene transcriptional activity (dos Reis et al. 2012). For plants, it is essential rapidly to re-programme their metabolic responses under environmental stress to maintain their functionality and structure when exposed, for example, to salinity (Cutler et al. 2010; dos Reis et al. 2012; Negrão et al. 2017).

Within plant cells, the mitochondrion is an essential organelle that aids in plant stress response, and it is the point for ATP generation, which involves cytochrome *c* oxidase or Complex IV dehydrogenase (DH). During the stress, alternative oxidase works to dissipate energy, thereby limiting the production of ROS (Moore & Albury 2008; Moore et al. 2013; Vanlerberghe 2013). Alternative oxidase in thermogenic plants produces heat for pollen germination during respiration (Grant et al. 2010). *Sauromatum guttatum* is the thermogenic plant species where the *AOX* gene was first cloned (Rhoads & McIntosh 1991; 1993). Monoclonal antibodies have been developed against the AOX proteins of *S. guttatum*, which can detect the proteins in other plant species (Elthon et al. 1989; Finnegan et al. 1999).

AOX genes have been found through the accessibility of several molecular techniques and sequenced genomes, and in some instances monocots and dicots have been functionally characterised, for example, barley, chickpea, rice and *Arabidopsis thaliana* (Borecký et al. 2006; Costa et al. 2014; 2017a; Sweetman et al. 2018; Wanniarachchi et al. 2018). The *AOX* gene has two discrete subfamilies, Type 1 (*AOX1*) and Type 2 (*AOX2*); Type 1 is found in both dicot and monocot species, and Type 2 has been found only in dicots. It is believed that Type 2 was found in ancient monocots (Considine et al. 2002; Costa et al. 2014, 2017a). *AOX1* genes

are known to be highly responsive to deviations in respiratory metabolism and highly responsive to stresses (Dinakar et al. 2016; Karpova et al. 2002; Saha et al. 2016). Conversely, *AOX2* showed only minimal evidence of a stress response, but is responsible for developmental processes such as fertility, germination and growth (Chai et al. 2010, 2012; Costa et al. 2007; Saisho et al. 2001).

Historically, genomic and transcriptomic data lacked the completeness necessary to understand metabolic reactions and the alternative respiratory pathway (Collakova et al. 2012). Technological advancements in bioinformatics tools have yielded increased access to sequence data. For instance, the genome of *Arabidopsis thaliana* was first published in 2000, and shortly thereafter, the genome of rice was also sequenced; since then, other plants have also been sequenced (AGI 2000; Bolger et al. 2014). The wide range of improved sequencing technologies has resulted in a significant increase in the publishing of reference genomes (Michael & Jackson 2013). However, despite technological advances in bioinformatics processing, certain issues affect analyses of genomic data (Schmutzer et al. 2017).

Next Generation Sequencing (NGS), a most recent technological advancement, has proved problematic, particularly for analysing crop plants (Schmutzer et al. 2017). This is due to their large genomes and high ploidy, which confuses NGS genome processing. Subsequently, to sequence the wheat genome, it was necessary to develop new tools to decrease the NGS data to a practical level for processing (Bolger et al. 2017). Recent technologies in genomic sequencing have paved the way for improving genomic resources (Thind et al. 2018), which has facilitated the development of an understanding of how genomes can improve various crops for production (Bolger et al. 2014).

Genomic sequencing has resulted in genome databases, which has provided a repository for new research conducted on *AOX*. It was believed that *AOX* was only found in protists, some fungi and plants, but the use of bioinformatics research has found that organisms across all kingdoms have *AOX* sequences (McDonald et al. 2003; McDonald & Vanlerberghe 2006). This includes animals, where the availability of bioinformatics tools and genome sequence data has revealed *AOX* genes in various taxa (McDonald et al. 2009).

In *Arabidopsis*, the alternative respiratory pathway has been extensively studied (Clifton et al. 2006; Smith et al. 2009; Thirkettle-Watts et al. 2003; Umbach et al. 2005; Vanlerberghe 2013; Zhang et al. 2010). This pathway has five *AOX* genes encoding proteins, and these are *AOX1a*,

1b, *1c*, *1d* and *AOX2*, with all five encoded proteins localised within the mitochondria (Thirkettle-Watts et al. 2003). Two of the initially cloned *AOX* genes found in wheat (Takumi et al. 2002) resulted in extensive biochemical work along with expression studies, which showed that they participated in developmental processes as well as stress response (Feng et al. 2008a; 2008b; Jacoby et al. 2010; Mizuno et al. 2008; Naydenov et al. 2008; 2010). Aspects that remain unclear about *AOX* genes are at the genome level, where the expression patterns and the number of *AOX* genes in wheat have not been determined. Having information about the wheat genes would enable genome-wide research of the *AOX* family across both ancestral diploid and hexaploid species of monocots (Clavijo et al. 2017; Jia et al. 2013; Ling et al. 2013).

The aim of this chapter is to identify and classify *AOX* genes in *Triticum aestivum* along with related species, and then evaluate their responses to abiotic stress *in silico*. Four *Triticum* species were studied as follows: *T. aestivum* (AABBDD), *T. turgidum* (AABB), *T. urartu* (A^uA^u) and *T. monococcum* (A^mA^m). Three *Aegilops* species were studied as follows: *Ae. speltoides* (SS), *Ae. sharonensis* ($S^{sh}S^{sh}$) and *Ae. tauschii* (DD). Multiple approaches were employed to undertake this aim, and this chapter describes the way this was conducted, using public databases and bioinformatics tools to determine the *AOX* genes that are prone to a stress response. No studies had been published on the *AOX* in *T. aestivum* and related species until this work commenced in 2014. However, Brew-Appiah et al. (2018) recently published an article on several *AOX* genes in *T. aestivum*, *T. urartu*, *Ae. speltoides* and *Ae. tauschii* species, which will be discussed later in this chapter. The present research was undertaken to extend the comparative analysis of gene sequences and structural diversity of identified *AOX* genes in wheat and its related species using publicly available databases and bioinformatics programs.

3.2 Results

3.2.1 Identification of AOX Candidates in *Triticum* and *Aegilop*s Species

3.2.1.1 *In Hexaploid Wheat (T. aestivum cv. Chinese Spring)*

At the beginning of 2014, the Chromosome Survey Sequences (CSS) were the first genome database made publicly available for bread wheat (Brenchley et al. 2012). Due to the lack of annotation data in CSS, the full contigs were obtained from the database and manually validated using *OsAOX1*, *WAOX1a* and *WAOX1c* as described in Section 2.4.1. In 2014, it was possible to identify the full length of eight *AOX1* genes in *T. aestivum* using the CSS database

(Table 3.1). Three additional *AOX1* isoforms were completed and confirmed using IWGSC-CSSv2; the improved assembly was released in July 2014. In 2015–2016, three assemblies, TGACv1, IWGSC-CSSv3 and IWGSC-WGA v0.4, of the bread wheat genome were made publicly available. Subsequently, two more *AOX1* isoforms have been found. During the first two years of this project (2014–2016), 13 full-length *TaAOX1* genes, including known *AOX1* genes (AB078882.1 and AB078883.1), were identified in *T. aestivum*. In January 2017, an updated version of IWGSC-RefSeq v1.0 was available at URGI under the terms of the Toronto agreement (Birney et al. 2009). Using the IWGSC-RefSeq v1.0, this study confirmed the existence of 13 full-length *TaAOX1* genes, with an additional eight partial *AOX1* genes, the first time they had been found (Table 3.2). These eight partial genes only partially encode the AOX protein domain.

Within the period of the current study, six versions of wheat genome assemblies have been used. This study has updated its findings continuously, as shown in Table 3.1. The results used in the final analysis were based on the most recent wheat assembly, IWGSC-RefSeq annotation v1.1, released in July 2018 without restriction. The current study identified and confirmed the existence of 21 *AOX* candidate genes, as noted above, in the IWGSC-RefSeq annotation v1.1. Additionally, within the 21 *AOX* candidate genes, there were eight partial *AOX* genes, as stated above (Table 3.1 and Table 3.2).

Table 3.1 A summary of the findings on *AOX1*, and updates of the bioinformatics data using improved assemblies, during 2014-2018.

A summary of *AOX1* candidate genes that were found in different databases, available at URGI. Overlapping genes ID, Contigs ID and Scaffold ID were indicated in each database. CSS was the first dataset released in 2012. IWGSC-RefSeq v1.0 was restricted by the Toronto agreement. IWGSC-RefSeq annotation v1.1 is the most recent wheat assembly released in July 2018. An asterisk (*) indicates that *AOX1* candidates were only partially identified. The years 2014 to 2018 refer to the time when the information was accessed from the databases.

***AOX* candidate genes**		**Databases**					
		2014		**2015**	**2016**	**Jun-16**	**2017-2018**
		CSS	**IWGSC-CSSv2**	**Wheat-TGACv1**	**IWGSC-CSSv3**	**IWGSC-WGA v0.4**	**IWGSC-RefSeq v1.1**
AOX1a	2AL	Traes_2AL_3FE5DF00C.1	Contig 2AL_6423394	Scaffold_093624_2AL	2AL_sc1297	Scaffold40216chr2A	TraesCS2A02G439400
	2BL	Traes_2BL_AE761AE4B.1*	Contig 2BL_8073369	Scaffold_132767_2BL	2BL_sc41	Scaffold38376chr2B	TraesCS2B02G459300
	2DL	Traes_2DL_21323959A.1*	Contig 2DL_9787485*	scaffold_159044_2DL	2DL_sc66611	Scaffold31560chr2D	TraesCS2D02G436700
AOX1c	6AL	Traes_6AL_E38401887.1	Contig 6AL_ 5832763	Scaffold_471250_6AL	6AL-sc31	Scaffold45172chr6A	TraesCS6A02G269100
	6BL	Not found	Not found	Scaffold_499881_6BL	6BL-sc243	Scaffold38171chr6B	TraesCS6B02G296400
	6DL	Traes_6DL_01E9A5EC9.1*	Contigs 6DL-3289573	Scaffold_528632_6DL	6DL-sc2360	Scaffold32591chr6D	TraesCS6D02G245800
AOX1d	2AL	Not found	Contig 2AL_6345448	Scaffold_093545_2AL	2AL_sc281	Scaffold40216chr2A	TraesCS2A02G438200
	2AL	Not found	Contig 2AL_6361262	scaffold_094717_2AL	2AL_sc4553	Scaffold40216chr2A	TraesCS2A02G439100
	2AL	Traes_2AL_2EFFD7933.1*	Contig2AL-6438699*	Scaffold_093545_2AL	2AL_sc3843	Scaffold40216chr2A	TraesCS2A02G438300
	2BL	Traes_2BL_EA2B95CF0.1*	Contig 2BL_8091682	Scaffold_129474_2BL	2BL_sc61	Scaffold38376chr2B	TraesCS2B02G459000
	2BL	Not found	Contig 2BL_8091682	Scaffold_129474_2BL	2BL_sc61	Scaffold38376chr2B	TraesCS2B02G459100
	2DL	Traes_2DL_0F0A9C3B9.1*	Contig 2DL__9847494	Scaffold_160654_2DL	2DL_sc1665	Scaffold31560chr2D	TraesCS2D02G436100
	2DL	Not found	Not found	Scaffold_162315_2DL	2DL-sc193373	Scaffold31560chr2	TraesCS2D02G436200

Table 3.2: Partial *AOX* candidate genes found in IWGSC-RefSeq annotation v1.0/v1.1

A summary of partial/like *AOX1* candidate genes found in IWGSC-RefSeq annotation v1.0 or IWGSC-RefSeq annotation v1.1, which were available at URGI. Overlapping genes ID indicated in each database. Low-confidence genes (LC) were based on the IWGSC-RefSeq annotation. The years 2017 and 2018 refer to the time when the information was accessed from the databases.

AOX candidate genes		Databases	
		2017	2018
		IWGSC-RefSeq v1.0	IWGSC-RefSeq v1.1
AOX1a-like/partial	3BS	TraesCS3B01G313800LC	TraesCS3B02G313800LC
	6BL	TraesCS6B01G496600LC	TraesCS6B02G496600LC
	7BL	TraesCS7B01G356600	TraesCS7B02G356600
AOX1d-like	2DL	TraesCS2D01G552300LC	TraesCS2D02G552300LC
	4AS	TraesCS4A01G037300LC TraesCS4A01G037400LC	TraesCS4A02G037300LC TraesCS4A02G037400LC
AOX1e-partial	3BL	TraesCS3B01G087900	TraesCS3B02G087900
	3DS	TraesCS3D01G072900LC	TraesCS3D02G072900LC
AOX-like/partial	4BL	TraesCS4B01G272200	TraesCS4B02G272200

3.2.1.2 *In Tetraploid Wheat (T. turgidum)*

Triticum turgidum is considered a valuable source for wheat improvement (Munns et al. 2012). Tetraploid wheat (*T. turgidum*) was used as a source of biofortification of Zn and Fe in a wheat breeding program (Velu et al. 2014). Recent advancement in the sequencing of *T. turgidum* subspecies has enabled this study to identify the *AOX* candidates in *T. turgidum*. Three genome resources for *T. turgidum* were used to investigate the genetic diversity of *AOX* (Section 2.4.1). The wheat *AOX* genes identified in the previous Section were used to find *AOX* orthologous genes in *T. turgidum*. In 2017, the genome assembly of wild emmer wheat (*T. turgidum* ssp. *dicoccoides* cv. Zavitan) was released (WEWSeq v1.0). This current study identified 16 *AOX* candidate genes in total, which included seven partial *AOX* in wild emmer wheat (Table 3.3 and Table 3.4). In durum wheat, two genome resources from different durum wheat cultivars were used (Section 2.4.1). When commencing this investigation in 2017, the transcriptome datasets of durum wheat cv. Kronos were the best resource available for conducting this bioinformatics analysis. In 2017, this research identified 14 *AOX* candidate genes in total in durum wheat cv. Kronos with six partial *AOX* (Table 3.3 and Table 3.4). In June 2019, a study on *T. turgidum* ssp. *durum* cv. Svevo was published, which updated the durum wheat *AOX* findings based on the Svevo genome assembly (Maccaferri et al. 2019). In the Svevo genome

assembly, one more candidate *AOX* was partially identified, in addition to the 14 main *AOX* genes similar to those identified in cv. Kronos (Table 3.3 and Table 3.4).

Table 3.3: Summary of findings of AOX1 candidates in T. turgidum using three genomic resources

A summary of *AOX1* candidate genes that were found in different genomic resources, which were available at the GrainGenes database. Overlapping gene IDs and Scaffold IDs were indicated in each database. The years 2017 and 2019 refer to the time when the information was accessed from the databases.

***AOX* candidate genes**		**Databases**		
		2017		**2019**
		Zavitan WEWSeq v.1.0	***Triticum turgidum* cv. Kronos EI v1.1**	**Durum wheat (cv. Svevo) RefSeq Release 1.0**
AOX1a	2AL	TRIDC2AG063170 TRIDC2AG063180 TRIDC2AG063160	Scaffold_007012	TRITD2Av1G252980
	2BL	TRIDC2BG067000	Scaffold_029145	TRITD2Bv1G214620
AOX1c	6AL	TRIDC6AG041320	Scaffold_022783	TRITD6Av1G170830
	6BL	TRIDC6BG048280	Scaffold_030043	TRITD6Bv1G157350
AOX1d	2AL	TRIDC2AG063080	Scaffold_050931	Td_Svvo|whe_Td_AB_Svevo_2A
	2AL	TRIDC2AG062980	Scaffold_027471	TRITD2Av1G252640
	2AL	TRIDC2AG062990	Scaffold_027471	TRITD2Av1G252650.1
	2BL	Scaffold70815	Scaffold_054600	TRITD2Bv1G214460.7
	2BL	TRIDC2BG066960	Scaffold_054600	TRITD2Bv1G214460.3

Table 3.4: Partial *AOX* candidate genes found in *T. turgidum* using three genomic resources

A summary of partial/like *AOX1* candidate genes that were found in three genomic resources, which were available at the GrainGenes database. Overlapping genes ID and Scaffold ID indicated in each database. Low-confidence genes (LC) were based on the IWGSC-RefSeq annotation. The years 2017 and 2019 refer to the time when the information was accessed from the databases.

AOX candidate genes		Databases		
		2017		2019
		Zavitan WEWSeq v.1.0	*Triticum turgidum* cv. Kronos EI v1.1	Durum wheat (cv. Svevo) RefSeq Release 1.0
AOX1a-like/partial	3BS	Scaffold115660	Scaffold_004545	Td_Svvo|whe_Td_AB_Svevo_3B
	5BL	TRIDC5BG064370	Not found	Not found
	6BL	TRIDC6BG042150	Scaffold_012051	TRITD6Bv1G135070
	7BL	TRIDC7BG056950	Not found	Td_Svvo|whe_Td_AB_Svevo_7B
AOX1d-like	4AS	TRIDC4AG005530	Scaffold_064813	Td_Svvo|whe_Td_AB_Svevo_4A
AOX1e-partial	3BL	Scaffold79340	Scaffold_023488	TRITD3Bv1G023890
AOX-like/partial	4BL	TRIDC4BG047100	Scaffold_006210	TRITD4Bv1G162220

3.2.1.3 *In Diploid Wheat (T. urartu cv. G1812 and T. monococcum L)*

In 2015, the current study investigated the existence of *AOX* genes in the progenitor of the A genome, in diploid wheats (*T. urartu* cv. G1812, and *T. monococcum*). The wheat *AOX* genes identified in Section 3.2.1.1 were used to find *AOX* orthologous genes in the diploid wheats. In red wild einkorn wheat (*T. urartu* cv. G1812), three genomic resources were used to identify *AOX* (Section 2.4.1). In 2015, only two genome assemblies were available for the red wild einkorn wheat, TGAC_WGS_urartu_v1, and ASM34745v1. This study identified five *AOX* candidate genes in the TGAC_WGS_urartu_v1 genome assembly; only four of them were found in ASM34745v1, and within the four *AOX* candidate genes, there were three partial sequences (Table 3.5). The most recent assembly (Tu2.0) was publicly available at NCBI as of April 2018 (Ling et al. 2018). All five *AOX* noted above were found in Tu2.0 genome assembly. Interestingly, the Tu2.0 genome assembly contains two copies of *AOX1c* on chromosome 4 and 6, which were 100% identical (Table 3.5). Only one genome resource is available at URGI TGAC for cultivated einkorn wheat (*T. monococcum*). This research identified four *AOX* candidate genes in *T. monococcum* (Table 3.5).

Table 3.5: *AOX1* candidates identified in *T. urartu* and *T. monococcum* during 2014–2018

A summary of *AOX1* candidate genes found in different assemblies that were available at URGI and NCBI databases. Contigs ID and accession ID were indicated in each database. Asterisk (*) indicates that *AOX1* candidates were partial sequences. The years 2014, 2015 and 2018 refer to the time when the information was accessed from the databases. # indicated the location based on the Tu.20 assembly.

AOX candidate genes	Databases				
	2014	2015	2018		2014
	TGAC_WGS_urartu_v1 at URGI	ASM34745v1 at NCBI (Accession)	Tu2.0 at NCBI	# Location	TGAC_WGS_monococcum_v1 at URGI
AOX1a	Contig-164999	Scaffold36070 (KD215045.1*)	MKGO01000002.1	Chr2A:668044523-668046449 (-)	Contig_70429
AOX1c	Contig-194071	Scaffold39236 (KD153788.1*)	MKGO01000006.1	Chr6A:463555983-463557309 (-)	Contig_904991
	Not found	Not found	MKGO01000004.1	Chr4A:290418919-290420245 (+)	
AOX1d	Contig-166732	Scaffold16660 (KD146645.1*)	MKGO01000002.1	Chr2A:668594498-668595490 (+)	Contig_926000 & Ccontig_96141
	Contig-137614	Scaffold33580 (KD149474.1)	MKGO01005341.1	ChrUn:4268-5212 (-) #	
AOX1d-like	Contig-350172*	Not found	MKGO01000004.1	Chr4:552583345-552599241 (+)	Contig_97604

3.2.1.4 In Aegilops Species (Ae. speltoides*, Ae. sharonensis and Ae. tauschii)*

The research was expanded to cover *Aegilops* species, including S genome *Aegilops*: *Ae. Speltoides* and *Ae. sharonensis*, the putative progenitors of the hexaploid wheat B genome (Salse et al. 2008). The wheat *AOX* genes identified in Section 3.2.1.1 were used to find *AOX* orthologous genes in *Aegilops* species. TGAC_WGS_speltoides_v1 and TSL_WGS_sharonensis_v1 databases were used to identify *AOX* in *Ae. speltoides* and *Ae. sharonensis*, respectively. In 2015, four *AOX* candidate genes were found in *Ae. speltoides*, whereas five *AOX* candidate genes were reported in *Ae. sharonensis*, and within the five *AOX* candidate genes, one partial *AOX* gene sequence was found (Table 3.6). For *Ae. tauschii*, three genome resources were used to identify *AOX* genes (Section 2.4.1). In 2014, only two genome assemblies were available for *Ae. tauschii*, TGAC_WGS_tauschii_v1 and ASM34733v1. This study found three *AOX* candidate genes in TGAC_WGS_tauschii_v1, and four *AOX* candidates in ASM34733v1; within the four *AOX* candidate genes, it found three partial sequences (Table 3.7). The most recent assembly (Aet v4.0) was publicly available at NCBI as of October 2017 (Luo et al. 2017). This study updated the *AOX* findings in *Ae. tauschii* based on the Aet v4.0 genome assembly. In total, six *AOX* candidate genes were found in *Ae. tauschii* using the Aet v4.0 genome assembly (Table 3.7).

Table 3.6: *AOX1* candidates identified in *Ae. speltoides* and *Ae. sharonensis* assemblies

A summary of *AOX1* candidate genes found in different assemblies that were available at URGI. Contigs IDs were indicated in each database. An asterisk (*) indicates that *AOX1* candidates were partially identified. The year 2015 refers to the time when the information was accessed from the databases.

Databases		
***AOX* candidate genes**	**TGAC_WGS_speltoides_v1 (2015)**	**TSL_WGS_sharonensis_v1 (2015)**
AOX1a	Contig_195745	Contig_144255*
AOX1c	Contig_239141	Contig_332219 Contig_ 2452024
AOX1d	Contig_1628212 Contig_1601667	Contig_161299
	Contig_403763	Contig_167302 Contig_1106212
AOX1e	Not found	Contig_1082850

Table 3.7: *AOX1* candidates identified in *Ae. tauschii* assemblies

A summary of *AOX1* candidate genes found in different assemblies that were available at URGI, ATGSP and NCBI databases. Contigs ID, scaffold ID and accession ID are indicated in each database. Asterisks (*) indicate that *AOX1* candidates were misannotated. The years 2014 and 2017 refer to the time when the information was accessed from the databases. Overlapping gene IDs were obtained from Ensembl Plants. # indicates the location based on Aet v4.0 assembly.

AOX candidate genes	Databases			
	2014	2017		
	TGAC_WGS_tauschii_v1 at URGI/ or ATGSP **	ASM34733v1 at NCBI (Accession)	Aet v4.0 at NCBI (Ensembl Plants)	# Location
AOX1a	Contig-HI244C14_RI339F10 **	Scaffold49178 (KD548317.1*)	CM008369.1 (AET2Gv20967100)	Chr2D:545909448-545911386 (+)
AOX1c	Contig_130240	Scaffold94414 (KD593434.1*)	CM008373.1 (AET6Gv20685500*)	Chr6D:376749751-376751062 (-)
AOX1d	Not found	Not found	CM008369.1 (AET2Gv20965600)	Chr2D:545533581-545534567 (+)
	Contigs-4230.6	Scaffold131071(KD630003.1*)	CM008369.1 (AET2Gv20965700)	Chr2D:545589298-545590290 (+)
AOX1d-like	Not found	Not found	CM008369.1 (AET2Gv20982300)	Chr2D:552549445-552570313 (+)
AOX1e	Not found	Scaffold60901 (KD560010.1)	CM008370.1 (AET3Gv20162700*)	Chr3D:36388531-36391038 (-)

3.2.2 Characterization of AOX genes

3.2.2.1 Classification of AOX1-subfamily Members in Triticum *and* Aegilops *Species*

The classification schemes for AOX proposed by Costa et al. (2014) were used to classify AOX protein sequences identified in wheat and related species. Specific amino acids and phylogenetic analysis were used to designate AOX types by using AtAOX1a (acc. no. At3g22370) protein sequence as a reference, as described in Section 2.4.2. First, AOX protein sequences were analysed to distinguish AOX1 from AOX2 using the most reliable amino acids (positions 229, 233 and 241) and secondary reliable amino acids (positions 112, 124, 129, 232 and 342) according to Costa et al. (2017b). Based on the amino acids specific for the AOX1, all identified AOX protein sequences in *Triticum* and *Aegilops* species were classified as AOX1, other than a partial AOX protein sequence found on the long arm of chromosome 4 on the B genome of *T. aestivum* and *T. turgidum*, which were designated as TaAOX-4BL and TtAOX4BL, respectively.

Second, the AOX1d type was determined, based upon the most reliable amino acids that differentiate monocots AOX1d from AOX1a–c/1e, at positions (175, 178 and 181), as well as the secondary reliable amino acids (positions 126, 142, 167, 180, 277, 230, 295 and 343). The most reliable and secondary reliable amino acids were both found on 27 AOX protein sequences identified in wheat and related species, including AOX1d-like isoforms, with the exception of the amino acids positioned at 295 of TaAOX1d2-2DL-like and AetAOX1d2-2DL-like (Figure 3.1). Note that in some cases, a gene was given the suffix '-like' if it had an internal stop codon and was given the suffix '-partial' if only a partial sequence was obtained from the database. The *AOX1d* gene has duplicated target sequences, which were numbered (1, 2 and 3) after the letter 'd' according to the percentage of identity in the target sequences compared with the *H. vulgare* AOX1d clades (Appendix C.1). Finally, phylogenetic analyses were used to support AOX classification into types and subtypes (Figure 3.2).

In *T. aestivum*, all *AOX1*-subfamily members were found and classified as *AOX1a*, *AOX1c*, *AOX1d* and *AOX1e*, including partial and *AOX*-like isoforms (Figure 3.2 and Table 3.8). For *AOX1a* isoforms, six candidates *AOX* sequences, including partial and *AOX*-like isoform, were classified as type *AOX1a*. They were designated as *TaAOX1a-2AL*, *TaAOX1a-2BL*, *TaAOX1a-2DL*, *TaAOX1a-3BS-like*, *TaAOX1a-6BL-like* and *TaAOX1a-7BL-partial*. For *AOX1c*, three candidate *AOX* sequences were fully identified and designated as *TaAOX1c-6AL*, *TaAOX1c-*

6BL and *TaAOX1c-6DL*. In addition, the *AOX1d clade* has nine duplicated sequences, including an *AOX-like* isoform; these were designated as *TaAOX1d1-2AL*, *TaAOX1d2-2AL*, *TaAOX1d3-2AL*, *TaAOX1d1-2BL*, *TaAOX1d2-2BL*, *TaAOX1d1-2DL*, *TaAOX1d2-2DL*, *TaAOX1d1-2DL-like* and *TaAOX1d1-4AS-like*. However, in addition to *TaAOX1d* clades, only two *AOX1e* partial sequences were found in *T. aestivum* and were designated as *TaAOX1e-3BL-partial* and *TaAOX1e-3DS-partial.*

In *T. turgidum*, all *AOX1*-subfamily members were found and classified as *AOX1a, AOX1c, AOX1d* and *AOX1e*, including partial sequences and *AOX*-like isoforms (Figure 3.2 and Table 3.8). For *AOX1a* isoforms, six candidate *AOX* sequences, including partial and *AOX*-like isoforms, were classified as *AOX1a* and designated as *TtAOX1a-2AL*, *TtAOX1a-2BL*, *TtAOX1a-3BS-like*, *TtAOX1a-5BL-partial*, *TtAOX1a-6BL-like* and *TtAOX1a-7BL-partial*. For *AOX1c*, only two candidate *AOX* sequences were fully identified and were designated as *TtAOX1c-6AL* and *TtAOX1c-6BL.* The *AOX1d* clade has six duplicated sequences, including an *AOX*-like isoform, which were designated as *TtAOX1d1-2AL*, *TtAOX1d2-2AL*, *TtAOX1d3-2AL*, *TtAOX1d1-2BL*, *TtAOX1d2-2BL* and *TtAOX1d1-4AS-like*. In *T. turgidum*, a single copy of *AOX1e* partial sequence was found and designated as *TtAOX1e-3BL-partial.*

In diploid wheats, *AOX1a*, *AOX1c* and *AOX1d* were identified in both *T. urartu* and *T. monococcum*. In *T. urartu* (Figure 3.2 and Table 3.8), the identified *AOX* candidates were designated as *TuAOX1a, TuAOX1d1-like, TuAOX1d2, TuAOX1d3* and two copies of *AOX1c (TuAOX1c-4AS, and TuAOX1c-6AL).* In *T. monococcum, AOX* candidates were designated as *TmAOX1a, TmAOX1c, TmAOX1d1-like* and *TmAOX1d2.* However, *AOX1e* was not found in both *T. urartu* and *T. monococcum.*

In *Aegilops* species, all *AOX1*-subfamily members, *AOX1a, AOX1c, AOX1d and AOX1e,* were found in *Ae. sharonensis* and *Ae. tauschii*, but no *AOX1e* type genes were found in *Ae. speltoides* (Figure 3.2 and Table 3.8). *AOX* candidates from *Aegilops* species were designated as *AesAOX1a, AesAOX1c, AesAOX1d1* and *AesAOX1d2* in *Ae. speltoides. AeshAOX1a-partial, AeshAOX1c, AeshAOX1d1, AeshAOX1d2 and AeshAOX1e* were identified in *Ae. sharonensis*. Further, *Ae. tauschii* has *AetAOX1a, AetAOX1c, AetAOX1d1, AetAOX1d2, AetAOX1d2-like* and *AetAOX1e.*

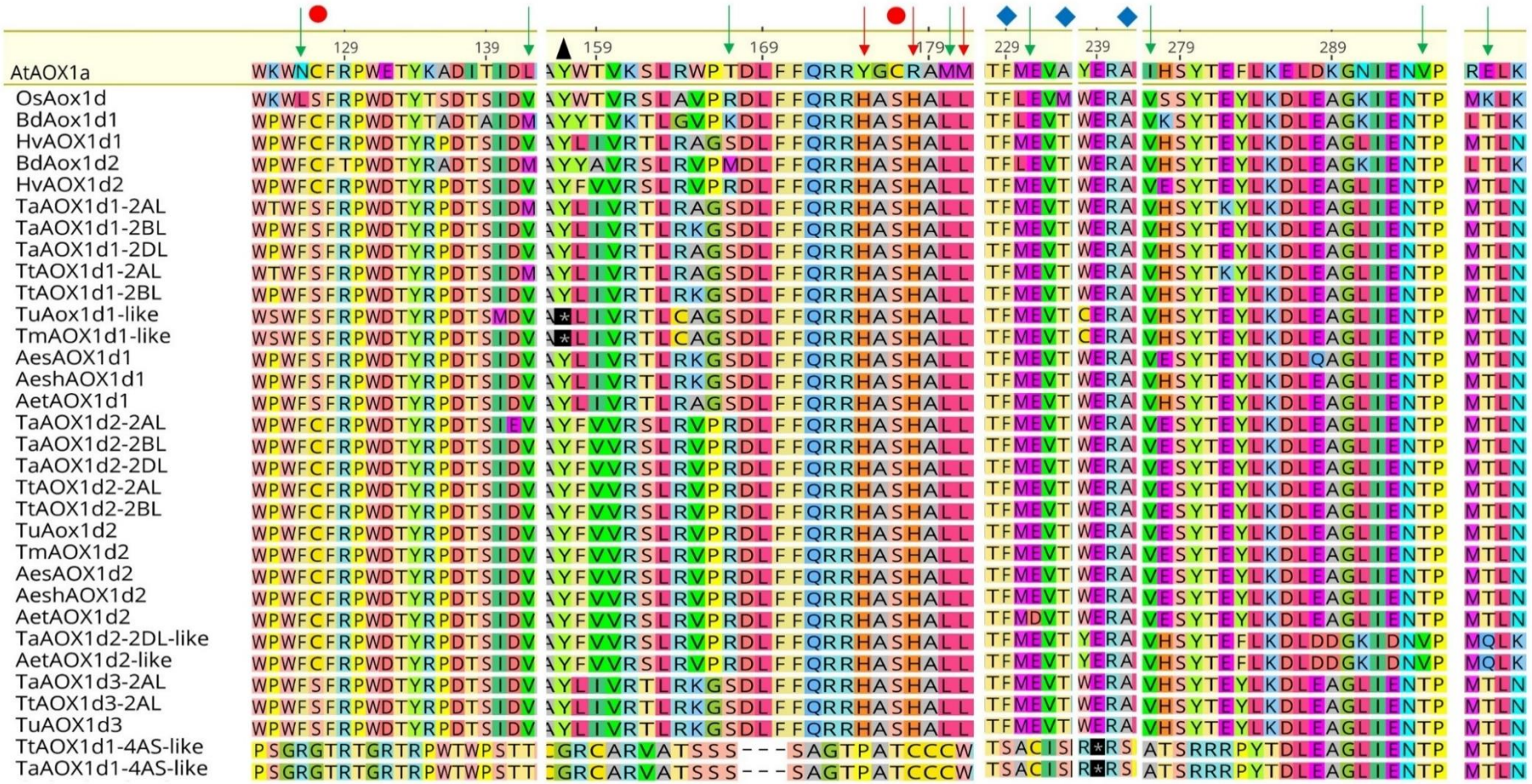

Figure 3.1: Alignment of protein AOX1d clade identified in *Triticum* and *Aegilops* species

The alignments of amino acid sequences aligned with orthologous protein from *O. sativa*, *H. vulgare*, and *B. distachyon* showing specific amino acids that were used to classify *AOX* into *AOX* subfamilies as described by Costa et al. (2017b). The blue diamond indicates the most reliable amino acids (positions 229, 233 and 241) that were used to distinguish AOX1 from AOX2. Red arrows indicate the positions of the most reliable amino acids that differentiate monocots AOX1d from AOX1a–c/1e (175, 178 and 181). Green arrows indicate the positions of the secondary reliable amino acids (positions 126, 142, 167, 180, 277, 230, 295 and 343). Red circles indicate CysI and CysII. Black triangle indicates the internal stop codon (shaded in black). *Arabidopsis thaliana* AOX1a (acc. no. AT3G22370) was used as reference.

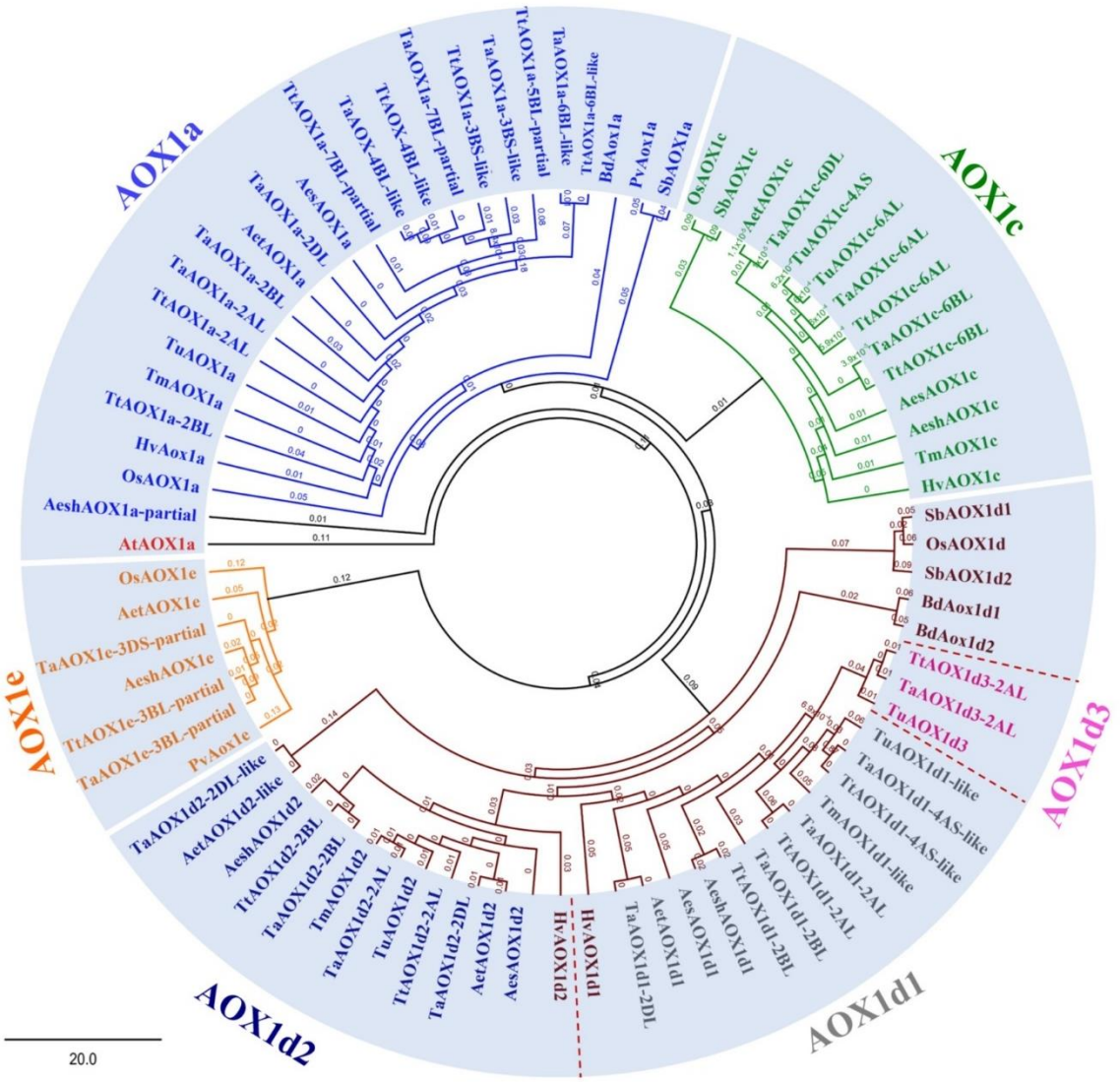

Figure 3.2: Phylogenetic relationship of AOXs in *Triticum* and *Aegilops* species

A neighbour-joining phylogenetic tree was constructed based on the full-length alignment of 62 putative AOX amino acid sequences, identified in *Triticum* and *Aegilops* species, which were aligned with 18 known AOX using the ClustalW2.1 plugin in Geneious Prime software version 2019.1.3 (Biomatters Ltd., Auckland, New Zealand). Bootstrap analyses were conducted using 1,000 replicates. Substitutions per site are shown on the branches. The tree was divided into four major clades, which indicated the AOX1-subfamily: AOX1a in blue, AOX1c in green, AOX1d in brown and AOX1e in orange. The accession numbers of selected AOX protein from *A. thaliana*, *H. vulgare*, *B. distachyon*, *S. bicolor*, *O. sativa* and *P. virgatum* are listed in Appendix C9.

Table 3.8: Numbers of *AOX1*-subfamily members in *Triticum* and *Aegilops* species

The number in parentheses indicates *AOX* genes that were partially/like identified; nd stands for no data.

AOX1 isoform	*T. aestivum* cv. Chinese Spring	*T. turgidum* ssp.			*T. urartu* cv. G1812	*T. monococcum*	*Ae. speltoides*	*Ae. sharonensis*	*Ae. tauschii* ssp. *strangulata*	Total
		dicoccoides cv. Zavitan	*durum* cv. Svevo	*durum* cv. Kronos						
AOX1a	3 (1)	2 (2)	2 (1)	1 (1)	1	1	1	(1)	1	12 (6)
AOX1c	3	2	2	2	2	1	1	1	1	15
AOX1d1	3	2	2	2	nd	nd	1	1	1	12
AOX1d2	3	2	2	2	1	1	1	1	1	14
AOX1d3	1	1	1	1	1	nd	nd	nd	nd	5
AOX1e	(2)	(1)	(1)	(1)	nd	nd	nd	1	1	2 (5)
AOX1a-like	(2)	(2)	(2)	(2)	nd	nd	nd	nd	nd	(8)
AOX1d1-Like	(1)	(1)	(1)	(1)	(1)	(1)	nd	nd	nd	(6)
AOX1d2-Like	(1)	nd	nd	nd	nd	nd	nd	nd	(1)	(2)
AOX-like	(1)	(1)	(1)	(1)	nd	nd	nd	nd	nd	(4)
Total	13 (8)	9 (7)	9 (6)	8 (6)	5 (1)	3 (1)	4	4 (1)	5 (1)	60 (31)

3.2.2.2 Characteristics of AOX Genes Identified in Triticum and Aegilops Species

The present research was able to identify the full or partial *AOX* sequences in *Triticum* and *Aegilops* species. In the full-length identified *TaAOX1* genes, the length of *AOX* ranged from 987 bp to 1963 bp, and the CDS ranged from 987 bp to 1044 bp (Table 3.9). Further, partial/like sequences ranged from 585 bp to 20871 bp, and the CDS ranged from 351 bp to 975 bp. Notably, most of these partial/like sequences were classified as low-confidence (LC) genes by IWGSC_Refseq v1.1 annotation (IWGSC-2018). *TaAOX1d2-2DL-like* and *TaAOX1e-3DS-partial* were the only two *AOX* genes longer than 3000 bp and classified as LC genes (Table 3.9).

In *T. aestivum*, the comparison of the genome structure revealed variations in exon-intron architecture within the *AOX* genes subfamily (Table 3.9; Figure 3.3). The genome structure of the *TaAOX1a* clade and the *TaAOX1c* clade comprises four exons interrupted by three introns, whereas the *AOX1d* clade contains no introns. However, *AOX*-partial/like sequences comprise up to five exons interrupted by four introns, such as *TaAOX1e-3BL-partial* (Table 3.9; Figure 3.3). Notably, the size of the last three exons was conserved (129, 489 and 57 bp, respectively), whereas the introns and first exon showed variable lengths among the *AOX1* subfamily.

The *AOX1*-subfamily genes identified in other *Triticum* and *Aegilops* species shared similar gene characteristics as noted in *T. aestivum* (for *T. turgidum*, Table 3.10 and Figure 3.4; for *T. urartu* and *T. monococcum*, Table 3.11 and Figure 3.5; for *Aegilops* species, Table 3.12 and Figure 3.6).

Table 3.9: Characteristics of *AOX1* subfamily genes identified in *T. aestivum*

Overlapping gene IDs were obtained from the latest annotation IWGSC_Refseq v1.1 * indicates the length of open reading frame (ORF). ** indicate ORF location in the chromosome. LC indicates that these *AOX* candidates were classified as low-confidence gene by the annotation.

AOX isoforms	Overlapping gene(s) ID	Gene length (bp)*	Location (Strand)**	CDS length (bp)	Exon-intron architecture
TaAox1a-2AL	TraesCS2A02G439400	1900	Chr2A:690738159-690740058 (+)	987	4/3
TaAox1a-2BL	TraesCS2B02G459300	1957	Chr2B:653745973-653747929 (+)	990	4/3
TaAox1a-2DL	TraesCS2D02G436700	1963	Chr2D:547250216-547252178 (+)	1011	4/3
TaAox1a-3BS-like	TraesCS3B02G313800LC	585	Chr3B:280583967-280584551 (+)	420	2/1
TaAox1a-6BL-like	TraesCS6B02G496600LC	606	Chr6B:464408149-464408754 (+)	441	2/1
TaAox1a-7BL-partial	TraesCS7B02G356600	586	Chr7B:616911618-616912203 (+)	387	3/2
TaAox1c-6AL	TraesCS6A02G269100	1327	Chr6A:495464128-495465454 (-)	1044	4/3
TaAox1c-6BL	TraesCS6B02G296400	1305	Chr6B:532150706-532152010 (-)	1038	4/3
TaAox1c-6DL	TraesCS6D02G245800	1312	Chr6D:348029192-348030503 (+)	1038	4/3
TaAox1d1-2AL	TraesCS2A02G439100	990	Chr2A:690409667-690410656 (+)	990	1/0
TaAox1d1-2BL	TraesCS2B02G459000	990	Chr2B:653610681-653611670 (+)	990	1/0
TaAox1d1-2DL	TraesCS2D02G436100	987	Chr2D:546872156-546873142 (+)	987	1/0
TaAox1d1-4AS-like	TraesCS4A02G037300LC TraesCS4A02G037400LC	966	Chr4A:33079374-33080339 (+)	966	1/0
TaAox1d2-2AL	TraesCS2A02G438200	993	Chr2A:689630507-689631499 (-)	993	1/0
TaAox1d2-2BL	TraesCS2B02G459100	993	Chr2B:653619281-653620273 (+)	993	1/0
TaAox1d2-2DL	TraesCS2D02G436200	981	Chr2D:546931974-546932954 (+)	981	1/0
TaAox1d2-2DL-like	TraesCS2D02G552300LC	20871	Chr2D:555113513-555134383 (+)	975	3/2
TaAox1d3-2AL	TraesCS2A02G438300	990	Chr2A:689709559-689710548 (-)	990	1/0
TaAox1e-3BL-partial	TraesCS3B02G087900	2818	Chr3B:56062841-56065658 (-)	756	5/4
TaAox1e-3DS-partial	TraesCS3D02G072900LC	17198	Chr3D:33297816-33315013 (-)	789	4/3
TaAox-4BL-like	TraesCS4B02G272200	586	Chr4B:548863261-548863846 (-)	351	2/1

Figure 3.3: Patterns of genomic structure variations of *AOX1* subfamily genes in *T. aestivum*.

Yellow bars represent exons, whereas the red lines represent introns. Gene structures were generated using GSDS web server v2.0.

Table 3.10: Characteristics of *AOX1* subfamily genes identified in *T. turgidum*

Overlapping gene IDs were obtained from the wild emmer wheat genome assembly WEWSeq v.1.0. * indicates the length of open reading frame (ORF). ** indicate ORF location in the chromosome. # indicates that these gene(s) IDs were classified as low-confidence gene by the annotation.

AOX isoforms	Scaffold/Gene(s) ID	Gene length (bp)*	Location (strand)**	CDS length (bp)	Exon-intron architecture
TtAox1a-2AL	TRIDC2AG063170 TRIDC2AG063180 # TRIDC2AG063160 #	1901	Chr2A:683996247-683998147 (+)	987	4/3
TtAox1a-2BL	TRIDC2BG067000	2237	Chr2B:650422390-650424626 (+)	996	4/3
TtAox1a-3BS-like	Scaffold115660	585	Chr3B:278665002-278665586 (+)	420	2/1
TtAox1a-5BL-partial	TRIDC5BG064370	586	Chr5:600998328-600998913 (+)	387	3/2
TtAox1a-6BL-like	TRIDC6BG042150	606	Chr6B:448686488-448687093 (+)	441	2/1
TtAox1a-7BL-partial	TRIDC7BG056950	586	Chr7B:625330077-625330662 (+)	387	3/2
TtAox1c-6AL	TRIDC6AG041320	1327	Chr6A:497463815-497465141 (-)	1044	4/3
TtAox1c-6BL	TRIDC6BG048280	1305	Chr6B:516664473-516665777 (-)	1038	4/3
TtAox1d1-2AL	TRIDC2AG063080	990	Chr2A:683667157-683668146 (+)	990	1/0
TtAox1d1-2BL	Scaffold70815	990	Chr2B:649987618-649988607 (+)	990	1/0
TtAox1d1-4AS-like	TRIDC4AG005530	966	Chr4A:33189888-33190853 (+)	966	1/0
TtAox1d2-2AL	TRIDC2AG062980	993	Chr2A:682901269-682902261 (-)	993	1/0
TtAox1d2-2BL	TRIDC2BG066960	993	Chr2B:649996080-649997072 (+)	993	1/0
TtAox1d3-2AL	TRIDC2AG062990	990	Chr2A:682979971-682980960 (-)	990	1/0
TtAox1e-3BL-partial	Scaffold79340	2846	Chr3B:62211878-62214723 (+)	756	5/4
TtAox-4BL-like	TRIDC4BG047100	586	Chr4B:548863051-548863659 (-)	351	2/1

Figure 3.4: Patterns of genomic structure variations of *AOX1* subfamily genes in *T. turgidum.*

Yellow bars represent exons, whereas the red lines represent introns. Gene structures were generated using GSDS web server v2.0.

Table 3.11: Characteristics of *AOX1* subfamily genes identified in *T. urartu* and *T. monococcum*

* indicates the length of open reading frame (ORF). ** indicate ORF location in the chromosome.

Species	*Aox* isoforms	Accession/Contig ID	Gene length (bp)*	Location (strand)**	CDS length (bp)	Exon-intron architecture
T. urartu	*TuAox1a*	MKGO01000002.1	1927	Chr2A:668044523-668046449 (-)	987	4/3
	TuAox1c	MKGO01000006.1	1327	Chr6A:463555983-463557309 (-)	1044	4/3
	TuAox1c	MKGO01000004.1	1327	Chr4A:290418919-290420245 (+)	1044	4/3
	TuAox1d1-Like	MKGO01000004.1	15897	Chr4:552583345-552599241 (+)	987	2/1
	TuAox1d2	MKGO01000002.1	993	Chr2A:668594498-668595490 (+)	993	1/0
	TuAox1d3	MKGO01005341.1	945	ChrUn:4268-5212 (-) #	945	1/0
T. monococcum	*TmAox1a*	Contig_70429	1973	7734-9706 (+)	987	4/3
	TmAox1c	Contig_904991	1320	1549-2868 (+)	1038	4/3
	TmAox1d1-like	Contig_97604	987	1114-2100 (-)	987	1/0
	TmAox1d2	Contig_926000 Ccontig_96141	993	1-170 (-) 235-1057 (-)	993	1/0

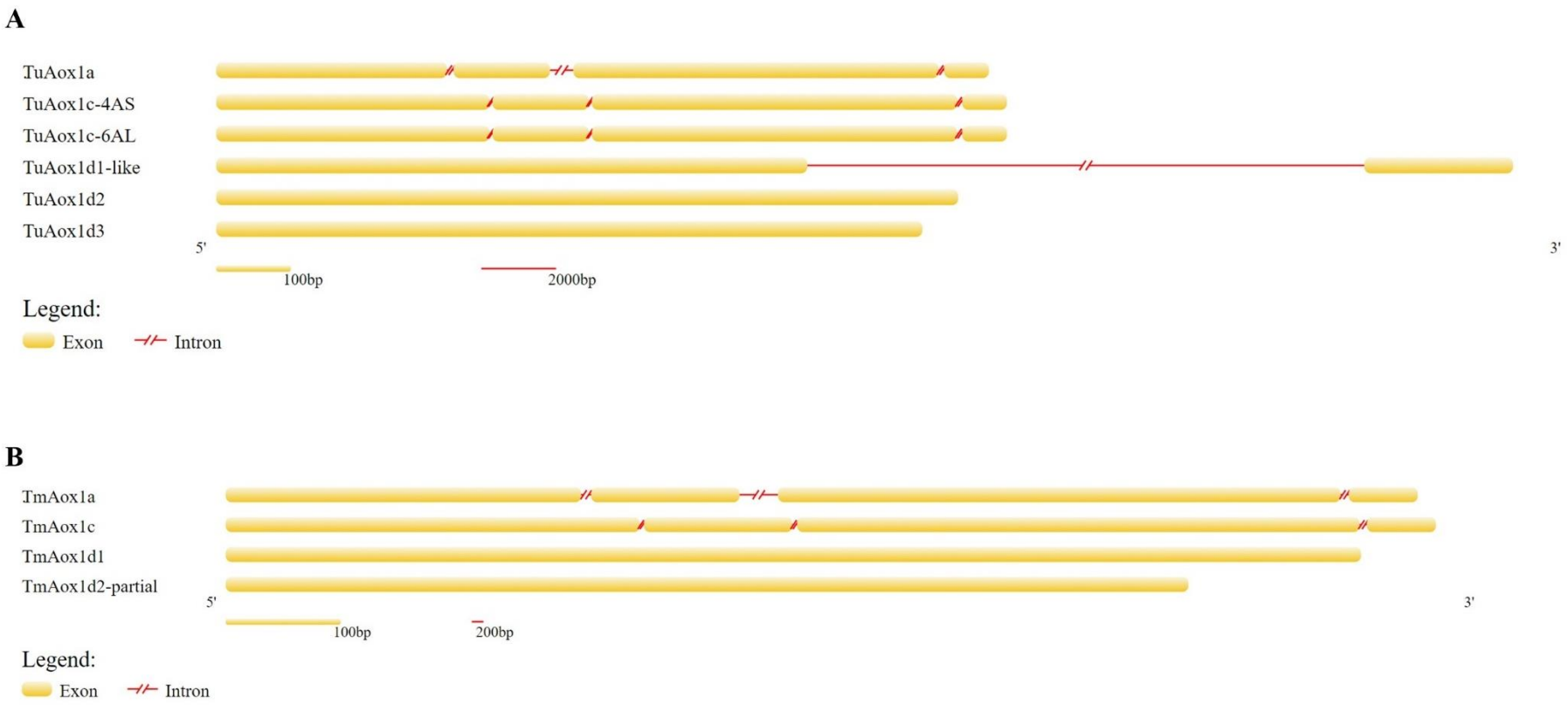

Figure 3.5: Patterns of genomic structure variations of *AOX1* subfamily genes in *T. urartu* and *T. monococcum*

(A)The genomic structure of *T. urartu AOX1* genes. (B) The genomic structure of *T. monococcum AOX1* genes. Yellow bars represent exons, whereas the red lines represent introns. Gene structures were generated using GSDS web server v2.0.

Table 3.12: Characteristics of *AOX1* subfamily genes identified in *Aegilops* species

* indicates the length of open reading frame (ORF). ** indicate ORF location in the chromosome.

Species	AOX isoforms	Contig (s)/Gene ID	Gene length (bp)*	Location (strand)**	CDS length (bp)	Exon-intron architecture
Ae. speltoides	*AesAox1a*	contig_195745	1974	5159-7132 (+)	996	4/3
	AesAox1c	contig_239141	1305	1765-3069 (-)	1038	4/3
	AesAox1d1	contig_1628212 contig_1601667	990	1-537 (-) 955-1419 (+)	990	1/0
	AesAox1d2	contig_403763	993	1346-2338 (-)	993	1/0
Ae. sharonensis	*AeshAox1a-partial*	contig_144255	1353	1-1353 (-)	570	3/2
	AeshAox1c	contig_332219 contig_ 2452024	1332	1251-2583 (+) #	1038	4/3
	AeshAox1d1	contig_161299	990	110-1099 (+)	990	1/0
	AeshAox1d2	contig_167302 contig_1106212	993	89-1091 (+) #	993	1/0
	AeshAox1e	contig_1082850	3475	116-3590 (+)	1044	5/4
Ae. tauschii	*AetAox1a*	AET2Gv20967100	1939	Chr2D:545909448-545911386 (+)	987	4/3
	AetAox1c	AET6Gv20685500	1312	Chr6D:376749751-376751062 (-)	1038	4/3
	AetAox1d1	AET2Gv20965600	987	Chr2D:545533581-545534567 (+)	987	1/0
	AetAox1d2	AET2Gv20965700	993	Chr2D:545589298-545590290 (+)	993	1/0
	AetAox1d2-like	AET2Gv20982300	20869	Chr2D:552549445-552570313 (+)	975	3/2
	AetAox1e	AET3Gv20162700	2508	Chr3D:36388531-36391038 (-)	876	5/4

Figure 3.6: Patterns of genomic structure variations of *AOX1* subfamily genes in *Aegilops* species

The genomic structure of *AOX1* genes in *Ae*. *speltoides*, *Ae. sharonesis* and *Ae. tauschii* are shown in A, B and D, respectively; yellow bars represent exons, whereas the red lines represent introns. Gene structures were generated using GSDS web server v2.0.

3.2.2.3 Characteristics of AOX Protein Sequences in Triticum and Aegilops Species

The current study revealed that the *Triticum* and *Aegilops* AOX1 gene subfamilies, which were fully identified and classified as high-confidence (HC) genes, encoded similar protein sizes, which ranged from 314 aa (TuAOX1d3) to 347 aa (AOX1c clade), with the molecular weights (Mw) ranging from 35 to 38 kDa and isoelectric points (pI) ranging from 6.7 to 8.9. The subcellular localisation analysis indicated that the *Triticum* and *Aegilops* AOX1-subfamily was targeted to the mitochondria. The N-terminal region exhibited significant variation within the *AOX* subfamily (Figure 3.7). The longest targeting peptide was observed in the AOX1c clade (~56 aa), apart from AOX1c on the D genome, which exhibited the smallest (27aa), whereas the AOX1a clade, AOX1d clade and AOX1e exhibited ~51, 46 and 43 aa lengths, respectively, with the exception of TuAOX1d3, which showed a 31 aa length (Figure 3.7; Tables 3.14 & 3.17).

Multiple alignments of the AOX coding regions revealed that *Triticum* and *Aegilops* AOX proteins are highly conserved within each isoform. For instance, CysI and CysII were conserved in the AOX1a clade, and the AOX1c clade of *Triticum* and *Aegilops* species, whereas the AOX1d clades possessed a serine residue instead of cysteine at the position of CysI. Comparatively, the AOX1d2 clade, in relation to the AOX1d1 clade and AOX1d3 clade, possesses another serine residue at the position of CysII, including AOX1d1-like in the diploid wheats (*T. urartu* and *T. monococcum*). Notably, the TmAOX1d2 had CysI and CysII as observed in AOX1a, and AOX1c orthologues (Figure 3.8; Table 3.13). This differentiation occurred in all *Triticum* and *Aegilops* AOX1d clades and is noted for the first time.

The analysis of the helix bundles, α2, α3, α5 and α6, which accommodated the di-iron carboxylate active site residues, E (glutamate) and H (histidine), indicated almost universal conservation across *Triticum* and *Aegilops* AOXs. However, there was one notable exception; the AeshAOX1e protein lacked the glutamate residue (E222) located in α3 (Figure 3.8). The analysis of region 3 revealed that E/DNV motifs were conserved within the AOX1-subfamily. The AOX1a clade, AOX1c clade, AOX1d clade and AOX1e clade contained DNV, ENV, ENT or DDV motif, respectively (Figure 3.8). However, there the exceptions were TaAOX1d2-2DL-like and AetAOX1d2-like, which have DNV motifs identical to AOX1a clade (Appendix B.1.1; B.2.3). Most of the residues required for AOX activity, such as T184, W211, Q247 and Y258, were conserved in *Triticum* and *Aegilops* species. However, R178 was substituted as

histidine in AOX1d clade only, but threonine residue ThrII (T279) was substituted as serine in all *Triticum* and *Aegilops* species (Figure 3.8; Table 3.13).

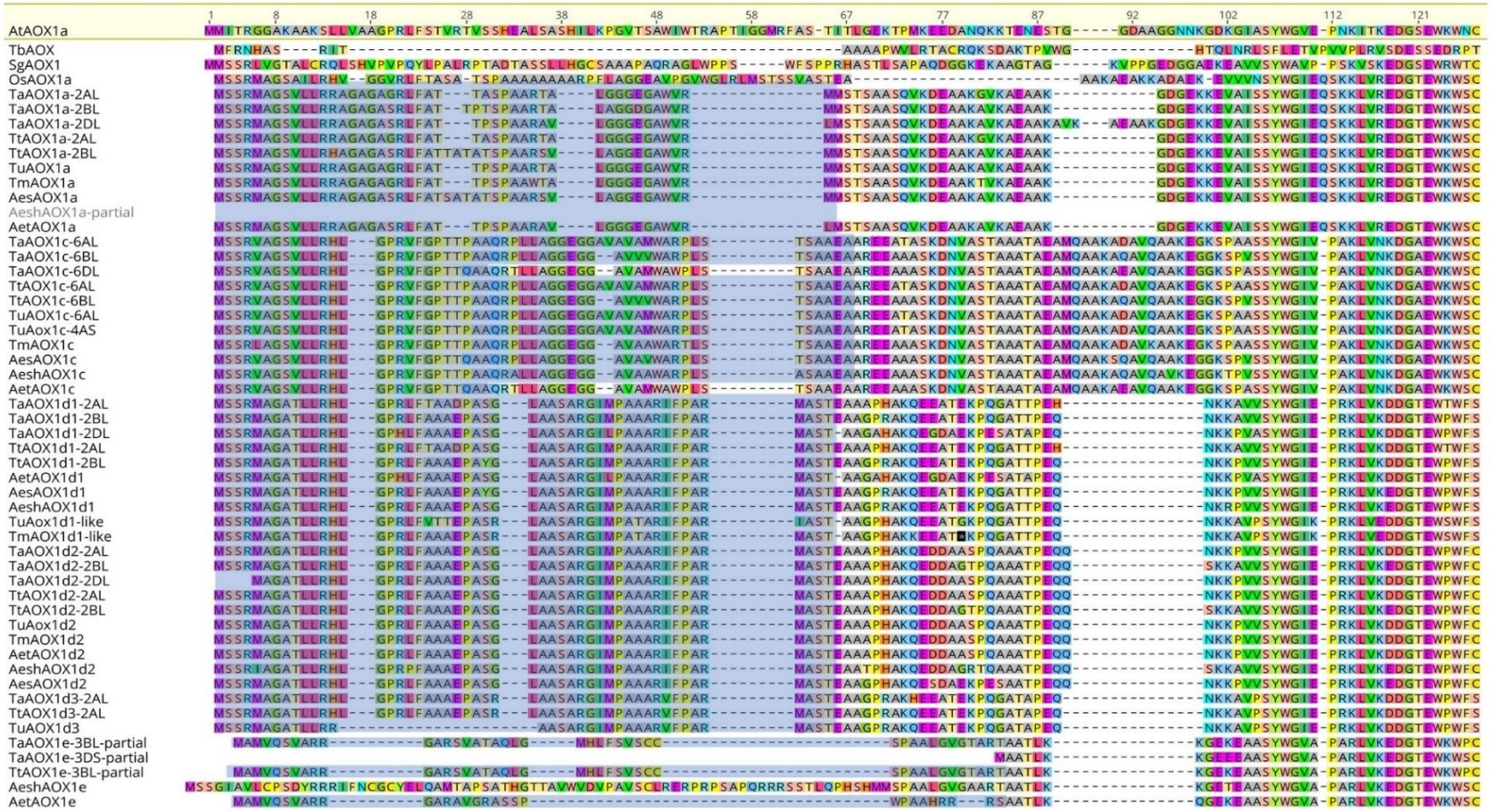

Figure 3.7: Multiple alignment of N-terminal region of AOX proteins from *Triticum* and *Aegilops* species and four other AOX sequences

The predicted length of the cleavage site of the mitochondrial targeting is shaded by light-blue rectangles. The alignment was performed using ClustalW2.1 plugin in Geneious Prime software version 2019.1.3 (Biomatters Ltd., Auckland, New Zealand). The predicted amino acid sequences used for comparison were *A. thaliana* (At.AOX1a; AT3G22370.1), *O. sativa* (AOX1a; LOC_Os04G51150), *S. guttatum* (SgAOX1; AAA34048.1) and *T. brucei* (TbAOX; XP_822944.1). Black triangles indicate the internal stop codon (shown on a black background).

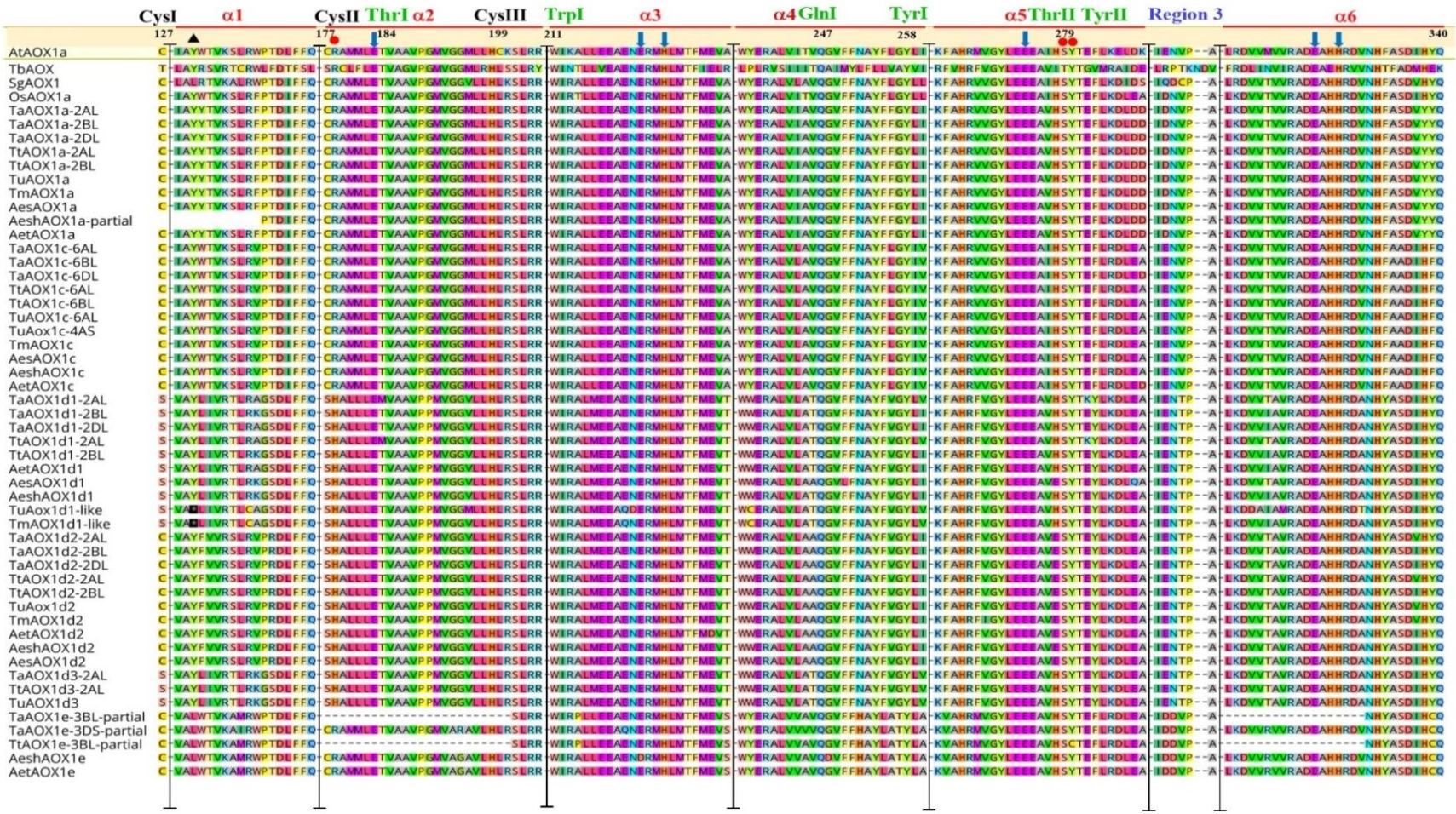

Figure 3.8: Multiple alignments of helices α1-α6 and region 3 in AOX protein from *Triticum* and *Aegilops* species and four other AOX sequences

Truncated alignment of the helices α1-α6 and region 3 were combined to show the similarity and the highly conserved important residue for AOX regulation, AOX motifs and AOX activity. The sites of cysteines (CysI, CysII and CysIII) were indicated in black font. The di-iron carboxylate active site residues, E (glutamate) and H (histidine), are

indicated by blue arrows. Berthold et al. (2000) defined motifs that were found in the regions of α3, α 5 and α 6 as highly conserved in AOX. Region 3 contains E/DNV residues, which were important to the pyruvate-sensitivity (Crichton et al. 2005). The amino acids required for AOX activity are indicated in green font: T184, W211, Q247, Y258, T279 and Y280; changes in an amino acid that are involved in AOX activity are indicated by red circles: R178, T279 and Y280. Deletion areas are shown by minus signs. The alignment was performed using ClustalW2.1 plugin in Geneious Prime software version 2019.1.3 (Biomatters Ltd., Auckland, New Zealand). The predicted amino acid sequences used for comparison were *A. thaliana* (At.AOX1a; AT3G22370.1), *O. sativa* (AOX1a; LOC_Os04G51150), *S. guttatum* (SgAOX1; AAA34048.1) and *T. brucei* (TbAOX; XP_822944.1). Numbering is based on the *A. thaliana* (At.AOX1a; AT3G22370.1) sequence.

Table 3.13: Summary of AOX protein residues involved in AOX regulation, characterisation or activity in *Triticum* and *Aegilops* species

Selected AOX proteins identified in the current study were analysed to explore AOX characteristics. All residues are numbered according to *A. thaliana* (At.AOX1a; AT3G22370.1). Highlighted in orange are the changes in conserved residues, whereas the yellow box indicates that data are not available owing to partial sequences. The residues in red are amino acids proposed to coordinate the iron-binding residues. AOX1-partial/like sequences were not long enough for protein analysis; therefore, they been excluded. The di-iron carboxylate active site residues, (E) and (H), are shown in red font.

	AOX regulation			AOX Motifs				AOX activity									AOX domain	
AOX isoforms	CysI (C127)	CysII (C177)	CysIII (199)	LETVAA	ERMHLMT	LEEEA	RADEAHH	R178	ThrI (T184)	TrpI (W211)	GlnI (Q247)	TyrI (Y258)	HisIII (H266)	ThrII (T279)	TyrII (Y280)	TyrIII (Y304)	Start (aa)	END (aa)
TaAOX1a-2AL	C	C	L	Yes	Yes	Yes	Yes	R	T	W	Q	Y	H	S	Y	Y	109	310
TaAOX1a-2BL	C	C	L	Yes	Yes	Yes	Yes	R	T	W	Q	Y	H	S	Y	Y	110	311
TaAOX1a-2DL	C	C	L	Yes	Yes	Yes	Yes	R	T	W	Q	Y	H	S	Y	Y	117	318
TaAOX1c-6AL	C	C	L	Yes	Yes	Yes	Yes	R	T	W	Q	Y	H	S	Y	Y	128	329
TaAOX1c-6BL	C	C	L	Yes	Yes	Yes	Yes	R	T	W	Q	Y	H	S	Y	Y	126	327
TaAOX1c-6DL	C	C	L	Yes	Yes	Yes	Yes	R	T	W	Q	Y	H	S	Y	Y	126	327
TaAOX1d1-2AL	S	S	L	LEMVAA	Yes	Yes	Yes	H	M	W	Q	Y	H	S	Y	Y	112	311
TaAOX1d1-2BL	S	S	L	Yes	Yes	Yes	Yes	H	T	W	Q	Y	H	S	Y	Y	112	311
TaAOX1d1-2DL	S	S	L	Yes	Yes	Yes	Yes	H	T	W	Q	Y	H	S	Y	Y	111	310
TaAOX1d2-2AL	C	S	L	Yes	Yes	Yes	Yes	H	T	W	Q	Y	H	S	Y	Y	112	312
TaAOX1d2-2BL	C	S	L	Yes	Yes	Yes	Yes	H	T	W	Q	Y	H	S	Y	Y	113	312
TaAOX1d2-2DL	C	S	L	Yes	Yes	Yes	Yes	H	T	W	Q	Y	H	S	Y	Y	109	308
TaAOX1d3-2AL	S	S	L	Yes	Yes	Yes	Yes	H	T	W	Q	Y	H	S	Y	Y	112	311
TaAOX1e-3DS-partial	C	C	L	Yes	Yes	Yes	Yes	R	T	W	Q	Y	H	S	Y	Y	41	244
TtAOX1a-2AL	C	C	L	Yes	Yes	Yes	Yes	R	T	W	Q	Y	H	S	Y	Y	109	310
TtAOX1a-2BL	C	C	L	Yes	Yes	Yes	Yes	R	T	W	Q	Y	H	S	Y	Y	112	313
TtAOX1c-6AL	C	C	L	Yes	Yes	Yes	Yes	R	T	W	Q	Y	H	S	Y	Y	128	329
TtAOX1c-6BL	C	C	L	Yes	Yes	Yes	Yes	R	T	W	Q	Y	H	S	Y	Y	126	327
TtAOX1d1-2AL	S	S	L	LEMVAA	Yes	Yes	Yes	H	M	W	Q	Y	H	S	Y	Y	112	311
TtAOX1d1-2BL	S	S	L	Yes	Yes	Yes	Yes	H	T	W	Q	Y	H	S	Y	Y	112	311
TtAOX1d2-2AL	C	S	L	Yes	Yes	Yes	Yes	H	T	W	Q	Y	H	S	Y	Y	113	312
TtAOX1d2-2BL	C	S	L	Yes	Yes	Yes	Yes	H	T	W	Q	Y	H	S	Y	Y	113	312
TtAOX1d3-2AL	S	S	L	Yes	Yes	Yes	Yes	H	T	W	Q	Y	H	S	Y	Y	112	311
TuAOX1a-2AL	C	C	L	Yes	Yes	Yes	Yes	R	T	W	Q	Y	H	S	Y	Y	109	310
TuAox1c-4AS	C	C	L	Yes	Yes	Yes	Yes	R	T	W	Q	Y	H	S	Y	Y	128	329
TuAOX1c-6AL	C	C	L	Yes	Yes	Yes	Yes	R	T	W	Q	Y	H	S	Y	Y	128	329
TuAox1d1-like	S	S	L	Yes	Yes	Yes	Yes	H	T	W	Q	Y	H	S	Y	Y	110	310
TuAox1d2-2AL	C	S	L	Yes	Yes	Yes	Yes	H	T	W	Q	Y	H	S	Y	Y	113	312
TuAOX1d3-2AL	S	S	L	Yes	Yes	Yes	Yes	H	T	W	Q	Y	H	S	Y	Y	97	296
TmAOX1a	C	C	L	Yes	Yes	Yes	Yes	R	T	W	Q	Y	H	S	Y	Y	109	310
TmAOX1c	C	C	L	Yes	Yes	Yes	Yes	R	T	W	Q	Y	H	S	Y	Y	126	327
TmAOX1d1-like	S	S	L	Yes	Yes	Yes	Yes	H	T	W	Q	Y	H	S	Y	Y	111	310
TmAOX1d2	C	C	L	Yes	Yes	Yes	Yes	H	T	W	Q	Y	H	S	Y	Y	61	260
AesAOX1a	C	C	L	Yes	Yes	Yes	Yes	R	T	W	Q	Y	H	S	Y	Y	112	313
AesAOX1c	C	C	L	Yes	Yes	Yes	Yes	R	T	W	Q	Y	H	S	Y	Y	126	327
AesAOX1d1	S	S	L	Yes	Yes	Yes	Yes	H	T	W	Q	Y	H	S	Y	Y	112	311
AesAOX1d2	C	S	L	Yes	Yes	Yes	Yes	H	T	W	Q	Y	H	S	Y	Y	113	312
AeshAOX1a-partial		C	L	Yes	Yes	Yes	Yes	R	T	W	Q	Y	H	S	Y	Y	2	171
AeshAOX1c	C	C	L	Yes	Yes	Yes	Yes	R	T	W	Q	Y	H	S	Y	Y	126	327
AeshAOX1d1	S	S	L	Yes	Yes	Yes	Yes	H	T	W	Q	Y	H	S	Y	Y	112	311
AeshAOX1d2	C	S	L	Yes	Yes	Yes	Yes	H	T	W	Q	Y	H	S	Y	Y	113	312
AeshAOX1e	C	C	L	Yes	DRMHLMT	Yes	Yes	R	T	W	Q	Y	H	S	Y	Y	127	329
AetAOX1a	C	C	L	Yes	Yes	Yes	Yes	R	T	W	Q	Y	H	S	Y	Y	109	310
AetAOX1c	C	C	L	Yes	Yes	Yes	Yes	R	T	W	Q	Y	H	S	Y	Y	126	327
AetAOX1d1	S	S	L	Yes	Yes	Yes	Yes	H	T	W	Q	Y	H	S	Y	Y	111	310
AetAOX1d2	C	S	L	Yes	Yes	Yes	Yes	H	T	W	Q	Y	H	S	Y	Y	113	312
AetAOX1e	C	C	L	Yes	Yes	Yes	Yes	R	T	W	Q	Y	H	S	Y	Y	71	273

Table 3.14: Characteristics of the AOX1 subfamily proteins identified in *T. aestivum*

AOX1-partial/like proteins are highlighted in orange. pI: isoelectric point; Mw: molecular weight; nd; no data available

AOX isoforms	Protein size (aa)	pI	MW (kDa)	Export probability to mitochondria (%)	Signal peptide size (aa)	Subcellular localisation
TaAOX1a-2AL	328	8.1	36.7	99.2	45	mTP
TaAOX1a-2BL	329	8.1	36.9	98.7	46	mTP
TaAOX1a-2DL	336	8.4	37.6	98.8	45	mTP
TaAOX1a-3BS-like	140	nd	nd	99.0	46	mTP
TaAOX1a-6BL-like	147	nd	nd	99.6	30	mTP
TaAOX1a-7BL-partial	129	nd	nd	98.7	46	mTP
TaAOX1c-6AL	347	8.5	38.4	86.1	56	mTP
TaAOX1c-6BL	345	8.9	38.3	83.5	54	mTP
TaAOX1c-6DL	345	7.8	38.4	77.2	27	mTP
TaAOX1d1-2AL	329	7.7	37.0	95.5	51	mTP
TaAOX1d1-2BL	329	7.6	37.0	97.2	51	mTP
TaAOX1d1-2DL	328	6.7	36.7	95.0	51	mTP
TaAOX1d1-4AS-like	321	nd	nd	not predictable	nd	OTHER
TaAOX1d2-2AL	330	6.8	37.1	97.2	51	mTP
TaAOX1d2-2BL	330	6.8	37.1	92.7	51	mTP
TaAOX1d2-2DL	326	6.8	36.7	90.3	47	mTP
TaAOX1d2-2DL-like	324	nd	nd	not predictable	nd	OTHER
TaAOX1d3-2AL	329	8.3	37.0	99.1	51	mTP
TaAOX1e-3BL-partial	251	7.9	28.2	99.4	43	mTP
TaAOX1e-3DS-partial	262	6.7	30.0	not predictable	nd	OTHER
TaAOX-4BL-like	117	nd	nd	96.0	46	mTP

Table 3.15: Characteristics of AOX1 subfamily protein identified in *T. turgidum*

AOX1-partial/like proteins are highlighted in orange. pI: isoelectric point; Mw: molecular weight; nd; no data available

AOX isoforms	Protein size (aa)	pI	MW (kDa)	Export probability to mitochondria (%)	Signal peptide size (aa)	Subcellular localisation
TtAOX1a-2AL	328	8.1	36.7	97.9	45	mTP
TtAOX1a-2BL	331	7.7	37.1	97.9	48	mTP
TtAOX1a-3BS-like	140	nd	nd	98.8	46	mTP
TtAOX1a-5BL-partial	129	nd	nd	90.9	46	mTP
TtAOX1a-6BL-like	147	nd	nd	99.8	46	mTP
TtAOX1a-7BL-partial	129	nd	nd	99.0	46	mTP
TtAOX1c-6AL	347	8.5	38.4	83.6	56	mTP
TtAOX1c-6BL	345	8.9	38.3	78.5	54	mTP
TtAOX1d1-2AL	329	7.7	37.0	95.4	51	mTP
TtAOX1d1-2BL	329	7.6	37.1	94.0	51	mTP
TtAOX1d1-4AS-like	321	nd	nd	not predictable	nd	OTHER
TtAOX1d2-2AL	330	6.8	37.1	97.2	51	mTP
TtAOX1d2-2BL	330	6.8	37.1	97.0	51	mTP
TtAOX1d3-2AL	329	8.3	37.0	98.6	51	mTP
TtAOX1e-3BL-partial	251	7.8	28.2	99.4	43	mTP
TtAOX-4BL-like	117	nd	nd	97.7	46	mTP

Table 3.16: Characteristics of AOX1 subfamily proteins identified in *T. urartu* and *T. monococcum*

AOX1-partial/like proteins are highlighted in orange. pI: isoelectric point; Mw: molecular weight

Species	AOX isoforms	Protein size (aa)	pI	MW (kDa)	Export probability to mitochondria (%)	Signal peptide size (aa)	Subcellular localisation
T. urartu	TuAOX1a	331	8.1	36.8	93.2	45	mTP
	TuAOX1c-4AS	347	8.5	38.4	73.3	56	mTP
	TuAOX1c-6AL	347	8.5	38.4	73.3	56	mTP
	TuAOX1d1-like	328	7.4	36.8	61.0	51	mTP
	TuAOX1d2	330	6.8	37.1	69.3	51	mTP
	TuAOX1d3	314	8.3	35.5	95.3	36	mTP
T. monococcum	TmAOX1a	328	7.7	36.8	95.9	45	mTP
	TmAOX1c	345	8.7	38.2	83.2	54	mTP
	TmAOX1d1-like	328	9.1	36.5	62.4	51	mTP
	TmAOX1d2	330	6.8	37.1	69.3	51	mTP

Table 3.17: Characteristics of the AOX1 subfamily proteins identified in *Aegilops* species

AOX1-partial/like proteins are highlighted in orange. pI: isoelectric point; Mw: molecular weight; nd; no data available

Species	AOX isoforms	Protein size (aa)	pI	MW (kDa)	Export probability to mitochondria (%)	Signal peptide size (aa)	Subcellular localisation
Ae. Speltoides	AesAOX1a	328	8.1	37.1	99.0	48	mTP
	AesAOX1c	345	8.9	38.3	85.2	54	mTP
	AesAox1d1	329	8.2	37.1	93.2	51	mTP
	AesAOX1d2	330	6.8	37.2	97.0	51	mTP
Ae. Sharonensis	AeshAOX1a-partial	189	6.1	21.7	Partial	nd	nd
	AeshAOX1c	345	9.1	38.2	82.7	54	mTP
	AeshAOX1d1	329	7.6	37.1	95.7	51	mTP
	AeshAOX1d2	330	7.1	37.2	96.9	51	mTP
	AeshAOX1e	347	7.8	39.0	18.2	nd	OTHER
Ae. tauschii	AetAOX1a	328	8.1	36.8	98.7	45	mTP
	AetAOX1c	345	7.8	38.4	77.2	27	mTP
	AetAOX1d1	328	6.7	36.7	94.1	51	mTP
	AetAOX1d2	330	6.8	37.1	97.5	51	mTP
	AetAOX1d2-like	324	nd	nd	not predictable	nd	OTHER
	AetAOX1e	291	8.6	32.9	99.7	31	mTP

3.2.2.4 Validation of AOX Candidate Genes in Triticum *and* Aegilops *Species*

The findings in Section 3.2.2.3 showed that several *AOX* sequences were only partially identified in *Triticum* and *Aegilops* species. Validation of *AOX* candidate genes was conducted by examining the AOX protein sequences in *Triticum* and *Aegilops* species. Thus, AOX domain composition analyses were conducted to identify AOX domains using the database of protein families (Pfam) (Finn et al. 2015), as described in Chapter 2, Section 2.4.1. AOX domain composition analyses revealed that AOX1d1-4AS-like from *T. aestivum* and *T. turgidum* contained a small sequence of AOX domain, ~54 aa, whereas most of AOX1a-partial/like did not contain the AOX domain (Section 3.2.2.3; Table 3.13). Those sequences which were designated as (-like/or -partial) were subjected to a pseudogene analysis.

The characteristics of *AOX*-partial/like isoforms set them apart from all other *AOX* genes. Thus, to ensure that all partial *AOX1* sequences predicted on IWGSC v1.1 are not truncated copies, a homology-based approach was used to identify the potential codon sequence region. In IWGSC-RefSeq v1.1 annotation, some of these candidate *AOX* genes were classified as HC genes, such as *TaAOX1a-7BL*-partial and *TaAOX-4BL-like*, or LC genes, such as *TaAOX1-3BS*-like, *TaAOX1a-6BL-like, TaAOX1-3BS-like* and *TaAOX1d1-4AS-like*. To identify the potential codon sequence region, *AOX*-like/or -partial fragments were compared with their ancestral counterparts. For instance, the bioinformatics analysis revealed that *TaAOX1d1-4AS-like* and *TtAOX1d1-4AS-like* were perhaps inherited from *TuAOX1d1-like*, as shown in Figure 3.9. Pairwise alignment of the ORF sequences revealed that *AOX1d1-4AS-like* from *T. aestivum* and *T. turgidum* were similar to *AOX1d1-like* that is found in the diploid wheat *T. urartu* and *T. monococcum*, 96% and 95%, respectively, at the gene level. However, at the protein level, they were very dissimilar, 35%, and 36%, respectively (Table 3.18). Of note, *TuAOX1d1-like* shared the same chromosomal location as other orthologues in *T. aestivum* and *T. turgidum*, which were located on the short arms of chromosome 4A. In addition, polymorphism analysis revealed that *TaAOX1-3BS-like*, *TaAOX1a-6BL-like*, *TaAOX1-3BS-like*, *TaAOX1d1-4AS-like* and their orthologues have an internal stop codon caused by polymorphic events such as single nucleotide polymorphisms (SNPs), or insertion and deletions (InDels), for instance in *TaAOX1d1-4AS-like* (Figure 3.9). Pseudogene candidates were tested for by PCR with cDNA as template, but no amplification was found. In agreement with the PCR results, *in silico* analysis failed to obtain any data from wheat EST or TSA at NCBI, which matched these *AOX*-partial/like genes, confirming that these are most likely pseudogenes.

Figure 3.9: Pairwise alignment of nucleotide and deduced amino acid sequences of AOX1d1-4AS-like sequences from Triticum species

Two InDels events are shown in red; the internal stop codon is shown in blue.

Table 3.18: Percentage identity of AOX1d1-like identified from *Triticum* species

The percentage identity between *AOX1d1-like* orthologues at gene sequence level is shown in light green and that between AOX1d1-like orthologous protein sequences is shown in light blue.

	TuAox1d1-like	*TmAox1d1-like*	*TtAox1d1-4AS-like*	*TaAox1d1-4AS-like*
TuAox1d1-like		94	35	35
TmAox1d1-like	97		36	36
TtAox1d1-4AS-like	96	95		100
TaAox1d1-4AS-like	96	95	100	

3.2.3 Chromosomal Localisation of AOX1-subfamily Members in *Triticum* and *Aegilops* Species

The chromosomal localisations of *AOX1* gene sequences were obtained from genome assemblies of wheat and its relatives. The *AOX* genes were unevenly distributed across the chromosomes of *Triticum* and *Aegilops* species (Table 3.19). The majority of *AOX1* genes were mapped on the long arm of chromosome 2 in all *Triticum* and *Aegilops* species (Table 3.19). Notably, *AOX1* genes across the three genomes of *T. aestivum* were widely spread in their distribution (Figure 3.10 and Table 3.19). The subgenome B possesses nine copies of *AOX1*, whereas A and D subgenomes have six *AOX1* each, including *AOX1-like/partial* isoforms (Table 3.9). The same pattern was observed in *T. turgidum* species, where the B subgenome has a higher number of *AOX1* copies than the A genome (Table 3.19). The current research revealed that *AOX1a* and *AOX1d* clades are in tandem arrangements in *Triticum* and *Aegilops* species. For instance, *AOX1* genes were found in the following order, *AOX1d2*, *AOX1d3*, *AOX1d1* and *AOX1a*, in the A subgenome as well as in the B and D subgenomes of *T. aestivum*, with the exceptions that *AOX1d3* genes were missing in the B and D subgenomes (Figure 3.10). The same pattern is conserved in *T. turgidum*, *T. urartu* and *Ae. tauschii* (Section 3.2.2.2; Tables 3.10–3.12). The chromosomal localisation of *AOX1*-subfamily members in *Triticum* and *Aegilops* species are conserved. For instance, in *T. aestivum*, *AOX1a* and *AOX1d* clades were mapped on the long arm of chromosome 2, *AOX1c* was mapped on the long arm of chromosome 6 and *AOX1e* was mapped on the long arm of chromosome 3 (Figure 3.10). In addition, other *AOX1*-partial/like copies were found on chromosomes 3, 4 and 7 (Figure 3.10). Each of the full-length *AOX1* isoforms had three homologues evenly distributed among *T. aestivum* subgenomes other than *AOX1d3*, which was found only in the A genome. In *T. turgidum* species, two homologues of the full *AOX1* isoforms were distributed into the A and

B subgenomes. In general, diploid species possess a single copy of *AOX1*-subfamily genes, which is located in proximity to the related orthologues from *T. aestivum* subgenomes.

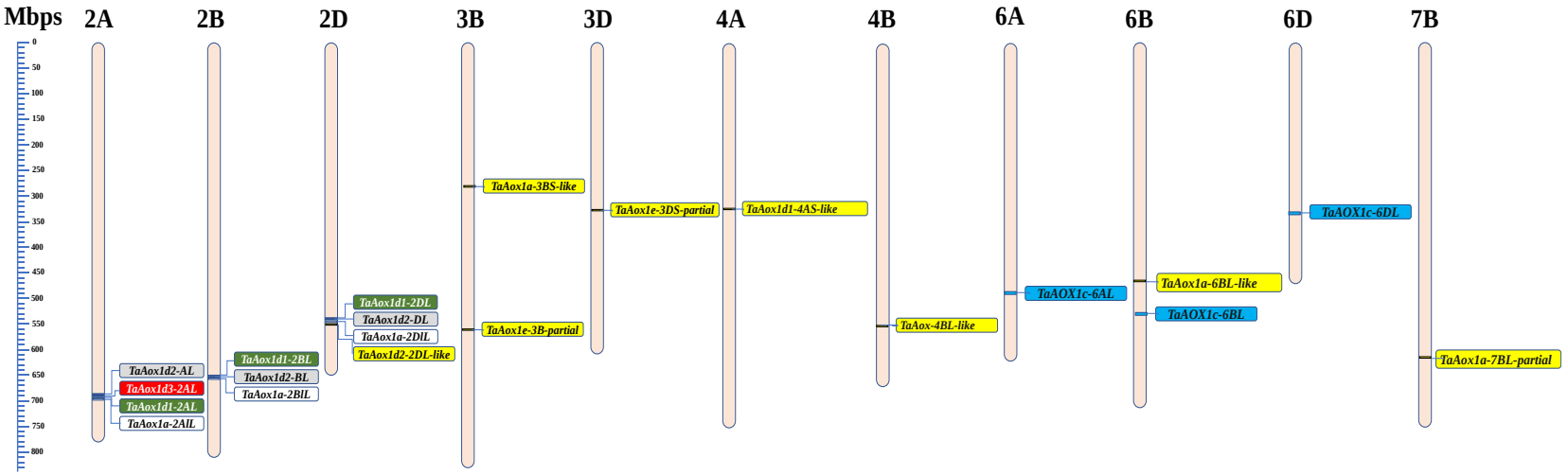

Figure 3.10: Chromosomal distribution of *AOX1*-gene subfamily members in *T. aestivum*

AOX1-subfamily members and its homologues that were fully identified are shown in the same colour, in white boxes (AOX1a), blue boxes (*AOX1c*), green boxes (*AOX1d1*), grey boxes (*AOX1d2*) and a red box (*AOX1d3*), whereas *Aox1e*, and *AOX-partial*/*like* are shown in yellow boxes. The scale represents the length of wheat chromosomes based on IWGSC-RefSeq annotation v1.1 released in July 2018.

Table 3.19: The number of *AOX* gene sequences distributed among *Triticum* and *Aegilops* species chromosomes

The chromosomal localisations are indicated by numbers in each subgenome of *Triticum* and Aegilops species. ***** indicates that *Aox* genes were assigned to an unknown chromosome.

Chromosome	*T. aestivum* cv. Chinese Spring			*T. turgidum* ssp.						*T. urartu* cv. G1812	*T. monococcum*	*Ae. speltoides*	*Ae. sharonensis*	*Ae. tauschii* ssp. *strangulata*	Total
				dicoccoides cv. Zavitan		durum cv. Svevo		durum cv. Kronos							
	A	B	D	A	B	A	B	A	B	A^u	A^m	S	S^{sh}	D	
Chr.1															0
Chr.2	4	3	4	4	3	4	3			2				4	31
Chr.3		2	1		2		2							1	8
Chr.4	1	1		1	1	1	1			2					8
Chr.5					1										1
Chr.6	1	2	1	1	2	1	2			1				1	12
Chr.7		1			1		1								3
* Unknown								14		1	4	4	5		28
Total	6	9	6	6	10	6	9	14		6	4	4	5	6	91

3.2.4 Characterisation of Putative Post-translational Modification Sites in the Coding Region of *AOX*

Plants are highly dependent on multiple cellular processes, whereby they rely on protein modifications such as glycosylation, acetylation and phosphorylation, and this could also apply to AOX (Blanco-Herrera et al. 2015; Gibbs 2015; Hartl & Finkemeier 2012; Hosp et al. 2017; Moore et al. 2013; Takano et al. 2015; Wagner et al. 2018; Zhang et al. 2009). Variability in the number of putative AOX post-translational modification sites was observed among all AOX proteins. For instance, AesAOX1a had 36 potential phosphorylation sites (18 Ser, 13 Thr, 5 Tyr), one acetylation site and one N-glycosylation site, whereas TuAOX1d3 had 22 putative phosphorylation sites (14 Ser, 4 Thr, 4 Tyr) only. Acetylation sites were predicted in TaAOX1a-2DL, TaAOX1d1-2AL, TuAOX1a, TmAOX1c, AesAOX1a and AetAOX1a. Notably, the N-glycosylation sites were predicted in the AOX1a clade only (Figure 3.11).

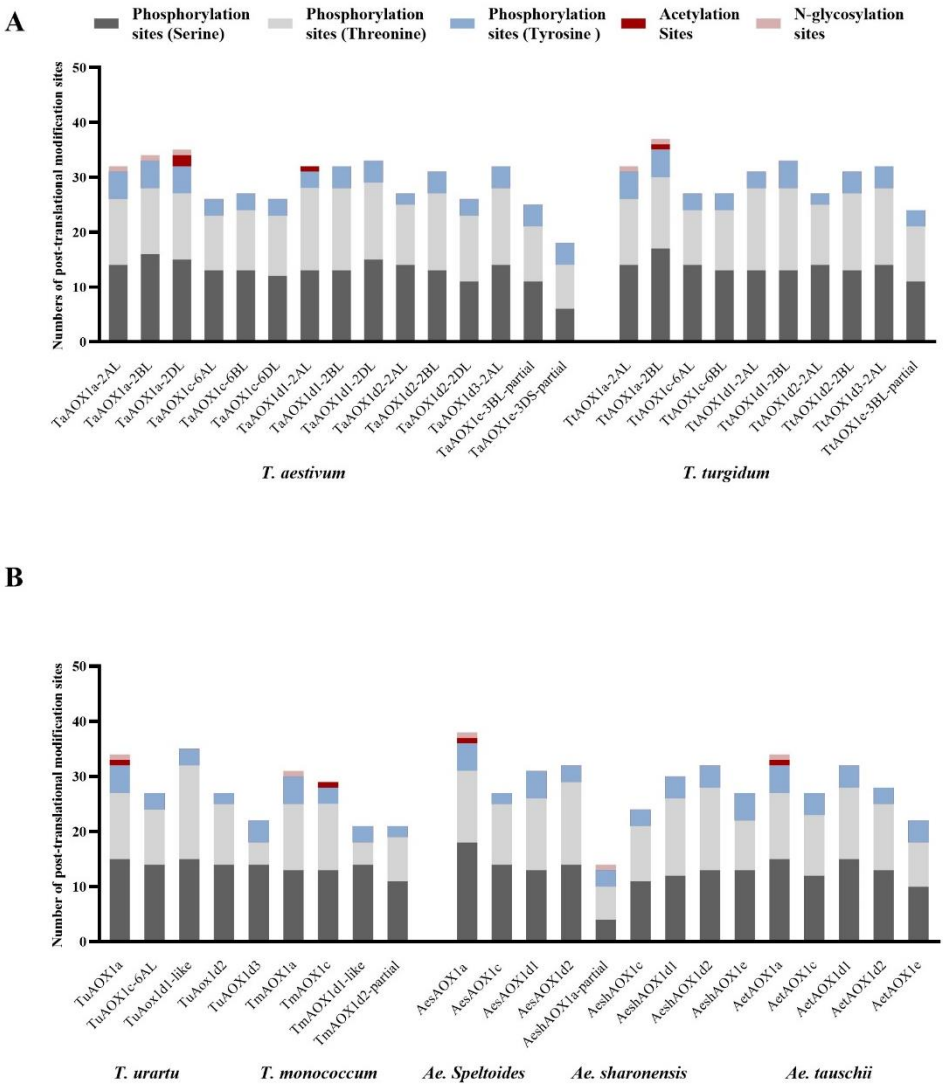

Figure 3.11: Putative post-translational modification sites of *AOX* from wheat and its ancestors

(A) The number of putative post-translational modification sites in *AOX* from hexaploid and from tetraploid wheats and (B) the number from diploid ancestors of wheat. Predictions of phosphorylation sites (serine, threonine or tyrosine) in AOX proteins were carried out using NetPhos 3.1 Server. The Musite prediction program was used to predict acetylation sites, and NetNGlyc 1.0 Server was used to predict putative N-glycosylation sites (Chapter2; Section 2.4.3).

3.2.5 Prediction of *cis*-regulatory Elements in Promoter Regions of *AOX* in *Triticum* and *Aegilops* Species

At the transcriptional level, *cis*-regulatory elements (CREs) in the promoter regions are crucial for understanding *AOX* gene regulation (Polidoros et al. 2009). In this study, 2 kb upstream regions from the translation start site (ATG) were analysed for CREs using the PlantCARE database (Lescot et al. 2002). The results of the promoter regions analyses are listed in

Appendix C2 and summarised in Figure 3.12, based on the CRE function. The findings revealed that all *AOX* genes contain similar CREs (Figure 3.12). Those CREs were classified by function into four groups: light- *cis*-responsive elements; abiotic stress-response *cis*-elements; growth- and development- related *cis*-elements; and hormone-response *cis*-elements. The differences between the *AOX* genes were in the type and number of CREs groups apart from the TATA and CAAT-box elements. In general, the majority of the CREs present in all *AOX* genes are involved in hormone response and then light-responsive elements, respectively, except for the *AOX1d* clade, which contained abiotic stress-response *cis*-elements in greater number than light-responsive elements. Notably, the promoter regions of *AOX1a* and *AOX1d* genes contained more abiotic stress response *cis*-elements than *AOX1c* with exceptions of *TaAOX1c-6BL* and *TtAOX1c-6BL*.

This study found that two types of hormone-response *cis*-elements were common between *T. aestivum AOX* genes and their orthologues in tetraploid and diploid species. These elements were involved in abscisic acid-response (e.g. ABRE) and MeJA-response. Similarly, at least three abiotic stress response CREs (e.g. WRE3, GC-motif, LTR, DRE1, MBS and TC-rich repeats) were identified in the promoters of all AOX genes, *TaAOX1d1-2AL*, and its orthologues contained only three types of abiotic stress response CREs (WUN-motif, ARE and GC-motif). Low-temperature responsiveness (LTR), which is involved in the cold response, was found in most of the *AOX* promoter regions in *Triticum* and *Aegilops* species (Appendix C2).

Intriguingly, some CREs were only limited to a specific *AOX* isoform; for example, P-box, which was involved in the gibberellin-responsive element. P-box was found only in *TaAOX1c-6AL*, and *TtAOX1c-6AL* promoter regains (Appendix Table C.2.1). Moreover, a gain or loss of CREs in the promoter regions of *TaAOX1* was found between *T. aestivum* and its ancestors. For instance, *TaAOX1a-2BL* gained unique CREs related to growth and development elements called AC-II, which were absent from the promoter regions of *TtAOX1a-2BL* and *AesAOX1a*. An example of the loss of CREs was found in the *TaAOX1a-2DL* promoter region where the LTR element and WRE3 were absent in *TaAOX1a-2DL* and present in its orthologous form of the D genome ancestor, *AetAOX1a* (Appendix Table C.2.3). In addition, this study revealed that there were CREs found in one homologue and absent in others. For instance, TC-rich repeats, which are involved in defence and stress responsiveness, were found only in *TaAOX1a-2AL* but absent in homologues located on the B and D genomes. Further, the MYB

binding site, which is involved in drought-inducibility (MBS), was found on *TaAOX1c-6BL* and absent in homologues located on the A and D genomes (Appendix Tables C.2.1–C.2.3).

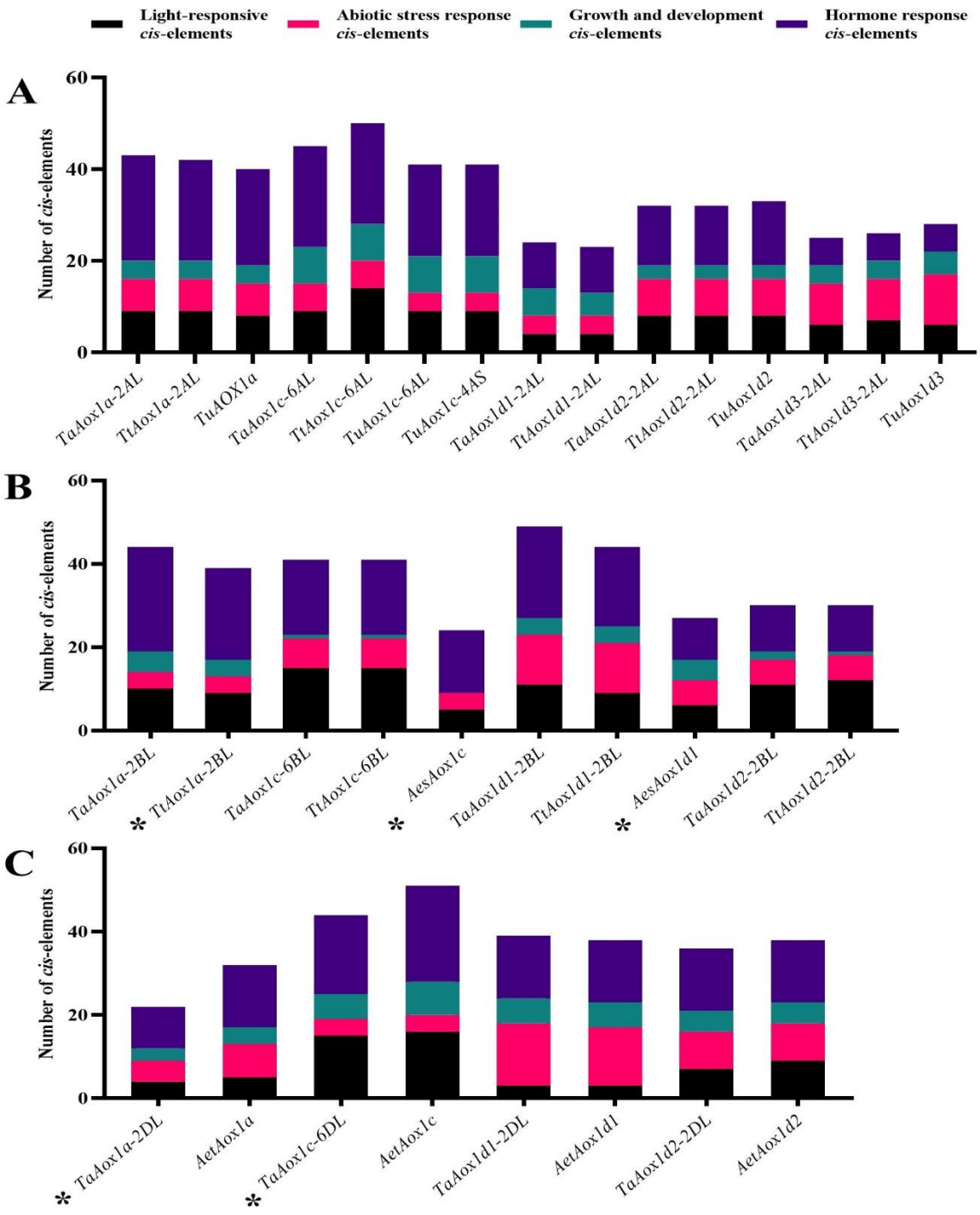

Figure 3.12: Putative numbers of *cis*-regulatory elements in the promoter regions of *AOX* from wheat and its ancestors

The number of CREs by function was determined as described in Chapter 2 (see Appendix C2). CREs on AOX1 genes from the A, B and D genomes are shown in (A), (B) and (C), respectively. CRE data were obtained from the PlantCARE database. * indicates promoter regions that were only partially identified.

3.2.6 *In silico AOX* Expression

The analysis of RNA-seq data, using the wheat database expVIP (Borrill et al. 2016; Ramírez-González & Borrill 2018), was conducted to study further the functionality of wheat *AOX* genes (Borrill et al. 2016). *In silico*, wheat *AOX1* expression was analysed in five different tissues (grain, spike, stem, leaf and root) at different developmental stages, as well as their responses to biotic and abiotic stresses, by using RNA-seq data as described in Section 2.3.7. In this Section, 13 genes were selected since they have been supported by the wheat EST and TSA databases at NCBI (Appendix Table C5.1). The biotic stresses included four fungal infections, fusarium head blight (*F. graminearum*), stripe rust (*P. striiformis*), powdery mildew (*B. graminis*) and septoria tritici blotch (*Z. tritici*), together with abiotic stresses, such as cold, drought, heat, drought–heat combined and phosphate starvation.

In general, the study's findings showed that there was a varied expression between the HC *AOX1* gene family members (Figure 3.13; Figure 3.14), whereas the truncated and LC genes had no expression or were very lowly expressed (Appendix 7.1). Notably, *TaAOX1d1-2AL* expression was undetectable, whereas its homologous genes, *TaAOX1d1-2BL*, and *TaAOX1d1-2DL*, were highly expressed (Figure 3.13; Figure 3.14). The results showed that particular *TaAOX1* genes were expressed at most developmental stages (seedling, vegetative and reproductive). For instance, the *TaAOX1a* clade and *TaAOX1d* clade were highly expressed in root and leaf at all stages, with the exception of *TaAOX1d1-2AL*. However, the *TaAOX1c* clade was highly expressed only in the leaf at the vegetative and reproductive stages (Figure 3.13).

Under biotic stresses, the expression of *TaAOX1a* clade and *TaAOX1d* clade were significantly expressed during fungal infections, at different times (Figure 3.14A). However, the *TaAOX1d1-2AL* homologue was not responsive to any biotic stresses. Further, *TaAOX1a*, *TaAOX1d2*, *TaAOX1d3* and *TaAOX1c* clades were significantly expressed by powdery mildew infection at 24h and then decreased as time progressed (Figure 3.14A).

While the expression level of the *TaAOX1a* clade and of*TaAOX1c-6BL* were up-regulated, the *TaAOX1d* clade was down-regulated under cold stresses (Figure 3.14B). Under drought and heat stresses, *TaAOX1a* and *TaAOX1d* clades were the most responsive isoforms at different times of the stresses. The *TaAOX1d* clade exhibited an early response, but the *TaAOX1a* clade constantly increased either under heat or the dual stress (Figure 3.14B). The findings showed

that *TaAOX* genes either had no response or low expression under phosphate starvation conditions (Figure 3.14B).

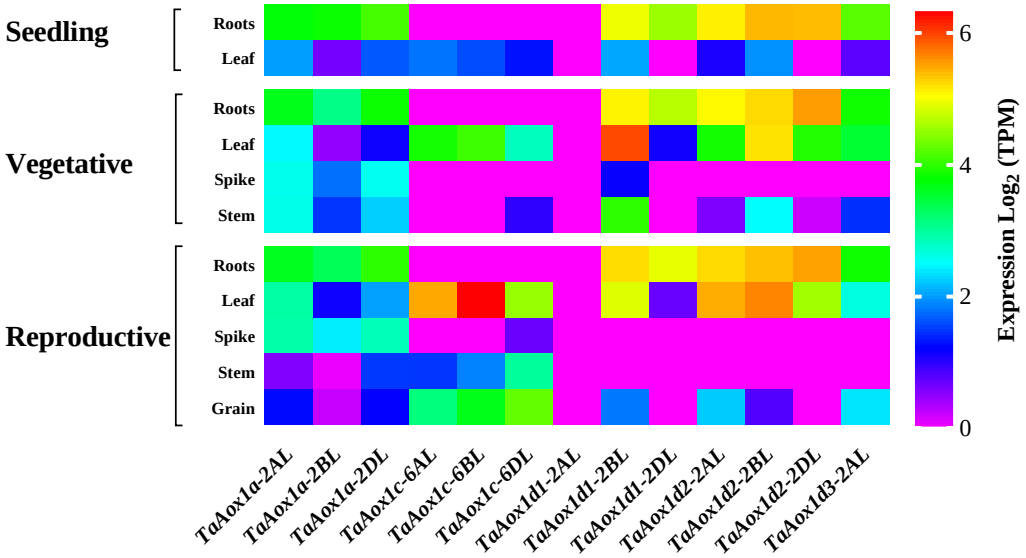

Figure 3.13: Gene expression profiles of selected wheat *AOX1* genes in different tissues during wheat development

Expression of wheat *AOX1* in five different tissues during wheat development; (root, leaf, stem, spike and grain) are shown as a heat map. RNA-seq data were retrieved from ExpVIP database (Borrill et al. 2016; Ramírez-González & Borrill 2018). The heat map was constructed using GraphPad Prism (8.1.2). The scale indicates expression values as log2 transcripts per million. The full details can be found in Appendix C7.1.

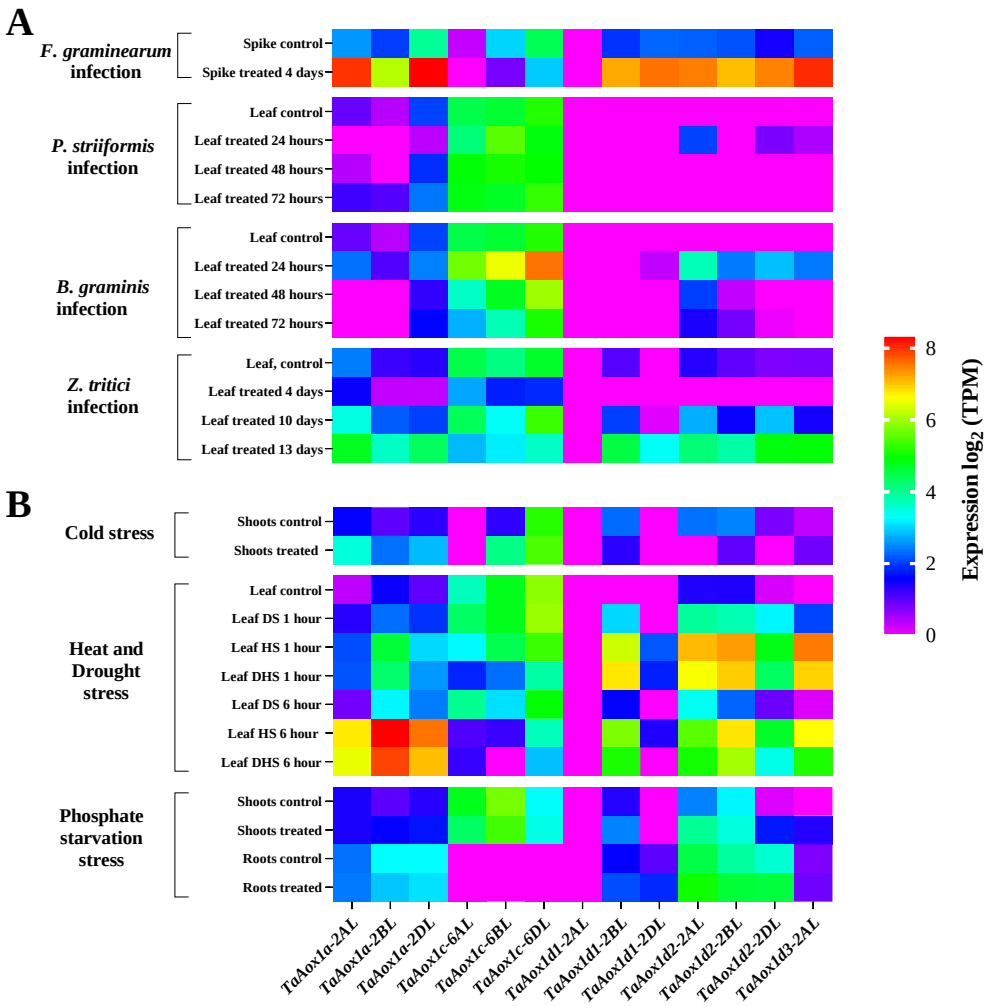

Figure 3.14: Gene expression profiles of selected wheat *AOX1* genes under various biotic and abiotic stresses

Heat map (A) shows the expression of wheat *AOX1* in response to four fungal infections, Fusarium head blight (*F. graminearum*), stripe rust (*P. striiformis*), powdery mildew (*B. graminis*) and Septoria tritici blotch (*Z. tritici*), after different time points. Heat map (B) shows the expression of wheat *AOX1* in response to various abiotic stress, namely, cold, drought (DS), heat (HS), drought–heat (DHS) and phosphate starvation. RNA-seq data were retrieved from ExpVIP database (Borrill et al. 2016; Ramírez-González & Borrill 2018). The heat map was constructed using GraphPad Prism (8.1.2). The scale indicates expression values as log2 transcripts per million. The full details can be found in Appendix C7.1.

3.2.7 Optimisation and Validation of qRT-PCR Assay

PCR, as well as reverse transcriptase-PCR (RT-PCR), were employed to confirm *AOX* genes and examine their expression at the transcript level to validate the bioinformatics findings. In this study, genome-specific primers were developed to amplify specific AOX gene sequences from *T. aestivum* and its orthologues in other *Triticum* and *Aegilops* species (Section 2.3.4). The current study confirmed the specificity of each primer set by gel electrophoresis of PCR

products and melting curve analysis at the end of the qRT-PCR amplification, which was confirmed by a single band (Figure 3.15) or peak (Figure 3.16).

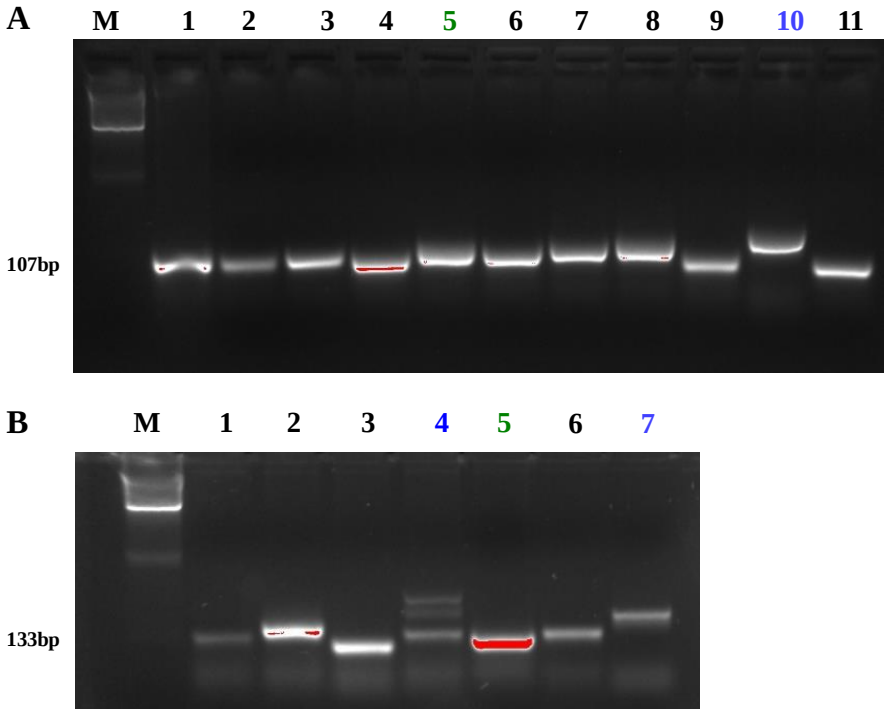

Figure 3.15: The qRT-PCR products, showing a single band of expected size amplified from *AOX1* gene isoforms from wheat after electrophoresis in 2% agarose gel

The blue font shows the result from old primer sets before optimisation. The green font shows the qRT-PCR product of *TaAOX1d2-2AL* in both the leaf and the root.

Gel (A) shows gene-specific primers, the *AOX1a* gene clade and *AOX1d* gene clade in leaf as indicated below:

M: 100bp marker

1: *TaAOX1a-2AL*, in leaf, expected size = 107bp

2: *TaAOX1a-2BL*, in leaf, expected size = 98bp

3: *TaAOX1a-2DL*, in leaf, expected size = 117bp

4: *TaAOX1d2-2AL*, in leaf, expected size = 110bp

5: *TaAOX1d1-2AL*, in leaf, expected size = 129bp

6: *TaAOX1d3-2AL*, in leaf, expected size = 134bp

7: *TaAOX1d1-2BL*, in leaf, expected size = 150bp

8: *TaAOX1d2-2BL*, in leaf, expected size = 158bp

9: *TaAOX1d1-2DL*, in leaf, expected size = 108bp

10: *TaAOX1d2-2DL*, in leaf, expected size = 219bp

11: *TaAOX1a-2AL*, in leaf, expected size = 107bp

Gel (B) shows gene-specific primers for *AOX1c gene* clade in leaf, and *AOX1d* gene clade in or root as below:

M: 100bp

1: *TaAOX1c-6AL*, in leaf, expected size = 133bp

2: *TaAOX1c-6BL*, in leaf, expected size = 182bp

3: *TaAOX1c-6BL*, in leaf, expected size = 107bp

4: *TaAOX1c-6DL*, in leaf, expected size = 146bp

5: *TaAOX1d1-2AL*, in root, expected size = 129bp

6: *TaAOX1d2-2DL*, in root, expected size = 140bp

7: *TaAOX1d2-2DL*, in root, expected size = 219bp

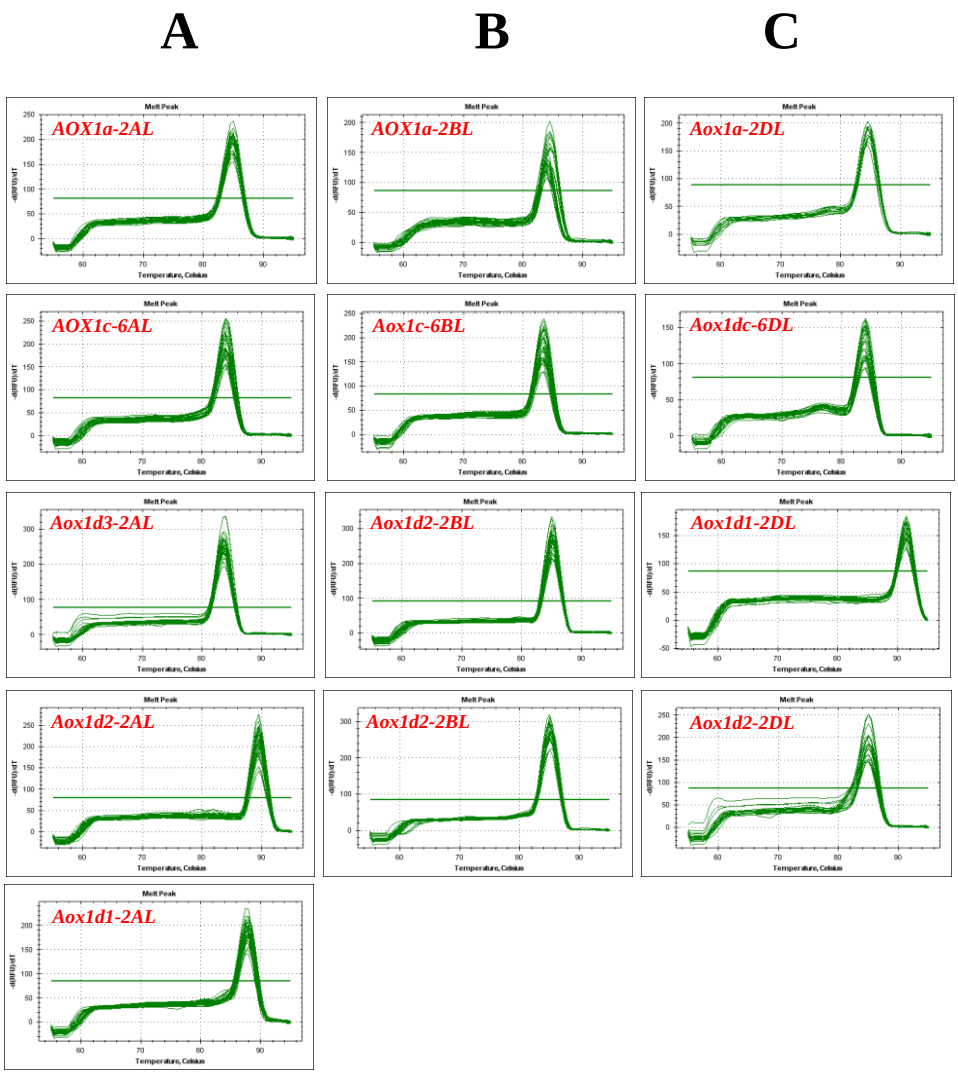

Figure 3.16: Melting curve profiles for *AOX* products derived from gene isoforms from *Triticum* and *Aegilops* species showing specific peaks

Column (A) shows *AOX1* profiles from the A genome from *Triticum* species; column (B) shows *AOX1* from the B genome from *Triticum* and *Aegilops* species; and column (C) shows *AOX1* from the D genome from *Triticum* and *Aegilops* species. Melting curve profiles confirmed the qRT-PCR product specificity and ensured that it is free of primer dimers and non-specific amplicons.

3.3 Discussion

3.3.1 Comparative Sequence Analysis with Previous Findings

When the current research commenced in March 2014, published data were unavailable about *AOX* genome structure in *Triticum* and *Aegilops* species, apart from *WAOX1a* and *WAOX1c* isoforms identified by Takumi et al. (2002). In November 2014, Costa et al. (2014) published AOX protein classification schemes, which contained two copies of the AOX1d protein. They identified them by using the wheat EST database at NCBI; these were designated as AOX1d1 and AOX1d2-partial (Costa et al. 2014; supplemental Table S2). The current study successfully identified 13 full-length isoforms of *TaAOX1* in *T. aestivum* during 2014–2015, using improved wheat assemblies. Nine out of the 13 *TaAOX1* genes encoding AOX1, including known *AOX1* genes (AB078882.1 and AB078883.1), were presented as a poster at the 7th International Crop Science Congress, which was held between 14–19 August 2016 in Beijing, China (Appendix D). In August 2018, Brew-Appiah et al. (2018) published their *AOX* findings on *T. aestivum*, *T. urartu*, *Ae. tauschii* and *Ae. speltoides*. Thus, this section compares the previous findings with findings in the current study.

The *AOX* gene sequences from both Takumi et al. (2002) and Costa et al. (2014) had not been assigned to the wheat subgenomes, whereas the current study has defined the localisation of the *AOX1* genes based on wheat genome annotations (Table 3.20). Compared with the results of Takumi et al. (2002), the current research showed that the *TaAOX1a-2AL* and *TaAOX1c-6AL* were identical, with the expectation of a few sequence errors (Table 3.1). Brew-Appiah et al. (2018; Table 1) obtained *TaAOX*, *TuAOX* and *AetAOX* from Ensembl Plants, and used URGI to obtain *AesAOX* copies. The Ensembl Plants platform hosted the TGACv1 assembly, which was annotated by the Earlham Institute (Clavijo et al. 2017). When comparing Brew-Appiah et al.'s (2018) *AOX* study in *T. aestivum* with the current research findings, the percentage identity of AOX1 proteins varied from 4 to 100% (Table 3.20).

TGACv1 was one of the updated wheat assemblies used in the current study. This study revealed that most of the *AOX* gene models in TGACv1 were probably incorrectly annotated. As an example, the current study successfully annotated *TaAOX1a-2BL* manually based on alignment results with known AOX1a protein sequences from *Oryza sativa* and *Hordeum vulgare* (Figure 3.17A). The manual annotations were confirmed by the latest IWGSC_Refseq v1.1 annotation (Figure 3.17C). Further, the TGACv1 automated annotation failed to identify

the genome structure of *TaAOX1a-2BL* correctly (Figure 3.17B). The same discrepancies between TGACv1 and IWGSC_Refseq v1.0 were found for *AOX1d* and *AOX1c* clades (Appendix C5). In terms of *AOX* classification and nomenclature, Brew-Appiah et al. (2018, Fig 1) incorrectly classified *TaAOX1d-2BL.2* and *TaAOX1d-2BL.1* owing to an error in their phylogenetic analysis. According to the current research, their *TaAOX1d-2BL.2* and *TaAOX1d-2BL.1* should be reversed to reflect the correct classification as *TaAOX1d1-2BL* and *TaAOX1d2-2BL*, which are shown in Section 3.2.2 (Figure 3.2). The results for *TaAOX1d1-2BL* and *TaAOX1d2-2BL* in this research are consistent with the Costa et al. (2014, supplemental Table S2) AOX classification scheme.

Table 3.20: Percentage identity of AOX1 sequences previously identified in *T. aestivum* compared with the Brew-Appiah et al. (2018) findings

(A) shows the AOX proteins identified by Takumi et al. (2002). (B) shows AOX proteins published by Costa et al. (2014). (C) shows AOX proteins published by Brew-Appiah et al. (2018) for *T. aestivum*.

* shows AOX protein that does not have any published data, apart from Ensembl Plants ID, although Brew-Appiah et al. (2018) designated as ne.TaAOX•-2AL.

# indicates two AOX proteins that were found in Costa et al. (2014; supplemental Table S2); there was no genomic structure published. ** indicates that the Ta.Fragment-7BL had no data to make a comparison.

Current nomenclature	Corresponding protein in published sources (% identity)		
	A	B	C
TaAOX1a-2AL	Waox1a (99.39%)		TaAOX1a-2AL.sv1 (100%) TaAOX1a-2AL.sv2 (97.56%)
TaAOX1a-2BL			TaAOX1a-2BL (71.99%)
TaAOX1a-2DL			TaAOX1a-2DL.sv1 (100%) TaAOX1a-2DL.sv2 (87.50%)
TaAOX1a-3BS-like			put.regTaAOX-3B (64.19%)
TaAox1a-6BL-like			put.regTaAOX-6BL (69.82%)
TaAox1a-7BL-partial			Ta.Fragment-7BL**
TaAOX1c-6AL	Waox1c (99.42%)		TaAOX1c-6AL (87.41%)
TaAOX1c-6BL			TaAOX1c-6BL.sv1 (80.05%), TaAOX1c-6BL.sv2 (75.64%), TaAOX1c-6BL.sv3 (80.05%)
TaAOX1c-6DL			TaAOX1c-6DL (87.34%)
TaAOX1d1-2AL			TaAOX1d-2AL.1 (89.36%)
TaAOX1d1-2BL		AOX1d1 (100%) #	ne.TaAOX1d-2BL.2 (100%)
TaAOX1d1-2DL			ne.TaAOX1d-2DL (100%)
TaAOX1d1-4AS-like			put.TaAOX1d-like-4AS (4.17%)
TaAOX1d2-2AL		AOX1d2 (partial) #	TaAOX1d-2AL.2.sv1 (100%) TaAOX1d-2AL.2. sv2 (100%)
TaAOX1d2-2BL			ne.TaAOX1d-2BL.1(100%)
TaAOX1d2-2DL			TaAOX1d-2DL (98.79%)
TaAOX1d2-2DL-like			TaAOX1a-like-2DL (51.9%)
TaAOX1d3-2AL*			ne.TaAOX•-2AL*
TaAOX1e-3BL-partial			regTaAOX-3B (38.65%)
TaAOX1e-3DS-partial			put.TaAOX1e-3DS (100%)
TaAOX-4BL-like			regTaAOX-4BL.sv1 (39.41%)

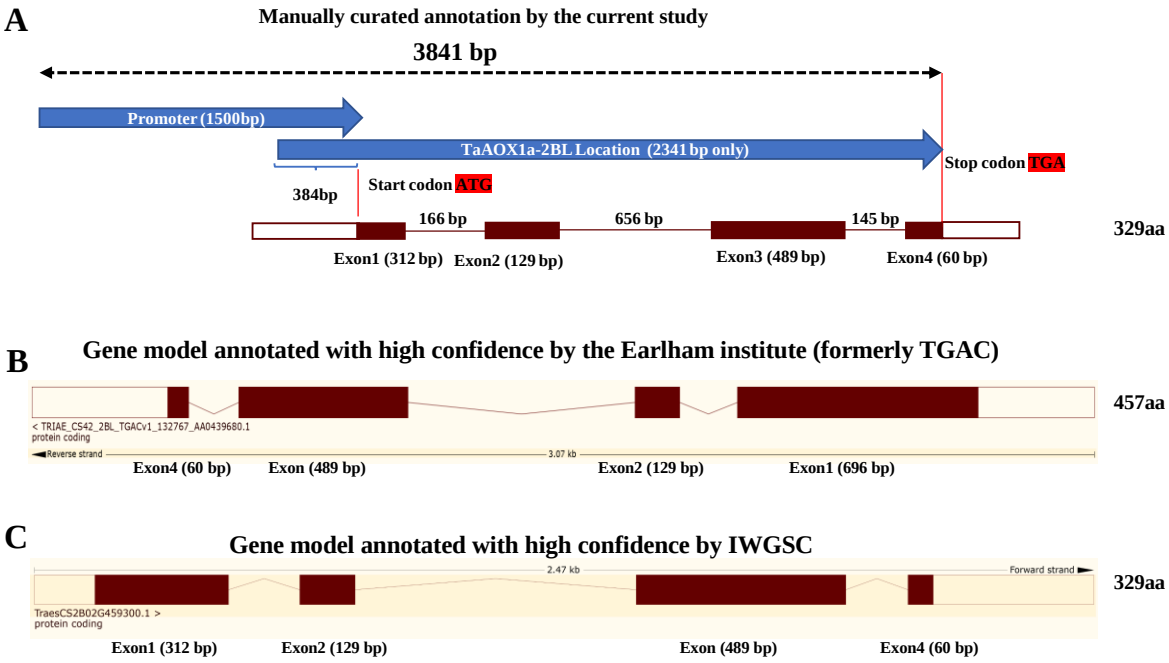

Figure 3.17: Comparative analyses of *TaAOX1a-2BL* between TGACv1 and current IWGSC_Refseq v1.1 gene models' annotations

(A) A diagram of manually curated annotations of *TaAOX1a-2BL* from the current study. Recognition of start/stop codons and genomic structure were based on alignment results with known AOX sequences from wheat, rice and barley and are indicated in red. (B) and (C) Discrepancies identified between TGACv1 and the current IWGSC_Refseq v1.1 gene models annotations, respectively. Brew-Appiah et al. (2018) used TGACv1 as the annotation to obtain their AOX findings. IWGSC_Refseq v1.1 is the latest version of wheat genome annotations, which confirmed the manually curated annotations in A. Exons are shown in brown-coloured boxes, introns are shown as lines, and untranslated regions are shown as empty boxes. The 384 bp segment in (A) shows the extra fragment that affected the TGACv1 annotation of *TaAOX1a-2BL*.

Using the ASM34745v1 assembly and annotation from April 2013 by Beijing Genomics Institute (BGI), Brew-Appiah et al. (2018) predicted four *AOX1* genes in *T. urartu:* TRIUR3_10307 (*TuAOX1a*), TRIUR3_08189 (*TuAOX1c*), TRIUR3_12374 (*TuAOX1d.1*) and TRIUR3_19476 (*TuAOX1d.2*). The gene lengths were 1383, 4469, 888 and 8777 bp, respectively. The genomic structures were three exons and two introns for *TuAOX1a*, seven exons and six introns for *TuAOX1c*, a single exon for *TuAOX1d.1* and three exons and two introns for *TuAOX1d.2* (Brew-Appiah et al. 2018, Table 3). The current study revealed that the ASM34745v1 assembly was fragmented and contained an inaccurate *AOX* annotation (Figure 3.18). Thus, those *AOX* genes identified by Brew-Appiah et al. (2018) were either overestimated or incomplete. In the study of Brew-Appiah et al. (2018), the genes ID TRIUR3_10307 and TRIUR3_12374 were affected by the existence of a gap assembly (Figure 3.18 A & C). These genes were designated as *TuAOX1a* and *TuAOX1d.1*, respectively (Brew-Appiah et al. 2018). However, the gene ID TRIUR3_08189 (*TuAOX1c*) showed an example of an overestimated gene (Figure 3.18 B).

Compared with the current study, *TuAOX1c* was truncated owing to the gaps in the ASM34745v1 assembly (Figure 3.18 B). In addition, *TuAOX1d3*, identified in the current study, was matched to exon number 3 (TRIUR3_19476-T1.exon3), which indicated another example of an overestimated gene length (Figure 3.18 D). Although Brew-Appiah et al. (2018) mentioned four copies of *AOX* found in *Ae. speltoides*, there was only one *AOX* sequence from this species published in their paper, *ne.AesAOX1d* (current name *AesAOX1d2* 100%). Some other *AOX* genes were not indicated, apart from their location in URGI. For *Ae. tauschii*, Brew-Appiah et al. (2018) used the ASM34733v1 sequence assembly. Compared with the findings in the current study, the ASM34733v1 assembly has inaccurate *AOX* gene model annotations. Thus, Brew-Appiah et al. (2018) identified incomplete *AOX* genes in *Ae. tauschii* (Figure 3.19). Although Brew-Appiah et al. (2018) did not find *AOX1c* or *AOX1d1*, the current study was able to clearly identify *AOX1c* and *AOX1d1* (Section 3.2.2.1; Section 3.2.2.2; Table 3.12).

Table 3.21: Percentage identity of AOX1 sequences identified in the current work, in diploid species, compared with Brew-Appiah et al.'s (2018) findings

AOX1 sequences identified by Brew-Appiah et al. (2018) were partial, indicated by (*), whereas # indicates lengths were overestimated. Most of the *Ae. speltoides* AOX sequences were not identified (indicated by light grey). RC: Reverse Complement. Note: This table represents only the corresponding protein and its location published by Brew-Appiah et al. (2018); for the current study's findings, see Tables 3.11 and 3.12.

Species	Current nomenclature	Corresponding protein in Brew-Appiah et al. (2018) (% identity)	Ensembl plants/IWGSC fragment location
T. urartu	TuAOX1a	TuAOX1a (100%) *	TRIUR3_10307
	TuAOX1c	TuAOX1c (55.9%)	TRIUR3_08189
	TuAOX1d2	TuAOX1d.1 (100%) *	TRIUR3_12374
	TuAOX1d3	TuAOX1d.2 (100%) #	TRIUR3_19476
Ae. speltoides	AesAOX1a	ne.AesAOX•	TGAC_WGS_speltoides_v1_contig_195745
	AesAOX1c	ne.AesAOX•	RC.TGAC_WGS_speltoides_v1_contig_239141
	AesAOX1d1	Fragment	TGAC_WGS_speltoides_v1_contig_1601667
	AesAOX1d2	ne.AesAOX1d (100%)	RC.TGAC_WGS_speltoides_v1_contig_403763
Ae. tauschii	AetAOX1a	AetAOX1a (100%) *	F775_17784
	AetAOX1d2	AetAOX1d (100%) *	F775_18387
	AetAOX1d2-like	AetAOX1d-like (50.5%)	F775_43125
	AetAOX1e	AetAOX1e (87.13%)	F775_11948

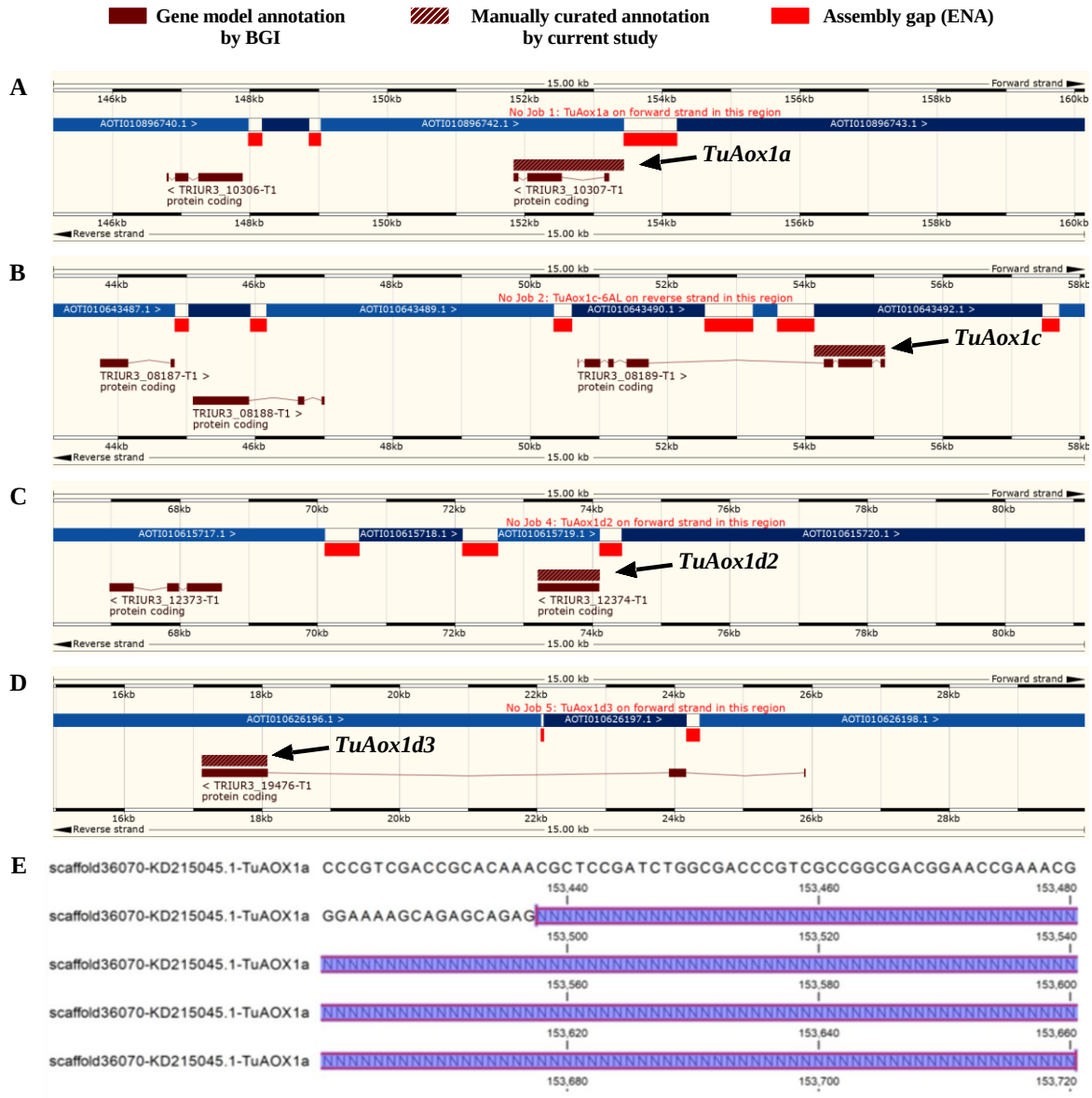

Figure 3.18: Comparative analyses of *TuAox1* gene model annotations predicted by BGI and the current study in *T. urartu* (Genome assembly: ASM34745v1, released in April 2013).

(A) and (E) Section from scaffold36070 that contains part of *TuAox1a*, which contains sequential N bases. (B) A section from scaffold39236, which contains part of *TuAox1c*. (C) A section from scaffold16660, which contains part of *TuAox1d2*. (D) A section from scaffold33580, which contains *TuAox1d3* on exon number 3 (TRIUR3_19476-T1.exon3). Detailed view panels were retrieved from Ensembl Plants release 45 in September 2019. BGI: Beijing Genomics Institute; ENA: European Nucleotide Archive.

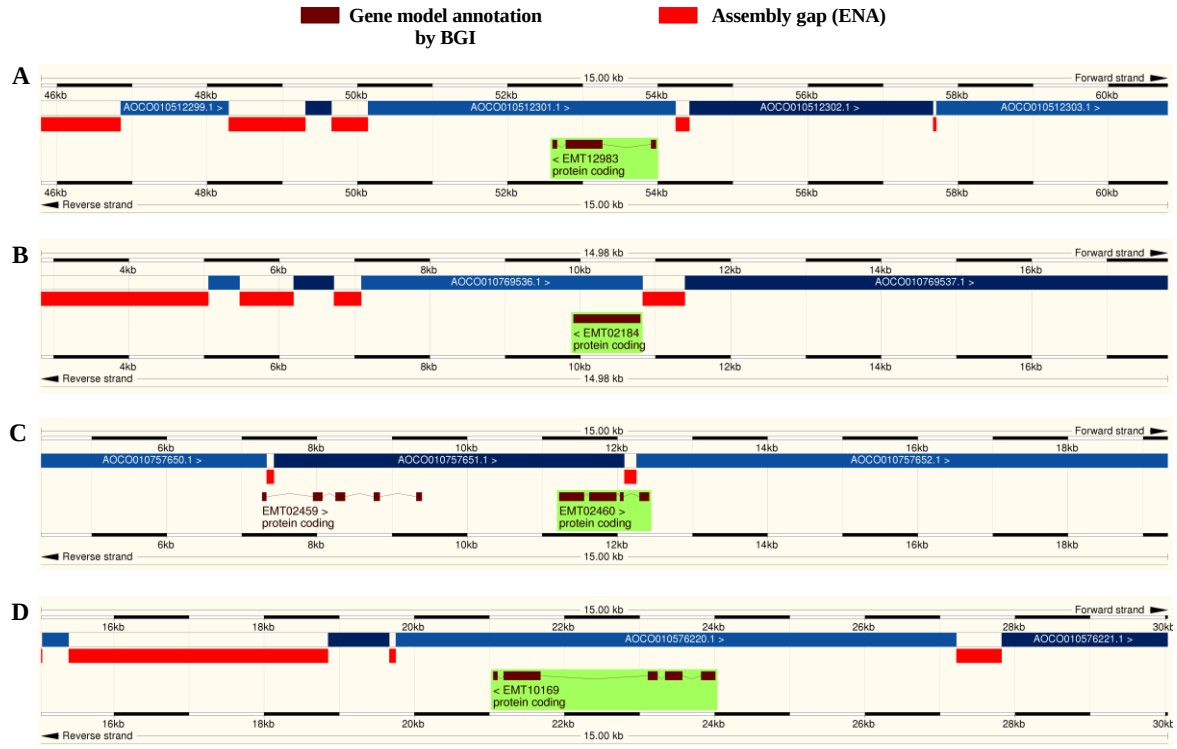

Figure 3.19: Comparative analyses of *AetAox1* gene model annotations predicted by BGI and the current study in *Ae. tauschii* (Genome assembly: ASM34733v1 released in December 2013).

(A) Section from scaffold49178, which contains part of *AetAox1a*. (B) A section from scaffold131071, which contains part of *AetAox1d2*. (C) A section from scaffold123987, which contains part of *AetAox1d2-like*. (D) A section from scaffold60901, which contains *AetAox1e*. Detailed view panels were retrieved from Ensembl Plants Archive release 35 in April 2017. BGI: Beijing Genomics Institute; ENA: European Nucleotide Archive.

In the current study of *T. turgidum* sequences, *AOX* orthologues were identified for the first time. However, there was an indication of incomplete gene models found in the current Ensembl Plants database, version 45 released in September 2019, which can be observed in Figure 3.20. Both *T. turgidum* ssp. *dicoccoides* cv. *Zavitan* (WEWSeq v1.0) and *T. turgidum* ssp. *durum* cv. Svevo (Svevo.v1) genome assemblies are hosted at Ensembl Plants. For instance, the best results of a *TtAOX1a-2AL* BLAST search, identified in the current study, revealed that the genome assembly of *T. turgidum* ssp. *dicoccoides* cv. Zavitan (WEWSeq v1.0) has three genes designated in the gene model annotation as TRIDC2AG063170, TRIDC2AG063180 and TRIDC2AG063160 (Figure 3.20 A). These gene IDs were classified as HC and LC gene models but failed to predict the full length of the AOX protein. However, a manual inspection revealed that there were no ambiguous bases (Ns) in the corresponding region. The second example of an incomplete gene model was found in the genome assembly of *T. turgidum* ssp. *durum* cv. Svevo (Svevo.v1). *TtAOX1d1-2AL* has not been assigned as an annotated gene (Figure 3.20B).

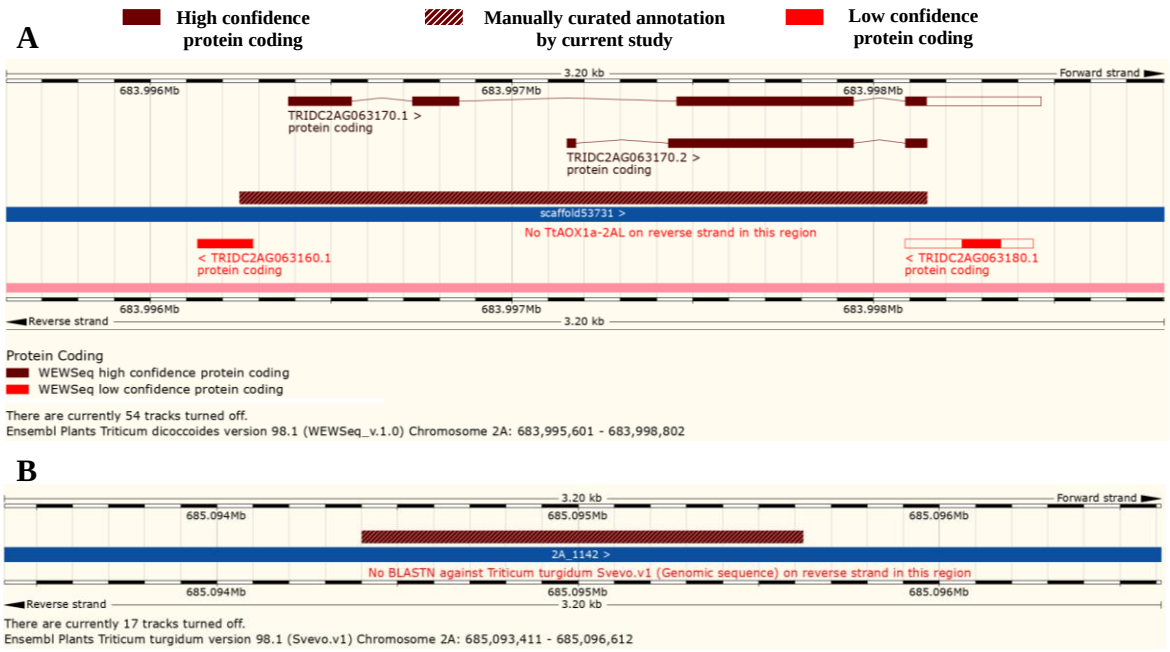

Figure 3.20: Comparative analyses of *TtAox1* with WEWSeq and Svevo.v1 gene model annotations

(A) Gene model annotations predicted by WEWSeq Consortium and (B) predicted by Svevo.v1, compared with the current study's manually curated annotations. In (A), *TtAOX1a-2AL*, identified in the current study, was aligned to the genome assembly of *T. turgidum* ssp. *dicoccoides* cv. Zavitan **(**WEWSeq v1.0) using the BLAST tool. In (B) *TtAOX1d1-2AL* was aligned with the genome assembly of *T. turgidum* ssp. durum cv. Svevo (Svevo.v1). Detailed view panels were retrieved from Ensembl Plants release 45 in September 2019.

3.3.2 General Discussion

This chapter aimed to identify and characterise *AOX* genes in *Triticum* and *Aegilops* species. The objective was to evaluate the evolution of the AOX protein by assessing its taxonomic distribution in *Triticum* and *Aegilops* species. A comparative assessment of all *AOX* gene sequences was conducted by using bioinformatics, *in silico*, and *in vitro* techniques to explore *AOX* gene structure, expression and phylogenetic relationships between *Triticum* and *Aegilops* species. The availability of high-quality genome assemblies for both species facilitated identification and characterisation of the *AOX* gene subfamily in *T. aestivum* and its related species (IWGSC 2018; Ling et al. 2018; Luo et al. 2017). In plants, AOX proteins are encoded by small gene families, *AOX1* and *AOX2* (Costa et al. 2014, 2017a). However, the number of *AOX* genes varies among different plant species. For instance, AOX in *Arabidopsis* is encoded by four *AOX1* (1a, 1b, 1c and 1d) and a single *AOX2* (Polidoros et al. 2009), whereas *C. arietinum* AOX is encoded by one *AOX1* and three *AOX2* (Sweetman et al. 2018). However, in *O. sa*tiva, and *H. vulgare*, AOX is encoded by only four *AOX1*-subfamilies, and *AOX2* was never found in monocot species (Wanniarachchi et al. 2018). The current study is the first to identify and characterise the number of *AOX1*-subfamily genes in *Triticum* and *Aegilops* species.

In hexaploid wheat (*T. aestivum*), there are three *AOX1a* gene copies that were fully identified and classified as HC genes. In tetraploid wheat (*T. turgidum*), two copies of *AOX1a* were found, whereas single copies of *AOX1a* were found in diploid *T. urartu*, *T. monococcum*, *Ae. speltoides*, *Ae. sharonensis* and *Ae. tauschii*. Regarding the *AOX1c* gene lineage, in hexaploid wheat (*T. aestivum*) there were three HC copies, with the tetraploid wheat (*T. turgidum*) having only two copies, and for almost all diploid species, a single copy of *AOX1c*, except for *T. urartu* which had two copies. Hexaploid wheat (*T. aestivum*), three HC copies of each *AOX1d1* and *AOX1d2* and a single copy of *AOX1d3* were found. In tetraploid wheat (*T. turgidum*), two copies were identified for each *AOX1d1* and *AOX1d2* genes, whereas a single copy of *AOX1d3* was found. Most of the diploid species had single copies of *AOX1d1* and *AOX1d2* with the exception of *T. urartu*, which had *AOX1d2* and *AOX1d3*, not including *AOX1d1-like*. As regards the *AOX1e* gene, two partial copies were found in the hexaploid wheat (*T. aestivum*), one copy was identified for the tetraploid wheat (*T. turgidum*) and only *Ae. sharonensis* and *Ae. tauschii* were found to have a single copy in diploid wheat. Interestingly, *AOX1*-subfamily genes existed in three homologue forms in *T. aestivum*, with the exception of *AOX1d3* and

AOX1e. Thus, this finding indicates that *AOX* genes were retained after two rounds of polyploidisation.

In the current study, the wheat *AOX1d* clade isoforms were classified into three groups. This assumption was validated by the observation of the separate *AOX1d* clades in the phylogeny. In addition, these findings concurred with the previously identified AOX1d1 protein by Costa et al. (2014; supplemental Table S2). Moreover, it was also supported by the alignment results of the *AOX1d* clade, identified in the current study, with *HvAOX1d1* and *HvAOX1d2*.

At the genome level, the findings revealed that *Triticum* and *Aegilops AOX* genes share a similar genomic structure and lengths as observed in *A. thaliana*, *O. sativa* and *H. vulgare* (Considine et al. 2002; Ito et al. 1997; Saisho et al. 1997; Wanniarachchi et al. 2018). In the current research, the *AOX1a* and *AOX1c* clades were found to consist of four exons interrupted by three introns. Concurring with the other *AOX* studies, the size of last three exons of the *AOX* genes were conserved 129 bp, 489 bp and 57 bp, respectively (Campos et al. 2009; Castro et al. 2017; Ding et al. 2018; Polidoros et al. 2005; Velada et al. 2016). However, the loss or gain of introns has been reported in the *AOX* subfamily (Polidoros et al. 2009). The current study revealed that the *AOX1d* clade had lost their introns. It determined the intron–exon boundaries of *TaAOX*, which were inspected manually by using known *AOX* structures from *O. sa*tiva, *H. vulgare*, *S. bicolor* and *B. distachyon*. In addition, results for the genome structure were in agreement with previously known wheat *AOX* genes: BAB88645.1 and BAB88646.1 (Takumi et al. 2002). Finally, the exon-intron structures of *TaAOX* were supported by the latest wheat annotation (IWGSC 2018).

The current study revealed that *AOX* coding region transcript lengths in *Triticum and Aegilops*, which were derived from fully identified genes, ranged from 987 bp to 1963 bp, with the encoded protein ranging from 314 aa to 347 aa and the molecular weights (Mw) ranging from 35 to 38 kDa. These characteristics were similar to known AOX1, such as from *O. sativa* (Ito et al. 1997). Similarly, in a number of species the molecular mass of AOX was approximately 30 to 41 kDa, including *S. guttatum, G. max, N. tabacum*, *O. sativa*, *A. maculatum*, *C. arietinum*, *O. europaea*, *C. sinensis* and *H. vulgare* (Day & Wiskich 1995; Ding et al. 2018; Elthon & McIntosh 1987; Hoefnagel & Wiskich 1998; Ito et al. 1997; Sweetman et al. 2018; Umbach & Siedow 1993; Velada et al. 2018; Wanniarachchi et al. 2018).

At the protein level, the current study revealed that AOX proteins in *Triticum and Aegilops* have highly conserved histidine (H) and glutamate (E) residues, which are used for their characterisation as a member of the di-iron carboxylate protein family (Berthold et al. 2002; Moore & Albury 2008; Vanlerberghe et al. 1998). These conserved residues (H and E) were found in AOX across many organisms in multiple kingdoms (McDonald 2008). In addition, *AOX1a*, *AOX1c* and *AOX1e* clades have two regulatory cysteines conserved, CysI and CysII (Berthold et al. 2000; Holtzapffel et al. 2003). In contrast, the *AOX1d* clade naturally possesses serine residues at either or both cysteine sites. Previous studies elsewhere have noted that AOX naturally possesses serine residues at either cysteine sites (CysI or CysII) or both sites, such as those found in *O. sativa*, *Z. mays* and *L. esculentum* (Holtzapffel et al. 2003; Ito et al. 1997; Karpova et al. 2002). When cysteine is modified, the AOX protein is activated by succinate (Djajanegara et al. 1999; Holtzapffel et al. 2003). From this, it can be hypothesised that most *AOX1d* clades could be dependent on succinate regulation since they have a serine residue in either CysI or CysII positions. The regulation of AOX activity and the ways in which this is affected by the CysII residue are still being researched (Umbach et al. 2006). Crichton et al. (2010) suggested that CysII could influence the enzyme catalytic cycle through oxygen interactions. In addition, mutagenesis studies revealed that the AOX protein was unable to be oxidised when CysI was replaced by Ala in *Arabidopsis* (Vanlerberghe et al. 1998).

Most of the residues involved in AOX catalytic activity were conserved in *Triticum and Aegilops* AOX proteins, such as W211, Q247, Y258 and Y304 (numbered according to At.AOX1a; AT3G22370.1) (Crichton et al. 2010; Neimanis et al. 2013). However, a threonine residue (T184) is conserved in all identified AOX proteins, except TaAOX1d1-2AL and TtAOX1d1-2AL, where it is replaced by methionine residue. Further, partial and/or entire attenuation of AOX activity could result from mutations of particular residues, which impede the di-iron centre of an active site (Crichton et al. 2010; May et al. 2017; Shiba et al. 2013; Young et al. 2014). In testing the recombinant *S. guttatum* AOX protein (rSgAOX) in a T179A substituted mutant (At.AOX1a; AT3G22370.1; T184), the activity was significantly reduced (Young et al. 2014). Hence, TaAOX1d1-2AL may possibly have a similar reduction in enzyme activity. Notably, the threonine residue (numbered according to At.AOX1a; AT3G22370.1; ThrII/T279) is replaced by serine in *Triticum* and *Aegilops* AOX proteins. This substitution has preserved the side-chain properties; however, the loss of the methyl group of this residue on the enzymatic outcome requires further investigation. Further, substitution (R178H) was found in all *AOX1d* clades (numbered according to At.AOX1a; AT3G22370.1). It was observed

previously that R178A and R178Q in rTbAOX eliminated almost all of the AOX functions despite some side-chain chemistry being conserved in latter mutations (Shiba et al. 2013). Given that R178H was observed in one clade, it indicates the importance of R178H for the functionality of *AOX1d* clade, which suggests that further research of R178H is necessary.

Acquiring this information enables increased understanding of the function of AOX proteins in grass and polyploid monocot species and also draws attention to the need for greater knowledge of the biochemical properties of additional AOX isoforms across more plant species. Owing to the comparative similarities of AOX isoforms, further research is required on their functionality in differing biological frameworks. This could be achieved by undertaking mutation studies, whereby the focus would be on the consequences of substitutions. From previous research conducted on site-directed mutagenesis in angiosperms, it is likely that AOX will be inactive because of the modification of key residues (Albury et al. 2002; Berthold et al. 2002).

The research conducted in this thesis has provided valuable information for AOX, specifically in *Triticum* and *Aegilops*, and suggested opportunities for further research in other areas of AOX. Given that the previous site-directed mutagenesis research has been conducted on AOX in angiosperms, this presents an opportunity for comparative research. AOX1a and AOX1c had CysI and CysII, which indicated these genes were likely post-translationally regulated by redox mechanisms and therefore stimulated by pyruvate (Vanlerberghe et al. 1998). However, AOX1d1 and AOX1d3 in *Triticum* and *Aegilops* had double substitutions at CysI and CysII that were replaced by serine residues. Interestingly, most of the *Triticum* and *Aegilops* AOX1d2 orthologues contained serine residue at CysI. However, TmAOX1d2 had CysI and CysII as observed in AOX1a, and AOX1c orthologues. *Triticum* and *Aegilops* possess different forms of CysI and CysII substitutions naturally, and investigating these would enrich post-translational regulation studies. Thus, further studies should consider *Triticum* and *Aegilops* species to study different types of post-translational regulation by succinate versus pyruvate.

Pseudogenes are an integral feature in the evolution of *Triticeae* genomes, including wheat, which contains 12% pseudogenes (Wicker et al. 2011). It is possible that the pseudogenes have been retained during wheat evolution. For instance, the AOX1d1-4AS-like pseudogene copies, which were found in *T. aestivum*, *T. turgidum* and *T. urartu*, shared the same nucleotide substitutions that caused the internal stop codon (TAA). Characteristics of AOX-partial/like isoforms set them apart from all other *AOX* genes. It is known that pseudogenes are unlikely to

be expressed (Wicker et al. 2011). In agreement with the PCR results outlined in the current work, *in silico* analysis failed to obtain any EST or TSA that supports the existence of functional *TaAOX1-like* genes. Further confirmation was obtained from the IWGSC-RefSeq annotation v1.1, where *TaAOX1-like* copies were assigned to LC genes class. It can be concluded that *AOX1-like* could be pseudogene or truncated copies and not functionally expressed ones.

Notably, it was found that the subgenome B possessed a higher number of *AOX1* genes in both *T. aestivum* and *T. turgidum* species. The observations were in accordance with a number of studies that reported that the B genome possesses a higher number of genes than either of the other subgenomes of *T. aestivum* (Akhunov et al. 2003; IWGSC 2014; Qi et al. 2004). The precise mechanisms that lead the B genome to possess a higher number of genes in *T. aestivum* remain elusive. However, one possible hypothesis could relate to the hexaploid genome and the evolutionary history of *T. aestivum* compared with its diploid ancestors (Qi et al. 2004). *T. aestivum* experienced two rounds of whole-genome duplication events from its diploid ancestor (Qi et al. 2004). Thus, the number of *T. aestivum AOX1* genes was significantly higher than that in its diploid ancestors and more than in other monocots, such as *O. sativa* and *H. vulgare*.

Moreover, differences in the number of *AOX1* genes were observed among the AOX homologues. The *AOX1d* and *AOX1c* homologue groups contained three times the number of *AOX* genes in the diploid donors. Three duplication events of *AOX1d* clades were predicted in the long arm of chromosome 2 in the A genome of *T. aestivum*, whereas two *AOX* clades were found on the long arm of chromosome 2 of other subgenomes. A polyploidisation event in wheat evolution history might have led to the expansion of the *AOX* gene family (Moore & Purugganan 2005). In addition, the current study found that there are three copies of genes of the *AOX1d* clade in chromosome 2 in the A genome of *T. aestivum* and *T. turgidum* species. However, *T. urartu* had only one copy of the *AOX1d* clade genes in chromosome 2. These duplication events may play crucial roles in biological functions (Jiang et al. 2013). The previous studies on rice and *Arabidopsis* showed that tandem-arranged genes were responsive to environmental stresses, such as low temperature (Costa et al. 2014; Ito et al. 1997; Rizzon et al. 2006). These current results suggest that tandem duplications played a crucial role in the expansion of the *AOX* gene family in *Triticum* and *Aegilops* species. The current study corroborates the hypothesis of convergent gene evolution, which was reported by Costa et al. (2014). In *Triticum* and *Aegilops* species, *AOX1d* experienced a duplication event and became

located in a tandem arrangement with *AOX1a*. In all investigated *Triticum* and *Aegilops* species *AOX1a* and *AOX1d* existed in an identical order as observed in other plants (Costa et al. 2014).

The current study revealed that TaAOX1 on the B genome had the highest number of polymorphism events (SNPs/InDels) compared with other AOX on A, and D genome (Appendix C6). The increasing number of polymorphisms in *AOX1* located in the B genome was postulated owing to the age of the lineage of the diploid donors. Qi et al. (2004) reported that an older lineage is more likely to exhibit more polymorphisms. *T. urartu* is the youngest lineage among the diploid donors (*ca.* 1 MYA), followed by *Ae. tauschii* (*ca.* 2.5 MYA), whereas *Ae. speltoides* is the oldest (*ca.* 4 MYA) (Qi et al. 2004). The significant difference between members of the *AOX1*-subfamily in terms of the number of predicted glycosylation, acetylation and phosphorylation suggests the prospect of mutable functionality and regulation, which bodes further investigation.

Studies of CREs in the gene promoter are vital to understanding gene regulations (Polidoros et al. 2009). Differential expression profiles of *AOX* in response to stress could be mediated by specific CREs. In *Arabidopsis*, it has been reported that duplicate genes shared similar CREs but had different gene expression (Haberer et al. 2004). It has been suggested that small changes in CREs could lead to neofunctionalisation or subfunctionalisation. Neofunctionalisation is a process in which one duplicate gene retains the ancestral function, whereas its paralog gains a new function post duplication. However, in subfunctionalisation, the duplication genes will randomly lose subfunctions, thereby retaining their ancestral function (Panchy et al. 2016).

In addition, co-expressed genes are more likely to be regulated by common CREs (Allocco et al. 2004). In this study, the *TaAOX1a-2Al* and *TaAOX1d* clades shared similar CREs, which may explain the relationship between them in terms of gene expression. In *Arabidopsis*, it has been reported that *AOX* responds differently to various stresses because of specific CREs (Clifton et al. 2005; 2006). Moreover, it has been reported that orthologous genes were more likely to have similar CREs (Clifton et al. 2006). The current research found that *TaAOX1d1-2AL* and it is orthologues tend to have the same abiotic stress response elements (WUN-motif, ARE and GC-motif). Notably, light-dependence *cis*-responsive elements, abiotic stress-response *cis*-elements and hormone-response *cis*-elements were previously reported to induce *AOX* (Bartoli et al. 2005; Feng et al. 2008a, 2008b; Garmash et al. 2015; Vanlerberghe 2013).

Fauteux and Strömvik (2009) reported that endosperm expression-related elements (e.g. Skn-1) played essential roles in seed germination. In agreement with RNA-seq analysis, CREs analysis showed variation in the type and number of CREs between *AOX* homologues. Loss or mutation of bases in the promoter regions could lead to decreased induction of *AOX* (Dojcinovic et al. 2005). For instance, the mutation in the G-Box element of *AtAOX1a* causes their reduction of expression in the presence of antimycin A (Dojcinovic et al. 2005). The current research has revealed that the LTR element and WRE3 were absent in *TaAox1a-2DL* but present in its orthologous form, the D genome ancestor, *AetAOX1a*. Zou et al. (2011) indicated that the presence and absence of CREs were essential predictors of stress-responsive transcription in *A. thaliana*. In the present study, it was observed that the *TaAOX1* genes gained or lost CREs, in some cases, compared with homologues and orthologue genes in wheat and its ancestors even when they possessed a conserved protein structure. Thus, the differential expression of *AOX* isoforms within wheat and among its ancestors might be caused by genome evolution in the gene promoter regions. Wittkopp and Kalay (2012) and Swinnen et al. (2016) stated that differential expression of members of the gene family might be attributed to divergence, duplication or mutation evolution in CREs. The prediction of multiple CREs in *AOX1* genes indicated that this gene might have multiple functions in light, growth and abiotic stress. Hence, understanding the integration of CREs into *AOX* expression and the related effects on regulation requires further research in this area.

Since commencing this study in 2014, researchers elsewhere have suggested that gene models in wheat genome assemblies had problems regarding accuracy. In any initial genome assemblies and annotations, they were characterised by having many gaps, missing genes and errors (Baptista & Kissinger 2019; Denton et al. 2014). For *T. aestivum*, the first gene models, which were released with the IWGSC Chromosome Survey Sequences (CSS), were built by Plant Genome and Systems Biology (PGSB, version 2.2) (IWGSC, 2014). However, due to the fragmented nature of the first wheat genome assembly (CSS), the predicted HC genes were frequently inaccurate or truncated copies. This study was able to compare several genome assemblies in *Triticum* and *Aegilops* species and thus quantify the errors found in the *AOX* gene model annotations among *Triticum* and *Aegilops* species. Surprisingly, the numbers and types of errors were almost identical in *AOX* genes predicted among *Triticum* and *Aegilops* assemblies. Vendramin et al. (2019) reported that some transcripts in the *T. turgidum* assembly (Svevo.v1) were not annotated or classified as HC genes because of the lack of matched protein coverage, or their expression level. Thus, it can be concluded that manual inspection is essential

in identifying unannotated genes. Zapata et al. (2016) raised the importance of manual data validation to avoid incorrectly annotated genes in the *A. thaliana* assembly.

The current research revealed that low-quality assemblies were the major cause of mistakes and errors, which concurred with Alkan et al.'s (2011) finding. A few examples of separation of *AOX* genes across multiple contigs were found in *Triticum* and *Aegilops* genome assemblies. Thus, the number of *AOX* genes and the exact genomic structure may not be perfect. Together, mistakes in assembly could lead to some incorrect annotations of *AOX* gene models in *Triticum* and *Aegilops* assemblies, which were found in Brew-Appiah et al.'s (2018) study. The current study examined gene annotation based on known *AOX* isoforms found in *O. sa*tiva, *H. vulgare*, *B. distachyon* and *S. bicolor*, and interspecies comparison within *Triticum* and *Aegilops* species. The results of the *AOX* genes model, which were annotated manually in the current study, are supported by the latest released IWGSC_Refseq v1.1 annotation (IWGSC 2018).

Comparative analysis with Brew-Appiah et al. (2018) revealed that the major cause of discrepant *AOX1* gene models was the automated annotation. *TaAOX* isoforms, identified by Brew-Appiah et al. (2018), were obtained from Ensembl Plants. At that time, Ensembl Plants was hosting TGACv1 as a genomic resource for *T. aestivum* genome. However, a number of gene annotation errors existed in the gene model in TGACv1. Brew-Appiah et al. (2018) were possibly misled by the gene annotation errors. The comparison between TGACv1 and IWGSC_Refseq v1.1 annotation can classify these errors into overestimation and underestimation of the length of *AOX* gene model. For instance, underestimation of AOX protein sequence length can be noted in *TaAOX1d-2AL*.1 (*TaAOX1d1-2AL* in the current study), which has 294 amino acids based on TGACv1 annotation but has 329 amino acid according to IWGSC_Refseq v1.1 annotation. Discrepancies identified between TGACv1 and the current IWGSC_Refseq v1.1 can be observed in *TaAOX1c* clade. For instance, TaAOX1c-6BL was overestimated in size by 128 amino acids in TGACv1. The discrepancies between TGACv1 and IWGSC_Refseq v1.1 can only be explained by annotation procedure variation.

A common probable cause of overestimating gene size in the study by Brew-Appiah et al. (2018) was the presence of ambiguous bases (Ns), which can lead to a single gene being split into multiple contigs. For instance, *T. urartu* AOX1c and AOX1d2, identified by Brew-Appiah et al. (2018) using *T. urartu* (ASM34745v1), were overestimated owing to the presence of ambiguous bases (Ns). However, the presence of ambiguous bases (Ns) could also have resulted in truncated copies, as observed in *T. urartu* AOX1a and AOX1d1. Similar

discrepancies were found between *Ae. tauschii* assemblies, ASM34733v1, and Aet v4.0. The most truncated gene model was observed in ASM34733v1 annotation relative to the current Aet v4.0 annotation. For instance, *AetAOX1a* and *AetAOX1d2* genes were truncated copies in ASM34733v1, and that was due to the fragmented nature of ASM34733v1. Thus, this study provides evidence that several of the AOX isoforms identified by Brew-Appiah et al. (2018) were inaccurate because of the low quality of *Triticum* and *Aegilops* assemblies and its annotation pipelines, although alternative high-quality assemblies existed and were publicly available (IWGSC 2014, 2018: Ling et al. 2013; 2018; Luo et al. 2017; Zhao et al. 2017b).

This study demonstrates the importance of manually annotated inferences across *Triticum* and *Aegilops* species to improve gene model annotations. The impact of genome annotation quality goes beyond just gene model annotation because it leads to subsequent problems in the gene evolutionary studies and in understanding the biological systems (Klimke et al. 2011). It should be noted that during the writing of this thesis, an updated version of the bread wheat genome (IWGSC-RefSeq v2.0) was released in July 2019 but is restricted under the Toronto agreement. Moreover, an improved version of the *T. turgidum* ssp. *dicoccoides* cv. Zavitan (WEW_v2.0) was released with no restrictions in March 2019 (Zhu et al. 2019). However, for both versions, they have yet to be annotated. Thus, the current *AOX* findings would contribute towards improving gene model annotations of *Triticum* and *Aegilops* species and produce better-annotated databases.

The expression of *AOX* genes was examined using RNA-seq data generated from five different tissues during bread wheat development as well as their responses to biotic and abiotic stresses. In general, 12 of 13 full-length intact *TaAOX1* genes were differentially expressed. In contrast, the expression levels of truncated *AOX* sequences, and pseudogene copies, were low or undetectable, presumably due to the instability of their transcripts at the post-translational level, which was regulated by the nonsense-mediated mRNA decay surveillance mechanism (Hug et al. 2016). In that research, a pair of *TaAOX1d1* homologues genes were expressed in different tissues during wheat development, and were responsive to fungal infections, heat and drought stress, whereas the A genome homologue, *TaAOX1d1-2AL*, was undetectable. However, those findings contradict the results of qRT-PCR in the current research, which showed that *TaAOX1d1-2AL* was expressed in leaf and root of bread wheat cultivars (Figure 3.15). In addition, the analysis of wheat EST and TSA databases at NCBI revealed that several EST and TSA hits confirmed the expression of *TaAOX1d1-2AL* (Appendix Table C5.1). The

contradiction in the findings can be explained by the expression level of *TaAOX1d1-2AL*, which could be expressed at a very low level that cannot be detected. In addition, this study revealed that a threonine residue (T184) was replaced by a methionine residue in *TaAOX1d1-2AL*. Young et al. (2014) discovered that the activity of recombinant *S. guttatum* AOX protein (rSgAOX) was significantly reduced owing to T179A substituted mutant (At.AOX1a; AT3G22370.1; T184). Thus, T184M substitution could impede the di-iron centre of an active site.

The analysis of RNA-seq data showed that *TaAOX1a* and *TaAOX1d* clades were the most responsive isoforms to both abiotic and biotic stresses. These findings concur with those of several studies showing that *AOX1a* and *AOX1d* genes were the most stress-responsive ones among *AOX* isoforms (Clifton et al. 2005; Huang et al. 2002; Saisho et al. 1997; Wanniarachchi et al. 2018). Costa et al. (2007) studied *AOX* gene expression in sensitive and tolerant genotypes of *V. unguiculata*. Their findings revealed significant variation of *AOX* expression between different genotypes. Thus, the expression of *TaAOX* genes may differ based on wheat cultivars, which would deviate from those found in this study. It is plausible that wheat *AOX* genes could contain diverse expression patterns due to differences in gene structures among the differing clades.

Moreover, numerous studies on plants have indicated that genes that have fewer introns or simple structures are prone to quickly activate and respond rapidly to environmental stress (Castro et al. 2017; Chung et al. 2006; Yan et al. 2017). Conversely, genes that have more complex structures will have a longer time to respond to stress, as found in rice and *A. thaliana* (Chung et al. 2006; Heyn et al. 2015; Ren et al. 2006). Expression varied between the *AOX* gene family members. The variations in expression within the *AOX* gene family could explain differences in functionality and response to different environmental conditions. In hexaploid wheat, different types of transcriptional regulation may have resulted from either physiological or polyploidisation events. Researching the *AOX* gene expression pattern in diploid species may increase knowledge of the evolution of *AOX* genes in polyploid species, as found in wild relatives of rice (Covshoff et al. 2016; Scafaro et al. 2016). Hence, certain germplasm sources likely exist that could be introgressed, and thereby provide opportunities to enhance wheat for the global market (Placido et al. 2013). In the current research, genome-specific primers were developed to amplify specific *AOX* gene transcripts in *T. aestivum* and its orthologues in other

Triticum and *Aegilops* species. Further research using qRT-PCR is justified because it could further increase the validation of *AOX* gene expression.

Chapter 4: Variations of AOX Expression in Response to Abiotic Stress in *T. aestivum*

4.1 Introduction

The production of wheat is dependent on both rain and irrigation to maximise yields, and yet, salinisation poses a considerable threat (Colmer et al. 2006; Ghassemi et al. 1995). Research has shown that wheat is typically characterised by Na^+ exclusion, whereby transport of Na^+ to the shoot is minimal (Colmer et al. 2005; Cuin et al. 2008; Munns & James 2003). Consequently, research has been focused on identifying strategies to increase crop production on arable land that has been subject to salinity.

The purpose of the current research was to increase scientific knowledge of the *AOX* gene response to abiotic stress and effects on respiration and plant growth in wheat. To ascertain the AOX isoform that responds to stress, the role of AOX as a part of the ETC components and its effect on changing ROS level in the mitochondria, first, this study investigated the effect of different respiratory inhibitors, KCN and antimycin A (AA) on the cytochrome and alternative pathways by examining AOX expression. Conducting this investigation first allowed identification of the AOX isoforms expressed under chemical stress that induce ROS formation. Further, this research investigated the physiological and biochemical effects of salinity stress in four bread wheat cultivars, Chinese Spring (CS), Opata M85, Gladius and Drysdale, as well as the *AOX* expression in two of these cultivars, which differs in response to the salinity stress.

Each of the four cultivars was subjected to 150 mM NaCl, and the AOX expression was measured in both the leaf and root for each cultivar, under laboratory conditions. Further, the respective salinity tolerances of the four wheat cultivars were identified and the following were assessed: *AOX* gene expression, AOX protein content, MDA level, Na^+ and K^+ ion concentration levels, K^+/ Na^+ ratio and physiological changes, for example, fresh weight (FW) and dry weight (DW). By investigating these, the effects on the cultivars' shoot growth were determined. Shoot growth has been identified as a vital component for plant salinity tolerance (Shavrukov et al. 2010). This research could provide insight for future crop breeding by identifying the AOX isoforms that might provide increased tolerance to salinity. In this regard, full understanding of *AOX* gene expression in wheat and its response to salinity requires the

use of integrated data from biochemistry, proteomics and physiology. Although the use of such data has been undertaken in two wheat varieties by Jacoby et al. (2010), research on the *AOX* gene family expression has not been conducted to date. Therefore, this research adopted the strategy of comparing tolerant and sensitive wheat cultivars subjected to salt to assess *AOX* gene expression and AOX protein abundance.

4.2 Results

4.2.1 Reference Gene Validation for Normalisation of qRT-PCR

To analyse AOX gene expression, it was first necessary to have consistent results from qRT-PCR, which is dependent on the accuracy of transcript normalisation (Guenin et al. 2009). Therefore, it is essential to utilise stably expressed genes for systematic validation to ensure the availability of reliable, accurate data for the qRT-PCR analysis. In addition, the accuracy of gene expression depends on other factors, such as RNA quality and quantity (Bustin et al. 2013; Fleige et al. 2006). In the current research, RNA purity was measured spectrophotometrically by using the ratio of absorbance at 260 nM to 280 nM as described in Section 2.2.1, and the quality of RNA was examined routinely by agarose gel electrophoresis (e.g. Figure 4.1).

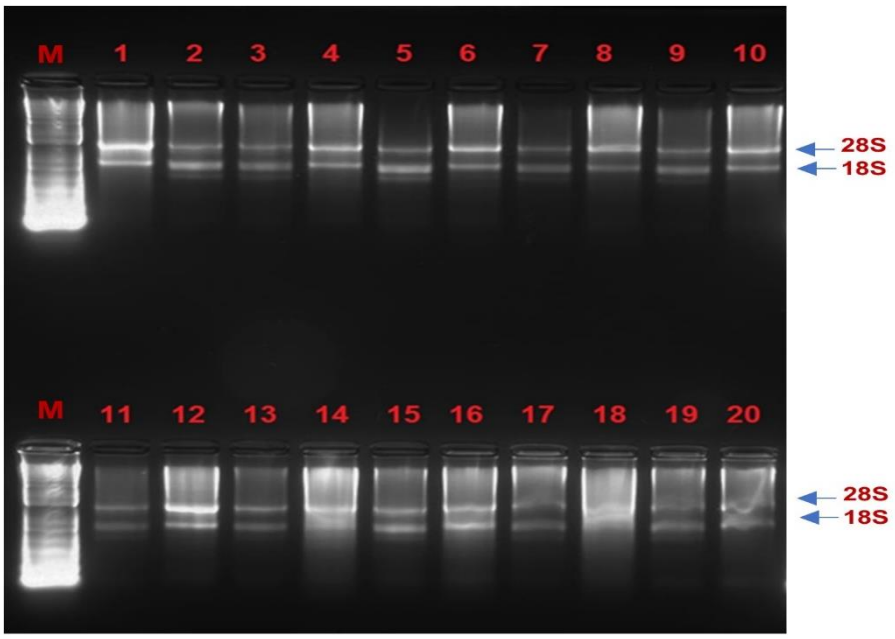

Figure 4.1: Total RNA analysis by agarose gel electrophoresis

Two µg of total RNA of leaf, 1–10, and root, 11–20, were run on a 1.5% denaturing agarose gel. The ribosomal RNA bands, 18S and 28S, were visible as an indicator of RNA integrity.

M: Ambion's RNA Millennium Markers™

Lanes 1–10: Chinese Spring (CS), leaf

Lanes 11–20: Chinese Spring (CS), root

To assess the stability of expression of five candidate reference genes in wheat, the qRT-PCR assay was used. This study has examined the well-known traditional reference genes, GAPDH (Ta30768) and Actin (Ta54825), used for higher plants (Paolacci et al. 2009; Pillitteri et al. 2004; Saraiva et al. 2014). Along with these two traditionally used genes, three novel reference genes were examined, ADP-ribosylation factor (Ta2291), cell division control protein, of the AAA-superfamily of ATPases (Ta54227) and RNase L inhibitor-like protein (Ta2776) as suggested by Paolacci et al. (2009). The qRT-PCR specific primers for the five candidate reference genes were taken from Paolacci et al. (2009) and are discussed in Section 2.3.4. In this study, the expression profile of candidate reference genes was analysed in 10 cDNAs prepared from RNA from leaf and roots tissues, which were harvested from the chemical and salinity stress treatments. Mean quantification cycle (Cq) values were obtained to determine the expression levels of each gene (Figure 4.2).

The research findings show that the traditional reference genes exhibited higher variability compared with the novel reference genes. Of the traditional reference genes, GAPDH had Cq values from 18.8 to 26.0 and Actin had Cq values from 15.2 to 21.6. For the novel reference

genes, Ta2291 had Cq values from 16.5 to 20.6; Ta54227 had Cq values from 17.9 to 21.9, and Ta2776 had Cq values from 23.01 to 26.9. The range of variation of Cq values was 4.1 for Ta2291, 4.02 for Ta54227 and 3.9 for Ta2776 (Figure 4.2). The low variation of Cq values for the novel genes might indicate a more stable expression, and these were chosen as the best reference genes. Agarose gel electrophoresis analysis was used to check the specificity of the primers (Figure 4.3). A single PCR product was confirmed for each set of reference gene primers, at the expected size, except Actin that had an additional amplification product along with the expected product (Figure 4.3D). In this study, the novel reference genes Ta2291, Ta54227 and Ta2776 showed low variability compared with GAPDH and Actin. Thus, they have been used subsequently for normalisation of qRT-PCR data from the three experimental conditions, salinity, KCN and AA.

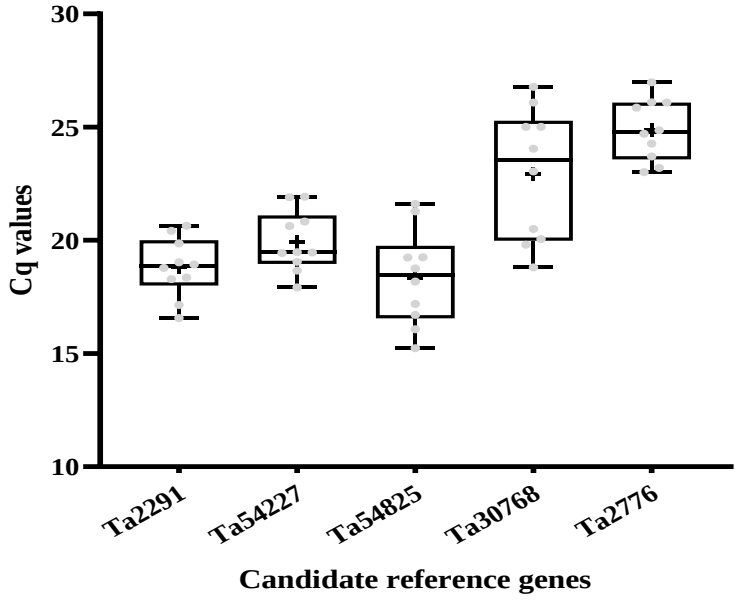

Figure 4.2: Distribution of transcript abundances of five candidate reference genes

The quantification cycle (Cq) values for 10 samples are shown as grey circles in the boxplot. Boxes indicate the 25th/75th percentiles. Whiskers represent the minimum and maximum Cq values, and + represent the mean. Five candidate reference genes were used: GAPDH (Ta30768), Actin (Ta54825), ADP-ribosylation factor (Ta2291), cell division control protein, AAA-superfamily of ATPases (Ta54227), and RNase L inhibitor-like protein (Ta2776).

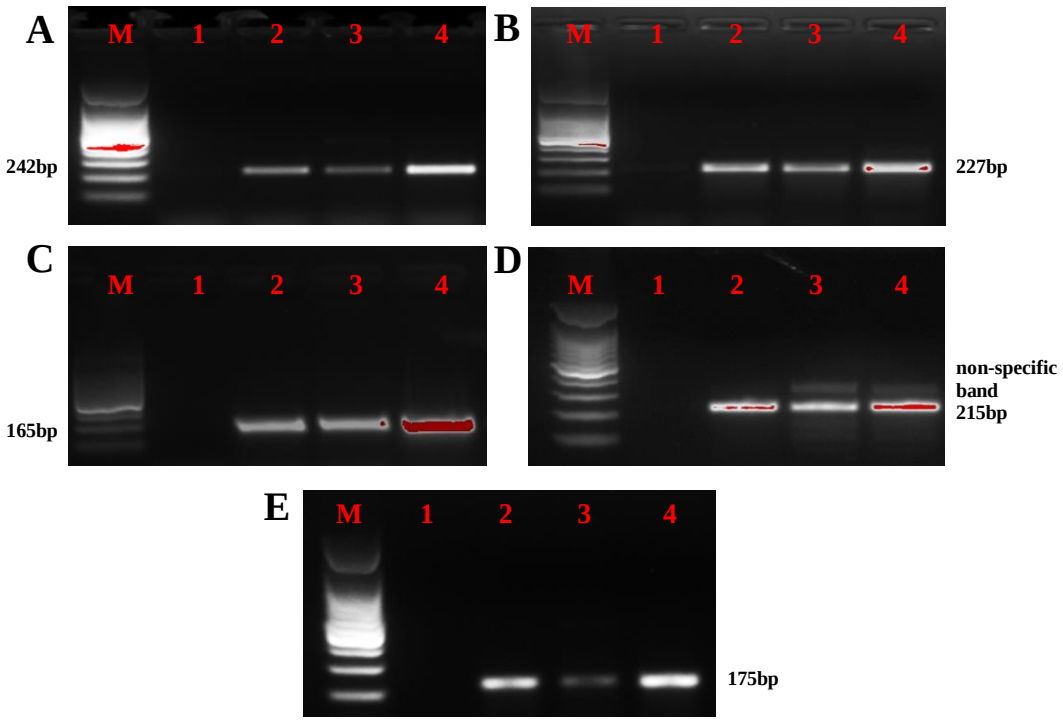

Figure 4.3: Amplification of a specific qRT-PCR product of the expected size for five candidate reference genes

The qRT-PCR product was run in 2% agarose gel to check the primer specificity for each candidate reference gene. (A) Cell division control protein AAA-superfamily of ATPases (Ta54227). (B) RNase L inhibitor-like protein (Ta2776). (C) ADP-ribosylation factor. (D) Actin (Ta54825). (E) GAPDH.

M:100bp

1: Empty

2: Leaf harvest from control

3: Leaf harvest from NaCl-treated

4: Root sample from NaCl-treated

4.2.2 Effect of Potassium Cyanide Stress on MDA Content

In plants, the amount of lipid peroxidation can be indicated by the level of MDA content (Del Buono et al. 2011). Cyanide has been shown to increase oxidative stress in plants (Vanlerberghe & McIntosh 1997). Knowing that MDA is a marker for measuring lipid peroxidation, it can be assumed that subjecting wheat cultivars to chemical treatments will induce oxidative stress, as shown by increased MDA content. When subjecting Chinese Spring to potassium cyanide (KCN), the MDA level was 38.3, which showed a significant increase above the control of 17.4, reflecting a 121% increase (Figure 4.4).

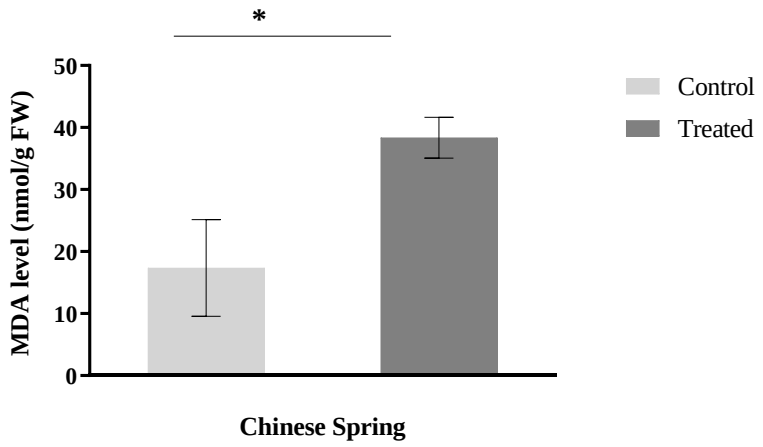

Figure 4.4: Effect of KCN on MDA content in bread wheat

The MDA content in bread wheat cv. Chinese Spring at the seedling stage following a treatment with 5mM of KCN for 24 h. Error bars represent ± SE of the mean of three biological replicates relative to the control. Two-way ANOVA was applied on the data to determine statistical significance. ***** indicates significant difference compared with the control at $p < 0.05$.

4.2.3 Effect of Potassium Cyanide Stress on Oxygen Consumption Rates of Sliced Leaf

Cyanide is a known inhibitor of the cytochrome pathway, whereby it is an efficient chemical compound that can induce AOX expression (Vanlerberghe & McIntosh, 1997). Thus, AOX respiration capacity is altered because of inhibition of the cytochrome pathway capacity. To determine leaf respiration rates in response to KCN, the oxygen uptake rate of the sliced leaf was measured using a Clark-type oxygen electrode, as outlined in Section 2.2.4. After applying a 5 mM KCN solution to a 5-day old seedling of Chinese Spring for 24 h, the results showed no significant change in the total respiration compared with the control. The rate of 0.2 nmol O_2/min/cm^2 was observed for both control and treated. However, there was a significant change in AOX capacity from that of the control (0.02 nmol O_2/min/cm^2) in the treated, which doubled to 0.04 nmol O_2/min/cm^2 (Figure 4.5).

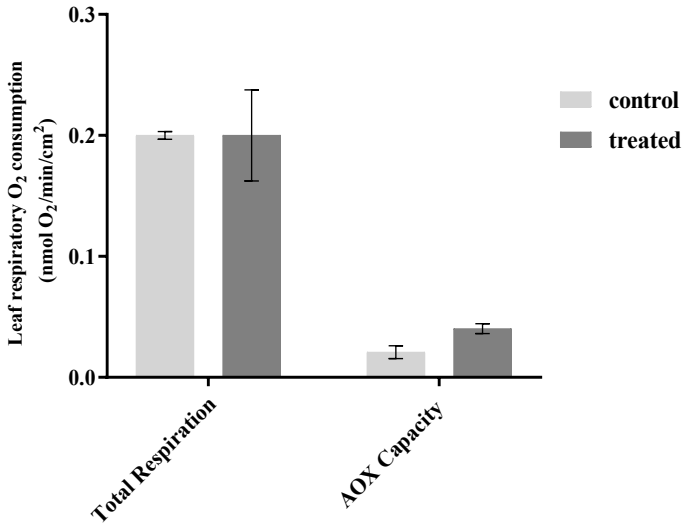

Figure 4.5: Total respiration and AOX capacity in sliced leaf from Chinese Spring under KCN treatment

Rates of oxygen uptake (nmol/h/cm2) in sliced leaf were measured with a Clark-type oxygen electrode. Light-grey bars represent the control, and dark-grey bars represent treated. Data represent the mean and standard error of the mean (SEM) of three biological replicates.

4.2.4 Alternative Oxidase Genes in Response to Chemical Stress

A common strategy to study *AOX genes* at the transcriptional level is to inhibit mETC chemically using inhibitors such as KCN and AA (Vanlerberghe & McIntosh 1997). In the current research, KCN and AA were used independently to identify the *TaAox1* isoforms that were responsive to the chemical inhibition of the mETC. KCN and AA treatments were applied to 7-day-old wheat seedlings cv. Chinese Spring seedlings. The effect of the chemical stresses on *Aox1* transcript abundance was measured in the roots and the leaves using qRT-PCR. Gene-specific primer pairs were developed for each genome; the amplification specificity of primer sets has been discussed in Section 3.2.10. In general, transcript abundance of *AOX1*a homologues, and *AOX1d* homologues, were significantly increased in both root and leaf in response to KCN and AA. However, the most interesting finding in this research is that the *TaAOX1c* clade was less responsive or not responsive in both chemical treatments.

Under KCN treatment, the transcript abundance of *TaAox1a-2BL* was the highest among *TaAox1* genes followed by TaAox*1d2-2BL* and *TaAox1d1-2DL*, in both root and leaf. The fold change of *TaAox1*a-2BL was 176.3 in leaf at 6 h and 201.4 at 3 h in root under KCN. The expression of both *TaAox1d2-2BL* and *TaAox1d1-2DL* were five times higher in the leaf compared with the root. *TaAox1a and TaAox1d* homologous genes both exhibited up-

regulation under KCN in both leaf and root. *TaAox1c-6BL* was tissue-specific, and it was expressed highly only in leaf. Under Antimycin A, the expression profile of *Aox1* genes was similar to the response with KCN. In both root and leaf, *Aox1*a homologues and *Aox1d* clade homologues were significantly increased. Although *TaAox1c-6AL* and *TaAox1c-6DL* were not responsive, transcript *TaAox1c-6BL* exhibited a reduction in the leaf at 3h and 6h before maintaining steady expression (Figure 4.6).

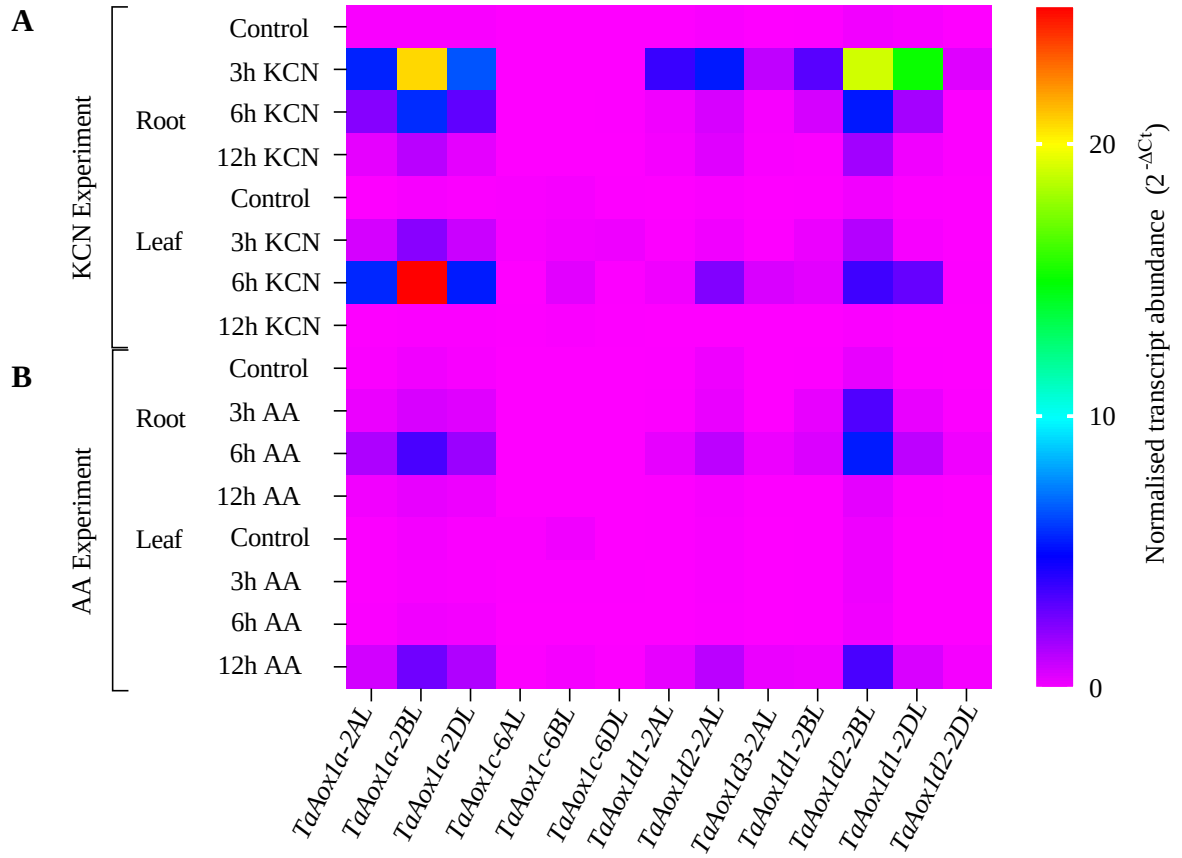

Figure 4.6: Heatmaps showing normalised transcript abundance of AOX1 in wheat cv. Chinese Spring under chemical stress

(A) Expression of *TaAOX1* in response to KCN stress. (B) Expression of *TaAOX1* in response to AA stress. Transcript levels of *AOX* were determined by qRT-PCR at four time points in both root and leaf of wheat cv. Chinese Spring. The heatmap was constructed using GraphPad Prism (8.1.2) from qRT-PCR data determined in this study. The $2^{-\Delta CT}$ methodology (Livak & Schmittgen 2001) was used to normalise expression data. Scale indicates normalised transcript abundance. Refer to Appendix C7.2 for the actual values.

4.2.5 Effects of Salinity Stress on Physiological and Biochemical Parameters of Wheat

The salt tolerance of four genotypes of *T. aestivum* (Chinese Spring, Opata M85, Drysdale and Gladius) were analysed for their relative sensitivity to NaCl (150mM) for five days and compared with the control (0 mM NaCl). To determine the salinity tolerance in the shoot, it was necessary to calculate the relative dry weight (RDW). RDW in the shoot is frequently used

to determine salinity tolerance (Shavrukov et al. 2010). Under controlled conditions, genotypes with a high RDW are typically the most salt-tolerant and exhibit rapid growth (Shavrukov et al. 2010). Typically, this will result in the leaves having an increased production of shoot biomass, and the Na^+ accumulation will be low to moderate (Shavrukov et al. 2010).

The RDW range for the four cultivars ranged from 38% to 81% (Figure 4.7A). The average was calculated at 61% (80.5 + 67.0 + 59.3 + 38.3/4); two cultivars, Drysdale and Gladius, had percentages above the mean and two, Opata M85 and Chinese Spring, below it, and hence, there was a normal distribution. Given that the mean was 61% and Opata M85 was only minimally under the mean (by 2%), it would be reasonable to infer that only Chinese Spring is salt-sensitive and that Opata M85 was moderately salt-sensitive whereas Drysdale and Gladius were salt-tolerant.

To measure the physiological effects of NaCl on the four cultivars in terms of plant height, the wheat seedlings' length was recorded at day 5. No significant differences were observed between salt-tolerant species Gladius and Drysdale, or salt-sensitive cv. Chinese Spring (Table 4.1). Gladius and Drysdale exhibited reduced height compared with their control, by about 2 cm and 5.4 cm on average, respectively. Chinese Spring showed a reduction in plant height under treatment by 5.2 cm on average compared with the control (Table 4.1).

In the current research, the accumulation of Na^+ in the third leaf varied among genotypes. When Gladius was treated with 150 mM NaCl, it had an average accumulation of 45 mM; Drysdale's accumulation was 38.8 mM, followed by Opata M85, which had 22 mM (Figure 7.1B). Chinese Spring had the least accumulation of NaCl at 21 mM. The mean was 31.7 mM, and there was a normal distribution because there were two cultivars above and two below the mean.

In addition, the relative salinity tolerance in relation to the K^+/Na^+ ratio for each cultivar was investigated (Figure 4.7C). Again, Gladius showed the lowest ratio of 8.96, Drysdale showed a 9.7 ratio, Opata M85 showed 14.6 and Chinese Spring had the highest ratio at 15.6. The mean was 12.2, and there was a normal distribution because there were two cultivars above and below the mean. Gladius has the highest percentage of Salinity Tolerance Index (STI) and yet the lowest K^+/Na^+ ratio, whereas Chinese Spring has the lowest STI and yet has the highest K^+/Na^+ ratio.

The salt tolerance index for each of the four cultivars and the standard error of the mean (SEM) are reported in Figure 4.7D and Table 4.1. The SEM is significant because it provides a

confidence interval for determining the distribution of the mean population within the range of the mean. Thereby, SEM indicates the accuracy of the estimated data (McDonald 2008). From the study, by calculating the quartile ranges based on the statistical results for the four cultivars, it is suggested that for a cultivar to be considered tolerant the STI needs to be above 70% and for sensitive cultivars, below 48%.

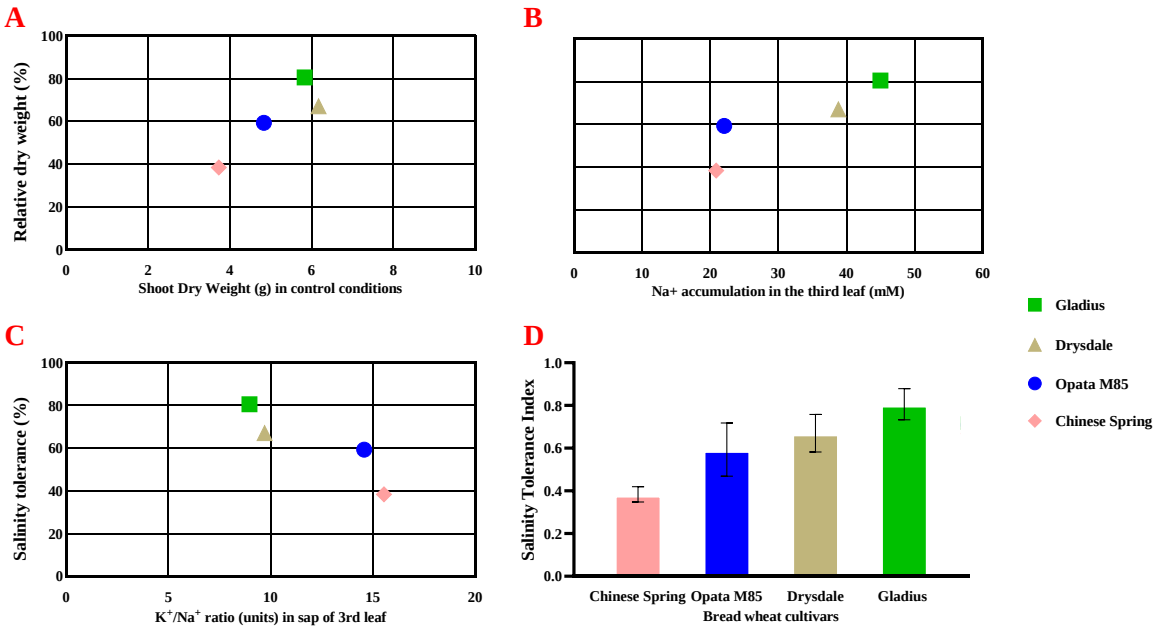

Figure 4.7: Effects of 150mM NaCl on four bread wheat growth parameters

(A) Growth rate in control conditions as shoot dry weight (SDW) and relative dry weight of the shoot. (B) Relationship between the Na^+ concentration in the third leaf and relative dry weight. (C) Relationships between ratio K^+/Na^+ and salinity tolerance. (D) Salinity Tolerance Index (STI) based on SDW in four bread wheat cultivars: Chinese Spring (CS), Opata M85 (Op.), Gladius (Gl.) and Drysdale (Dr.) RDW was calculated according to Section 2.2.1

Table 4.1: Mean values of effects of salinity stress on physiological and biochemical parameters of four wheat cultivars

Four bread wheat cultivars were grown under control and 150 mM salt-treated conditions for 5 days in a hydroponic system. Seedlings were harvested and physiological and biochemical parameters were measured according to Section 2.2.1 and Section 2.2.2 Ion concentration was measured in mM in the sap of the 3rd leaf for seedlings grown for 5 days at 150 mm NaCl. Data are presented as mean ± standard error for four biological replicates. The significant differences in treated to control are represented by colours as follows: light orange ($p \leq 0.05$), light red ($p \leq 0.0003$), and light green ($p < 0.0001$) by two-way ANOVA.

Cultivar	**Chinese Spring**		**Opata M85**		**Drysdale**		**Gladius**	
Parameters	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Root fresh weight (g)	1.1 ± 0.4	0.9 ± 0.12	2.4 ± 0.1	2.1 ± 0.3	2.7 ± 0.4	1.9 ± 0.2	2.2 ± 0.2	1.2 ± 0.1
Root dry weight (g)	0.06 ± 0.01	0.04 ± 0.01	0.08 ± 0.02	0.06 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.05 ± 0.002
Shoot fresh weight (g)	7.97 ± 0.2	4.7 ± 0.4	8.5 ± 0.1	6.43 ± 0.1	10.33 ± 0.5	8.1 ± 0.3	9.4 ± 0.3	7.9 ± 0.02
Shoot dry weight (g)	3.7 ± 0.4	1.4 ± 0.3	4.8 ± 0.4	2.9 ± 0.2	6.2 ± 0.9	4.1 ± 0.3	5.8 ± 0.8	4.7 ± 0.6
Plant height (cm)	28.6 ± 0.5	23.3 ± 1	35.2 ± 1.9	26.8 ± 2.7	36.7 ± 1.7	31.3 ± 2.6	32.2 ± 0.6	30.2 ± 0.5
Leaf K^+ content (mM)	247.5 ± 22	297.8 ± 9.6	261.7 ± 23.5	309.2 ± 9.67	213.5 ± 27.1	376.8 ± 39.5	243.8 ± 9.01	400.1 ± 31.8
Leaf Na^+ content (mM)	3.6 ± 0.5	20.9 ± 2.9	3.9 ± 0.9	22.05 ± 2.3	6.1 ± 0.9	38.8 ± 3.7	5.9 ± 0.6	45 ± 1.2
Leaf K^+/N^+ ratio	44.2 ± 19	15.6 ± 2.4	66.35 ± 17.7	14.6 ± 1.4	34.8 ± 5.7	9.7 ± 0.2	41.2 ± 6.5	8.96 ± 0.7
STI based on $SDW_{control}$	N/A	0.38 ± 0.04	N/A	0.59 ± 0.1	N/A	0.67 ± 0.1	N/A	0.8 ± 0.1
Relative shoot dry weight (%)	N/A	38.4	N/A	59.3	N/A	67.02	N/A	80.6

131

4.2.6 Changes to Malondialdehyde (MDA) Caused by Salinity Stress

In plants, the amount of lipid peroxidation can be indicated by the level of MDA accumulation (Del Buono et al. 2011). Plants subjected to oxidative stress undergo a lipid peroxidation process, which results in the production of MDA (Wang et al. 2008). MDA levels are determined using TBARS reflecting the exposure of a plant to oxidative stress (Moller et al. 2007). Increases in TBARS reflect a rise in MDA levels due to salinity stress (Del Buono et al. 2011). Salinity stress can induce higher levels of MDA (AbdElgawad et al. 2016; Kumar et al. 2017). This study found that all four cultivars had an increases MDA when subjected to salt stress (Figure 4.8 and Table 4.2). The range of increase for all four cultivars was from 5.3% to 42.3%. The two salt-tolerant cultivars Gladius and Drysdale had the least percentage increase of 5.3% and 6% respectively, and Chinese Spring exhibited the highest percentage increase in MDA at 42.3%.

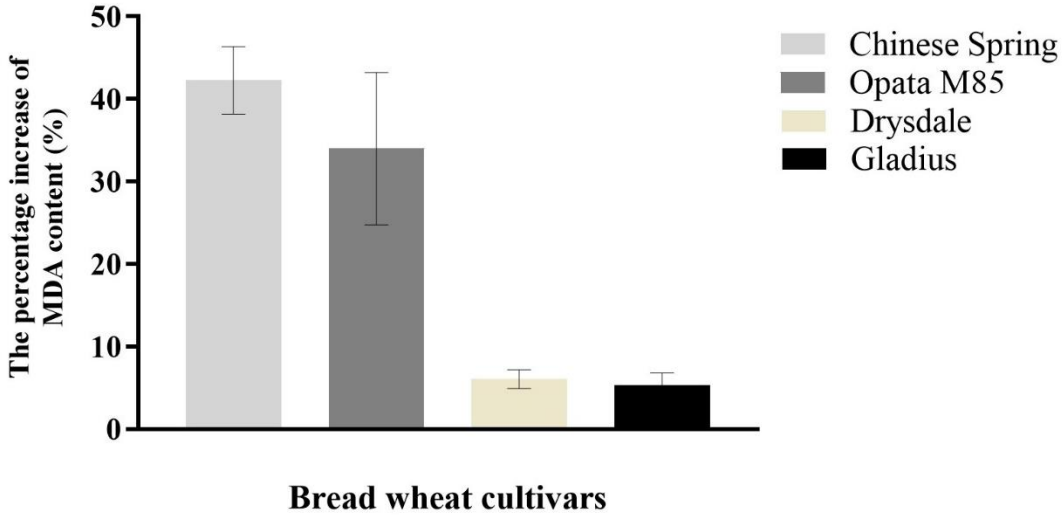

Figure 4.8: Effect of salt stress on MDA content in bread wheat

The percentage increase of MDA content in four bread wheat cultivars Chinese Spring, Opata M85, Gladius and Drysdale, which were subjected to salinity (150 mM NaCl), compared with controls. The leaf was harvested at 5 days after treatment. Error bars represent ± SE of the mean of three biological replicates relative to the control.

Table 4.2: Effect of 150 mM NaCl on MDA content in bread wheat

Four bread wheat cultivars grown under either 150 mM for 5 days in the hydroponic system, along with control seedling (no treatment). Seedlings were harvested and treated according to Section 2.2.3. Data are presented as mean ± standard errors of three biological replicates. MDA content represented as nmol per g FW.

Parameters	150mM NaCl Experiment			
	Chinese Spring	Opata M85	Drysdale	Gladius
Control	17.9 ± 2	39.4 ± 6.8	20.9 ± 4.3	16.4 ± 1.6
Treated	25.5 ± 5.1	52.8 ± 10.2	22.2 ± 2.1	17.3 ± 2.5
Change (%)	42.3 ± 4.1	34 ± 9.2	6.1 ± 1.1	5.3 ± 1.5

4.2.7 Alternative Oxidase Genes in Response to Salinity Stress

To identify the genes responsive to biological stress in modern *T. aestivum* bread wheat, the transcript response of the *AOX* gene family was determined by qRT-PCR. Gene-specific primer pairs were developed for each genome, and the amplification specificity of primer pairs has been discussed in Section 3.2.10. Based on the physiological and biochemical analysis, the two cultivars that differed most under salinity stress were chosen to study the *AOX1* gene expression, cv. Chinese Spring (salt-sensitive) and Drysdale (salt-tolerant). Wheat seedlings were grown hydroponically, as described in Section 2.1. To assess the *AOX1* response to salinity, wheat seedlings at the tillering stage (Zadok's scale Z20) were exposed to 150 mM NaCl and harvested at three time points 0, 12h and 72h, after the salt was applied. The transcript abundance of *TaAox1* isoforms varied between sensitive and tolerant cultivars and tissue types (Figure 4.9; Appendix C7.3). In general, this study noted that the salt-sensitive cultivar exhibited higher transcript abundances of *TaAox1* than the tolerant cultivar. In the leaf, the transcripts of *TaAox1c-6BL* were abundant in the salt-sensitive cultivar, and increased ~111.33-fold, followed by *TaAox1d2-2BL*, *TaAox1c-6AL*, *TaAox1a-2AL* and *TaAox1d1-2AL* after treatment. In the leaf, the tolerant cultivar showed a similar expression. However, the transcripts of *TaAOX1a* homologues and the *TaAOX1d* clade homologues were decreased at the 12h before a steady expression level was maintained. In roots, the expression patterns were similar to the expression in the leaves. However, the *TaAOX1c* clade genes were not expressed in roots at the tillering (Z20) stage (Figure 4.9).

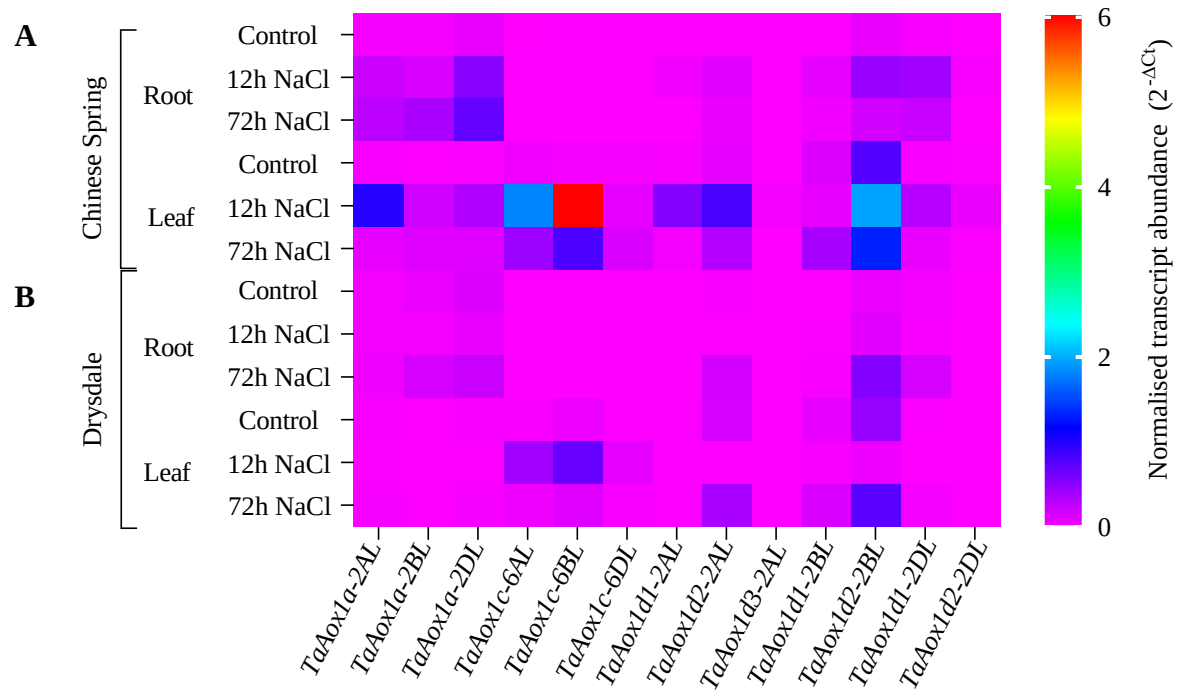

Figure 4.9: Heatmaps showing normalised transcript abundance of *AOX1* in wheat cultivars in response to salinity

(A) Transcript levels of *TaAOX1* were determined by qRT-PCR at three time points in both root and leaf of Chinese Spring (salt-sensitive), and (B) in Drysdale (salt-tolerant). Bread wheat cultivars Chinese Spring, and Drysdale, were subjected to two salinity levels (0 and 150 mM NaCl). The heatmap was constructed using GraphPad Prism (8.1.2) from qRT-PCR data determined in this study. The $2^{-\Delta CT}$ methodology (Livak & Schmittgen 2001) was used to normalise expression data. The scale indicates normalised transcript abundance. Refer to Appendix C7.3 for the actual values.

4.2.8 Abundance of AOX Protein under Salinity or KCN Stress

Immunoblot analysis was used to determine whether changes in the transcript level were reflected as changes in AOX protein content. To determine that equivalent amounts of protein were loaded, two approaches have been compared to decide the best method. The two approaches were the stain-free technique and the traditionally used loading control (Porin). Typically, traditional loading control has been used; however, research has shown that it has potential inaccuracies (Dittmer & Dittmer 2006). Therefore, the stain-free technique using Sypro Ruby (SR) and β-actinin is considered superior to the traditional loading control because the former has higher speed, fewer steps and lower consumption of materials, as well as improved quality of electrophoresis (Colella et al. 2012).

Additional research has also shown that stain-free detection outperforms traditional use of housekeeping proteins or Ponceau S staining as a loading control alternative for Western blot

testing (Rivero-Gutiérrez et al. 2014). Further, the commercially obtained pre-prepared Bio-Rad gel, when compared against Ponceau and housekeeping protein methods, is superior in terms of consistency (Rivero-Gutiérrez et al. 2014). For the current research, a comparison was conducted (Appendix C8). It showed that the stain-free technique performed better in terms of quantifying AOX protein and was hence selected to study AOX protein.

Total protein was extracted from the shoot tissue of bread wheat exposed to salinity stress or KCN (Section 2.3.8). The treatment time of tissue collections was different from that of gene expression and activity measurements (Section 2.3.8). Previous research has shown that the AOA monoclonal antibody has been effective in detecting AOX protein in several plant species, including *T. aestivum* (Jacoby et al. 2010; Sugie et al. 2006; Takumi et al. 2002; Wanniarachchi et al. 2018). This study also used the monoclonal antibody AOA to detect and verify the variations to AOX protein response when exposed to salinity stress in *T. aestivum.* For this study, and for ensuring an accurate estimation, each lane was loaded with an equal amount of total protein. The predicted size of AOX in reduced form is shown at ~36 kDa (Figure 4.10), the reduced (monomer) band allowing an estimation of the total amount of AOX protein. The quantity of AOX protein was calculated by using Bio-Rad's Image Lab software (Section 2.3.8).

The quantification analysis confirmed that AOX protein significantly increased compared with the control under both stresses, salinity and KCN. Notably, the effect of KCN treatment for 24 h on AOX protein was ~1.5-fold change compared with salinity for five days. Under KCN, the fold change was ~1.8 at 24h compared with the control and less effect was noted in shorter exposure, ~1.3 at 18 h. Under salinity stress for five days, the fold change was ~1.2 compared with the control. The quantification analysis shown in Figure 4.10C corresponds to the increased levels in the transcript abundance of cv. Chinese Spring under KCN and salinity.

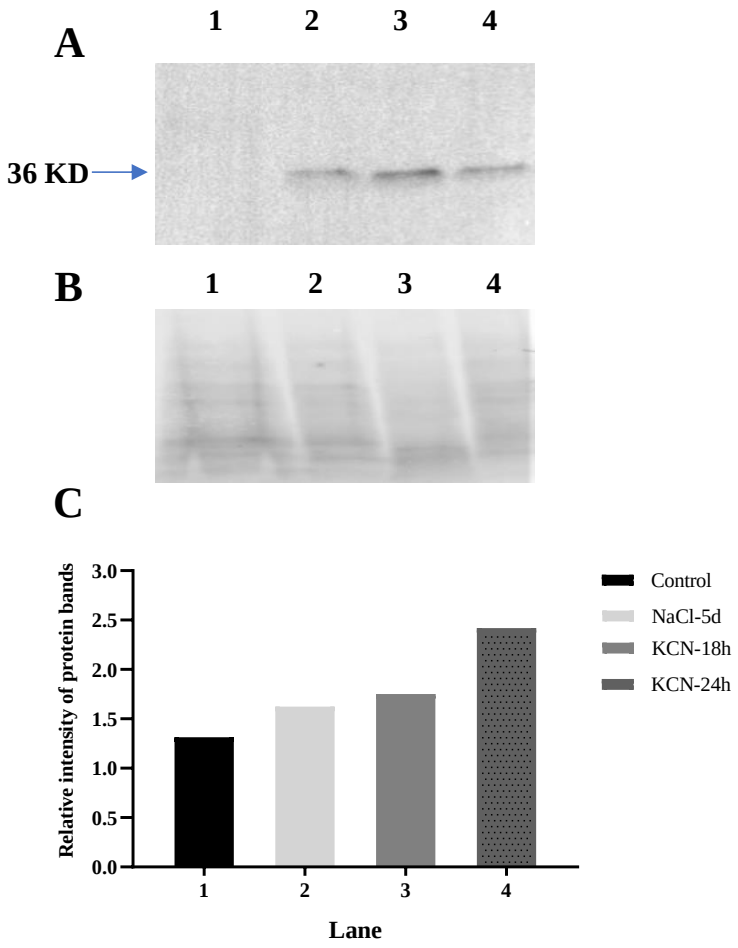

Figure 4.10: Western blot of AOX protein in shoot tissue from *T. aestivum* cv. Chinese Spring under salinity stress or KCN, using the stain-free method as loading controls

A) Detection of AOX by the monoclonal AOA antibody.

It shows the detection of the reduced (monomer) band.

B) Stain-Free membrane after transfer

Total protein was detected by the stain-free (SF) technique in the membrane after the transfer, which was used as a loading control.

C) Quantification of AOX Western blot

Lane 1: Control

Lane 2: Salt treated for 5 days

Lane 3: KCN treated for 18h

Lane 4: KCN treated for 24h

4.3 Discussion

At the transcript level, obtaining reliable and accurate data for the qRT-PCR depends on the accuracy of the transcript normalisation (Guenin et al. 2009). Therefore, this research has validated the stability of five reference genes under salinity, KCN and AA, including traditional reference genes GAPDH (Ta30768) and Actin (Ta54825) along with the three novel reference genes ADP-ribosylation factor (Ta2291), cell division control protein AAA-superfamily of ATPases (Ta54227) and RNase L inhibitor-like protein (Ta2776) as suggested by Paolacci et al. (2009). Concurring with Paolacci et al.'s (2009) study, this study's findings revealed that Ta2776 was the most stable reference gene under experimental conditions followed by Ta54227 and Ta2291. Of the traditional reference genes, the GAPDH gene had poor expression stability, compared with Actin. Thus, the findings are in accordance with those of Paolacci et al. (2009) and Long et al. (2010), which showed that GAPDH was not stable in wheat seedlings under stress.

In agreement with Paolacci et al.'s (2009) study, this research's findings revealed that Actin produced multiple amplification products, which indicated it had a poor specificity. Thus, neither GAPDH nor Actin may be the best choice as reference genes. Several studies have shown that the stability of expression of these traditional genes varies under different conditions (Gutierrez et al. 2008; Long et al. 2010; Paolacci et al. 2009). Thus, validating the stability of candidate reference genes under different experimental conditions is key to obtaining reliable, accurate data from the qRT-PCR assay. Using multiple reference genes is an effective way to achieve more robust, accurate data (Le et al. 2012; Løvdal & Lillo 2009). Thus, in this research, the three reference genes, Ta2776, Ta54227, and Ta2291, were the most stable and are more appropriate for normalisation of *AOX1* gene expression under the three experimental conditions, salinity, KCN and AA.

Cyanide has been shown to increase oxidative stress in plants (Vanlerberghe & McIntosh 1997). The current study's findings revealed a significant increase in MDA content as well when bread wheat cv. Chinese Spring was subjected to KCN by more than doubling its level from control. KCN has been shown to induce an increase in ROS, resulting in oxidative stress in plants (Moller 2001; Umbach et al. 2012). An increase in ROS production diminishes a plant's resistance to abiotic stress (Mhadhbi et al. 2013; Miller et al. 2010). The increased ROS results from electron leakage and causes inefficiency in the mETC (Jastroch et al. 2010). Plants must minimise damaging alterations to their mETC to survive under abiotic stress conditions

(Amirsadeghi et al. 2006; Atkin & Macherel 2009; Rhoads et al. 2006). Moller (2001) noted that AA and KCN are both recognised as mETC inhibitors; hence, they induce an increase in mROS. ROS has been shown to influence gene expression and thereby can influence abiotic stress responses and growth (Gill & Tuteja 2010).

After treatment with 5 mM KCN, the sliced leaf of cv. Chinese Spring exhibited no change in total respiration. However, a significant change occurred in the AOX capacity, which increased considerably. In this case, the increase in AOX supports observations in the literature that AOX expression in plants increases when subjected to KCN (Abu-Romman et al. 2012; Li et al. 2008; Polidoros et al. 2005; Takumi et al. 2002). KCN is known to inhibit respiration by restricting the proton translocation site at Complex IV of the cytochrome pathways (Grabel'nykh et al. 2011; Takumi et al. 2002). Given that the cytochrome pathway is highly responsive to inhibitors of the mETC, especially KCN and antimycin A, it has been determined that the alternative respiratory pathway is tolerant to these chemical agents (Campos et al. 2009; Fu et al. 2010; Grabel'nykh et al. 2011). The findings indicate that AOX is an essential factor in the regulation of cyanide-resistant respiration. Cyanide-insensitive oxygen uptake was not found in the mitochondria of durum wheat seedlings (Goldstein et al. 1981); Pastore et al. (2006) noted that oxygen uptake was completely blocked and that there was no immediate AOX activity.

The findings from this research were that the cultivars Gladius and Drysdale were both more salt-tolerant, which confirms the results of Genc et al. (2007). Gladius is known to have a high Salinity Tolerance Index, and this was the case here at 81%, and supports the literature that states that it is an efficient Na^+ excluder (Genc et al. 2007). Drysdale too is an efficient Na^+ excluder but is considered to have a lower efficiency to Gladius (Fleury et al. 2010; Genc et al. 2007; Shavrukov et al. 2006). This study confirmed that Drysdale had an RDW of 17% less than Gladius (80.57 − 67.02 = 13.55/80.57). Further, this study confirmed that Chinese Spring is salt-sensitive, which is in accord with the findings of Dvorák and Ross (1986), Dvorák et al. (1988), Colmer et al. (2006) and Jacoby et al. (2013).

Given these findings, this raises the question that since Gladius is the most salt-tolerant and Chinese Spring the least, what is the relative importance of NaCl accumulation in the leaves, given that salt-tolerant Gladius had a higher level of Na^+ accumulation in the leaf when subjected to 150 mM NaCl, in determining the genotype's overall salt tolerance? (Figure 4.7B). It is known that bread wheat is more salt-tolerant than durum wheat, which is most likely due

to more efficient Na^+ exclusion (Colmer et al. 2006). However, barley is more salt-tolerant than bread wheat and durum wheat even though the Na^+ concentration levels in the leaf are similar to that in durum wheat (Genc et al. 2007; Maas 1986). Genc et al. (2007) suggested that this was possibly due to increased tissue tolerance to Na^+.

Given that durum wheat is more salt-sensitive and yet accumulates more Na^+ in the leaves, similar to barley that is salt-tolerant, it can be deduced that Na^+ accumulation in the leaf does not signify a determinant factor for overall salt tolerance. The results showed that all four cultivars had high Na^+ accumulations. The salt-sensitive cv. Chinese Spring had the lowest accumulation, which ranged from 3.6 mM to 20.9 mM. The three known salt-tolerant cultivars all had higher Na^+ accumulations than the known salt-sensitive cv. Chinese Spring.

These findings support the assertions made by Munns and James (2003) and Munns and Gilliham (2015) that high levels of Na^+ accumulation in the leaf do not correlate to the plant being salt-tolerant. Munns and Gilliham (2015, p. 669) stated, 'In general, salt-tolerant species have high Na^+ and Cl^- concentrations in leaves'. Since all four *T. aestivum* cultivars exhibited high concentration levels of Na^+, and coupled with the evidence that supports this is not associated with salt tolerance, it can be deduced that Na^+ accumulation in the leaf is not directly related to effects on SDW and growth rate.

Albacete et al. (2008) and Munns and Tester (2008) noted that the plant growth rate could be significantly reduced without Na^+ reaching phytotoxic levels. Since the Na^+ accumulation was high in all four cultivars, both tolerant and sensitive, it suggests other factors must be responsible for determining a plant's tolerance level. Greenway and Munns (1980) and Tavakkoli et al. (2010) suggested that Cl^- could have a significant role in SDW growth rate. Yet, if accepting Munns and Gilliham's (2015) assertion that salt-tolerant species have high concentrations of Cl^-, this also suggests other factors must affect salt-tolerant species. Therefore, tolerant cultivars may have a coping mechanism against salinity stress that is reduced, or is non-existent, in sensitive species.

Historically, a lower Na^+/K^+ ratio meant that the plant was more salt-tolerant (Chhipa & Lal 1995). Despite the presumed salinity tolerance relationship with K^+/Na^+ ratio, Munns and James (2003) found no correlation. Genc et al. (2007), on conducting two experiments, could not demonstrate any association between salinity tolerance and the K^+/Na^+ ratio in bread wheat. These studies (Genc et al. 2007; Munns & James 2003) contradict previous findings (Chen et

al. 2007; Dvorák et al. 1994; Gorham et al. 1987, 1997). The results in this thesis support the research findings of Munns and James (2003), Genc et al. (2007) and Jacoby et al. (2013), who stated that Chinese Spring was salt-sensitive and Gladius was salt-tolerant. Thus, these contradictory results outlined could be explained by the differing mechanisms for salt tolerance of the examined wheat cultivars and their respective levels of Na^+ exclusion (Colmer et al. 2005).

From the two ranges of STI based on SDW for Gladius and Drysdale, the potential for overlap can be observed, because Gladius could have at the lower end of the SEM range an STI mean of 0.73, while Drysdale could have an upper end of the range STI based on SDW of 0.75. Thus, this would suggest that Drysdale would be considered more salt-tolerant than Gladius, in line with the findings of Takahashi et al. (2015). On observing the range of Opata M85, it too could be considered highly salt-tolerant, rather than moderately, were the high end of the range, 0.71, to be realised, which is comparable to that of Gladius. Since the STI of Chinese Spring is considerably lower at 0.38 and it is known to be salt-sensitive, it can conclude that the three cultivars in this study are a cluster, and that Gladius, Drysdale and Opata M85 are all salt-tolerant.

It has been shown that wheat genotypes exhibit distinct adaptive physiological mechanisms when subjected to abiotic stresses (HongBo et al. 2005). Measuring MDA using TBARs provides a measure of the effects of the abiotic stress damage (Moller et al. 2007). MDA levels have been shown to increase when pea leaves were subjected to abiotic stress (Taylor et al. 2002). Increases in MDA content can also indicate a change in osmotic stress response when subjected to salt stress. MDA content has been shown to increase significantly in maize when subjected to increased solutions of NaCl (AbdElgawad et al. 2016). In a study involving two rice cultivars, one salt-tolerant and another salt-sensitive, it was found that when subjected to salinity stress lipid peroxidation increased in both cultivars (Khan & Panda 2008). The findings also noted a distinct variation in levels of lipid peroxidation between the tolerant and sensitive cultivars, which indicated different coping mechanisms in each cultivar studied (Khan & Panda 2008). The salt-tolerant cultivar had lower levels of lipid peroxidation in terms of MDA content in comparison with the salt-sensitive cultivar. Thus, the salt-tolerant cultivar exhibited a better protection mechanism as opposed to the salt-sensitive cultivar, thereby indicating that different cultivars have different response mechanisms to salinity stress. The lower MDA content of Gladius and Drysdale, the most tolerant wheat cultivars in this research, was an indication of

their ability to minimise the effect of the oxidative damage due to a salinity environment, which explains their relative performance in terms of growth rate.

The results of this research showed that MDA levels increased for all four cultivars when subjected to salinity stress. The two salt-tolerant genotypes had the least increase, at 5.3% for Gladius and 6.1% for Drysdale, which confirms the findings of Rao et al. (2013), who noted that salt-tolerant cultivars had lower percentage increases in MDA content than salt-sensitive cultivars. Rao et al. (2013) used growth cut-off percentages to determine salt tolerance levels, deeming percentages >42% as salt-sensitive and <35% as salt-tolerant. In this case, cv. Chinese Spring would be classified as salt-sensitive. Simova-Stoilova et al. (2010) found that sensitive wheat cultivars had distinct damage to lipids as a result of oxidative stress. This suggests that cv. Chinese Spring, as a sensitive cultivar, should have had more pronounced oxidative damage, and as a result, lipid peroxidation, as indicated by MDA content would be increased, which was the finding in this study.

In the case of Opata M85, it would also fall into the salt-tolerant range based on Rao et al.'s (2013) classification method. Zou et al. (2016) also found that MDA levels increased by 35%, in the leaves of *T. aestivum* wheat seedlings when subjected to salinity for five days, which based on the Rao et al. (2013) classification would suggest that those wheat seedlings were salt- tolerant. Hasanuzzaman et al. (2017) similarly found that MDA content increased in wheat seedlings by 60% and 73%, which would imply that those seedlings were salt-sensitive cultivars. A lower MDA content is significant in terms of salt tolerance and indicates a lower level of lipid peroxidation (Borzouei et al. 2012). A low lipid peroxidation level indicates a plant's capacity to reduce oxidative damage when subjected to salinity, thus making it salt-tolerant and able to maintain its growth (Borzouei et al. 2012). In this study, the most tolerant wheat cultivars, Gladius and Drysdale, showed an indication of their ability to minimise the effects of oxidative damage caused by salinity stress. In addition, this explains their growth and performance as measured by their respective SDW measurements.

Taylor et al. (2002) suggested that plants have three mechanisms to cope with oxidative stress. Their first suggestion is that the MDA increased because of abiotic stress due to defence proteins in mitochondria, as a mechanism for coping with oxidative stress (Taylor et al. 2002); this scenario was not investigated in this research. The second mechanism suggested is that there is a correlation between AOX induction and lipid peroxidation damage (Taylor et al. 2002). This research confirmed that there was a significant correlation between AOX induction

and lipid peroxidation damage at the transcript level when *T. aestivum* was subjected to KCN. The research on salinity stress found that there was a significant correlation between AOX induction and lipid peroxidation damage in salt-sensitive and salt-tolerant species at the transcript level. Taylor et al.'s (2002) third suggestion is that AOX induction is too slow to save specific highly susceptible mitochondrial sites. The present study found that the *Aox1a gene* clade and the *Aox1d2* clade were the most responsive isoforms when exposed to salt stress. These results are the first to identify the *Aox1a* clade and the *Aox1d2* clade in *T. aestivum.* However, AOX induction could be effective in delaying oxidative damage to the mitochondrial membrane (Taylor et al. 2002).

From this research, it can be concluded that in those salt-sensitive cultivars that were highly susceptible to oxidative damage, there was a significant induction of AOX in a possible attempt to delay cell death. For the less susceptible, more salt-tolerant cultivars, AOX induction was less than in the salt-sensitive ones. However, the induced AOX protein in the tolerant cultivars was correlated with a reduction in MDA content, thereby delaying damage caused by oxidative stress. Given that Drysdale had a lower AOX expression at the transcript level and that the AOX protein levels were not measured, it follows that Drysdale may have another mechanism for coping with oxidative stress that does not induce AOX expression. This presents an opportunity for further research on Drysdale to confirm whether AOX delays damage caused by oxidative stress. In addition, further study is needed to investigate the correlation between the genes that encode the mitochondrial protein defence mechanism and *Aox1* gene expression.

To date, study has not been undertaken of the *Aox1a* or *Aox1c* genes in wheat, when subjected to a variety of stresses, such as metal toxicity, drought, salinity, heat stress, light stress, ozone effects or nutrient limitation (Feng et al. 2013). In this study, 13 *Aox1* genes were identified and examined at the transcript level in *T. aestivum* bread wheat under chemical and salinity stress. The findings as regards both types of stress were in accordance with those of Gray et al. (2004) and Clifton et al. (2006) who reported that when tobacco was subjected to abiotic stress or when the cytochrome pathway respiratory was inhibited, *Aox1* genes were expressed.

Concerning KCN-induced stress, this study's findings were in agreement with those of Takumi et al. (2002). In their study, they subjected *T. aestivum* seedlings to a 3 mM KCN solution for 24 h. In this study, a 5 mM solution was used for the same period. Both Takumi et al. (2002) and the present study showed that KCN inhibits respiration rate through the cytochrome pathway, which resulted in an increase in AOX activity. Takumi et al. (2002) noted differing

transcript levels between *WAox1a* and *WAox1c* (renamed in this study as *TaAox1a-2AL*, and *TaAox1c-6AL*, respectively), such that only *WAox1a* increased in response to the change in the cytochrome pathway activity. Moreover, Takumi et al. (2002) asserted that the difference between the two *WAox1* genes was due to differing transcriptional control from different regulatory pathways. This finding suggested that the two genes were responsive to different abiotic stresses. In this study, 13 full-length genes were studied at the transcript level, and it was found that *Aox1a* from the three subgenomes, A, B and D, were significantly increased under KCN treatment, whereas *Aox1c* from A, B and D were not responsive. This finding indicates the mechanism of *Aox1* subfamily gene expression differs based on various stress conditions (Takumi et al. 2002). Similarly, Arabidopsis leaves subjected to KCN caused the cytochrome pathway to be chemically inhibited, whereby the AOX overexpression reduced the production of ROS and this resulted in no additional oxidative damage (Umbach et al. 2005).

The AOX transcript has been induced in numerous plant species, including *Arabidopsis,* when subjected to salinity stress (Kreps et al. 2002; Seki et al. 2002). AOX transcript response as a result of salinity stress in *Arabidopsis* resulted in increased protein levels and AOX activity as a defence mechanism (Smith et al. 2009). Plants undergoing salinity stress minimise the damaging effects by inducing responsive measures, such as AOX activity. In salt-stressed *Arabidopsis*, AOX activity brought about decreases in ROS, resulting in a reduction in oxidative damage (Smith et al. 2009; Umbach et al. 2005). In addition, Jacoby et al. (2013) similarly reported that when the *T. aestivum* cytochrome respiratory pathway was inhibited by a high concentration of salt, AOX protein was induced. In the current study, a higher expression profile of *Aox1* was found in the salt-sensitive cv. Chinese Spring than in the salt-tolerant cv. Drysdale. The difference found in the *Aox1* gene expression in the two cultivars in response to salinity stress is consistent with the observations of Kong et al. (2001), showing that AOX capacity increased fourfold in the salt-sensitive genotype compared with the salt-tolerant genotype. In addition, this study's findings confirm those of previous research that AOX is responsive and AOX capacity increased for wheat, orange, soybean and barley under salinity stress (Ferreira et al. 2008; Hilal et al. 1998; Jolivet et al. 1990).

In general, this study revealed that *TaAOX1a* and *TaAOX1d2* homologues were the most responsive isoforms among *Aox1* genes studied in both root and leaf under chemical and salinity stresses. *TaAox1d2-2DL* was the exception, which was less responsive compared with *TaAox1d2-2AL* and *TaAOX1d2-2BL* under salinity stress. The *TaAox1c* homologues were not

responsive to the chemical treatment, with exceptions of *TaAox1c-6BL*, which was expressed at a very low level for both the leaf and the roots under KCN. Conversely, the expression of *TaAox1c-6BL* was the most expressed isoform under salinity at the tillering stage (Z20). This corresponds to the findings for barley, which showed that *HvAOX1a*, *HvAOX1d1* and *HvAOX1d2* were responsive to KCN under stress but *HvAOX1c* was not stress-responsive (Wanniarachchi et al. 2018).

Zhang et al. (2016) studied two wheat cultivars 'Chinese Spring' and 'Qing Mai 6', analysing these for their transcriptional response to salt stress, and found that these differed significantly in their respective salinity responses. They found differential expression patterns between the two wheat cultivars. Chinese Spring had 2,537 salt-responsive genes when subjected to salt stress. Qing Mai 6 had 2,624 salt-responsive genes. It was found that 85% of the tandem duplicated genes were diverged and expressed dynamically over the salinity exposure time (Zhang et al. 2016). In this research, the *TaAox1a* and *TaAox1d* clade genes were tandemly arranged in wheat chromosomes. Their expression appeared to be diverged and expressed dynamically during the exposure to salinity stress. Differential expression patterns were observed among the *TaAox1* gene isoforms, and their homologues, which were in line with previous findings on other wheat gene families (Kaur et al. 2017; Nan et al. 2018). Unequal contributions of wheat subgenomes towards gene expression were reported (Kaur et al. 2017; Nan et al. 2018). The distinctive expression patterns of *TaAox1* genes could indicate that *TaAox1* genes play divergent roles in regulating oxidative stress.

In the current study, analysis of *TaAOX1a* homologues revealed variability in the length of the mTP among the TaAOX1a clade protein isoforms at the N-terminal region (Section 3.2.2.3). The bioinformatics analysis identified an InDel event at exon 1 of *TaAox1a-2DL*, which could affect its expression. The effect of the variability in the N-terminal region in AOX1 protein isoforms on the regulation of *AOX1* gene expression, or protein transport or activity, remains unknown (Campos et al. 2009). Interestingly, *TaAox1d1* and *TaAox1d3* homologues were less responsive to the abiotic stresses, or had no response, but *TaAox1d2* was highly responsive to both chemical and salt stress. This difference might be due to the single-nucleotide polymorphisms (SNPs), or other differences in the promoter regions, which can affect gene expression (Nogales et al. 2016).

In the bioinformatics analysis (Section 3.2.2.3), this study revealed that CysI, CysII and leucine at the CysIII position were all identified in predicted TaAOX1a and TaAOX1c clades proteins.

However, TaAOX1d clades was the exception, since *TaAOX1d1* and *TaAOX1d3* homologues had a serine at the CysI and CysII positions, whereas *TaAOX1d2* only had serine in the position of CysII. These substitutions have been shown to affect AOX1 activity and response to metabolites (Selinski et al. 2017). In the case where the cysteine residues (CysII) were replaced by Ser (CSL), there was an increased expression of AtAOX1d-CSL (Selinski et al. 2017). The double substitutions in CysI and CysII (AOX1D-SSL) showed a decrease in activities when compared with the single substitutions. Given that *TaAox1d1* and *TaAox1d3* had either less or no response, it can be presumed that they had significantly reduced activities because of the double substitutions in CysI and CysII.

Rhoads and McIntosh (1992) reported that oxidative stress induces AOX activity, whereby AOX protein becomes expressed in *S. guttatum*. It was proposed AOX expression could be induced when ROS was generated due to abiotic stress in plants (Wagner & Krab 1995). In this research, abiotic stress caused an alteration in the *AOX1* gene expression, which brought about a change in AOX capacity (Vanlerberghe & McIntosh 1994). Further research on *AOX* gene expression has been undertaken in soybean, rice and Arabidopsis (Finnegan et al. 1997; Ito et al. 1997; Saika et al. 2002; Saisho et al. 1997). The alleviation of abiotic stress in plants is associated with AOX gene expression (Fiorani et al. 2005; Giraud et al. 2008; Murakami & Toriyama 2008; Panda et al. 2013; Smith et al. 2009; Sugie et al. 2006; Wang et al. 2011; Wang & Vanlerberghe 2013). Consistent with the present study's findings at the transcript level and AOX capacity, the immunoblot analysis revealed that AOX abundance differed between KCN and salinity stress. The AOX protein abundance was higher in the KCN-treated seedlings than in those under salinity stress. AOX protein abundance was induced by salinity stress, consistent with the proteomic studies by Jacoby et al. (2010, 2013), in which they studied both tolerant and sensitive wheat cultivars under salinity stress. Moreover, this study's findings support those of Takumi et al. (2002), which showed that the AOX protein abundance was higher in seedlings under KCN treatment than in seedlings under cold stress.

Jacoby et al. (2010) found that the sensitive wheat cultivar Janz and the tolerant wheat cultivar Wyalkatchem showed significant differences between the control and treated measurements. For the salt-tolerant wheat cultivar, Wyalkatchem, they showed that there was significantly less AOX abundance when treated was compared with control. The difference in AOX abundance in the sensitive cultivar Janz after treatment was approximately 33% and can be explained by the fact that in the sensitive cultivar, the increased AOX expression response is due to the

exposure to salinity (Jacoby et al. 2010). The control level of AOX protein abundance in Wyalkatchem was double that of Janz under control conditions, which indicated a more significant level of AOX prior to being exposed to salinity stress. At the transcript level, similar observations were present in the current research for the two cultivars, sensitive wheat cv. Chinese Spring, and tolerant Drysdale. The cultivars had different responses with the salt-tolerant increasing by more than 200%. The salt-tolerant wheat cultivars have an advantage over salt-sensitive cultivars, such as cv. Chinese Spring, in responding to salinity stress. Thus, further enquiry is needed into AOX protein level and its role in salinity tolerance. Any future research would also need to confirm the post-translational regulation of AOX under salinity.

To sum up, this study is the first that has investigated the relative contributions of *Aox1* gene expression from all three homeoloci of *T. aestivum* (A, B and D) in response to chemical and salinity stresses. This chapter discussed the *Aox1* gene expression in two *T. aestivum* cultivars exposed to different abiotic stresses. In the first experiment under chemical exposure to KCN and AA, *T. aestivum* cv. Chinese Spring was treated with 5 mM and 20 µM, respectively. In the second experiment, *T. aestivum* cultivars cv. Chinese Spring and Drysdale were exposed to 150 mM of NaCl. The results showed that the *AOX* gene expression differed under each of the abiotic stresses. For cv. Chinese Spring, the *TaAox1a*- and *TaAox1d* clades were the most responsive to KCN toxicity. Similarly, for AA, both *TaAox1a*- and the *TaAox1d* clades were the most responsive in *Aox1* gene expression. It should be noted that *TaAox1c* clades were non-responsive in both cases.

Under salinity stress, the *Aox1* gene expression differed between Chinese Spring and Drysdale cultivars. *TaAox1c-6BL* expression in the root, in the salt-sensitive cv. Chinese Spring, was the most responsive under salinity stress, whereas the expression of *TaAox1c-6BL* was 88% less responsive in the salt-tolerant cv. Drysdale than cv. Chinese Spring. Therefore, it can be concluded that *TaAox1c-6BL* has a role in functioning to reduce ROS in salt-sensitive *T. aestivum* cv. Chinese Spring. The *TaAox1a* clade and *TaAox1d* clades were also highly expressed in both leaf and root. However, it should be noted that this was for seedlings at the tillering stage (Z20). To further understand the role of *AOX* gene expression, it is necessary to investigate its hereditary ancestors to identify the one that has a higher tolerance to abiotic stress. In doing so, it will expand understanding of the role of AOX and knowledge of which wild ancestor's introgression influenced modern cultivated *T. aestivum*. By understanding *AOX* genes in wild wheat ancestors, it could expand knowledge of the ways in which different

genomes contribute to gene expression and also the understanding of the role of AOX towards reducing oxidative stress in the ancestral species.

Chapter 5: Variations of AOX1 Expression *T. aestivum* Ancestors under Salinity stress

5.1 Introduction

Typically, *T. aestivum* (bread wheat) has been found to be more salt-tolerant than *T. turgidum* ssp. (durum wheat) (Colmer et al. 2006; Francois et al. 1986; Maas & Greive 1990; Rawson et al. 1988). The direct ancestors of *T. aestivum*, *Ae. tauschii* and *T. dicoccoides*, and their direct ancestors, *T. urartu* and *Ae. speltoides*, each have differing responses to salinity (Colmer et al. 2006). This chapter aims to study the effects of salinity stress and the responsive expression of *Aox1* genes for the four direct wild *Triticum and Aegilops* ancestors of *T. aestivum*. Respiratory flux through AOX has been hypothesised to be an energetically wasteful pathway and thus could potentially negatively affect plant growth. If this is the case, it raises an important question. During the domestication of wheat, was there a selection away from AOX to improve yields, or is AOX vital to plant growth and adaptation and hence has been retained and expressed to a similar extent?

Investigating *Aox1* gene expression in *T. aestivum* and ancestors will increase the understanding of the mechanisms used in those ancestral species that exhibit higher tolerance to abiotic stresses. Identifying the *AOX* family genes that are most highly expressed will increase the knowledge of which wild ancestor's introgression influenced modern cultivated bread wheat. Further, by increasing the understanding of *AOX* genes in wild wheat ancestors, ways may be found to improve cultivated wheat tolerance to oxidative stress through ROS minimisation. To conduct this research, the following four ancestors (noting their respective accessions) were used: *T. urartu* (IG45626), *Ae. speltoides* (AUS.21650), *Ae. tauschii* (AUS-24119) and *T. turgidum* ssp. *dicoccoides* (Daliyya). For comparison, two bread wheat cultivars, Chinese Spring (CS) and Opata M85, were used. CS was selected as the salt-sensitive cultivar since it is well-known and documented (Colmer et al. 1995; Jacoby et al. 2013; see Chapter 4). In the case of Opata M85, it was selected based on the major experiment in Chapter 4. It was assumed that *AOX1* response would be more pronounced when subjected to salt; however, Drysdale, a relatively high salt-tolerant cultivar, showed a delayed response in *AOX* expression. Hence, Opata M85 was chosen based on STI data from this study, since it showed that it was a moderately salt-tolerant cultivar, to determine its *AOX1* responses.

5.2 Results

5.2.1 Effects of Salinity Stress on Physiological and Biochemical Parameters

5.2.1.1 Biomass Measurement

Salt tolerance was analysed by assessing the relative sensitivity to NaCl in the ancestors of *T. aestivum*, namely, *T. urartu* (IG45626), *Ae. speltoides* (AUS.21650), *Ae. tauschii* (AUS-24119) and *T. turgidum* ssp. *dicoccoides* (Daliyya). The four ancestral species were measured for their biomass and NaCl accumulation when subjected to 150 mM salinity for three days; their responses were compared with those of the two bread wheat cultivars, Chinese Spring and Opata M85. To determine relative salinity tolerance in each ancestor, the relative dry weight (RDW) needed to be calculated. The RDW was calculated as a ratio from the average shoot dry weight (SDW), according to Shavrukov et al. (2010) (Section 2.2.1).

After the salt stress, Chinese Spring and Opata M85 had, respectively, RDWs of 45% and 83%, which indicates that Chinese Spring was salt-sensitive and Opata M85 was moderately tolerant. For the wild ancestors, *Ae. speltoides* had the highest RDW at 88% and *T. urartu* had the lowest RDW at 41%. The three ancestors, *Ae. speltoides*, *Ae. tauschii* and *T. dicoccoides*, were all salt-tolerant, whereas *T. urartu* was salt-sensitive (Figure 5.1 and Table 5.1).

The results showed that the largest decreases in the SDW were observed with the two known salt-sensitive genotypes *T. urartu* and cv. Chinese Spring. *T. urartu* was decreased by 59%, whereas cv. Chinese Spring was decreased by 55%. The two genotypes, *Ae. speltoides* and Opata M85, had the lowest decreases by 11% and 17% respectively (Table 5.1).

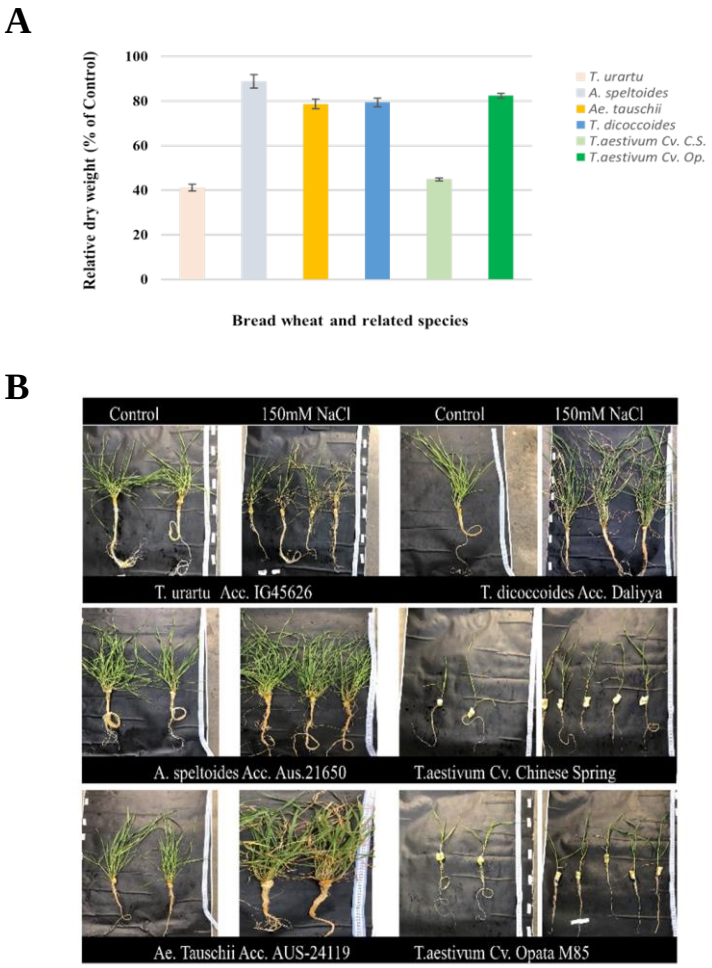

Figure 5.1: Effects of salinity stress on biomass of wheat cultivars and its wild ancestors

(A) RDW for wheat cultivars and its wild ancestors. (B) Overview of the extent of salt damage to bread wheat and related species. Four wild related species, *T. urartu* (acc. IG45626), *Ae. speltoides* (acc. AUS.21650), *Ae. tauschii* (acc. AUS-24119) and *T. turgidum* ssp. *dicoccoides* cv. Daliyya, and two bread wheat cultivars, Chinese Spring and Opata M85, were subjected to two salinity levels (0 and 150 mM NaCl). Pictures were taken at 4 days after treatment. *T. aestivum* (bread wheat) was harvested at the seedling stage, as opposed to the four wild ancestor species, which were harvested at the stem elongation stage. This was due to their slow growth, which resulted in the very low biomass of the wheat genotypes. Refer to Table 5.1 for the absolute values. Four biological replicates were collected for each species.

Table 5.1: Mean values of effects of salinity stress on biomass measurements of wheat cultivars and its wild ancestors

Two bread wheat cultivars and four of its direct ancestors were grown under control or under 150 mM salt-treated conditions for 3 days in a hydroponic system. Seedlings were harvested and the physiological changes recorded in terms of shoot fresh weight (SFW), shoot dry weight (SDW) and Salinity Tolerance Index (STI) based on SDW (control) and relative dry weight (RDW) as discussed in Section 2.2.1. Data represent the mean and standard error of the mean (SEM) of the four biological replicates.

Parameters	T. *urartu* - acc. IG45626		*Ae. speltoides* - acc. AUS.21650		*Ae. tauschii* - acc. AUS-24119		*T. turgidum* ssp. *dicoccoides* cv. Daliyya		*T. aestivum* cv. Chinese Spring		*T. aestivum* cv. Opata M85	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated
SFW (g)	5.1 ± 0.8	2.7 ± 0.4	10.9 ± 2.3	9.3 ± 1.3	9.5 ± 1.2	7.1 ± 1	8.9 ± 1.4	6.8 ± 1	1.6 ± 0.2	0.9 ± 0.3	1.1 ± 0.2	0.9 ± 0.3
SDW (g)	3.6 ± 0.6	1.5 ± 0.2	6.2 ± 1.7	5.5 ± 0.4	5.1 ± 2	4 ± 0.4	4.5 ± 1	3.6 ± 0.4	0.83 ± 0.1	0.37 ± 0.03	0.92 ± 0.05	0.76 ± 0.03
STI based on $SDW_{control}$	N/A	0.4 ± 0.04	N/A	0.88 ± 0.1	N/A	0.78 ± 0.05	N/A	0.8 ± 0.05	N/A	0.5 ± 0.02	N/A	0.8 ± 0.03
RDW (%)	N/A	41.3 ± 1.5	N/A	88.3 ± 3	N/A	78 ± 2.2	N/A	79.9 ± 2	N/A	44.9 ± 0.7	N/A	82.8 ± 1

5.2.1.2 Na^+ and K^+ Concentration in Leaves

The range for Na^+ was from 19 mM to 176 mM; cv. Chinese Spring had the lowest and Daliyya had the highest concentration levels. The mean was 74 mM, and there was an even distribution with three values above and below the mean (Figure 5.2A). For the K^+ concentration, the range was from 211 mM to 466 mM; *Ae. speltoides* had the lowest and *T. urartu* the highest levels and the mean was 305 mM (Figure 5.2B). The K^+/Na^+ ratio was calculated for the six genotypes as represented in Figure 5.2C. The K^+/Na^+ ratio ranged from 1.9 to 16.7. The mean of the ratio was 8.6, and there was a normal distribution with three above and below the mean. The genotype with the highest average ratio was Chinese Spring at 16.7, and the lowest was cv. Daliyya at 1.9.

The correlation coefficient is a measure of the linear association between two sets of variables. The *r*-value range is between −1 and +1, where an *r*-value of −1 would indicate a negative correlation, a value of zero would indicate no correlation and an *r*-value of +1 would indicate high correlation (Ware et al. 2013). In this comparison, the two sets of data correlated were the change in SDW and the change in Na^+ concentration levels among the various species, which yielded an $r = 0.23$, and thus a weak positive correlation. Although the correlation coefficient measurement does not represent causality (cause and effect), it does indicate a level of association, namely, that one set of variables correlates with another set (Figure 5.2D).

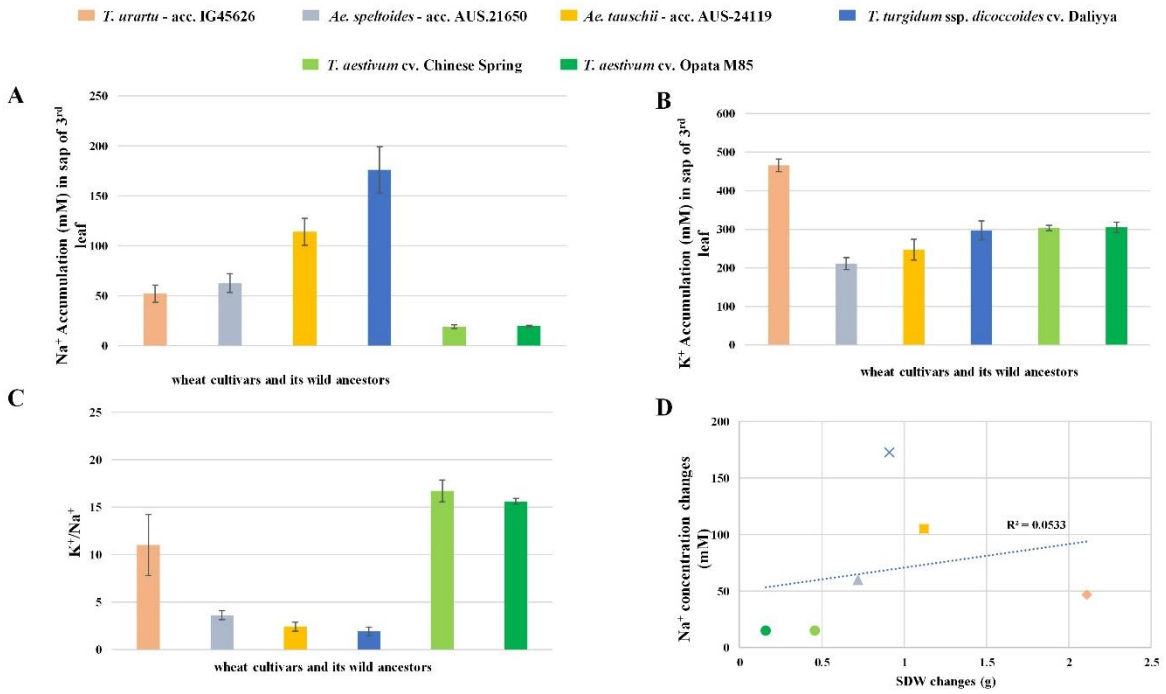

Figure 5.2: Na^+, K^+ accumulation and K^+/Na^+ ratio in sap of the 3rd leaf of bread wheat and wild ancestors

(A) Na^+ accumulation; (B) K^+ accumulation; (C) K^+/Na^+ ratio in sap of the 3rd leaf; and (D) the correlation of the change in SDW and the change in Na^+ concentration levels. Pearson's correlation coefficient (*r*) was calculated from the r^2 value. Four wild ancestor species, *T. urartu* (acc. IG45626), *Ae. speltoides* (acc. AUS.21650), *Ae. tauschii* (acc. AUS-24119) and *T. turgidum* ssp. *dicoccoides* cv. Daliyya, and two bread wheat cultivars, Chinese Spring and Opata M85, were subjected to two salinity levels (0 and 150 mM NaCl). K^+/Na^+ is a weighted average based on four replicates.

5.2.2 Effect of Salt Stress on MDA Content in Bread Wheat and Wild Ancestors

The MDA content level reflects the amount of lipid peroxidation found in plants following oxidative stress (Del Buono et al. 2011). This measure is used to determine the amount of oxidative stress plant have undergone, whereby there is an induced lipid peroxidation process resulting in MDA (Zou et al. 2016). The TBARs method is used to measure MDA levels and provide information on the abiotic stress response (Moller et al. 2007). Corresponding increases in MDA levels are indicated by rises in TBARs value, resulting from chemical or salinity stress (Del Buono et al. 2011). It has been previously reported that in plants, exposure to salinity stress leads to oxidative stress causing higher levels of MDA to be present (AbdElgawad et al. 2016; Kumar et al. 2017).

The research findings in this study can be separated by whether the species are considered salt-sensitive or tolerant. In this case, *T. urartu* and *T. aestivum* cv. Chinese Spring, the two known sensitive genotypes, showed increases in MDA levels as did *T. urartu* (Figure 5.3A). The response in *T. urartu* (29% increase) was the highest increase of all six genotypes studied. For *T. aestivum* cv. Chinese Spring was marginally increased by 7.5% above the control treatment (Figure 5.3A).

In Chapter 4, cv. Chinese Spring showed a 42% increase; however, the significant difference between these two findings can be explained by the age of the plant (tillering stage in Chapter 4 versus seedling stage in Chapter 5). In addition, the exposure period in Chapter 4 was five days, whereas in Chapter 5, it was three days (Section 4.2.6). This study revealed that there was no significant change in MDA content level for the three tolerant ancestors (Figure 5.3A). The correlation between MDA and RDW was assessed, and the coefficient was calculated between the change in MDA from control to treated and the RDW. The *r*-value of −0.61 indicates a strong negative correlation (Figure 5.3B). This suggests an inverse association. From this, it can be deduced that as the MDA content level increases, the RDW would decrease correspondingly, as would be expected under abiotic stress.

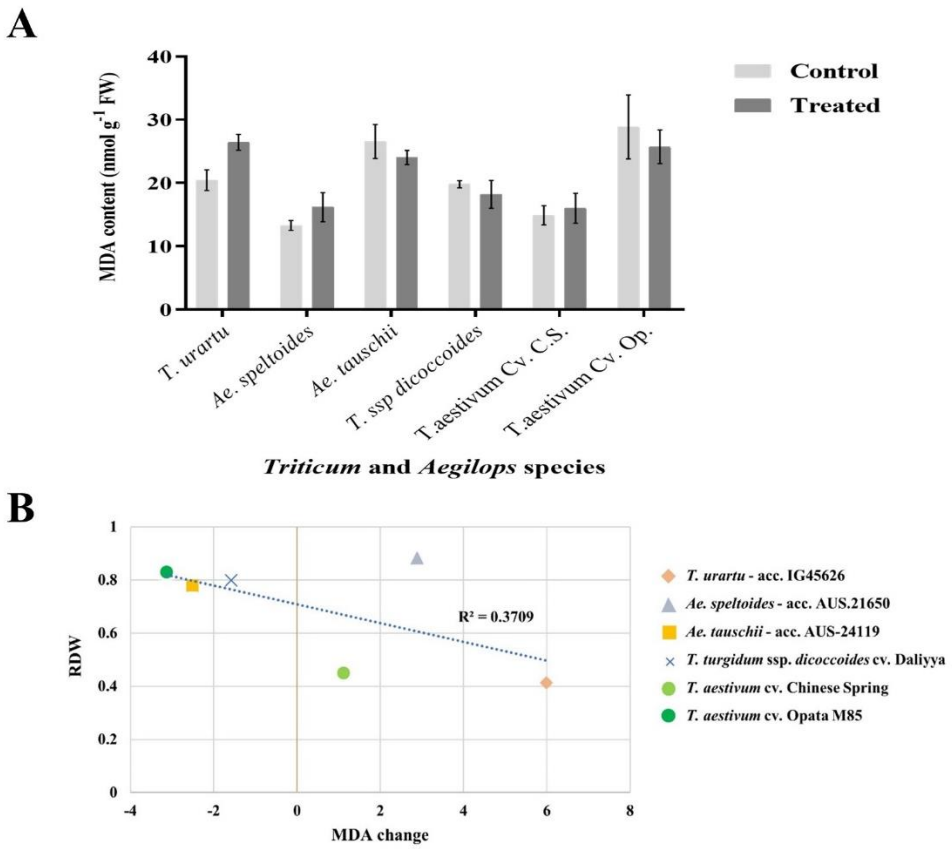

Figure 5.3: Effect of salt stress on MDA content in bread wheat and wild ancestors

(A) The effect of salt stress on MDA content. (B) Correlation of the change in MDA and the RDW. Four wild related species, *T. urartu* (acc. IG45626), *Ae. speltoides* (acc. AUS.21650), *Ae. tauschii* (acc. AUS-24119) and *T. turgidum* ssp. *dicoccoides* cv. Daliyya, and two bread wheat cultivars, Chinese Spring and Opata M85, were subjected to two salinity levels (0 and 150 mM NaCl). The latest fully developed leaf was harvested at three days after treatment. Pearson's correlation coefficient (*r*) was calculated from the r^2 value. Three replicate samples were used.

5.2.3 Effect of Salinity Stress on Tissue Respiration

Total leaf respiration and capacity of respiration to be facilitated by alternative oxidase was determined polarographically and expressed on a FW basis (Figure 5.4A). Under control conditions, the total leaf respiration rates varied for the six genotypes studied from 0.17 to 0.37 (nmol/h/cm^2). Interestingly, in response to salinity stress, *T. urartu* had a significant increase in total respiration, whereas other wild ancestors had insignificant changes (Figure 5.4A). Further, *Ae. speltoides* had the largest increase in AOX capacity, which more than doubled, whereas *Ae. tauschii* and *T. turgidum* ssp. *dicoccoides* cv. Daliyya had insignificant changes (Figure 5.4A). In addition, *T. urartu* and cv. Chinese Spring exhibited marginal increases in AOX capacity (Figure 5.4A). In comparing the change between MDA content levels and the change in AOX capacity for all six species, there was a positive *r*-value of 0.44, which indicates a relatively strong association (Figure 5.4B).

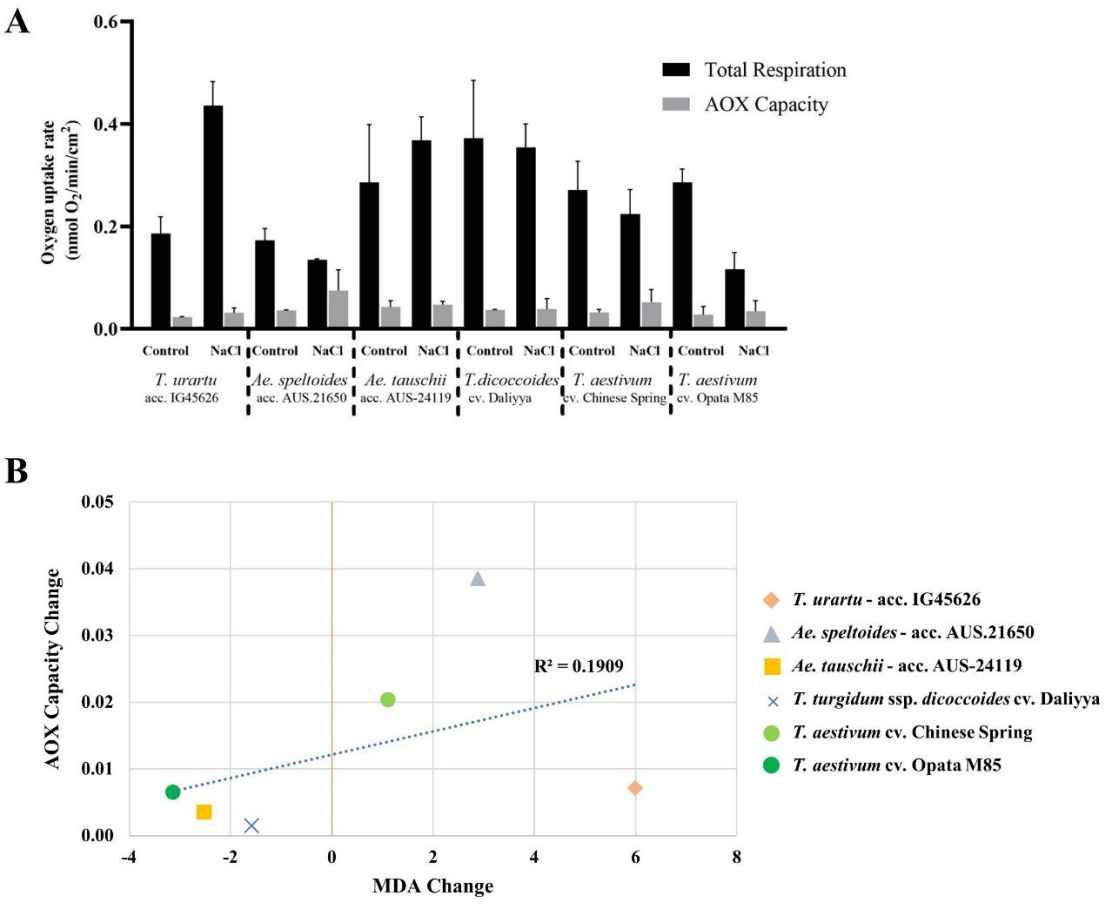

Figure 5.4: Effect of salinity stress on tissue respiration

(A) Total Respiration and AOX Capacity in sliced leaf from bread wheat and wild ancestors following control and salt treatments. (B) The correlation of the change between MDA content levels and the change in AOX capacity for all six species. Rates of oxygen uptake (nmol/h/cm^2) in sliced leaf were measured with a Clark-type oxygen electrode. Black bars represent total respiration, and grey bars represent AOX capacity. Data represent the mean and standard error of the mean (SEM) of three biological replicates. Pearson's correlation coefficient (*r*) was calculated from the r^2 value.

5.2.4 Transcript Abundance of *AOX1* in Response to Salt Stress

To identify the stress-responsive *AOX* genes in wild ancestors of modern bread wheat, the transcript response was determined by qRT-PCR. Gene-specific primer pairs were developed for each genome, and amplification specificity and efficiency of primers were set for each *AOX* gene isoforms, as discussed in Section 3.2.10. *AOX1* isoforms were present and expressed in the ancestors in a similar pattern to the commercial cultivars under control conditions (Figure 3.16).

The expression of *AOX1*-subfamily genes varied in terms of species, tissue types and exposure time (Figure 5.5 and Appendix C7.4). In general, the *AOX1a* clades and *AOX1d2* clades were the most responsive genes in all species studied. For instance, in the shoot, the transcript of the

AOX1a-2AL isoform was increased in *T. urartu* (wheat A-genome progenitor) by 2.5-fold. Similar expression patterns were observed in the commercial cultivar Chinese Spring (3.6-fold) at 12 h after salt was applied. Interestingly, the fold change expression of *AOX1a-2AL* was higher in the sensitive species compared with the tolerant even at 72 h, with a 3.5-fold change in *T. urartu* and 5.2-fold change in Chinese Spring (Figure 5.5). In the roots, *AOX1a-2AL* showed similar expression patterns as noted in the shoots. It was highly expressed in the sensitive species *T. urartu*, in bread wheat sensitive standard Chinese Spring by a 3.5-fold change and in Opata M85 by a 2.9-fold change.

Similar to *AOX1a-2AL*, *AOX1c-6AL* is also universally expressed in the roots and shoots, but at very low levels. In the shoot, the fold changes were the highest at 72 h in the sensitive diploid ancestral, *T. urartu* (11.6-fold), but not in the wheat standard Chinese Spring (2-fold change). Of note, *AOX1c-6AL* in Opata M85 maintained an increased expression at 72 h in shoot samples. However, the *AOX1c-6AL* expression in roots was highly expressed in the sensitive species *T. urartu* at 72 h. *AOX1d1-2AL* and *AOX1d3-2AL* had the lowest expression among the *AOX1* isoforms from the A genome in both roots and shoots of all the ancestral species and wheat standards, apart from *AOX1d3-2Al* in *T. urartu* at 12 h. In contrast to *AOX1d1-2AL* and *AOX1d3-2AL*, *AOX1d2-2AL* transcripts were the highest among *AOX1* isoforms on the A genome in all the species studied in both root and shoot. The fold changes at 12 h were 3.9-fold for *T. urartu*, 3.2-fold for *T. turgidum* ssp. *dicoccoides* and 3.6-fold for Chinese Spring. It is noted that *AOX1d2-2AL* for the shoot of *T. urartu* was almost 10 times higher than *AOX1a-2AL*, which made it the second highest expressed isoform from the A genome (Figure 5.5).

For the B genome, the transcript abundance of *AOX1a-2BL* had different expression patterns compared with the homologous gene, *AOX1a-2AL*. In the shoot, *AOX1a-2BL* was increased significantly in the diploid closest species, *Ae. speltoides* by a 3.8-fold change, but not in the tetraploid ancestor *T. turgidum* ssp. *dicoccoides*, which showed a -5.8-fold change at 72 h. In the shoot of the salt-sensitive standard Chinese Spring, *AOX1a-2BL* showed no real change. Further, Opata M85 showed no significant changes at 12 h or 72 h in these. In the root, *AOX1a-2BL* expression was very high in the diploid ancestor *Ae. speltoides*, and the wheat sensitive standard Chinese Spring. In the shoot, the expression *AOX1c-6BL* was significantly increased in *Ae. speltoides* at 72 h by a 2.6-fold change, However, in the tetraploid ancestor *T. turgidum* ssp. *dicoccoides* , the expression increased by 3.5-fold at 12 h. In the wheat sensitive standard Chinese Spring, the *AOX1c-6BL* was induced by 2.3-fold. The expression profile of *AOX1d1-*

2BL was lower in the diploid ancestor compared with the tetraploid ancestor, and the bread wheat standards in both shoots and roots. However, the *AOX1d2-2BL* expression profile was increased significantly in the ancestors *Ae. speltoides*, *T. turgidum* ssp. *dicoccoides*, as well as the wheat standards, Chinese Spring and Opata M85, in both roots and shoot (Figure 5.5).

For the D genome, the transcript abundance of *AOX1a-2DL* had similar expression patterns as observed in the homologous gene, *AOX1a-2AL*. In the shoot, *AOX1a-2DL* was highly expressed in the diploid ancestor, *Ae*. *tauschii*, at 72 h (2.7-fold). However, in the shoot of the wheat sensitive standard Chinese Spring, *AOX1a-2DL* was increased by a 3.1-fold change at 12 h. In the root, *AOX1a-2DL* expression had insignificant changes in the diploid ancestor *Ae*. *tauschii*, and in both wheat standards (Figure 5.5). As observed in the homologue gene of *AOX1c* on the A and B genomes, *AOX1c-6DL* was expressed at a low level in the shoot. However, an early response of *AOX1c-6DL* was noted in the sensitive wheat standard, which had a 2.3-fold change at 12 h, whereas the tolerant wheat standard OpataM85, showed a late response at 72 h by 2.7-fold. In contrast to the *AOX1d1* homologous gene on the A and B genomes, *AOX1d1-2DL* was significantly increased compared with *AOX1d2-2DL* in both the shoot and roots. Interestingly, the expression of *AOX1d1-2DL* and *AOX1d2-2DL* were positively correlated under control and stress condition, $R^2 = 0.99$ in the shoot and $R^2 = 0.98$ in the root of *Ae. tauschii*. The expression of *AOX1d1-2DL* was ~4 times higher than that of *AOX1d2-2DL* in the shoots of *Ae. tauschii* (Figure 5.5). Notably, *AOX1d1-2DL* in the diploid ancestor *Ae. tauschii* was significantly increased in the shoot when compared with its orthologue genes in wheat standards under control and stress conditions (Figure 5.5).

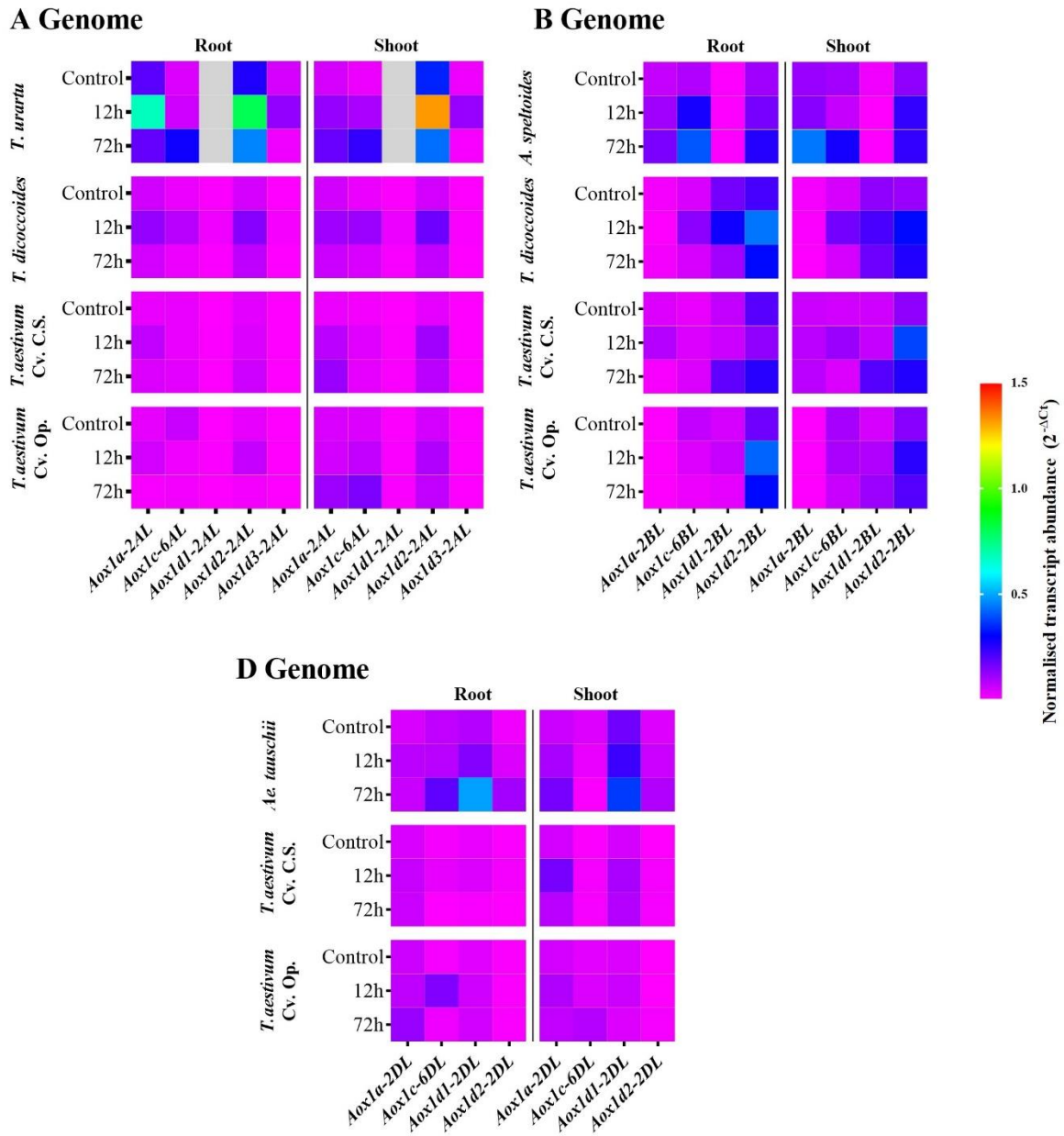

Figure 5.5: Heatmaps showing normalised transcript abundance of *AOX1* in wheat cultivars and its wild ancestors.

The expression of *AOX1* from the A, B and D genomes are shown separately. Transcript levels of *AOX* were determined by qRT-PCR at three time points in both root and shoot of four wild ancestors' species, *T. urartu* (acc. IG45626), *Ae. speltoides* (acc. AUS.21650), *Ae. tauschii* (acc. AUS-24119), *T. turgidum* ssp. *dicoccoides* cv. Daliyya, and two bread wheat cultivars, Chinese Spring, and Opata M85, after being subjected to two salinity levels (0 and 150 mM NaCl). The heatmap was constructed using GraphPad Prism (8.1.2). The $2^{-\Delta CT}$ methodology (Livak & Schmittgen 2001) was used to normalise expression data. Scale indicates normalised transcript abundance.

5.2.5 Changes in AOX Protein Abundance in Response to Salinity Stress

Immunoblot analysis was used to confirm whether changes that occurred in AOX activities and at the transcript level were reflected in AOX protein level. The total protein was extracted from

the shoot tissue of bread wheat (Figure 5.6A) and its ancestors (Figure 5.7A) grown under control or salinity conditions (Section 2.3.8). To quantify the AOX protein, the stain-free technique was used instead of the traditional loading control (Porin), since this is the most accurate loading control approach (Dittmer & Dittmer 2006). A comparison study of both methods, the stain-free technique and the traditional loading control, is presented in the previous chapter (Appendix C8). Numerous studies have shown that the monoclonal antibody (AOA) was successfully used to detect AOX protein across many plant species, including wheat (Jacoby et al. 2010; Sugie et al. 2006; Takumi et al. 2002; Wanniarachchi et al. 2018).

In this study, the AOA antibody was used to confirm the changes in AOX proteins in response to salinity in bread wheat and its ancestors. To achieve an accurate estimation, an equal amount of total extracted tissue protein was loaded in each lane (Section 2.3.8). For bread wheat cultivars, the expected size of AOX in its reduced form is ~36 kDa (Figure 5.6A). Immunoblot analysis confirmed that salinity has differing effects on the AOX protein content in sensitive and tolerant cultivars. The quantity of protein AOX was calculated by using Bio-Rad Image Lab software (Section 2.3.8). The quantification analysis confirmed that AOX significantly increased compared with control in the salt-sensitive wheat, Chinese Spring (2-fold change), and that the increase was lower in the more tolerant cultivar, Opata M85, where it did not significantly change. (Figure 5.6C). In the wheat ancestors, an AOX in reduced form is shown at ~36 kDa, as a single band (Figure 5.7A). The quantification analysis corresponded to the increased patterns noted in the transcript abundance of wheat ancestors. The highest increase in AOX protein in response to salinity was found in *T. urartu* (1.4-fold), followed by *T. turgidum* ssp. *dicoccoides* (1.14-fold), *Ae. tauschii* (1.1-fold) and *Ae. speltoides* (1.1-fold change) (Figure 5.7C). Comparing the association between the change in AOX capacity and the change in AOX protein levels revealed an *r*-value of 0.17. This indicates a weak positive correlation, which suggests that AOX is regulated at a post-transcriptional level (Figure 5.8)

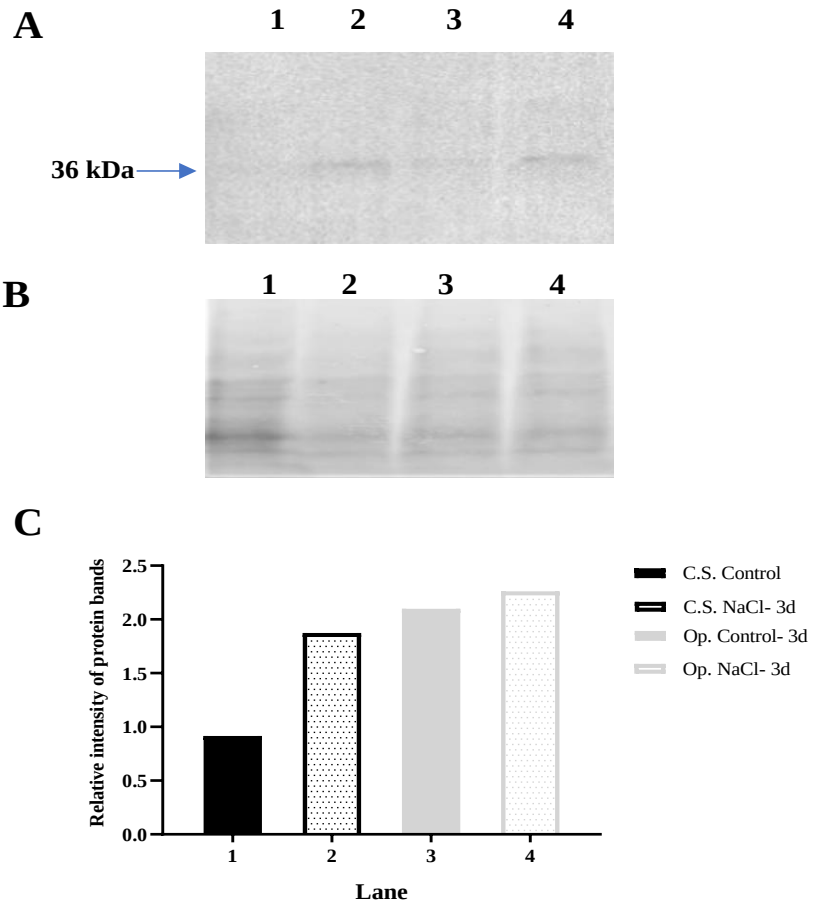

Figure 5.6: Western blot of AOX protein in shoot tissue from wheat under salinity stress using stain-free method as loading controls

20 µg of total protein extracts from shoot of bread wheat cultivars Chinese Spring and Opata M85.

A) Detection of AOX by the monoclonal AOA antibody

The reduced (monomer) band is indicated by arrow (36 kDa)

B) Stain-Free membrane after transfer

Total protein was quantified by the stain-free (SF) technique in the membrane after the transfer, which was used as a loading control.

C) Quantification of AOX Western blot

1: *T. aestivum* cv. Chinese Spring under control conditions

2: *T. aestivum* cv. Chinese Spring under 150mM NaCl for 3 days

3: *T. aestivum* cv. Opata M85 under control conditions.

4: *T. aestivum* cv. Opata M85 under 150mM NaCl for 3 days

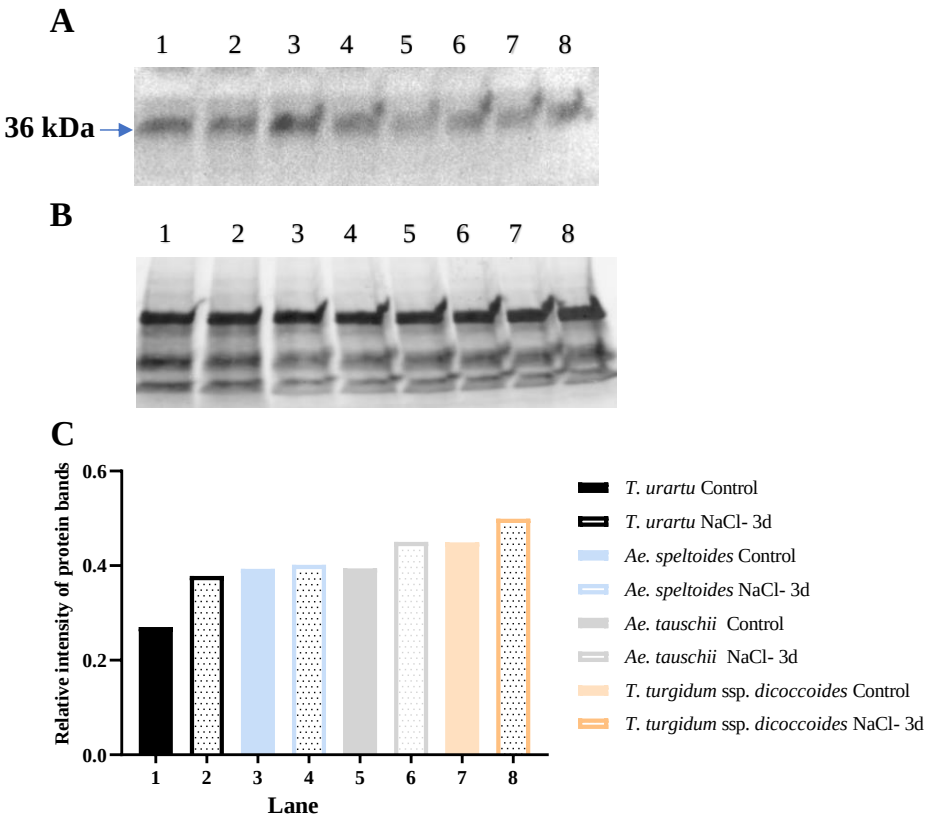

Figure 5.7: Western blot of AOX protein in shoot tissue from wheat progenitors under salinity stress using stain-free method as loading controls

A) 25 µg of total protein extracts from shoot of *Triticum urartu*, *Aegilops speltoides*, *Aegilops tauschii* and *T. turgidum* ssp. *dicoccoides* cv. Daliyya

Detection of AOX by the monoclonal AOA antibody

B) The reduced (monomer) band is indicated by arrow (36 kDa)

Stain-free membrane after transfer

Total protein was quantified by the stain-free (SF) technique in the membrane after the transfer, which was used as a loading control.

C) Quantification of AOX Western blot

1: *Triticum urartu* (acc. IG45626) under control conditions

2: *Triticum urartu* (acc. IG45626) under 150mM NaCl for 3 days

3: *Aegilops speltoides* (acc. AUS.21650) under control conditions

4: *Aegilops speltoides* (acc. AUS.21650) under 150mM NaCl for 3 days

5: *Aegilops tauschii* (acc. AUS-24119) under control conditions

6: *Aegilops tauschii* (acc. AUS-24119) under 150mM NaCl for 3 days

7: *T. turgidum* ssp. *dicoccoides* cv. Daliyya under control conditions

8: *T. turgidum* ssp. *dicoccoides* cv. Daliyya under 150mM NaCl for 3 days

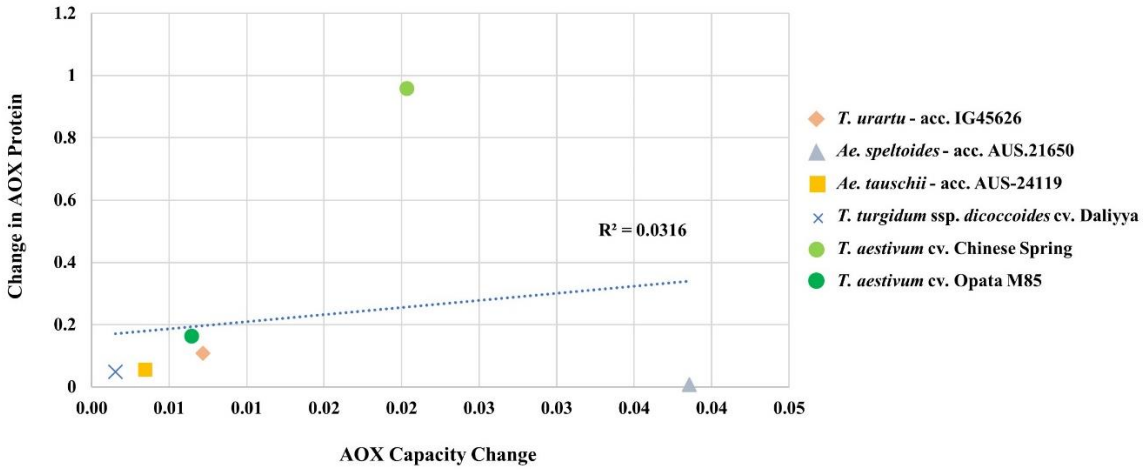

Figure 5.8: Correlation of the change in AOX capacity and in AOX protein levels

Pearson's correlation coefficient (r) was calculated from the r^2 value.

5.3 Discussion

It has been established that there is a correlation between SDW growth and high concentration of Na^+ in the leaf of salt-sensitive cultivars (Greenway & Munns 1980). The wild emmer wheat ancestor *T. dicoccoides* with a relatively high RDW displayed a high degree of salt tolerance and also has increased growth rates (Shavrukov et al. 2010). The cultivar, Chinese Spring, is known to be salt-sensitive from previous research (Colmer et al. 2006; Dvorák et al. 1988; Dvorák & Ross 1986; Jacoby et al. 2013), and the current research found that Chinese Spring was salt-sensitive with a low RDW. Opata M85 has been considered moderately salt-tolerant, and yet here, the percentage of RDW was 83%, which according to Rao et al.'s (2013) scale would mean it is highly salt-tolerant. The comparative results of the four ancestors in terms of RDW and STI showed that *T. urartu* was the only salt-sensitive ancestor, which confirmed the findings of Gorham et al. (1991) and Shavrukov et al. (2009). The other three ancestors in this study, *Ae. speltoides*, *Ae. tauschii* and *T. dicoccoides*, showed high levels of salt tolerance based on their respective STIs in this study.

For *Ae. speltoides*, the STI was the highest of the ancestors studied at 88% and had an RDW of 88.3%. These results are similar to those published by Noori (2005), which that stated *Ae. speltoides* was salt-tolerant and that it had the potential to be the genetic source for salinity tolerance as suggested by Ahmadi et al. (2018). Further, it answers the question posed by Colmer et al. (2006) that *Ae. speltoides* needed to be confirmed as salt-tolerant. This was because Farooq et al. (1989) and Gorham et al. (1991) had found poor survival and tolerance

to salt in *Ae. speltoides.* It also supports the proposed hypothesis published by Wang et al. (2003b) that *Ae. speltoides* was the potential source for the Ph^I line, which was salt-tolerant, as the cultivar AJDAj5. Wang et al. (2003b) proposed that *Ae. speltoides* is the closest related species as the B genome donor for *T. aestivum,* and since the Ph^I line is believed to have an ABD genomic sequence, which is similar to *T. aestivum.*

T. dicoccoides recorded the second highest STI at 80% and an RDW of 80 % among ancestors. These findings concur with those of Shavrukov et al. (2010), who reported a 90% RDW at 100 mM salt and 60% at 200 mM. To determine a reasonable comparison, it was necessary to calculate an average. It can be calculated at approximately 75% by adding and dividing the two numbers, which approximately assumes a concentration average of 150 mM over the 10 days. It should be noted that this research was conducted over three days, and the difference between the findings of both studies of 5% could be explained by the longer exposure period of the comparative study. Shavrukov et al. (2010) also noted significant variability within the various *T. turgidum* spp. *dicoccoides* genotypes examined according to the factors measured, including RDW, growth rate and Na^+ accumulation.

The RDW of *T. turgidum* spp. *dicoccoides* Daliyya, of 80% in this research, and it would suggest that this accession had a higher salt tolerance due to the exposure time, which was less, as compared with the five accessions, which had a longer exposure time in the study by Shavrukov et al. (2010). Accession Daliyya had the lowest RDW of the wild emmer accessions as reported by Shavrukov et al. (2010), and yet, it was identified as salt-tolerant by Nevo et al. (1992). Moreover, the average data were based on the 100 mM and 200 mM NaCl application levels and a longer period of exposure (Shavrukov et al. 2010). However, the variances between each of the five accessions of *T. dicoccoides* could have affected the overall mean (Shavrukov et al. 2010). Peleg (2008) showed that several accessions of the wild emmer, including *T. dicoccoides,* are highly drought tolerant. That study, coupled with both this research and previous studies, have shown an increased salt tolerance based on RDW (Shavrukov et al. 2010). This suggests that there could be a correlation between plant responsiveness to drought and salt tolerance for *T. dicoccoides*.

The *T. turgidum* ssp. *dicoccoides* cv. Daliyya had a leaf Na^+ concentration level of 176 ± 23 mM under 150 mM NaCl, which was the highest in the range studied. In comparison, a previous study recorded 172 mM for 100 mM NaCl and 217 mg NaCl for 200 mM NaCl salinity reported in the same accession (Shavrukov et al. 2010). If the two published figures are averaged, the

concentration in leaves of cv. Daliyya under salt stress is 195 mM NaCl (Shavrukov et al. 2010). Therefore, the results in this study were within the same range as previously published (Shavrukov et al. 2010). The comparative similarities in the level of Na^+ concentration would exclude this genotype as being salt-tolerant based on the Shavrukov et al. (2010) determination since the value is not in the low-to-moderate concentration level range. However, Shavrukov et al. (2010, p. 434) stated that the best salt-tolerant genotypes had 'high growth rate/SDW production under control conditions, high RDW and low-to-moderate Na^+ accumulation'. The plant exhibiting the increased production of shoot biomass, Na^+ accumulation could be relatively low to moderate (Shavrukov et al. 2010). Their conclusions noted that Daliyya had the highest Na^+ accumulation and was unlikely to be salt-tolerant (Shavrukov et al. 2010). Given that growth rate and a high RDW were the most essential factors in determining salt tolerance, the measurement of Na^+ should not be considered so important in determining Daliyya's salt tolerance level (Shavrukov et al. 2010). Further, given that significant reductions in growth can occur before levels of Na^+ attain phytotoxic levels (Albacete et al. 2008; Munns & Tester 2008) this would suggest that the cumulative effect of Na^+ accumulation is not correlated to SDW. This then implies that other factors, such as Cl^-, could have a significant role in SDW growth rate (Greenway & Munns 1980; Tavakkoli et al. 2010).

The measurement of RDW is one of the three criteria required to determine salinity tolerance (Shavrukov et al. 2010). The other two criteria are normal or rapid growth in terms of SDW biomass, and low-to-moderate Na^+ accumulation in leaves (Shavrukov et al. 2010). However, it should be noted that 'A combination of two key factors (normal or rapid growth rate and high RDW) could provide evidence for genuine tolerance to salinity' (Shavrukov et al. 2010). Therefore, it can be considered that high concentrations of Na^+ in the leaves are not an indication of salt tolerance, as suggested previously (Munns & Gilliham 2015; Munns & James 2003). This can bring us to the question about the relative importance of Na^+ accumulation in the leaves as a criterion, as suggested by Shavrukov et al. (2010). Further, the measurement of Na^+ and thus also the K^+/Na^+ ratio may not be a valuable indicator for determining salinity tolerance as confirmed earlier (Munns & Gilliham 2015; Munns & James 2003), whereby the focus should be on the growth rate and RDW as concluded by Shavrukov et al. (2010).

The efficiency of Na^+ exclusion is believed to be the reason that *T. aestivum* is more salt-tolerant than durum wheat (Colmer et al. 2006). However, this viewpoint would seem to be in question based on the hypothesis suggested above. It is known that barley is a more salt-tolerant

crop and has similar Na^+ concentration levels in the leaf as durum wheat, a salt-sensitive crop (Genc et al. 2007; Maas 1986). Since barley and durum wheat accumulate more Na^+ in the leaf, despite salt tolerance of barley plants, it can be deduced that the accumulation of Na^+ is not a significant factor in a crop's overall salt tolerance when evaluated separately. This would confirm the proposed hypothesis that Na^+ is not a good indicator of salt tolerance and affirms the assertion made by Ashraf and McNeilly (1988), or that there may only be a weak correlation (Hollington 2000; Huang et al. 2006). The current study has shown that both Daliyya and *Ae. tauschii* had the highest levels of Na^+ and yet, both genotypes are known to be salt-tolerant (Nevo et al. 1992; Nevo et al. 1993; Shavrukov et al. 2010; Shavrukov et al. 2009). Thus, this researcher's findings support the assertion that the relatively high levels of Na^+ do not reflect the salt tolerance capability.

For *Ae. tauschii*, the results here support Shah et al. (1987) and Schachtman et al. (1991, 1992). The same accession (AUS 24119) for *Ae. tauschii* used earlier was used, and the Na^+ concentration of 132 ± 35 mM was found in leaf sap (Shavrukov et al. 2009). In this research, the Na^+ concentration of 113.7 ± 13.5 mM was found. The increased salinity concentration can be explained by the exposure time, which was 10 days for Shavrukov et al. (2009) and only three days in this study. In both studies, *Ae. tauschii* accession AUS 24119 was subjected to a saline solution of 150 mM. There was significant variation between the 16 *Ae. tauschii* accessions, which ranged from 58 to 585 mM Na^+ with an average of 213 mM Na^+ (Shavrukov et al. 2009). Their findings indicated that accession AUS 24119 had the fourth-lowest concentration and was thus in the upper quartile of salt tolerance (Shavrukov et al. 2009). Given the current study's findings of a similar mM Na^+ concentration when adjusted for the SEM at the low end of the range (113.7 ± 13.5 mM Na^+ compared with 132 mM ± 35 mM Na^+ in leaf sap), the difference was insignificant, thus confirming that this accession *Ae. tauschii* (AUS 24119) is salt-tolerant.

This current research used the same *Ae. tauschii* accession AUS-24119 as previously published (Shavrukov et al. 2009), with 132 mM of Na^+, 267 mM of K^+ concentrations and a K^+/Na^+ ratio of 2.0. Using the same accession, the results in this study yielded similar findings of 113.8 mM of Na^+, 246.6 mM of K^+ and a K^+/Na^+ ratio of 2.4. From this comparison, *Ae. tauschii* accession, AUS-24119, confirmed the upper part of salinity tolerance among the 16 accessions studied (Shavrukov et al. 2009). The presented results on Na^+ and K^+ accumulation in leaves

and K^+/Na^+ ratio are similar to that published earlier (Munns & James 2003) and their conclusion that *Ae. tauschii* is known to be a salt-tolerant species.

Munns and Gilliham (2015) noted that salt-sensitive species would exhibit low concentration levels of Na^+ in the leaves. This research found that cv. Chinese Spring had the lowest levels at 18.6 mM and is known to be salt-sensitive (Colmer et al. 2006; Dvorák et al. 1988; Dvorák & Ross 1986; Jacoby et al. 2013). Shah et al. (1987), Gorham et al. (1991) and Colmer et al. (2006) also noted that *T. aestivum* exhibited low Na^+ with high K^+ concentration levels, which are similar to the findings of this research study for both varieties, Chinese Spring and Opata M85. However, taking into account that Na^+ and K^+/Na^+ are not good indicators of ST, this suggests the low importance of these two measurements in determining the value for assessing salt tolerance in *T. aestivum*.

It has been noted that low K^+/Na^+ ratios occur for both genotypes, *Ae. speltoides* and *T. dicoccoides* (Colmer et al. 2006; Gorham et al. 1991; Shah et al. 1987), consistent with this research. Munns and James (2003) stated that there was no association between salt tolerance and K^+/Na^+ ratio, even though a lower ratio had previously been associated with a higher salt tolerance (Chhipa & Lal 1995). Further research by Genc et al. (2007) using *T. aestivum* also found no correlation between these two properties, supported by the findings from this research. The results of this research confirm data published by Munns and James (2003) and Genc et al. (2007), but it contradicts the assertions made by other studies (Chen et al. 2007; Dvorák et al. 1994; Gorham et al. 1987, 1997).

The K^+/Na^+ ratio for *T. urartu* in this research was found to be 11.1 ± 3.2 in a range between 7.9 to 14.3, similar to those defined by Gorham et al. (1991). Similar results were found earlier with 22 studied accessions with an average K^+/Na^+ ratio of 11.4 ± 3.1. (Shavrukov et al. 2009), compared with the single accession used in the current study. Despite the difference in the number of studied accessions and the probable variations in Na^+ and K^+ levels among accessions, perhaps there is limited variability of the K^+/Na^+ ratio in plants of *T. urartu* species.

The weak *r*-value that was calculated for the correlation between SDW and Na^+ indicates the low association between the effects of salt on SDW growth and the concentration levels of NaCl in the leaves studied. This would confirm Genc et al.'s (2007) finding that there was no correlation between maintaining growth rate and Na^+ exclusion in the leaf. Given that there was only one accession for each species studied, the individual *r* values for each species could

not be computed. If there were more accessions for each species, then the individual *r* values for each could have been correlated, which might reveal more accurate associations of the effects of Na^+ on SDW for those known salt-sensitive and tolerant genotypes.

MDA significantly increased when subjected to salinity, which induces an osmotic stress response (AbdElgawad et al. 2016). Khan and Panda (2008) noted that there was variability in the levels of lipid peroxidation between salt-sensitive and salt-tolerant cultivars. Lower levels of lipid peroxidation, as shown by MDA content levels, are associated with salt tolerance (Borzouei et al. 2012). This has been interpreted as reflecting different response mechanisms associated with each cultivar. Low levels of peroxidation are an indicator of a plant's coping mechanism to reduce oxidative damage after salinity stress, whereby growth can be sustained when subjected to salt (Borzouei et al. 2012). It is known that plants subjected to salinity stress induce higher levels of MDA (AbdElgawad et al. 2016; Kumar et al. 2017).

Previously, it was reported plants of *T. urartu* subjected to 100 mM NaCl rapidly suffered senescence (Shavrukov et al. 2006). Some of the 22 accessions of *T. urartu* studied showed symptoms of salt toxicity even after exposure to only 50 mM NaCl (Shavrukov et al. 2006). The current study used a 150 mM NaCl solution, and the salt-sensitive ancestor *T. urartu* showed a significant increase in MDA content, suggesting an inability to cope with osmotic stress and suffer oxidative damage. This affirms that *T. urartu* is salt-sensitive.

The three wild ancestors, *Ae. speltoides*, *Ae. tauschii* and *T. turgidum* ssp. *dicoccoides* Daliyya, are all considered salt-tolerant (De León et al. 2011; Munns 2002; Nevo et al. 1992; Nevo et al. 1993; Noori 2005; Shavrukov et al. 2010). In this study, there was no significant change in MDA content level for the three ancestors under salt treatment. However, it should be noted that this study only used three replicates, and to determine greater statistical significance, further investigation would require more replicates. It is also known that MDA levels *in T. aestivum* and pea plants increase significantly when subjected to a high concentration of NaCl (Borzouei et al. 2012; Moran et al. 1994; Rao et al. 2013; Taibi et al. 2016; Zou et al. 2016). Knowing that MDA content levels vary between salt-sensitive and salt-tolerant genotypes, it can suggest the presence of different coping mechanisms under salt stress based on the studied genotype (Khan & Panda 2008).

The total respiration response is linked to salt tolerance in plants. Respiration rates have been shown to differ between cultivars and can be significant, particularly for *T. aestivum* genotypes

(Moud & Maghsoudi 2008). The impact of salinity induces alterations to the respiratory rate, which correlated with a reduction in growth rate, due to osmotic stress, for example, in the leaf of *Sorghum bicolor* (McCree 1986). In the case of durum wheat, significant decreases in respiration rates have been observed under salt stress (Flagella et al. 2006). The osmotic component of salinity stress is considered to have a less toxic effect than that of the ionic effects of salinity stress (Jacoby et al. 2011). In the current study, both bread wheat genotypes showed decreased respiration with a mean rate of 38.7%. Opata M85 had a more significant decrease of 60%, whereas cv. Chinese Spring decreased by 17%. Che-Othman et al. (2019) most recently noted that salt-treated wheat leaves had significantly higher O_2 consumption after 10 days and exhibited a 10-fold increase in Na^+ accumulation. Those findings contradict the results presented in this current research. However, the length of exposure to salinity has resulted in the elevated Na^+ concentration level that has correspondingly increased toxicity and thus respiration rate.

An apparent contradiction among observations of the changes in respiratory rates on salt treatment is evident from the results of numerous studies (Carillo et al. 2008; Kafi 2009; Kasai et al. 1998; Kong et al. 2001; Rawson 1986). Rawson (1986) found that there was a significant decrease in respiratory rate of wheat under differing concentration levels of NaCl, including 150 mM. Rawson (1986) noted that wheat showed a lower respiratory rate than barley and that there was a correlation between the increased concentration of NaCl solution and the decrease in respiratory rate. Kasai et al. (1998) reported that when cv. Chinese Spring was subjected to a 400 mM NaCl solution, there was a significant decrease in photosynthetic CO_2 fixation when calculated using the rate of O_2 evolution. Kasai et al. (1998) reported that the total O_2 consumption rate for cv. Chinese Spring was increased due to salinity stress, which had increased the level of activity in the cytochrome pathway.

The difference in findings of Kasai et al. (1998) could be attributed to the high concentration of applied NaCl. This statement can be confirmed by the findings of Kong et al. (2001), who reported differing respiratory rate results for a salt-sensitive cultivar and salt-tolerant wheat cultivars. Moreover, the variation has been found within one salt-sensitive cultivar based on the levels of salinity stress. For the salt-sensitive cultivar, the respiratory rates initially increased with a 0.5% NaCl but immediately started decreasing after the application of 1% to 2% NaCl (Kong et al. 2001). In contrast, the salt-tolerant cultivar did not show an appreciable change in respiratory rate during elevated concentration of applied NaCl (Kong et al. 2001).

This suggests that salt-sensitive cultivars suffer from increased oxidative damage caused by higher Na^+ accumulation resulting in a decrease in respiratory rate. This supports the findings in the current research for the salt-sensitive cultivar, Chinese Spring, but does not explain the results for the moderately salt-tolerant cultivar, Opata M85.

Carillo et al. (2008) reported a 1.2-fold increase in respiration in durum wheat under salt stress, particularly in younger leaves. In the present research, the respiration in the wild ancestor *T. turgidum* ssp. *dicoccoides* (Daliyya) showed an unchanged level of respiration in the seedlings.

The change in the respiration in Daliyya could be explained by the concentration of 300 mM NaCl over a 10-day exposure, which resulted in a significant increase in respiratory rate. In addition, Carillo et al. (2008) used *Triticum durum* Desf. cv. Ofanto, a known drought-sensitive cultivar as compared with Daliyya, a relatively salt-tolerant cultivar. Thus, the difference could be explained by the relative sensitivity to toxicity. *Triticum durum* Desf. cv. Ofanto is sensitive, and hence, this reduces the uptake of water caused by NaCl concentration, which in turn causes abiotic stress in the form of physiological drought condition resulting in an increased respiratory rate. Flagella et al. (2006) used the same genotype *Triticum durum* Desf. cv. Ofanto and found that there was a significant decrease in respiratory rates under seawater stress. These results concurred with those of Trono et al. (2004) that the respiratory rates in *Triticum durum* Desf. cv. Ofanto were decreased based on oxygen uptake and toxicity levels.

The sodium toxicity of seawater compared with that of pure NaCl would unquestionably induce toxic effects within plants. Hence, it can be inferred that higher concentrations of salt will have a severe, rapid effect on plant respiratory rates. For example, Kafi (2009) reported no change at 200 mM salt, but a decrease in respiratory rates for three wheat cultivars at 300 mM. Kafi (2009) studied two known salt-tolerant types and one sensitive type. All three cultivars decreased in respiratory rates from 0-100 mM salt treatment. However, one salt-tolerant cultivar increased the respiratory rate from 100 mM to 200 mM salt, whereas one salt-tolerant and the salt-sensitive cultivar both remained unchanged under the same 200 mM. At 300 mM, all three cultivars experienced significant decreases (Kafi 2009).

The unpredictability of the effects of salinity stress on respiratory rate indicates that the lack of clear-cut correlation between these two factors. Some cultivars have shown increases and decreases within the same experiment, dependent on the levels of NaCl treatment (Kafi 2009). In several instances, results in one study have contradicted those of another. Not only the levels

of NaCl concentration, but also the exposure time and speed at which salt absorption occurs can greatly influence respiratory rates. In case of increased respiratory rates, the expended carbon is unavailable to be used for synthesis and thereby limits new growth (Poorter et al. 1990). Some cultivars show decreases in respiratory rates when subjected to salinity stress (Flagella et al. 2006; Kafi 2009; Kong et al. 2001; Rawson 1986; Trono et al. 2004). Hence, it might be concluded that even taking into account differing levels of NaCl toxicity, other mechanisms within the plant could be working to reduce the effects of salinity. For example, the use of stored carbon to increase tissue tolerance. The variability in respiratory response to salinity stress suggests that a multitude of factors are contributing to salt tolerance; thus, further investigation is required to understand the mechanisms that aid in reducing the effects of salt stress.

AOX plays a significant role by increasing the AOX capacity in response to salinity stress (Hilal et al. 1998; Jolivet et al. 1990). Plants undergoing salinity stress that are able to increase AOX capacity could maintain a better growth rate and also reduce the level of ROS (Smith et al. 2009). Further, Na^+ accumulation in leaves was significantly lower when AOX capacity was increased (Smith et al. 2009). It is known that AOX capacity increases in a variety of plants on salt treatment, such as rice, chickpea, soybean, peas, tobacco, *Arabidopsis* and durum wheat (Andronis & Roubelakis-Angelakis 2010; Marti et al. 2011; Pastore et al. 2001; Ribas-Carbo et al. 2005; Smith et al. 2009; Sweetman et al. 2018; Wanniarachchi et al. 2018). The present research confirms that of the species studied, *T. urartu, Ae. speltoides* and *T. aestivum* cv. Chinese Spring all showed a change in AOX capacity.

Salt stress in several plant species, including *Arabidopsis,* has resulted in the induction of AOX transcript (Kreps et al. 2002; Seki et al. 2002), and ethylene can also play a role in inducing AOX activity (Vanlerberghe 2013). In addition, salinity induces oxidative stress in *Arabidopsis* and AOX transcript response will increase both capacity and protein levels as a means of protection (Smith et al. 2009). AOX activity is a responsive measure plants undertake to minimise the effects of salinity stress. For instance, *Arabidopsis* subjected to salinity resulted in increased AOX activity and a corresponding decrease in ROS, which reduced oxidative damage (Smith et al. 2009; Umbach et al. 2005). When *Arabidopsis* leaves were subjected to KCN, this resulted in the chemical inhibition of the cytochrome pathway, whereas up-regulation of *AOX* was directly related to the reduction of ROS and the minimisation of oxidative damage (Umbach et al. 2005). It was reported that there was a substantial reduction

in the cytochrome pathway respiration in pea leaves when subjected to 14-day salinity stress, but that AOX capacity was maintained (Marti et al. 2011). When tobacco plants were affected by abiotic or biotic stress and had suffered respiratory dysfunction, *AOX1* genes were expressed (Clifton et al. 2006). In fact, respiratory conditions have been shown to cause high levels of AOX protein in leaves of tobacco plants (Vanlerberghe et al. 1999).

The findings of the current study showed an increase in AOX capacity for both cultivars of bread wheat. The salt-sensitive cultivar cv. Chinese Spring showed a significant increase of AOX capacity of 64%, which corresponds to an almost fourfold higher level than those reported by Kong et al. (2001) in another salt-sensitive cultivar. The salt-tolerant cv. Opata M85 increased AOX capacity by 23%, a result similar to those presented by Kong et al. (2001). For the ancestors, AOX capacity varied with both *T. urartu* and *Ae. speltoides* and showed significant increases, and *Ae. tauschii* and Daliyya had insignificant changes in AOX capacity. From this result alone, it can be concluded that AOX is responsive to salinity regardless of concentration level. Studies of AOX response to salinity stress in Arabidopsis, barley, soybean, and orange have shown that AOX capacity increased (Ferreira et al. 2008; Hilal et al. 1998; Jolivet et al. 1990; Smith et al. 2009).

Studies have shown that plant growth may be linked to AOX activity (Arnholdt-Schmitt et al. 2006; Vanlerberghe 2013). Salt stress alters AOX protein expression, resulting in an increase in AOX capacity. Rhoads and McIntosh (1992) are the first to publish data on the transcripts from *AOX* gene expression in *S. guttatum*, followed by Vanlerberghe and McIntosh (1994) who published on AOX transcripts in tobacco. They reported that the cytochrome pathway activity response resulted in changes to *AOX1* gene expression, which affected the alternative pathway capacity (Vanlerberghe & McIntosh 1994). Exploration of the *AOX* gene expression continued with studies being conducted in rice, *Arabidopsis* and soybean (Finnegan et al. 1997; Ito et al. 1997; Saika et al. 2002; Saisho et al. 1997).

Abiotic stress tolerance in plants can be regulated through *AOX* gene expression (Fiorani et al. 2005; Giraud et al. 2008; Murakami & Toriyama 2008; Panda et al. 2013; Smith et al. 2009; Sugie et al. 2006; Wang et al. 2011; Wang & Vanlerberghe 2013). For example, the effects of cold stress on *Arabidopsis thaliana* was related to AOX activity, which can be vital in determining shoot acclimation (Fiorani et al. 2005). AOX overexpression can lead to decreased ROS when subjected to cold temperatures, and therefore, plant growth may be improved (Fiorani et al. 2005). Smith et al. (2009) reported that plants subjected to salinity stress could

increase AOX capacity. Further, when synthesised, the overexpression of *Ataox1a* gene showed a decrease in Na^+ accumulation in the leaves, lower concentration of ROS and a corresponding improvement in plant growth rates of 30–40% (Smith et al. 2009). Their study on *Arabidopsis* suggested that increased AOX activity in shoots and roots corresponded to better salinity tolerance (Smith et al. 2009). They concluded that AOX played a vital role in how plants adapt to abiotic stress, particularly when induced by salinity.

Takumi et al. (2002) are the first to isolate *WAox1a* and *WAox1c* (renamed as *TaAOX1a-2AL* and *TaAOX1c-6AL*, respectively) that encode AOX proteins in *T. aestivum. WAox1a* transcript increased when the plant was subjected to chemical treatment with KCN chemical treatment (Takumi et al. 2002). They concluded that various stress conditions would result in differing regulation of *AOX1* genes in *T. aestivum* (Takumi et al. 2002). Feng et al. (2013) noted the lack of studies on *Aox1a* or *Aox1c* conducted in wheat under salinity, drought, light stress, heat stress, ozone effects, metal toxicity or nutrient limitation.

At the transcriptional level, the current study showed that wheat and wild ancestors' *AOX1* isoforms were stress-responsive under salinity stress in both shoots and roots. Of these, *AOX1a* homologues and *AOX1d2* homologues were the most responsive isoforms in both diploid ancestors *T. urartu*, *Ae. speltoides*, and *Ae. tauschii*, and a tetraploid ancestor, *T. turgidum* ssp. *dicoccoides*. The exception was *AOX1d2-2DL* which appeared to be less responsive than *AOX1d1-2DL,* in both the diploid ancestors, *Ae. tauschii* and the wheat standards, Chinese Spring and Opata M85. In contrast, *AOX1c* homologues were expressed at very low levels in both shoots and roots. Similar findings were reported by Wanniarachchi et al. (2018) for barley, a *Triticeae* member, who found that *HvAOX1d1* and *HvAOX1d2* were highly responsive to salinity stress. In addition, *OsAOX1a* and *OsAOX1d* were the highest responsive isoforms found in rice, and *AOX1c* was not stress-responsive in either rice or barley (Wanniarachchi et al. 2018).

Gene expression can be affected by various factors, such as *cis*-acting regulatory elements (CREs), gene structure and single-nucleotide polymorphisms (SNPs) (Goebels et al. 2013; Heyn et al. 2015; Nogales et al. 2016; Oswald & Oates 2011). The promoter analyses discussed in Section 3.2.8 revealed that the most well-known CREs, which play a role in response to environmental stress, were present in the *AOX1* gene family in both bread wheat and wild ancestors' species (Figure 3.12). In addition, some varieties were observed in terms of the number of CREs that were involved in the developmental and environmental responses among

AOX1 isoforms and between *AOX1* orthologous genes. The promoter of both wheat and its ancestors had CREs that effect AOX expression, such as the positive regulator WRKY, DNA-Binding Protein 63, AtWRKY63, ANAC017 (Van Aken et al. 2013; De Clercq et al. 2013).

Gene structure is viewed as a vital regulator since both the length and number of exons and introns play an important role in influencing gene expression (Heyn et al. 2015). For instance, a plant gene with fewer introns has an advantage in responding rapidly for short cell cycles (Das & Bansal 2019). Conversely, plants with genes that have long introns will be at a disadvantage by having a delayed response (Heyn et al. 2015). Introns not only perform a regulatory response in gene expression but also affect gene transcription (Brinster et al. 1988). Sequence differences in nucleotides within a gene, such as insertions, deletions and SNPs, can influence gene expression.

Variation in nucleotides of AOX has been detected among the wheat and its ancestors' genes, including variation in the N-terminal, coding regions and intronic regions (Section 3.2.2.3). Notably, nearly all AOX1-predicted proteins identified in this study have CysI, CysII and leucine at the CysIII position. The exception was the AOX1d clades, where AOX1d1 and AOX1d3 homologues have serines at the CysI and CysII positions, while AOX1d2 only had serine in the position of CysII. It is known that CysI, CysII and CysIII are involved in AOX regulation. Selinski et al. (2017) reported that when the cysteine residue (CysII) was replaced by Ser (CSL), the expression of *AtAOX1d-CSL* increased. The double substitutions in CysI and CysII (AOX1D-SSL) revealed decreased activities compared with single substitutions. In *AOX1d1* and *AOX1d3* derived proteins in wheat and its ancestors, activities were lost, presumably due to double substitutions in CysI and CysII whereas the *AOX1d2* protein activity was increased.

A study of the effects of low temperature was conducted on two wheat cultivars, one cold-tolerant and one sensitive (Mizuno et al. 2008). It measured the effects of low temperature on the mitochondrial respiration activity and whether AOX was expressed (Mizuno et al. 2008). It was found that in the tolerant cultivar, the alternative pathway respiration capacity increased significantly, and there was an increase in AOX protein as compared with that of the sensitive cultivar (Mizuno et al. 2008). In the present study, consistent with the findings at the transcript level and AOX capacity, immunoblot analysis revealed that AOX abundance varied between the salt-sensitive and tolerant species. The commercial bread wheat cultivars also had higher AOX protein levels than the ancestors under control conditions. The AOX abundance was

higher in the salt-sensitive species *T. urartu* and Chinese Spring than in the tolerant ancestors *Ae. speltoides, Ae. tauschii,* and *T. turgidum* ssp. *dicoccoides,* and *T. aestivum* Opata M85. These findings are consistent with the proteomic studies by Jacoby et al. (2010; 2013), who studied both salt-tolerant and sensitive wheat cultivars under salinity stress and found that AOX response was different between cultivars. The 2010 study that compared the tolerant wheat cultivar Wyalkatchem against the sensitive cultivar Janz indicated that there was a significant change between cultivars from control to treated (Jacoby et al. 2010). The AOX abundance in Wyalkatchem, the tolerant cultivar, was significantly less when treated was compared with control, than that of the sensitive cultivar Janz. AOX abundance of Wyalkatchem increased by approximately 25% compared with that of Janz, which increased by 33%, which reflects a 33% difference in response between cultivars. The increase suggested that AOX expression was higher in the sensitive cultivar when subjected to salinity stress (Jacoby et al. 2010). However, it should be noted that the control level of AOX abundance in Wyalkatchem was almost twofold that of Janz, which thus had a higher base amount of AOX before being exposed to salinity. In the current study, a similar observation was made between Chinese Spring (sensitive) and Opata M85 (tolerant).

In a different study, salt-sensitive wheat cv. Chinese Spring was compared with the tolerant cv. AMP cultivar and both were exposed to salinity. Similar results were obtained, namely, that the tolerant cultivar had a higher amount of AOX abundance compared with the sensitive cultivar (Jacoby et al. 2013). The cultivars, when exposed to salt, similarly had different responses. The increase in the salt-tolerant AMP was less than that in the sensitive cultivar cv. Chinese Spring, which showed a significant increase of more than doubling its original AOX abundance (Jacoby et al. 2013). While the AMP response to salinity showed a greater growth tolerance than that of cv. Chinese Spring, it illustrates that the increased presence of AOX under control conditions could be a determining factor of salinity tolerance. The increased abundance of AOX in the tolerant cultivars suggests some unknown biological mechanism to respond to osmotic stress and thereby indicates some protein-level response to salt. This significant difference gives those tolerant cultivars a distinct advantage in responses to salinity stress. Further investigation is needed to determine which particular AOX protein(s) contributes more to salinity tolerance, by using mass spectrophotometry to obtain the protein sequence (Aebersold & Mann 2016). In the current study, AOX abundance was studied by examining the monomer under reducing conditions only. However, a future investigation should examine possible post-translational regulation of AOX under salinity.

To the present researcher's best knowledge, this chapter serves to present and discuss for the first time the comparative findings on *AOX* gene expression in two *T. aestivum* cultivars and four direct wild ancestors. The presented research found that *AOX* was salt-responsive across all species studied. The level of *AOX1* expression differed between species and *AOX* isoforms. The two most highly expressed *AOX* genes were *AOX1a* and *AOX1d2* across all species. The wild ancestor *T. urartu* had the highest expression of *AOX1d2*, followed by *AOX1a*. This indicates that those two AOX isoforms found in the wild ancestor *T. urartu* almost certainly integrated into *T. aestivum*. The next highest expression was found in the species cv. Chinese Spring. This research, along with other studies, has shown that both *T. urartu* and cv. Chinese Spring are both salt-sensitive. The findings suggest that the elevated *AOX* expression in both sensitive species under-treated conditions reflects the role of AOX in the plant's attempt to minimise ROS when subjected to salinity stress. In contrast, tolerant species exhibited higher pre-existing AOX protein levels than salt-sensitive species, which gives salt-tolerant species an advantage in coping with oxidative stress.

Chapter 6: The Main Conclusions and Future Research

6.1 Introduction

The overall aim of this research was to identify and characterise the *AOX* gene family in *T. aestivum*. In more detail, it sought to (1) identify the gene candidates in commercial bread wheat; (2) assess which of these genes, when exposed to chemical and a biological stress, such as salinity, had a stress response; and (3) assess *AOX* gene family members and expression to determine differences, if any, between *T. aestivum* and its wild ancestors during its evolution. The purpose of undertaking this research was to increase the understanding and knowledge of how *AOX* genes can increase stress tolerance in wheat by minimising ROS production, thereby potentially increasing crop yields.

Chapter 1 provided a literature overview and outline of the *Poaceae* taxonomy and genomes. Chapters 2 discussed the methods and materials used to conduct the experimentation, and Chapters 3, 4 and 5 presented the investigations in detail. Chapter 3 identified and characterised the expression of *AOX* gene family members in *Triticum* and *Aegilops* species and their discovery into a recent historical context. Chapter 4 specifically investigated AOX expression in *T. aestivum* to measure the response to real-world abiotic stress. Chapter 5 examined the expression of *AOX* genes in two bread wheat cultivars, Chinese Spring and Opata M85, and the likely ancestral species, of *T. aestivum* when exposed to salinity stress.

6.2 Significant Findings

6.2.1 A New *AOX* Subfamily Identification in *Triticum* and *Aegilops* Species

This research undertook to identify and characterise *AOX* gene families in *Triticum* and *Aegilops* species allowing evaluation of their evolution, by assessing their classification, genomic structure and expression. A comparative assessment of all AOX sequences was conducted to explore the similarities and differences at the *AOX* transcript and protein level between *Triticum* and *Aegilops* species. The first significant finding was the identification of 58 novel intact *AOX1* subfamily genes in *T. aestivum, T. turgidum, T. urartu, T. monococcum, Ae. speltoides, Ae. sharonensis and Ae. tauschii*. Further, 31 novel partial/like *AOX1* subfamily genes were found in these species. Thus, this research has shown that AOX exists in a relatively large number of isoforms in both the *Triticum* and *Aegilops* species**.**

Moreover, this study is the first to prove the expansion of the AOX1-subfamily genes. *T. aestivum* has three homologues forms, except for *AOX1d3* and *AOX1e*. The intact *AOX1*-subfamily contained three times the number of *AOX* genes than occur in the diploid donors, which indicates that AOX1 was retained after two rounds of polyploidisation. Given Costa et al.'s (2014) proposed hypothesis of convergent gene evolution of AOX, this current study's findings corroborate that the *AOX1d* clade experienced a duplication event and was located in a tandem arrangement with *AOX1a.* These results suggest that tandem duplications played a crucial role in the expansion of the *AOX* gene subfamily in *Triticum* and *Aegilops* species.

Another important finding from this research was the importance of manual annotations of AOX in *Triticum* and *Aegilops* species. In 2014, this thesis identified the accurate gene models of AOX by using manual annotations, which has been confirmed by the current IWGSC 2018 annotation. AOX expression in *Triticum* and *Aegilops* was confirmed by the RT-PCR and by searching the wheat EST and TSA databases at NCBI. AOX protein analysis showed that essential amino acids were necessary for AOX activity, which has been entirely conserved in *Triticum* and *Aegilops*.

6.2.2 Variations of the AOX1 Expression in *T. aestivum* under Abiotic and Biotic Stresses

The analysis of AOX transcript data, *in silico*, identified that the *TaAOX1a* clade and *TaAOX1d2* clade are the most responsive isoforms to abiotic and biotic stresses. This study extended the understanding of *T. aestivum* and the *AOX* gene family, and in particular, of *AOX1* gene expression responses under the three experimental conditions, KCN, AA and salinity, by using qRT-PCR. In the current research, genome-specific primers were successfully designed to study the *AOX1*-subfamily members individually. For the first time, 13 *AOX1* genes in *T. aestivum* were investigated by using qRT-PCR. In addition, the research explored their orthologues in both *Triticum* and *Aegilops* species at the transcript level. The results were consistent with *in silico* findings. Previous research had shown that KCN chemically inhibits the cytochrome pathway in *Arabidopsis* and Tobacco. This research has shown that KCN had a similar effect, inducing *AOX1* at the transcript level in *T. aestivum.*

An *AOX1* gene expression response also occurred when the two *T. aestivum* cultivars, Chinese Spring and Drysdale, were subjected to salinity stress. Despite the significant differences in response between the two cultivars, the findings affirm those of previous research and signify the active role that *AOX1* has as a defensive mechanism against oxidative stress. From this

research, it can be asserted that there is consistency across multiple plant species, *Arabidopsis,* tobacco, carrot, soybean, barley, and now, wheat, in that when subjected to stressful conditions *AOX1* genes are expressed.

Brew-Appiah et al. (2018) published studies suggesting that four *AOX1* genes were classified as non-expressed (*ne*) genes. However, in contrast, this research proved, *in vivo*, that genes of the *TaAOX1d* clade are expressed under all three experiments when exposed to salinity, KCN and Antimycin A stress. Further, *in silico*, this research found that all *AOX1d* clades were expressed in different tissues using the EST and TSA databases.

6.2.3 Variations of AOX1 Expression in *T. aestivum* Ancestors under Salinity Stress

In the current research, the salt-sensitive wild ancestor *T. urartu* had the highest expression of *AOX1d2,* followed by *AOX1a*. It can be concluded that although they may not be the only responsive genes when subjected to stress, the fact remains that these two highly responsive genes are most likely inherited in bread wheat from their wild ancestors. Given that the *AOX1a* and *AOX1d2* genes had the highest expression in both *T. aestivum* and its wild ancestors, and that these two genes have also been found in both rice and barley, it appears that these two genes play a very significant role in stress response. This presumably is because rice, barley and wheat all share a common ancestor. Despite *T. aestivum* and its wild ancestors having varied morphologies and physiologies, AOX has not lost its AOX isoforms and still maintains its expression. Since the AOX response is conserved and that, despite its evolution, AOX has not lost its gene family or its expression, it shows the importance of its role in better growth and yield of commercial cultivars.

6.3 Future Research

There is an opportunity to manipulate *AOX1a,* and *AOX1d2* genes for agricultural and biotechnological purposes, to overexpress the genes in plants and possibly breed plants that have greater stress tolerance. Given that *AOX1a* and *AOX1d2* genes in bread wheat were the most responsive, it presents opportunities to specifically design cultivars that are adaptive for environmental stresses, particularly given the impact of climate change.

The wild ancestors, *T. turgidum* ssp. *dicoccoides*, *Ae. speltoides* and *Ae. tauschii*, were very salt-tolerant. Thus, future research could be extended to study other oxidative stress enzymes, as well as to study *AOX1a* and *AOX1d2* under different abiotic stresses. Recently, the Wheat

10+ Genomes Project released reference sequences of multiple wheat cultivars. Thus, future research could be undertaken to examine polymorphisms in the *AOX* subfamily within bread wheat cultivars and ascertain whether any are associated with beneficial phenotypes.

The research conducted in this thesis has provided valuable information on *Triticum* and *Aegilops* AOXs, enabling comparative research with previous site-directed mutagenesis studies on AOX in angiosperms. This research found that AOX1a and AOX1c had conserved CysI and CysII, whereas *AOX1d* clades in *Triticum* and *Aegilops* had single or double substitutions at CysI and CysII, which were replaced by serine residues. However, most of the *Triticum* and *Aegilops* AOX1d2 orthologues comprised a serine residue at CysI. TmAOX1d2 had CysI and CysII as observed in AOX1a, and AOX1c orthologues. *Triticum* and *Aegilops* possess different forms of CysI and CysII substitutions naturally, a finding which would enrich post-translational regulation studies. Thus, further research could be conducted to verify whether AOX activity is affected by the substitution of CysI and CysII.

Owing to time constraints, the research was unable to investigate AOX1 protein levels in Drysdale, which would have provided a comparative analysis with cv. Chinese Spring. This analysis would have enabled a comparison of AOX1 protein capacity and MDA content levels for the salt-sensitive versus salt-tolerant varieties. In addition, further research could be conducted into the AOX protein of *Triticum* and *Aegilops* species using non-reducing SDS-PAGE and native PAGE analyses, where the mitochondria are subjected to different oxidising and reducing agents. Further research could also be undertaken using various methods to validate the number of *AOX* gene copies by using the Southern blot analysis.

This study demonstrated the importance of manually annotating across the *Triticum* and *Aegilops* species to improve gene model annotations. Given that this study provided accurate manual annotations and the later confirmation by the IWGSC-RefSeq v1.1 annotation, it will not only benefit gene models, but also improve the comprehension of biological systems, and thereby, inform future gene evolutionary studies. As an example, the current Ensembl Plants release 45 from September 2019, still shows incorrect AOX annotations. The manual annotations of the AOX data presented from this research findings will contribute towards improving the gene model annotations of *Triticum* and *Aegilops* species and produce a well-annotated database.

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Appendices

Appendices were combined as a separate PDF file.