Engineering Wheat (*Triticum aestivum* L.) for Abiotic Resilience by Manipulating Small Ubiquitin-Like Modifiers
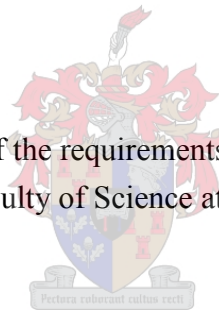
Marlon Luke Le Roux

Dissertation presented in fulfilment of the requirements for the degree Master of Science (M.Sc) in Genetics in the Faculty of Science at Stellenbosch University

Promoter: Professor Anna-Maria Botha-Oberholster

Co-Promoter: Doctor Christell van der Vyver

March 2016

Dedicated to

Luan Africa

Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

December 2015

i

Abstract

Research is becoming increasingly more focused on innovative strategies to improve wheat (*Triticum aestivum* L.) to meet current and future consumer demands. With the number of countries facing extreme climate variability increasing, drastic steps need to be taken to ensure local food security. Although genetic engineering of staple crops has been explored widely, they require challenging platforms with highly efficient explants with enhanced regenerative abilities for callus formation and somatic embryogenesis to consistently yield plantlets with altered attributes. Thus, this study describes the necessary steps to obtain transgenic wheat with relative ease, with the goal of improved abiotic stress tolerance. The developed *in vitro* regeneration and cryopreservation system was then used to introduce genes into bread wheat, namely Overly Tolerant to Salt 1 (*OTS1*) and *OTS2* (both are Small Ubiquitin-like Modifier (SUMO) protease genes) and transcription factor known as Inducer of CBF expression 1 (*ICE1*). The class of molecules have emerged as an influential mechanism for targeted protein management, as SUMO proteases play a vital role in regulating pathway flux and are therefore ideal targets for manipulating stress-responsive SUMOylation. This study thus describes the isolation and cloning of three genes and its manipulation into constitutive and drought inducible vectors. The latter makes use of a unique promoter which was characterized *in silico*, after which it was then applied. Finally this study also demonstrated the compatibility of the vectors within wheat as it was confirmed that genetic modification of wheat was achieved by particle bombardment.

Uittreksel

Navorsing lê toenemend meer klem op die ontwikkeling van innoverende strategieë met die oog op die verbetering van koring (*Triticum aestivum* L.) opbrengste om aan die huidige sowel as die toekomstige behoeftes te voldoen. Met die toenemende klimaat veranderinge wat talle lande in die gesig staar, moet drastiese stappe geneem word om plaaslike voedselsekuriteit te verseker. Alhoewel die genetiese modifisering van stapelvoedsel gewasse al wyd verken is, is daar 'n behoeftes vir uitdagende platforms met die vermoë om begin material (bv. onvolwasse embrios) doeltreffend om te skakel in kallus en somatiese embrios om ten einde deurlopend plante met verbeterde eienskappe te lewer. Dus, die huidige studie beskryf die noodsaaklike stappe wat gevolg moet word om transgeniese koring met relatiewe gemak te verkry met die doel om strestolerasie te verbeter. Die ontwikkelde *in vitro* sisteem, was aangewend om bepaalde gene, naamlik "Overly Tolerant to Salt 1" (*OTS1*) and *OTS2* (beide SUMO protease gene), in broodkoring uit te druk. Dieselfde stappe is gevolg met 'n addisionele transkripsie factor genaamd "Inducer of CBF expression 1" (*ICE1*). Die SUMO ("Small Ubiquitin-like Modifier") klas van molekule het na vore gekom as 'n invloedryke meganisme vir geteikende proteinbestuur, aangesien SUMO protease 'n noodsaaklike rol speel in die regulering van biochemiese pad verloop en is dus ideale teikens vir die manipulering van stress-verwante SUMO-lasie. Hierdie studie handel dus oor die isolasie en klonering van drie gene en hul manipulasie in konstitutiewe en droogte induserende vektore. Laasgenoemde maak gebruik van 'n unieke promoter wat in silico gekarakteriseer is. Laastens word die bruikbaarheid van die vektore in koring gedemonstreer nadat suksesvolle genetiese modifisering bevestig is.

Research Outputs

1) <u>**Submitted manuscript:**</u>

Marlon L. le Roux, Christell van der Vyver and Anna-Maria Botha (2015). *In vitro* regeneration and cryopreservation by encapsulation-dehydration of Southern African bread wheat cultivars (*Triticum aestivum* L.). South African Journal of Botany. Submitted and currently under review (Ref: SAJB_2015_15)

2) <u>**Provisional Patent**</u>:

Marlon L. le Roux, Anna-Maria Botha and Christell van der Vyver (2015). Method of enhancing stress tolerance of monocotyledonous plants with drought inducible vector. Stellenbosch University (Ref: P3053ZA00SCnc)

3) <u>**Conference Contributions**</u>

    a. <u>**National meetings:**</u>

        i. Marlon L. le Roux, Anna-Maria Botha and Christell van der Vyver (**2014**) Cryopreservation of South African spring wheat, using a novel explant. South African/Kenya NRF Bilateral Bioinformatics Workshop. Stellenbosch Institute for Advance Study. <u>(Presentation)</u>

        ii. Marlon L. le Roux, Christell van der Vyver and Anna-Maria Botha (**2013**) Establishment of a genetic engineering platform for wheat (*Triticum aestivum* L) studies. Winter Cereal Trust Annual Meeting CSIR, Pretoria, South Africa. <u>(Presentation)</u>

        iii. Marlon L. le Roux, Christell van der Vyver and Anna-Maria Botha (**2014** and **2015**). Innovative strategies to enhance drought tolerance in *Triticum aestivum* L. by using biotechnology advances. Winter Cereal Trust Annual Meeting, CSIR, Pretoria, South Africa. <u>(Presentation)</u>

4) **<u>International Conference Poster:</u>**

    i. Marlon L. le Roux, Anna-Maria Botha and Christell van der Vyver (2015) Engineering wheat (*Triticum aestivum* L.) for abiotic resilience. The 9th International Wheat Conference, Sydney, Australia.

5) **<u>International Presentation:</u>**

    i. Marlon L. le Roux, Anna-Maria Botha and Christell van der Vyver (2016) Sumolyation and its influences on wheat (*Triticum aestivum* L.) flowering time. 22nd International Plant Resistance to Insects Symposium South Africa-Accepted (http://ipri2016.co.za/)

List of Abbreviations

2,4 D	2,4 Dichlorophenoxyacetic acid
2,4,5-T	2,4,5-Trichlorophenoxyacetic acid
ABA	Abscisic acid
35s	Promoter derived from cauliflower mosaic virus
ABRE	Abscisic acid-responsive elements
ATP	Adenosine triphosphate
BAP	6-Benzylaminopurine
BA	6-γ-γ-Dimethylaminopurine (2iP)
bar	Phosphinotricin acetyltransferase
BLAST	Basic Local Alignment Search Tool
bp	Base pair(s)
°C	Degree Celsius
CaCl2	Calcium Chloride
CAF	Central Analytical Facilities
CaMV 35S	Cauliflower mosaic virus 35 S
CBF	C-repeat binding factors
CRT	C-repeat
cDNA	Complementary DNA
cm	Centimetre(s)
Dicamba	3,6-Dichloro-o-anisic acid
DMSO	Dimethyl sulfoxide
Dn	*Diuraphis noxia*
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DRE	Drought-responsive element

DREB	Drought-responsive element binding
E. coli	*Escherichia coli*
EDTA	Ethylenediamine tetra acetic acid
EIE	Immature seeds embryos
EIS	Immature seeds
EtOH	Ethanol
gDNA	Genomic DNA
g/l	Gram per litre
GRA	GC rich Rab Activator
IAA	Indole-3-acetic acid
ICE1	Inducer of CBF Expression 1
Induc A	Callus induction media with Murashige and Skoog vitamins
Induc B	Callus induction media Gamborg Vitamins
Kinetin	N-(2-furanylmethyl)-1H-puring-6-amine
kPal	Kilopascals
LBA	Luria Broth Agar
LBL	Luria Broth Liquid
MS	Murashige and Skoog medium
MSSA-reg	Murashige and Skoog with vitamins
MSSB-reg	Murashige and Skoog Gamborg Vitamins
NAA	1-Naphthaleneacetic acid
NCBI	National Centre for Biotechnology Information
ng	Nanogram
npt II	Neomycin Phosphotransferase II
OTS1	Overly Tolerant to Salt 1
OTS2	Overly Tolerant to Salt 2
PCR	Polymerase Chain Reaction

PEG	Polyethylene Glycol
PLACE	Plant *cis*-acting regulatory DNA elements Database
Pg	Picogram
Pmol	Picomole
PTM	Post translational modifications
R	Resistant gene
Rab17	Drought inducible promoter
RD	Restriction digest
RDZA	Regeneration media with Murashige and Skoog
RDZ B	Regeneration media with Gamborg Vitamins
RMC	Relative moisture content
RPM	Revolution Per Minute
RWA	Russian Wheat Aphid
SUMO	Small Ubiquitin-like Modifier
T_0	Parent generation
T_1	First generation plants
TF	Transcription Factor
U	Unit
Ubi 1	Maize ubiquitin promoter
WT	Wild type
Zeatin	6-(4-hydroxy-3-methyl-2-butenylamino) purine

Table of Contents

List of Figures

Chapter 2

Chapter 3

Chapter 4

Appendix A

Appendix B

List of Tables

Appendix B

Acknowledgements

To my father John – thank you for always having an open door, and motivating me to further myself. Tough love is what every son need, to realize what is important in life and how to overcome every situation in life. Lastly thank you to the entire Le Roux family for the random socials and get togethers - All has shown me the great value of family

To my brothers Kurt and Grant – thank you for always reminding me of what is important and not to lose myself in my career. I have and will always looked up to the both of you. Thank you to Le-Che and Alison- your kind words and motivation has always stayed with me, and lastly thank you to my little nephews Codi, Keyan, Kayden and my niece Payton for the random laughs.

To my 3 little boys, Thorn, Bentley and Siegfried, thank you for just being you. Each one of you has always showed me that there is more to life than just a career.

Last, but most certainly not least, to the love of my life, Luan Africa. You have been such an amazing support system for me throughout this entire study, from sitting up late in the lab with me, to helping me make sense of data, reading through ALL my reports (including this thesis), and listening to ALL my presentations throughout this degree. Thank you for always showing me the silver lining to every bad experiment and situation. Lastly thank you so much for blessing me with our 3 little ones (Thorn, Bentley and Siegfried). I dedicate this entire body of work to you my love.

CHAPTER 1

1.1 Introduction

Wheat (*Triticum aestivum* L.), the second largest staple food crop in the world, has been a target of genetic engineers trying to improve its qualitative and quantitative traits. Some of these traits focus on abiotic stress tolerance due to the fact that climate change is expected to have major effects on global agricultural production. A a high degree of confidence is associated with temperature estimates, with average increases of 2ºC or more predicted in most regions by 2050 (Jarvis *et al.*, 2010). In addition to this, the world population is increasing and is suspected to reach 9.7 billion by 2050 (Becker, 2015). This is concerning as the wheat industry is already threatened by various abiotic (drought, salt) and biotic (pathogens and pests) stressors. Ultimately this can result in a dramatic decrease in wheat yield, which directly affects farmers and jeopardises food security. It is therefore important to explore biotechnological strategies to improve this crop to a level where it is able to tolerate abiotic and biotic stress factors, in order to sustain the global demand for this crop.

Improving wheat has been a challenging task and transformation of this crop is not routine practise and protocols are not widely available, especially in Southern Africa. Various problems exist in the genetic transformation of wheat one being recalcitrance towards tissue culture procedures (Harwood, 2012). In addition to this, the explants with the high regeneration potential are season dependent, and for this reason cryopreservation (essentially meaning storage of biological material at ultra-low temperatures) may be a viable option for a constant supply of explants all year round.

With the aforementioned platforms established, transformation of wheat may be tackled, however a prerequisite for transformation is gene isolation following vector construction. The compatibility of the vector with the genome is of great importance, often vectors that are too large in size may be rejected by plant cells (Carrillo-Tripp *et al*., 2006; Peretz *et al*., 2007). The Method of transformation, i.e. particle bombardment, require vigorous optimization, as the use of inappropriate parameters might lead to complete destruction of

1

explants. Since the first description of transgenic wheat by Vasil *et al*., (1992) a plethora of literature has become available on wheat trangenics and its application; however, the transformation efficiency is still very low, usually between 0.3-1% (Li *et al*., 2012).

Despite the low transformation efficiency of wheat, research still progresses in search of novel ways to improve this crop. Recently a key mechanism used by plants i.e. *Arabidopsis Thaliana* to tolerate abiotic stresses, known as SUMOylation, has been described (Park and Yun, 2013). SUMOylation is a post translational modification process, similar to that of ubiquitination. Literature only expands on SUMOylation in the context of the model plant *Arabidopsis thaliana* or mammalian systems. Ultimately SUMOylation refers to the use of a Small Ubiquitin-like Modifier (SUMO) for polyubiquitination of a given substrate, thus signalling for proteosomal degradation, but also for retargeting and reprofiling (Johnson, 2004). This places SUMOylation as a likely central regulator of signalling in eukaryotes, and hence is an ideal focal point for manipulating complex molecular responses such as drought and heat tolerance. However, the tagging of a substrate by SUMO is a reversible process by the use of SUMO proteases, which are suspected to play an integrated role in regulating SUMOylation.

 SUMO proteases such as Overly Tolerant to Salt 1 (*OTS1*) and *OTS2* are attractive targets for manipulating stress responsive SUMOylation. Furthermore, stress response regulators, e.g. DREB and ICE1 transcription factors (controlling cold and drought stress) have clear SUMO attachment sites, indicating that SUMO plays a direct role in the stress mechanisms controlled by these regulators. Unfortunately, crop plants over expressing *DREB* have a yield penalty even under non-stressed conditions (Bihani *et al*., 2010), illustrating the adverse effect of constitutively activating metabolically costly stress response pathways - a concept that still needs validation within a hexaploid such as wheat (*Triticum aestivum* L.). However, using a drought inducible promoter proven not to be leaking in a monocot such as wheat, could perhaps overcome yield penalty.

The overall hypothesis of this study is that SUMOylation directly influences wheat (*Triticum aestivum* L.) development, ultimately affectively important agronomical attributes. Thus the long term objective of this study is to increase drought tolerance in South African wheat by manipulating and altering the SUMOylation

pathway without any yield penalty. However, prior to generating transgenic wheat populations, emphasis was placed on the following objectives:

1) Fully optimize an *in vitro* regeneration system which will serve as platform for subsequent transformation.

2) Establish a novel protocol to cryopreserve wheat explants (immature seeds) essentially assuring a constant supply of explants for transformation.

3) Isolation and cloning of three key stress response regulators from *Arabidopsis thaliana*, e.g., *ICE1*, *OTS1* and *OTS2* followed by cloning into a plant constitutive expression vector.

4) Construct a novel drought inducible vector and validated promoter *in silico*, prior to vector construction.

5) Genetically transform immature wheat embryos with vectors and confirm transgene integration followed by basic phenotyping of the T_0 generation.

This dissertation consists of five chapters. Chapters three and four are the research chapters, each divided into an introduction, materials and methods, results, discussion and conclusion. The content of each chapter is as follows:

CHAPTER 1:

Introducing the study by stating the rational followed by hypothesis and objective.

CHAPTER 2:

Literature review: Expanding on current trends in the field of *in vitro* regeneration, cryobiology, and transformation. Current literatures are discussed and compared to motivate why certain techniques were applied in this dissertation / study. Drought associative genes and transcription factors and their influences on post-translation modification are also discussed, validating why certain genes were used in this study.

CHAPTER 3:

Focus on *in vitro* regeneration of six South African wheat cultivars, and detailed description of a novel cryopreservation methodology, validated by comparative analysis. The chapter also illustrates the quantification of explant development during different phases of *in vitro* regeneration and cryopreservation.

CHAPTER 4:

Explains gene isolation and cloning into plant constitutive expression vectors. It also elaborates on the construction of a drought inducible vector, outlining promoter analysis *in silico*. The chapter proceeds with description of transformation of wheat embryos and obtaining transgenic wheat, with basic molecular and phenotypic analysis of transgenic plants (T_0).

CHAPTER 5:

An overview of the dissertation and its outcome, stating difficulties and possible solution to certain outcomes. Finally suggesting future studies to substantiate the results obtain in the study.

1.2 References

Becker R (2015). World Population Expected to Reach 9.7 Billion by 2050. News. Nationalgeographic .com (http://news.nationalgeographic.com/2015/07/world-population-expected-to-reach-9-7-billion-by-2050/, accessed on 1 October 2015)

Bihani P, Char B, Bhargava S (2010) Transgenic expression of sorghum DREB2 in rice improves tolerance and yield under water limitation. Journal of Agricultural Science. **149**: 95-101

Carrillo-Tripp J, Shimada-Beltrán H, Rivera-Bustamante R (2006). Use of geminiviral vectors for functional genomics. Current Opinion Plant Biology. **9**: 209-15

Johnson ES (2004) Protein modification by SUMO. Annual Review of Biochemistry. **73**: 355–82

Jarvis A, Ramirez J, Anderson B, Leibig C, Aggarwal P (2010). Scenarios of climate change within context of agriculture. *In*: MP Reynolds (ed.) climate change and crop production. CAB International, Wallingford, United Kingdom. pp 9-37

Harwood WA (2012). Advances and remaining challenges in the transformation of barley and wheat. Journal of Experimental Botany. **63**: 1791–1798

Li J, Ye X, An B, Du L, Xu H (2012). Genetic transformation of wheat: current status and future prospects. Plant Biotechnology Reports. **6**: 183–193

Peretz Y, Mozes-Koch R, Akad F, Tanne E, Czosnek H, Sela I (2007). A universal expression/silencing vector in plants. Plant Physiology. **145**: 1251-1263.

Park H, Yun D (2013). New insights into the role of the small ubiquitin-like modifier (SUMO) in plants. International Review of Cell and Molecular Biology. **300**: 161-209

Vasil V, Castillo AM, Fromm ME, Vasil IK (1992). Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus. Nature Biotechnology. **10**: 667 - 674

CHAPTER 2

Literature Review

2.1.1 The past and present of wheat

Throughout human history, wheat (*Triticum aestivum* L*.)* has always been a major staple food crop. It serves as a primary source of protein (9 -12%), starch (60-80%) and micro-nutrients, which are essential substrates for healthy human metabolism (Jones, 2005; Heyns, 2010). Currently, this crop is grown globally on more than 17% of the designated cultivatable land (Jones, 2005; Xia *et al*., 2012) located in a vast range of climatic environments (James, 2012). In contrast to maize and rice, there is no genetically modified wheat in the commercial industry (Ioset *et al*., 2007; Lupi *et al*., 2013). However, plant biotechnologists have enhanced this crop's qualitative and quantitative traits, allowing for the opportunity for commercialization in the near future.

Bread wheat's history dates back nearly 10,000 years, and according to reports (Harlan and Zohary, 1966; Haider, 2013) its domestication initially occurred in the fertile crescent of the Trigris-Euphrase basin in South-Western Asia (Haudry *et al*., 2007), which is regarded as the centre and origin of distribution and diversity of the wild progenitors of cultivated wheat (Shewry, 2009). In more recent years, specifically the period between 1965 and 1990, wheat production increased nearly threefold. This period is known as the Green Revolution (Loyola-Vargas, 2006).

Green-revolution-wheat was resistant to rust and fungal disease, but it require large amounts of fertilizer, pesticides, and irrigation to achieve the high yields for which it is famous. However, and more importantly, these breeding varieties (semi dwarf varieties) were able to grow in almost any part of the world, regardless of ecological conditions. Unfortunately the demand for wheat yield and production increases at a very fast rate, in addition to increases in world population. Subsequently wheat production could not keep with the demands

6

due to occurrence of pathogens, pest and weeds, in addition to post-harvest losses during storage (Loyola-Vargas, 2006).

2.1.2 Wheat: Current status and trends

2.1.2.1 Global and local statistics

With an ever increasing world population, expected to reach 9 billion in 2050 (Noack and Pouw, 2015), conventional breeders are investigating ways to produce more wheat to supply the demand (Oerke and Dehne, 1997; Loyola-Vargas, 2006). This is evident from the annual production of wheat, which has increased from 560 million tonnes in the year 2005 to 713 million tonnes in 2013 globally (FOA, 2013). According to predictions by the International Food Policy Research Institute (IFPRI), the world's wheat demand is expected to reach 841 million tonnes in the year 2020. However, in recent years (2000-2013) South Africa's wheat production decreased due to decreasing profit which subsequently effects total production (Commodity Intelligence Report, 2014). The major global wheat exporters are Argentina, Australia, Canada, Kazakhstan, Russian Federation, Ukraine and the United State (FAO, 2013). South Africa on the other hand is not considered a major exporter, since it imports wheat to supplement domestic production. This is attributed to dry conditions in parts of South Africa where the major wheat fields are located. These unpredictable and unfavourable climatic conditions that farmers experience leads to the country having to import more than what it exports (Harvey *et al*., 2014), resulting in a decline in production in South Africa over the past decade (van der Vyver, 2013). African agriculture is considered extremely vulnerable to changing climate because of its heavy dependence on rain-fed production (Challinor *et al*., 2007; Mertz *et al*., 2009). In addition to the abiotic stress, biotic stress has also put strain on this crop, a prime example being Russian wheat aphid (*Diuraphis noxia*) infestation (Zaayman *et al*., 2009).

2.1.2.2 Transgenic wheat and its global status

Although improved tillage and irrigation practices can increase production by conserving water, enhancing the genetic tolerance of crops to drought and heat stress is considered a crucial component for increasing crop production in the coming decades (Edmeades *et al*., 2001; Reynolds *et al*., 2010). Given the unpredictable nature of precipitation patterns, a necessary attribute of stress-tolerant varieties is that the engineered crop carry no yield penalty under both favorable and unfavorable moisture conditions.

Genetic modification (GM) technology has been used to develop many varieties of crops with enhanced attributes which aid in improving productivity, quality and stress tolerance (Yi *et al*., 2015). As per example: in 2013 Tanzania approved the commercial release of three drought-tolerant maize hybrid varieties to all local farmers (Clive, 2014). The list of GM crops for commercial release for improved drought tolerance has expanded in 2014. Crops such as GM sugarcane have been approved in the Philippines and rice in China (Clive, 2014; Normile, 2014).

GM-wheat field trials have been approved in the past and present, but only for research purposes (Fox, 2009; Malcolm, 2013), with Australia being the most open-minded towards GM-wheat field trails. The Australian Government has granted 14 licences (more than any other country) for small field trials of GM wheat in 2014, for wheat with modified traits such as increased salt tolerance, drought tolerance, altered starch content or nutrient-use efficiency (Australian Department of Health, 2015). Despite the favourable outcome of these trails, it has been made clear that the GM-wheat is not for any human nor animal consumption but solely for research purposes (Australian Department of Health, 2014). In spite of the governmental limitation on GM-wheat, research has made substantial progress, for example the development of "Golden Wheat" (Cong *et al*., 2009), which has improvement in its β-carotenoid content to address the vitamin A deficiency in malnourished human populations. The first generation of the "Golden wheat" had a 13.5-fold increase in vitamin A which could have a major impact on human populations, since the relative daily consumption of wheat-based products is very high (Tang *et al*., 2009). Furthermore, Rong *et al.* (2014) demonstrated that over expression of the endogenous ethylene-response factor (ERF) significantly regulates stress associated genes in wheat when exposed to salt or drought conditions. Also, Syngenta has developed a *Fusarium*-resistant wheat, which was

developed to combat crown rot and head blight, but due to first world politics and social concerns the project was suspended in 2007 (Dunwell, 2013).

2.1.3 Fundamentals to engineering wheat

Transgenic wheat studies have been a challenging task, and transformation of the *Gramineae* family of crops is not routine or widely established, especially in southern Africa. Gene transfer to higher plants requires robust tissue culture protocols from which viable and fertile transgenic plants can be regenerated. However, various problems such as explant production and availability, explant recalcitrance towards tissue culture procedures (media composition), geographical origin and physiological status of the donor plants, as well as interactions between all of the aforementioned need to be overcome when introducing new wheat cultivars *in vitro* (Vasil and Vasil 2006, Jones *et al*., 2005). A detailed discussion of these factors will follow in the sections below. To date, wheat cultivars for which *in vitro* regeneration systems have been established mostly include European, Asian, North African and North American cultivars, none of which are suitable for the climate of the Southern hemisphere (Rasco-Gaunt *et al*., 2001; Barro *et al*., 1999; Moghaieb *et al*., 2010; He *et al*., 2010).

2.1.3.1 Donor plant

Donor plants are defined as the mature wheat plant carrying the explant of choice. The developmental stages of the wheat plant can extend to ±110 days in total, depending on the season and cultivar (Nerson *et al*., 1980). Southern African spring wheat, i.e. Gamtoos and Palmiet, development is shorter (±121 days) then the winter wheat i.e. Tugela (±243 days), which requires vernalization (Ahrens and Loomis, 1963). However, irrespective of the cultivar, factors such as soil type, light, humidity and temperatures dictate the phonological development. A key stage in wheat development is initiation of terminal spikelet (heading) (Figure 2.1) as this signifies the end of initiation of spikelet primordia and thus potential seed/embryo sites, given that the explant of choice is embryos or anthers (Slafer and Savin, 1991). But the environmental temperature should always remain within the 25-26°C range (Asseng *et al*., 2011). Increases in temperature during early spikelet development reduce

the number of spikelets and subsequently the number of seed/embryo within a spikelet (Porter and Gawith, 1999). Although temperature sensitivity varies during the wheat plant's developmental stages, change in temperature can results in unwanted effects, such a complete sterility (Porter and Gawith, 1999).

	Establishment	Tillering	Heading	Flowering	Ripening
Spring wheat	15-20 days	10-20 days	20-30 days	15-20 days	10-15 days
Winter wheat	(*DP)10-15 days	15-25 days	40-50 days	30-35 days	10-15 days

DP* dormancy period for wheat can take up to 90 + days
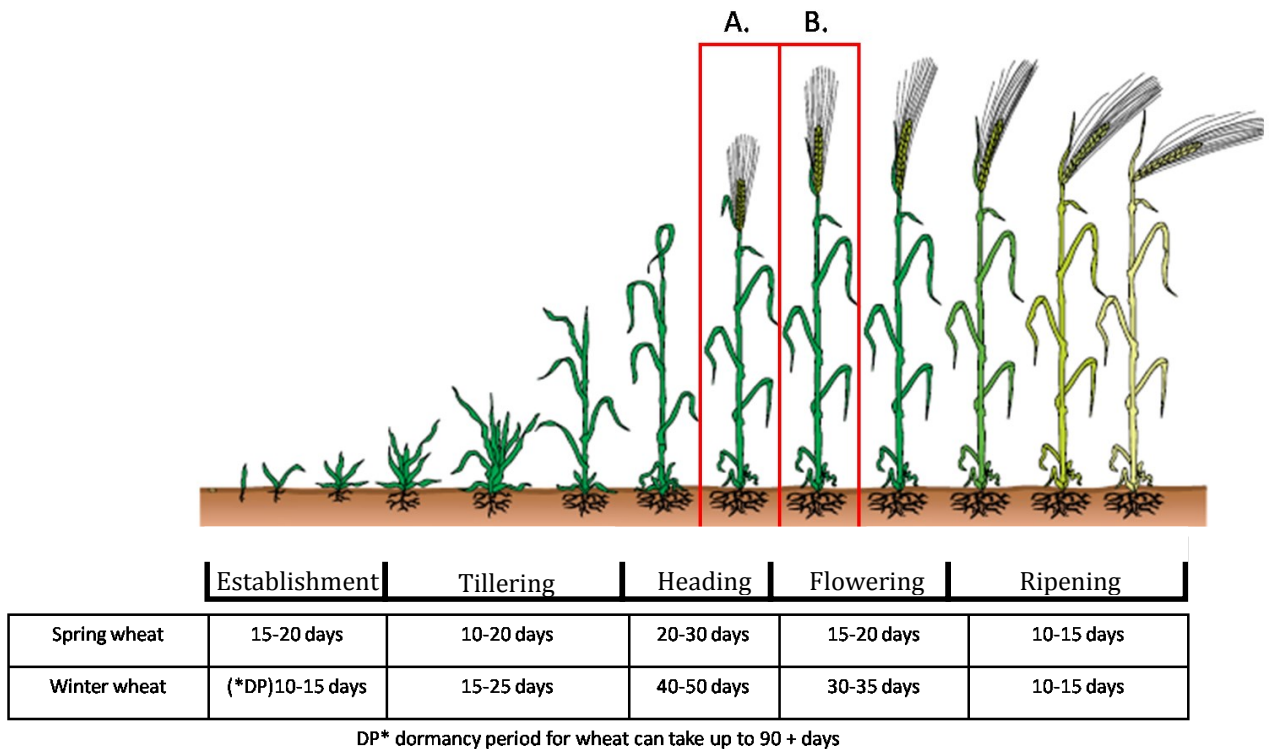
Figure 2.1 Wheat developmental stages until maturity for both spring and winter wheat with approximate days. (A) Refers to the start of spikelet development and; (B) start of anthesis. (Adapted from Screenivasula and Schnurbursch, 2012).

2.1.3.2 Anthesis

Anthesis is marked by the extrusion of anthers from the spikelets (Figure 2.2). This stage signifies pollination and fertilization, which occurs over the course of 7-10 days, leading to the formation of an embryo. Anthesis begins in the central part of the spikelet and continues towards the basal and apical parts during a three- to five-day period (Law and Worland, 1997; Borras-Gelonch *et al.,* 2012). The proximal florets of the central spikelet are fertilized two to four days earlier than the distal florets. These seeds usually have a greater weight (Simmons and Crookston, 1979). After floret fertilization, cellular division is rapid, during which the

10

endosperm cells and amyloplasts are formed. More importantly this is also the time when the embryo is formed. The identification of this growth stage (Figure 2.2) is very important for the *in vitro* regeneration based research - thus anthesis is a bench mark for embryo maturity and generally gives an indication if the embryo still possesses totipotency (Delporte *et al.*, 2012). The consensus across *in vitro* research is to isolate embryos 12-16 days post anthesis. During this time the embryo is regarded as immature with high totipotency. Exposure to sub- or super-optimal temperatures during anthesis can lead to infertile florets or sterile pollen, all of which lead to no embryo development (Borras-Gelonch *et al.,* 2012).
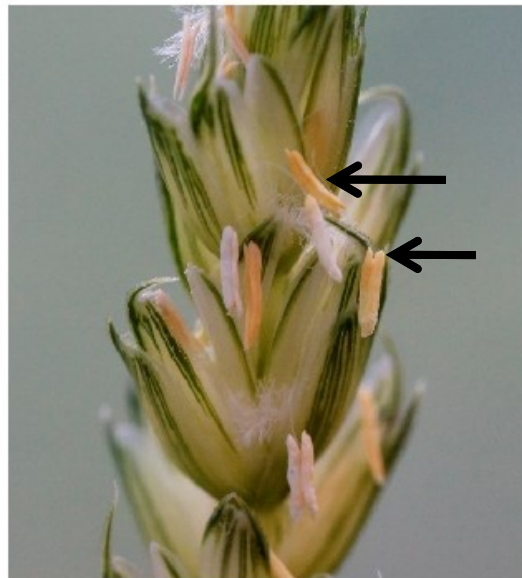
Figure 2.2 Wheat spikelet post anthesis. Extrusion of anthers is observed as yellow and white outgrowths as shown with black arrows (The University of Nottingham, 2015).

2.1.3.3 Explant type

Explants are defined as the primary source of tissue, extracted from the donor plant used to initiate the *in vitro* culture. Essentially an explant is chosen due to the nature of its totipotency and performance *in vitro*. To date, the wheat plant has been successfully regenerated from various explants including mature embryos, microspores, shoot tips, leaves, endosperm, seeds, etc. (Jones *et al*., 2005; Viertel and Hess, 1996; She *et al*., 2013; Yin *et al*., 2011; Ponya and Barnabas, 2003; Kavas *et al*., 2008).

11

From all the explants tested, the immature embryo (Figure 2.3B) is most efficient at callus formation, somatic embryogenesis and subsequent plant regeneration (organogenesis). However, this only applies to a few cultivars, due to the fact that regeneration is highly genotype-dependent (Tinak-Ekom *et al.,* 2014). Elite cultivars usually have a low regeneration capacity, although their performance is economically and agriculturally outstanding in terms of productivity, adaptability, tolerance to abiotic stress and disease-resistance (Wei *et al.,* 2015). These cultivars are generally very appealing for conventional and non-conventional breeding i.e. transgenic breeding.

A.   B.
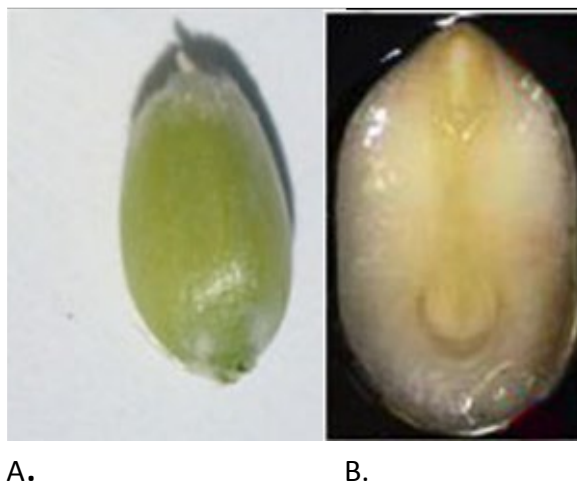
Figure 2.3 Immature seed (A) the host of the immature embryo (B).

2.1.3.4 *In vitro* regeneration regime

Callus (a mass of undifferentiated cells grown *in vitro*) is a product of exposing an explant to a designed medium containing auxins, carbon and amino acids. The formation of callus is simply a stepping stone, as the goal is to induce unipolar structures i.e. organogenesis or bipolar structure known as somatic embryos. The nature of regeneration is also referred to as indirect morphogenesis, as the explant only displays morphogenic patterns after callus formation. However, if an explant, i.e. leaf or stem, were to develop shoots with no callus interface, this would be direct morphogenesis. The induction of callus and subsequent plant formation is influenced by culture media composition. The basic nutrient requirements of cultured plant cells are very

similar to those of whole plants. The main components of the media consist of some or all of the following constituents: macronutrients, micronutrients, vitamins, amino acids or other nitrogen supplements, sugar(s), solidifying agents, and growth regulators.

2.1.3.5 *In vitro* growth media requirements

Macronutrients and micronutrients

Across all tissue or cell culture media, there is basic composition mixtures which is derived from formulations described by various scientist such as White (1963), Murashige and Skoog (1962), Gamborg B5 (1968), Schenk and Hilderbrandt (1972) and Lloyd and McCown (1980). These media were formulated to serve as general culture media for basic plant tissue culture with the intention of mimicking the plant's macro- and micronutrient requirements. Therefore these media differ in macronutrients and micronutrients exclusively and does not contain any hormones. As is the case with any other living system, macronutrients are essential, and without it the no development will occur. Plant tissue culture requires the addition of macronutrients at optimum concentration, however it varies considerably among species. There are six major macronutrients, i.e. nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), and sulphur (S). All these elements play a vital role in processes such as electron-transportation, photosynthesis, maintenance of water potential gradients, CO_2 uptake and O_2 expulsion, all of which help facilitate cell proliferation and differentiation. In conjunction with macronutrients (mM), micronutrient are also vital, but needed in smaller quantities (uM). Their presence is important as they are key factors in various physiological processes. There are seven micronutrients, namely iron (Fe), manganese (Mn), zinc (Zn), boron (B), copper (Cu), and molybdenum (Mo). Fe is most important as it serves as an activator for biochemical processes i.e. photosynthesis (Miller *et al*., 1993). However, Fe is generally used in its chelated form in culture media since iron citrate and tartrate are difficult to dissolve and frequently precipitate after medium is prepared (Saad and Elshahed, 2015). Murashige and Skoog (1962) used an ethylene diaminetetraacetic acid (EDTA)-iron chelate to bypass this problem. Furthermore, the other micronutrients also hold high value as lack of any can lead to no/poor callus formation and lack of embryogenicity, interveinal chlorosis of young leaves and necrosis (Carvajal *et al*., 1996).

Carbon and energy sources

Plants have the unique ability to generate their own carbohydrates by CO_2 assimilation during photosynthesis. However, since cells (from explants) are not all fully autotropic, the addition of carbohydrates to media is crucial throughout the entire *in vitro* regeneration process given that sufficient light are proved. The preferred carbohydrate is sucrose at concentrations of 2-3% for wheat and barley tissue culture (Roberts-Oehlshlager, 1990; Ball *et al*., 1992; Mahmood *et al.,* 2012). Lactose, galatose, maltose, starch, glucose and fructose may be a substitute in some cases, however many were reported as less effective, with the exception of glucose, which was shown to be as effective as sucrose (Indrianto *et al.,* 1999; de Paiva Neto and Otoni, 2003; Yaseen *et al*., 2013). This is attributed to the fact that plant cells use glucose first, followed by fructose (glycolysis). Methods of sterilizing the carbon source has been shown to influence tissue culture, for example, autoclaving sucrose leads to hydrolyzation, resulting in more utilizable sugars such as fructose, result in faster growth of the cells (Jain *et al.,* 1997).

Vitamins

Like many other plants, wheat synthesizes its own vitamins required during growth and development (Dupont, 2008). Vitamins such as thiamine (vitamin B1), nicotinic acid and pyridoxine (vitamin B6) serves as catalysts in various biochemical reactions. However, vitamins can limit the growth of cells *in vitro* since the explants are usually taken from the donor plant, which is responsible for the synthesis of vitamins. A key vitamin required during cell or tissue regeneration is thiamine (vitamin B1) and generally the concentration ranges from 0.1 to 10 mg.l^{-1}. Exceedingly high vitamin concentrations in culture often lead to necrosis, thus it is recommended to add a carbohydrate, such as myo-inositol (Saad and Elshahed, 2015). Myo-inositol is known to play a role in cell division as it is easily broken down to ascorbic acid and pectin (which are vitamins) and incorporate into phosphoinositides and phosphatidyl-inositol.

Amino acids

Cells in culture generally have the capacity to synthesize their own required amino acids, however it is advisable to add correct amino acids, hereby facilitating further cell growth. Additional amino acid ultimately

provides an immediate source of nitrogen although inorganic nitrogen (micronutrient) is added the latter still needs to be efficiently broken down prior to assimilation. Organic nitrogen is commonly added, by using a mixture such as casein hydrolysate, L-glutamine, L-asparagine, and adenine. When the aforementioned amino acids are added caution is advised since it often leads to growth inhibition. In most cases L-glutamine and casein (hydrolysate) are preferable for wheat *in vitro* regeneration (Sangwan and Sangwan-Norreel, 1996). This is due to the fact that wheat explants, in particular embryos, require high demand of energy *in vitro*. Glutamine is a major source of amino acid which can be metabolize into energy by rapidly dividing cells, furthermore casein is the ultimate source of several microelements, vitamins and up to 18 amino acids, more recently it speculated that casein can mimic growth promoting factors (George *et al*., 2008; Ageel and Elmeer, 2011).

Solidifying agents

Gelling agents mimic the natural ground in a firm state, thus agar is the most preferred gelling agent to obtain semisolid and solid media. Agar is stable at temperatures exceeding 100°C and often the media needs to be exposed to such high temperature to ensure complete eradication of microbial contaminants (Martin, 1975). However the firmness of the agar is due to the concentration (0.5 and 1%) in addition to the pH (5.8) of the media. A substitute for agar is GELRITE, which is a synthetic agent, and can be used at half that of the amount of agar needed (1.25-2.5 g/litre). An additional advantage of using GELRITE is the clearness once the media as solidified, hereby making the detection of microbial contamination easy (Shungu *et al*., 1983). Whether explants grow best on agar or on other supporting agents varies from one species of plant to the next.

2.1.4 *In vitro* growth regulators

"Plants growth regulators" is a collective name for hormones which are responsible for adequate proliferation, differentiation and subsequently morphing into a plant. There are four well characterized groups known as auxins, cytokinins, gibberellins and abscisic acid, each of which differ considerably in function related to plant species and genotype (Kiba *et al*., 2010; Linkies and Leubner-Metzger, 2011; Vanstraelen and Benková, 2012).

15

Auxins are generally combined with cytokinins, however the ratio influences the extent of callus formation, somatic embryogenesis and organogenesis.

2.1.4.1 Auxins and somatic embryogenesis

Somatic embryos arise from individual cells from the explant, however it has no vascular connection to the maternal tissue of the explant. Induction of somatic embryogenesis is preferable as it leads to a high rate of clonal formation making this ideal for transgenic studies. When explants such as immature or mature embryos are used, the physical removal of axes is a prerequisite for somatic embryogenesis as shown in Figure 2.4 (Rasco-Gaunt *et al*., 1999; Ekom *et al*., 2014). The removal of the axis also prevent precocious germination.
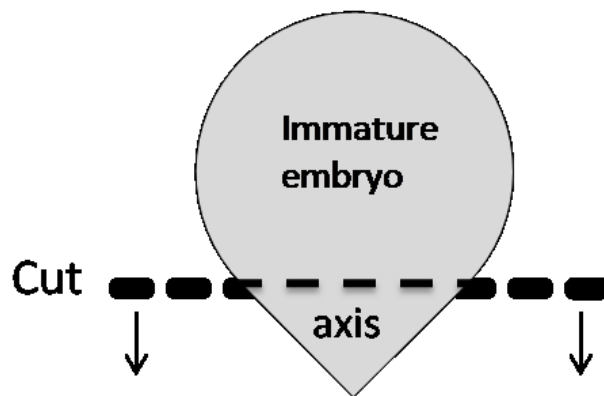
Figure 2.4 Immature embryos and location of axis which need to be removed prior to *in vitro* regeneration.

To further improve the efficiency of the explant, culture media composition requires fine tuning. Thus auxins play a vital role in the induction of callus and subsequent embryogenic tissue. Auxins such as 2,4-D (2,4-dichlorophenoxyacetic acid), 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) or dicamba (3,6-dichloro-o-anisic acid) and NAA (1-Naphthaleneacetic acid) are synthetic auxins which mimic the functioning of the natural plant hormone known as indole-3-acetic acid (IAA) which is known to play a role in cell proliferation (Zhao, 2012). These synthetic hormones are nearly 3-fold more effective in their functioning when compared to the natural hormone (Saad and Elshahed, 2015). These synthetic compounds were designed to induce embryogenesis, thus attempting to eliminate auxins from the induction media can result in no callus formation

(Turhan and Baser, 2004). The effects of auxins are not consistent across plant species or genotype. Perhaps this is due to many researchers often combining auxins such as 2,4-D (4 mg/l) and NAA (1 mg/l) for a higher frequency of callus formation (Coenen and Lomax, 1997; Ezeibekwe *et al*., 2009). It is not uncommon to combine auxins with chlorinated systemic herbicides such as picloram (4-Amino-3,5,6-trichloropyridine-2-carboxylic acid) to increase the efficiency callus formation even further (Barro *et a.,l* 1999; Azghandi *et al*., 2007).

2.1.4.2 Cytokinins

Cytokinins are a class of phytohormones which promote cytokinesis, ultimately leading to the development of shoots, hence their function is related to cell differentiation (Coenen and Lomax, 1997). Cytokinins commonly used in the *in vitro* culture media include 6-benzylaminopurine or 6-benzyladenine (BAP, BA), 6-γ-γ-dimethylaminopurine (2iP), N-(2-furanylmethyl)-1H-puring-6-amine (kinetin), and 6-(4-hydroxy-3-mehty-trans-2-butenylamino) purine (zeatin). Zeatin and 2iP are considered to be naturally occurring cytokinins, while BA and kinetin are synthetically derived cytokinins (Saad and Elshahed, 2015). Many plant species displays the absolute need for cytokinins *in vitro* for morphing of cells to organogenesis. Importantly these hormones are not exclusive for shoot development; often the ratio of cytokinins to auxins is crucial throughout the *in vitro* process. When the ratio of cytokinins to auxins is low, callus initiation, embryogenesis and root initiation are observed. In contrast, high cytokinin to auxin ratio leads to adventitious and axillary shoot proliferation (Haberer and Kieber, 2002). However there are some plant cells/tissues that can be cultured in a cytokinin-independent manner.

2.1.5 Genetic transformation

Early in the 20th century transgenic plants were merely spoken of in theoretical terms, and it was only in the early 1980s that the first of these transgenic plants was produced (Kruger-lebus, 1985; Bhalla *et al*., 2006). Ever since then transformation of wheat has been explored with the aim of improving the crop for better endurance of its environment, the results thus far have demonstrated that GM-technology is promising, as many laboratories have moved from greenhouse trials to field trials with exciting outcomes. However considerable debate remains among researchers about the respective advantages and disadvantages of methods to transform wheat.

The methods used for wheat transformation include polyethylene glycerol (PEG)-mediated transformation, pollen tube pathway transformation, *Agrobacterium tumefaciens*-mediated or biolistics techniques (Yi *et al*., 2015). However the latter remains the most frequent used global method for wheat. Biolistics, also referred to as particle bombardment, has achieved stable expression of various trans-genes in wheat through the application of this technique (Southgate *et al*., 1995; Rasco-Gaunt *et al*., 1999; Folling, 2001; Jackson *et al*., 2013). It involves the use of a helium driven system, which propels DNA-coated tungsten or gold particles into target tissue, leading to the establishment of mature transgenic plants. Particle bombardment is dependent on many factors which often require optimization relative to the genotype (Lacock and Botha, 2000). These parameters include DNA format and quantity, propellant force, target distance and helium pressure leading to effective DNA delivery. However, this technique is not without its pitfalls (Lacock, 1990; Southgate and Davey, 1995), one of which is tissue damage and also the integration of complex trans-gene patterns, which ultimately lead to difficulties in subsequent analysis.

Wheat is one of the most difficult crops to transform with an average stable transformation efficiency of 0.3-4% for stable expression irrespective of method of transformation (Jones *et al*., 2005; Li *et al*., 2012). This low rate of transformation has necessitated the use of a selection system which promotes the preferential survival of transformed cells/plantlets and killing non-transformed cells/plantlet. A selection system is comprised of two components: a selection agent (antibiotic or herbicide) which is added to the media and the use of a

particular gene which confers selective advantage on specific media (selection genes are incorporated into the transformation vector). Commonly used selection agents for wheat in particular are those based on formulations containing the herbicide phosphinothricin, and on aminoglycoside antibiotics i.e kanamycin, neomycin, gentamycin, and hygromycin, all of which inhibit protein synthesis (Goodwin *et al*., 2004). The antibiotics can be inactivated by phosphotransferases encoded by various genes including *npt*II (neo) and *hpt* (aphIV) (Bevan *et al*., 1983; Vandenelzen *et al*., 1985; Waldron *et al*., 1985; Jones, 2005).

2.1.6 Cryopreservation

With an *in vitro* transformation system established, a constant supply of explants is required, thus making cryopreservation the most viable option in the context of wheat. Cryopreservation is defined as the storage of biological material at sub-zero temperatures (Benson, 2008). These sub-zero temperatures create the platform for cellular and metabolic processes to enter their quiescent state, and in theory plant material can be preserved at these temperatures for an indefinite period of time, without the concern of becoming genetically unstable (Benson, 2008; Harding, 2010; Ogawa *et al*., 2012; Wang *et al*., 2012). Cryopreservation is tissue dependant and genotype specific, therefore a personalized cryopreservation protocol is crucial (Engelmann, 2004). Often the most concerning obstacle during cryopreservation is intracellular crystallization of water with associated deleterious effects.

2.1.6.1 Intracellular water

When water freezes it expands, but contrary to popular belief the expansion does not lead to cell damage/injury. When water is exposed to sub-zero temperature it undergoes a purification process, meaning water freezes as a pure substance, hereby excluding all else (Wowk, 2007). Instead of allowing all molecules to freely mix within it, thus remaining a solvent, the water molecules cluster together into a crystal pushing everything else out, consequently freezing leads to damaging by two distinct mechanisms (Figure 2.5):

i) Mechanical damage, which refers to the shape of the cells that becomes distorted due to intracellular crystallization;

ii) Solution-effects injury, as a consequence of chemical and osmotic effects, concentrated solutes in the residual unfrozen water between ice crystals.

A.   **B.**
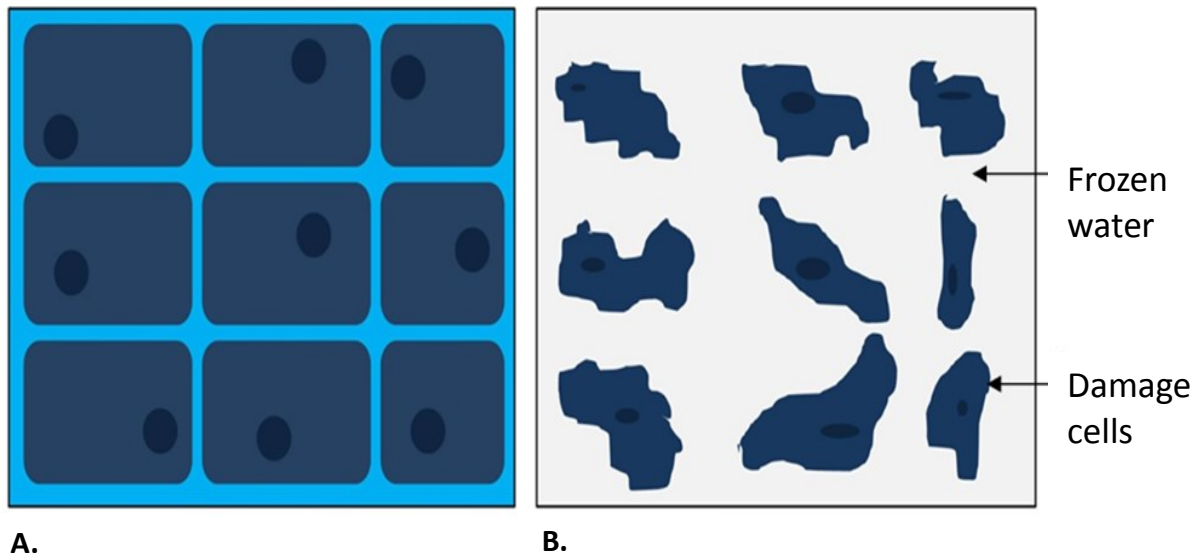
Figure 2.5 Cells damaged due to exposure to sub-zero temperatures. (A) Cells before freezing; (B) after freezing.

2.1.6.2 Cryopreservation techniques

Several protocols for cryopreservation of cultured plant cells and tissues have been developed for dicotyledonous and monocotyledonous plants since the initial cryopreservation of flax (*Linum usitatissimum*) suspension-cultured cells (Quatrano, 1968). Often these techniques take a classical approach, such as pre-freezing, desiccation, vitrification and slow freezing (Withers and King, 1980; Langis *et al*., 1989; Uragami *et al*., 1989; Sakai, 2008; Ogawa *et al*., 2012). Most of the aforementioned make use of sophisticated and expensive programmable freezers, in addition to the lengthy preparation, thus there is a great need for a simplified cryopreservation procedure. The latest and most important technique utilizes a combination of the listed techniques, while avoiding the use of expensive and sophisticated apparatus, i.e. Encapsulation / Vitrification.

To avoid intracellular crystallization of water, explants should undergo dehydration and vitrification. The latter refers to explants being exposed to highly concentrated cryo-protectants e.g. dimethyl disulfoxide (DMSO) or glycerol, which solidify into a metastable glass, without crystallization upon exposure to sub-zero temperatures (Sakai *et al*., 2008). However, highly concentrated cryo-protectants can be toxic to explants, thus samples are treated with a cryo-protective solution such as sucrose to induce mild plasmolysis, in preparation for dehydration-vitrification (Sakai *et al*., 2008). Encapsulating explants in alginated beads is also an additional means of cryoprotection, as its matrix serves as a barrier for chemical toxicity and osmotic stress during the vitrification process (Sakai *et al*., 2008; Chapman *et al*., 2008). In comparison to classical techniques, such as slow freeze/non-dehydration, vitrification allows explants to be cryopreserved by flash freeze in liquid nitrogen (Fahy *et al*., 2004; Sakai *et al*., 2008; Fábián *et al*., 2008).

2.1.6.3 Immune response post cryopreservation

It is known that a cellular immune response is elicited post thawing, with the consequent production of free radicals and activated oxygen, which in turn damage the cell membranes and membrane bound enzymes, resulting in lipid peroxidation (Funnekotter *et al*., 2012). This oxidation process ultimately results in the formation of malondiadehyde, which is regarded as highly toxic to plant tissues (Uchendu *et al*., 2010). To counteract this process and to minimize potential deleterious effects, antioxidants (ascorbic acid, sorbitol ect.) are used in a pre-treatment step (Fábián *et al*., 2008; Wang *et al*., 2012).

2.1.6.4 Wheat and cryopreservation

Wheat – a complex monocot, requires a defined cryopreservation methodology as the explant with highest regeneration capacity i.e. immature embryo (Harwood, 2011), is not only limited annually but contains high levels of intracellular free water, which leads to damage upon freezing, subsequently affecting regeneration. Gnanapragasam and Vasil (1992) were the first to successfully cryopreserve immature wheat embryos. They achieved 66% survival post cryopreservation by using an expensive programmable slow freezer. No survival was maintained after direct plunging of plant material into liquid nitrogen. More recently, Solov'eva *et al*.

(2011) cryopreserved 3 week old callus derived from mature seeds, achieving a survival rate of 90.5% for regeneration, but plantlet formation was not achieved. It is evident that there are cryopreservation methods for wheat, however the protocols are not user friendly. Therefore institutions such as the Svalbard Global Seed Vault (also known as the world seed bank) are constantly searching for methods to successfully cryopreserve wheat explants which do not require lengthy and complex protocols (Wageningen, 2015).

2.1.7 Abiotic stress

With an effective *in vitro* regeneration protocol, and a continuous supply of explant material, introducing genes into the wheat genome can be addressed with relative ease. The ability to alter the genome in a predictable manner is a powerful research tool for functional genomics, thus allowing us to address concerns such as abiotic stress, more specially drought tolerance, in a cereal crops such as wheat. Abiotic stress is defined as the negative impact of non-living factors on living organisms in a specific environment (Ciarmiello *et al*., 2009). Unfavourable climatic shifts are considered a major abiotic threat to nearly all crop production worldwide. Drought is considered one of the most important abiotic stress factors. Essentially it is a naturally occurring phenomena that occurs at any giving time point without any prejudice to its location. Furthermore it always impacts the ecosystem which varies with intensity, duration and socio-economic impact (Hunt *et al*., 2014). Recurrent droughts over the past 20 years have scorched millions of hectares of food crops in Southern Africa, the horn of Africa, and the Sahel belt from Mauritania to Sudan, with Niger and Chad especially severely impacted (Figure 2.6) (Rice, 2010).
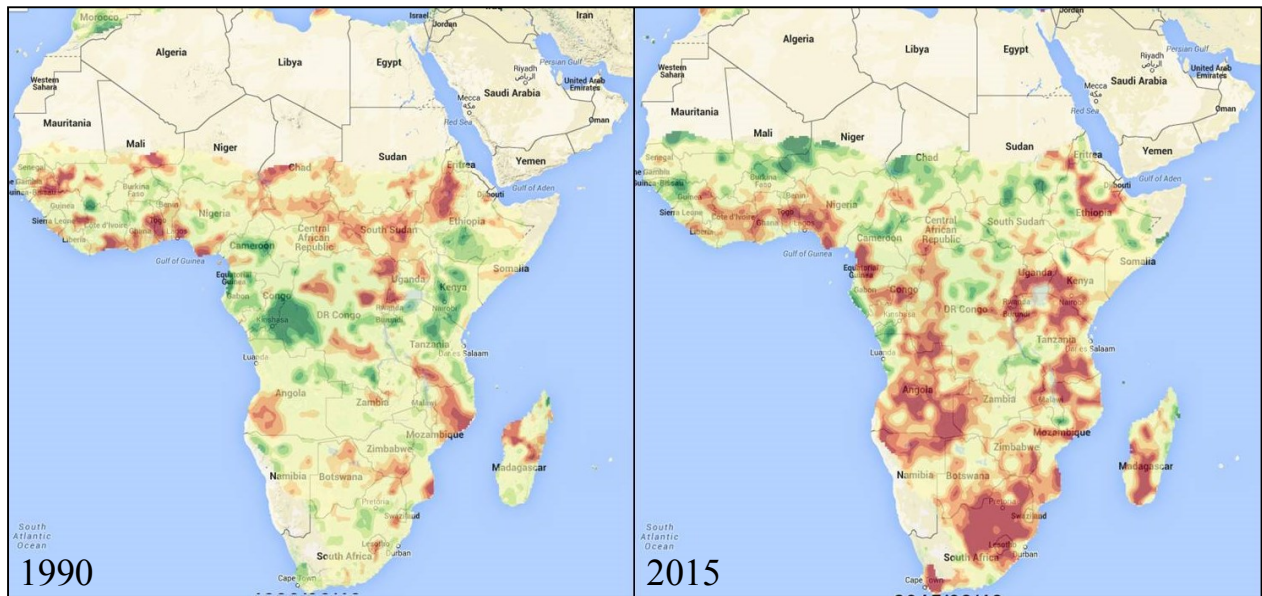
Figure 2.6 Depiction of areas affected by drought in 1990 and 2015. The intensity of drought are indicated by red whereas green indicate ideal condition for vegetative growth i.e. wheat. (A) Drought was relatively manageable and the intensity and duration was not as concerning as in 2015 (B) (http://stream.princeton.edu/AWCM/WEBPAGE/).

2.1.7.1 Plants strategies to manage drought

Plant growth is mediated by a variety of complex interlinked environmental conditions, which can either be conducive or destructive to physiological, phenological and morphological mechanisms in the plant which is controlled by a multitude of genes, proteins, metabolites, and their respective regulatory networks (Ahuja *et al*., 2010; Barnabas *et al*., 2008). Plants can respond to changes in their environment in a slow or rapid manner alternately allowing them to adapt and subsequently survive. A physiological response to drought is secondary which involves reduced growth rate, reduced photosynthesis, and stomatal closure (Yordanov *et al*., 2003). The primary response occurs at a transcriptome, proteome, etc., level, which mediates the unique response on a physiological and morphological level. The central dogma is the basic tenet in understanding the flow of genetic information within any biological system. However, the complexity is often reduced by excluding other factors which regulate the flow of genetic information. In the case of abiotic stress responses, plants require a unique set of metabolic proteins such as those involved in synthesis of osmoprotectants and of

23

regulatory proteins operating in the signal transduction pathways, such as kinases or transcription factors (TF) (Cengiz *et al*., 2014).

2.1.8 Transcription factors and promoters linked to drought stress

TFs are proteins which have DNA-binding domains, which allow them to uniquely bind to complementary *cis*-elements present within a promoter upstream of a respective target gene. They induce or suppress the activity of RNA polymerases, thus regulating gene expression (Latchman, 1997). The presence or absence of a TF often involves a whole cascade of signalling events determined by tissue type, developmental stage and environmental conditions (Latchman, 1997).

Comprehensive elucidation of dynamic coordination of drought and salt responsive TFs in interacting pathways based on systemic and mutant analyses allow for the general acceptance that drought and salt responses are very similar in crops (Bartels and Sunkar, 2005). The consensus is that changes in membrane integrity and modulation of lipid synthesis are key factors in the primary sensing of drought and salt (Golldack *et al*., 2014). Subsequently the plasma membranes are affected, which is driven by osmotic stress induced signalling, effecting the H+ -ATPase and Ca+ -ATPase activities that lead to changes in Ca+ influxes, cytoplasmic pH and apoplastic production of reactive oxygen species (Golldack *et al*., 2014). The aforementioned can be partially or completely linked to the phytohormone abscisic acid (ABA), which is regarded as the "god hormone" within drought and salt plant responses (Golldack *et al*., 2014). This hormone essentially influences many drought associative genes i.e. the C-repeat binding factor / Drought-responsive element binding factor (*CBF/DREB*) genes.

2.1.8.1 *ICE1* a regulator for water stress.

C-repeat (CRT) binding factors (CBFs) or drought-responsive element (DRE) binding (DREB) proteins have been extensively study in the context of cereal crops. The aforementioned proteins are responsible for a cascade of reactions ultimately allowing tolerance to high levels of abiotic stress i.e. drought. The *DREB/CBF* gene contains *cis*-elements within its promoter which allows for the binding of a TF to subsequently activate the *DREB/CBF* genes. *DREB/CBF* has a canonical MYC (myelocytomatosis ongene) (CANNTG) and MYB (myeloblastosis oncogene) (C/TAACNA/G) recognition site in its promoter (Chen *et al*., 2005). *ICE1* encodes a MYC-type basic-helix-loop-helix (dHLH), and binds to *MYC* of *DREB/CBF* promoter. Essentially this induces the expression of *DREB/CBF* allowing for cold acclimation (Figure 2.7) (Chinnusamy *et al*., 2003; Miura *et al*., 2007). However, *ICE1* association are not exclusive to cold regulation alone, as it was recently confirmed by Xu *et al*., (2014) that *ICE1* can also regulate drought tolerance. Although these authors essentially conducted gene stacking which involve *ICE1* and *DREB/CBF*, however the exact role of *ICE1* in drought response still requires elucidation. It has been documented that *ICE1* is constitutively expressed in all tissues, and contrary to popular belief, *ICE1* is only slightly up regulated by cold temperatures (Chinnusamy *et al*., 2003; Feng *et al*., 2012). Essentially, cold induce the modification of the ICE1 protein by SUMOlyation (ZhaoBo and JianKang, 2015). It is also speculated that transcription co-factors are perhaps a necessity for *ICE1* to bind to *MYC-CBF/DREB* (Chinnusamy *et al*., 2003; Miura and Ohta, 2010).
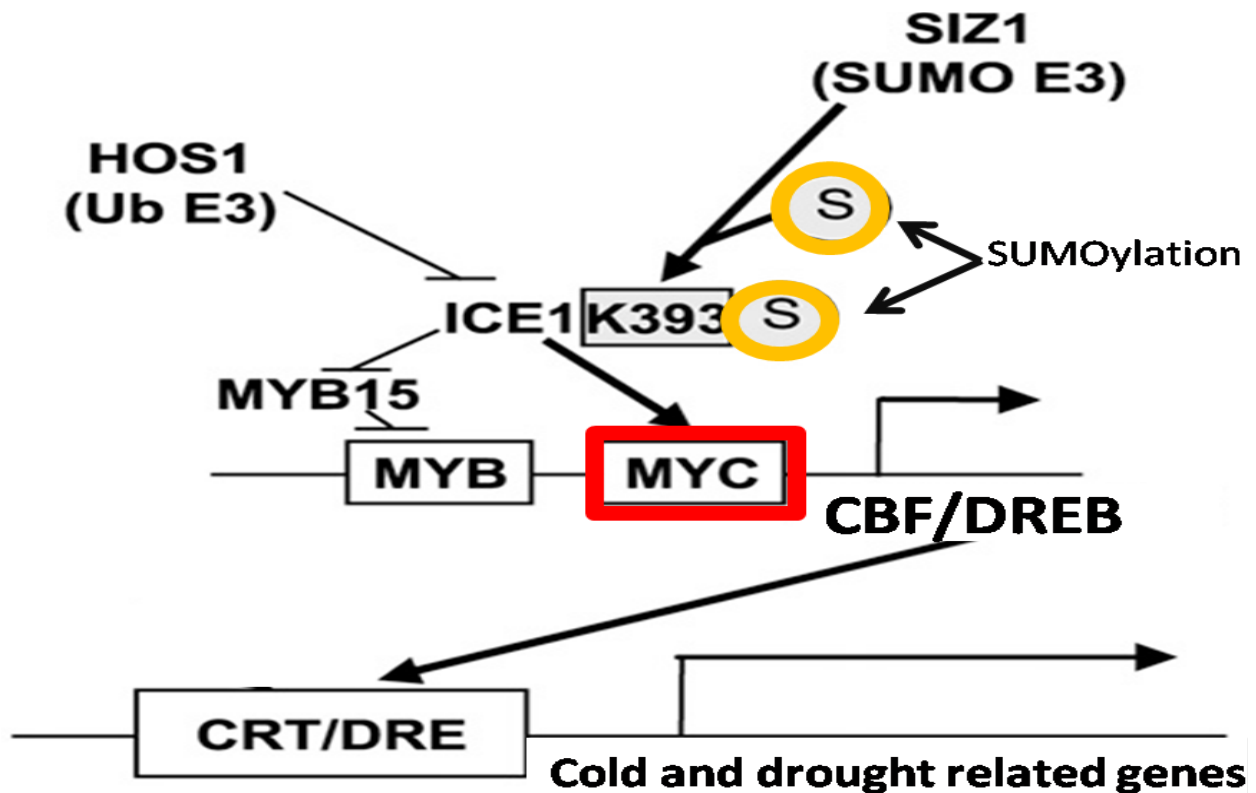
Figure 2.7 Broad overview of DREB/CBF regulon. ICE1 TF binds to *MYC* in the promoter to allow for the up regulation of the *CBF/DREB* gene. The CBF/DREB TF then binds to a conserve sequence upstream of the stress associated genes, allowing for the adaption to the adverse environmental conditions. However, for ICE1 to become active it requires to be SUMOylated by SIZ1 at K939 (Figure adapted from Miura *et al.,* 2007).

2.1.8.2 MYB15 TF a negative regulators of the *ICE1* pathway

The promoter of *CBF/DREB* has unique conserved regions which allow MYB DNA binding of which MYB15 TF is one, subsequently inhibiting the expression of *CBF/DREB* transcription (Figure 2.7), hereby negatively regulating freezing tolerance and perhaps other abiotic adaptations (Chen *et al*., 2005; Golldack *et al*., 2014). ICE1 TF physically interacts with *MYB15* promoters and attenuates *MYB15* expression, hereby preventing the inhibition of the *CBF/DREB* regulons (Figure 2.7). A recent study discovered a negative regulator of ICE1 TF known as *HOS1* (high expression of osmotically responsive gene) (Dong *et al*., 2006). In a study conducted by Dong *et al.* (2006), a knock-out line of *HOS1* in *A. thaliana* was developed and it was found that a reduction

26

in the *CBF/DREB* gene expression during cold stress ultimately affects cold tolerance. The obtained results can be explained as follows: upon cold stress, *HOS1* is exponentially unregulated, resulting in the migration of the TF to the location of where ICE1 TF is present (e.g. nucleus), where it physically interacts with ICE1 TF. This interaction leads to poly-ubiquitination of ICE1 for proteosomal degradation. Consequently *DREB/CBF* regulons are not transcribed since *ICE1* cannot activate the transcription of *DREB/CBF* (Dong *et al*., 2006). Therefore *HOS1* is considered an upstream yet direct regulator of the *CBF/DREB* regulon (Figure 2.7).

2.1.8.3 Abiotic stress related promoters

All the above mentioned TFs (ICE1, HOS1, MYB15 and CBF/DREB) have great potential in cereal engineering to overcome abiotic stress, however constitutive over expression of some genes can lead to the plant suffering a great deal of metabolic overload which essentially results in growth retardations and other unwanted phenotypic attributes (Mattoo *et al.,* 2015). The application of stress inducible promoters to confine over-expression to specific abiotic conditions has proven to work effectively in dicotyledonous and monocotyledonous plants (Liang *et al*., 2014). The *Rd29a* ABA-responsive promoter is well characterized, and was used for drought improvement in *A. thaliana*, tobacco, wheat, sugarcane and potato (Sun and Chen, 2002; Kasuga *et al*., 2004; Gao *et al*., 2005; Zhang *et al*., 2005; Behnam *et al*., 2006; Wu *et al*., 2008). However, leakiness of this promoter in wheat has been suspected which often translates into undesired phenotypes (Lopato and Langridge, 2011; Morran *et al*., 2011). The maize *Rab17* gene is induced by water deficit and increase levels of ABA (Vilardell *et al*., 1990; Morran *et al*., 2011b). *In planta* footprinting (DNA-protein interaction) and transient expression of *Rab17* in maize embryos, has revealed a promoter region which is important for optimum functioning and activation of the gene (Kizis and Pagès, 2002). Furthermore, two *cis*-elements was located within the promoter, which is drought responsive, known as *DRE2* with core sequence ACCGAC. Interestingly, the *DRE2* architecture is similar to the well documented DRE and C-repeat elements commonly referred to as *DRE/CRT*, which are the conserved areas that the CBF/DREB TF contains.

2.1.8.4 Post-translation modification

All previously mentioned, stress responsive genes, transcription factors and respective promoters are regulated by molecular signals which are key to understanding what drives specific responses. The elucidation of the aforementioned will provide us with insight into how wheat perceives varied environmental cues and transduce them into molecular and biochemical changes, leading to a coordinated tolerance response. Stress responsive genes can be successfully transcribed, however the protein may not be active until post-translational modification occurs (Kurepa *et al.*, 2003; Barrero-Gil and Salinas, 2013). The attachment of a small polypeptide to a target protein is an example of such a modification especially in eukaryotes e.g. ubiquitination and SUMOylation. It has been demonstrated that major regulons such as *DREB/CBF* are regulated by ubiquitination and SUMOylation, thus elucidation of these pathways, more specifically the amino acid sequences known as SUMO signatures, are pivotal to understanding abiotic stress.

2.1.9 SUMOylation

SUMOylation is an additional, yet essential post-translational modification and is in fact related it's in amino acid sequence to ubiquitin and is equally important in target protein management. However, it functions as a vital counterpoise to ubiquitination, adding a layer of control above ubiquitination with respect to substrate availability, stoichiometry, competition for targets and prevention of ubiquitin dependent protein degradation (Miura *et al.*, 2005).

Small Ubiquitin-like Modifier (SUMO) targets proteins and covalently binds to the lysine residues. This binding is reminiscent to those biochemical steps of ubiquitination, however SUMOylation has its own set of analogous E1, E2 and E3 enzymes that are involved in activation, conjugation and ligation (Conti *et al*., 2008; Miura and Ohta, 2010). SUMOylation responds to signals to maintain cellular protein activity as it has been proposed that SUMOylated proteins can undergo three general formations, based on yeast and mammalian systems (Li *et al*., 2005; Conti *et al.*, 2008; Miura and Ohta, 2010). First, SUMO can affect the target proteins

ability to interact with partner proteins. Secondly, it can also provide a binding site for an interacting partner to recognize (Miura and Hasegawa, 2010). An example of the target is a transcription factor and as it becomes SUMOylated it can thus enhance the TF ability to recruit chromatin–remodelling factors or inhibitory factors to repress the expression of downstream genes (Miura and Hasegawa, 2010). Finally, SUMOylation can also lead to a conformational change of a target protein (Miura and Hasegawa, 2010). Thus, SUMOylation appears to strategically influence target proteins in a very distinct manner often leading to the alteration of target protein, cellular location, stability or by antagonizing their degradation via the proteasome, e.g. by blocking ubiquitin attachment sites (Johnson, 2004).

 SUMOylation in plants is a complex process, with its importance spanning from abiotic stress tolerance (Chosed *et al*., 2006), flowering time (Jin *et al*., 2007), abscisic signalling (Miura and Ohta, 2010) and pathogen defence (Roden *et al*., 2004). In addition to this, plants lacking the SUMO conjugated proteins will not undergo normal development, as has been shown by the embryo-lethal phenotype associated with mutation in either SAE1/2 encoding E1 or SCE1 encoding conjugating E2 enzymes (Saracco *et al*., 2007). Low and high temperature, salt, drought and oxidative stress (pathogens) induce the conjugation of SUMO to protein substrates (Miura and Hasegawa, 2010). In addition to this phenotypic plasticity it is also regulated by SUMOylation, which is regulated to withstand cold temperature, basal thermo-tolerance and phosphate starvation responses (Miura *et al*., 2005; Yoo *et al*., 2006; Miura *et al.*, 2007b).

2.1.9.1 De-SUMOylation

SUMOylation is a reversible post-translation modification process, and the reverse reaction is referred to as de-SUMOylation. This process is driven by SUMO proteases that consist of a variable N-terminal domain and a conserved C-terminal protease domain. At least eight SUMO proteases were found in *A. thaliana*, and these were grouped into different families (Kurepa *et al*., 2003; Chosed *et al.,* 2006). SUMO proteases act as isopeptidases that specifically cleave the SUMO-substrate linkage to recycle free SUMO. At any given time plants have a small fraction of protein substrates that are SUMOylated. This small fraction is induced under normal environmental conditions, and is maintained by the process of de-SUMOylation driven by active

SUMO proteases (Mazzucotelli *et al.*, 2008; Miura and Hasegawa, 2010). The aforementioned was confirmed by the SUMO protease-deficient *A. thaliana* mutant "Early in short days4" (*esd4*) (Johnson, 2004; Conti *et al.*, 2008). These mutant plants (*eds4*) presented with an elevation in SUMOylated proteins, expressed early flowering, reduction of growth, as well as other inflorescence development defects. SUMO proteases also play a vital role in plant-pathogen interaction. This was confirmed by a study conducted by Roden *et al*. (2004) who investigated a phytopathogenic bacterium (*Xanthomonas pv campestris)* and its interaction with proteins *in planta*. Essentially their results indicated that SUMO proteases decrease SUMO conjugation onto targeted proteins, ultimately to subvert defence in host cells.

2.1.9.2 *OTS1* and *OTS2*

De-SUMOylation has been shown to play a vital role in salt tolerance in *A. thaliana*. Two SUMO proteases, namely *OVERLY TOLERANT TO SALT1* (*OTS1*) and *OTS2,* have redundant functioning, and facilitate salt tolerance in *A. thaliana*. A study conducted by Conti *et al.* (2008) confirmed that *OTS1* and *OTS2* are nucleus-localized. Furthermore, increased salt hypersensitivity was observed in *A. thaliana* double mutants lacking *ots1* and *ots2* compared with wild type and single mutant *ots1* and *ots2*. The *ots1* and *ots2* single mutants had a higher level of SUMO1/2 conjugated proteins than the wild type, however the double mutant *ots1 ots2* showed a dramatic increase in SUMO1/2 conjugated proteins. In contrast, over expression of the *OTS1* and *OTS2* appears to have enhanced the plant's ability to withstand high concentrations of salt. These results show that *OTS1* and *OTS2* play a pivotal role in the regulation of SUMOylation/de-conjugation of target proteins especially during stress conditions such as increased salt in the soil. Researchers at the University of Warwick have recently also shown the involvement of OTS1/2 in biotrophic pathogen defence with the context of salicylic acid signalling (Bailey, 2014). In the present study OTS1 and OTS2 from *Arabidopsis thaliana* was introduced into wheat in an effort to enhance stress tolerance.

2.2 References

Ageel S, Elmeer K (2011). Effects of Casein Hydrolysates and Glutamine on Callus and Somatic Embryogenesis of Date Palm (Phoenix dactylifera L.) New York Science Journal. **4**: 7

Ahuja, I, deVos R, Bones AM, Hall RD (2010). Plant molecular stress responses face climate change. Trends in Plant Science. **15**: 12

Ahrens J, Loomis W (1963). Floral Induction and Development in Winter Wheat. Crop Science. **3**: 463

Anandan S, Khan AA, Ravi D, Sai Bucha Rao M, Ramana Reddy Y, Blümmel M (2013).Identification of a superior dual purpose maize hybrid among widely grown hybrids in South Asia and value addition to its stover through feed supplementation and feed processing. Field Crops Research. **153**: 52-57

Asseng S, Foster I, Turner N (2011). The impact of temperature variability on wheat yields. Global Change Biology. **17**: 997-1012

Australian Government (2014). Department of Health, Office of gene technology regulator. GM wheat filed-trails approvals. Annual Reports. **1**: 2

Australian Government (2015). Department of Health, Office of gene technology regulator. GM wheat filed-trails approvals. Annual Reports. **1**: 1-3

Azghandi A, khosravi S, Mojtahedi N, Haddad R (2007). *In vitro* Propagation of *Lilium longiflorum Var. Ceb-Dazzle* through Direct Somatic Embryogenesis. Pakistan Journal of Biological Sciences. **10**: 2517-2521

Bailey M (2014). An investigation into the role of SUMO proteases *OVERLY TOLERANT to SALT*1 and -2 in salicylic acid mediated defence signalling in *Arabidopsis thaliana*: toward understanding the role of SUMOylation in SA signalling. Ph.D. University of Warwick

Ball ST, Zhou H, Konzak CF (1992). Sucrose concentration and its relationship to anther culture in wheat. Crop Science. **32**: 149-54

Barnabas B, Jager K, Feher A (2008). The effect of drought and heat stress on reproductive processes in cereals. Plant, Cell and Environment. **31**: 11-38

Barro F, Martin A, Lazzeri PA, Barcelo P (1999). Medium optimization for efficient   somatic embryogenesis and plant regeneration from immature inflorescences and immature scutella of elite cultivars of wheat, barley and *tritordeum*. Euphytica. **108**: 161-167

Barrero-Gil J, Salinas J (2013). Post-translational regulation of cold acclimation response. Plant Science. **205**: 48–54

Bartels D, Sunkar R (2005). Drought and salt tolerance in plants. Critical Reviews in Plant Science. **24**: 23–58

Behnam B, Kikuchi A, Toprak FC, Yamanaka S, Kasuga M, Yamaguchi-Shinozaki K, Watanabe KN (2006). The *Arabidopsis DREB1A* gene driven by the stress-inducible *rd29A* promoter increases salt-stress tolerance in proportion to its copy number in tetrasomic tetraploid potato (*Solanum tuberosum*). Plant Biotechnology. **23**: 169–177

Benson EE (2008). Cryopreservation of phytodiversity: a critical appraisal of theory & practice. Critical Reviews in Plant Sciences. **27**: 141–219

Bevan MW, Flavell RB, Chilton M (1983). A chimaeric antibiotic resistance gene as a selectable marker for plant-cell transformation. Nature. **304**: 184–187

Bhalla PL, Ottenhof HH, Singh MB (2006). Wheat transformation – an update of recent progress. Euphytica. **149**: 353–366

Borras-Gelonch G, Rebetzke GJ, Richards RA, and Romagosa I (2012). Genetic control of duration of pre-anthesis phases in wheat (*Triticum aestivum* L.) and relationships to leaf appearance, tillering, and dry matter accumulation. Journal of Experimental Botany. **63**: 69–89

Carvajal M, Cooke DT, Clarkson DT (1996). Responses of wheat plants to nutrient deprivation may involve the regulation of water-channel function. Planta. **199**: 372-381

Cengiz M, Inal B, Kavas M, Unver T (2014). Diverse expression pattern of wheat transcription factors against abiotic stresses in wheat species. Gene. **550**: 117–122

Challinor A, Wheeler T, Garforth C, Craufurd P, Kassam A (2007). Assessing the vulnerability of food crop systems in Africa to climate change. Climatic Change. **83**: 381–399

Chapman PD, Oliveira T, Livingston AG, Li K (2008). Membranes for the dehydration of solvents by pervaporation. Journal of Membrane Science. **318**: 5–37

Chen R, Ni Z, Nie X, Qin Y, Dong G, Sun Q (2005). Isolation and characterization of genes encoding Myb transcription factor in wheat (*Triticum aestivem* L.). Plant Science. **169**: 1146–1154

Chinnusamy V, Ohta M, Kanrar S, Lee BH, Hong X, Agarwal M, Zhu JK (2003*). ICE1*: A regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis* development. Gene Development. **17**: 1043–1054

Chosed R, Mukherjee S, Lois LM, Orth K (2006). Evolution of a signalling system that incorporates both redundancy and diversity: *Arabidopsis* SUMOylation. Biochemistry Journal. **398**: 521–529

Ciarmiello LF, Woodrow P, Fuggi A, Pontecorvo G, Carillo P (2011). Plant genes for abiotic stress. *In* Shanker A, Venkateswarlu B, ed, Abiotic Stress in Plants - Mechanisms and Adaptations, 1st ed. InTech, Croatia, pp 283-308

Clive J (2014). Global Status of Commercialized Biotech/GM Crops: ISAAA Brief No. 46. ISAAA. (http://www.isaaa.org/kc/cropbiotechupdate/article/default.asp?ID=12071, accessed on 19 June 2015)

Coenen C, Lomax T (1997). Auxin-cytokinin interactions in higher plants: old problems and new tools. Trends in Plant Science. **2**: 351-356

Commodity intelligence report (2014). Record Wheat Yields Expected for South Africa's 2014/15 Wheat Crop, 1st ed. **1-3** (http://pecad.fas.usda.gov/highlights/2014/09/SAfrica/index.htm, accessed on 5 October 2015)

Cong L, Wang C, Chen L, Liu H J, Yang G X, He G Y (2009). Expression *of phytoene synthase*1 and *carotene desaturase crtl* genes result in an increase in the total carotenoids content in transgenic elite wheat (*Triticum aestivum* L.). Journal of Agricultural and Food Chemistry. **57**: 8652–8660

Conti L, Price G, O'Donnell E, Schwessinger B, Dominy P, Sadanandom A (2008). Small ubiquitin-like modifier proteases *OVERLY TOLERANT TO SALT* 1 and -2 regulate salt stress responses in *Arabidopsis*. Plant Cell. **20**: 2894–908

de Paiva Neto V, Otoni W (2003). Carbon sources and their osmotic potential in plant tissue culture: does it matter? Scientia Horticulturae. **97**: 193-202

Delporte F, Jacquemin JM, Masson P and Watillon B (2012). Insights into the regenerative property of plant cells and their receptivity to transgenesis. Plant Signalling and Behaviour. **7**: 1608-1620

Dong CH, Agarwal M, Zhang Y, Xie Q, Zhu J-K (2006). The negative regulator of plant cold responses, *HOS1* is a RING E3 ligase that mediates the ubiquitination and degradation of *ICE1*. Proceedings of the National Academy of Sciences. **103**: 8281–8286

Dunwell JM (2013). Transgenic cereals: current status and future prospects. Journal of Cereal Science **59**: 419-434

Dupont FM (2008). Metabolic pathways of the wheat (*Triticum aestivum* L.) endosperm amyloplast revealed by proteomics. BioMedical Central Plant Biology. **8**: 39

Ekom DC, Udupa SM, Benchekroun MN, Ennaji MM, Abdelwahd R, Iragi D (2014). Immature embryo-derived of two bread wheat (*Triticum aestivum* L.) varieties transformation using particle bombardment method. Annual Research and Review in Biology. **4**: 3904-3914

Engelmann F (2004). Plant cryopreservation: progress and prospects. *In vitro* Cellular & Developmental Biology. **40**: 427–433

Edmeades G, Cooper M, Lafitte R, Zinselmeier C, Ribaut J (2001). Abiotic stresses and staple crops. *In*: Nosberger J, Geiger, HH, Struik PC, Edition, Crop Science: Progress and prospects. CABI International, Wallingford, UK, pp 137-154.

Ezeibekwe IO, Ezenwaka CL, Mbagwu FN, Unamba CI (2009). Effects of combination of different levels of auxin (NAA) and cytokinin (BAP) on *In vitro* propagation of *Dioscorea rotundata L*. (White Yam). Journal of Molecular Genetics. **2**: 18-22

Fábián A, Jäger K, Darkó É, Barnabás B (2008). Cryopreservation of wheat (*Triticum aestivum* L.) egg cells by vitrification. Acta Physiologiae Plantarum. **30**: 737–744

Fahy GM, Wowk B, Wu J, Phan J, Rasch C, Chang A, Zendejas E (2004). Cryopreservation of organs by vitrification: perspectives and recent advances. Cryobiology. **48**: 157–78

FAOSTAT (2013). Food and Agriculture Organization of the United Nations (http://faostat.fao.org, accessed on 3 September 2015).

Fretz A, Lorz H (1995). Cryopreservation of *in vitro* cultures of barley (Hordeum *vulgare* L. and H. *murinum* L.) and transgenic cells of wheat (*Triticum aestivum* L). Plant Physiology. **146**: 489–496

Folling LO (2001). Transformation of wheat (*Triticum aestivum* L.) microspore-derived callus and microspores by particle bombardment. Plant Cell Reports. **20**: 629–636

FOX JL (2013). Volunteer GM wheat, mischief or carelessness? Nature Biotechnology. **31**: 669–670

FOX JL (2009). Whatever happened to GM wheat? Nature Biotechnology. **27**: 974–976

Funnekotter A, Kaczmarczyk A, Menon A, Ye-Phang P, Al-Hanbal A, Bunn E, Ricardo LM (2012). Current issues in plant cryopreservation. Current Frontiers in Cryobiology. **4**: 417–438

Gamborg OL, Miller RA, Ojima K (1968). Nutrient requirements of suspension culture of soybean root cells. Experimental Cell Research. **50**: 151-158

Gao SQ, Chen M, Ma YZ (2005). Activity of *rd29A* promoter in wheat immature embryonic calli. Acta Physiologiae Plantarum. **31**: 150–153

Gao SQ, Chen M, Xia LQ, Xiu H J, Xu ZS, Li LC, Zhao CP, Cheng XG, Ma YZ (2009). A cotton (*Gossypium hirsutum*) DRE-binding transcription factor gene, *GhDREB*, confers enhanced tolerance to drought, high salt, and freezing stresses in transgenic wheat. Plant Cell Report. **28**: 301–311

George E, Hall M, De Klerk G (2015). Plant propagation by tissue culture. 3rd Edition, pp115-120

Gnanapragasam S, Vasil IK (1992). Cryopreservation of immature embryos, embryogenic callus and cell suspension cultures of gramineous species. Plant Science. **83**: 205–215

Goasdoue N, Goasdoue C, Dardoize FS, Couffignal R, Chimie LD, Curie M, Jussieu P (1993). Changes in fatty acid and lipid composition of wheat when treated with a chemical hybridizing agent. Phytochemistry **34**: 375–380

Golldack D, Li C, Mohan H, Probst N (2014). Tolerance to drought and salt stress in plants: Unravelling the signalling networks. Frontiers Plant Science. **5**: 151

Goodwin J, Pastori G, Davey M, Jones H (2004). Selectable markers: Antibiotic and herbicide resistance. *In* L Peña, ed, Transgenic plants: methods and protocols, 10th ed. Humana Press, Totowa, pp 191-201

Haberer G, Kieber JJ (2002). Cytokinins: new insights into a classic phytohormone. Plant Physiology. **128**: 354-362

Haider N (2013). The origin of B-genome of bread wheat (*Triticum aestivum* L.). Russian Journal of Genetics. **49**: 263–274

Harding K (2007). Plant and algal cryopreservation: issues in genetic integrity, concepts in cryobionomics and current applications in cryobiology. Molecular and Biology Evolution. **18**: 151-154

Harwood W (2011). Advances and remaining challenges in the transformation of barley and wheat. Journal of Experimental Botany. **63**: 1791-1798

Harlan JR, Zohary D (1966). Distribution of wild wheat and barley. Science. **153**: 1074–1080

Harvey CA, Rakotobe ZL, Rao NS, Dave R, Razafimahatratra H, Rabarijohn H, Rajaofara H, Mackinnon JL, B PTRS (2014). Extreme vulnerability of smallholder farmers to agricultural risks and climate change in Madagascar. Philosophical Transactions of the Royal Society. **369**: 19691–19696

Malcolm S (2013) Trial of genetically modified wheat approved. ABC Rural. http://www.abc.net.au/news/2013-11-01/gm-wheat-trial-approved/5064146 accessed on 4 January 2016

Haudry A, Cenci A, Ravel C, Bataillon T, Brunel D, Poncet C, Hochu I, Poirier S, Santoni S, Glémin S (2007). Grinding up wheat: a massive loss of nucleotide diversitysince domestication. Molecular and Biology Evolution. **24**: 1506–17

He Y, Jones HD, Chen S, Chen XM, Wand DW, Li KX, Wand DS, Xia LQ (2010). *Agrobacterium*-mediated transformation of durum wheat (*Triticum turgidum* L. var. *durum* cv *Stewart*) with improved efficiency. Journal of Experimental Botany. **61**: 1567-1581

Heyns IC (2010). Mapping and restructuring of an *Ae. kotschyi* derived translocation segment in common wheat. Ph.D. Stellenbosch University

Hunt ED, Svoboda M, Wardlow B, Hubbard K, Hayes M, Arkebauer T (2014). Monitoring the effects of rapid onset of drought on non-irrigated maize with agronomic data and climate-based drought indices. Agricultural and Forest Meteorology. **191**: 1–11

Indrianto, Erwin Heberle-Bors, Alisher Touraev (1999). Assessment of various stresses and carbohydrates for their effect on the induction of embryogenesis in isolated wheat microspores. Plant Science. **143**: 71-79

Ioset JR, Urbaniak B, Ndjoko-Ioset K, Wirth J, Martin F, Gruissem W, Hostettmann K, Sautter C (2007). Flavonoid profiling among wild type and related GM wheat varieties. Plant Molecular Biology. **65**: 645–54

Jackson MA, Anderson DJ, Birch RG (2013). Comparison of *Agrobacterium* and particle bombardment using whole plasmid or minimal cassette for production of high expressing, low-copy transgenic plants. Transgenic Research. **22**: 143–51

Jain RK, Davey MR, Cocking EC, Ray Wu (1997). Carbohydrate and osmotic requirements for high frequency plant regeneration from protoplast-derived colonies of indica and japonica rice varieties. Journal of Experimental Botany. **48**: 751-758

Jin J, Jin Y, Lee J, Miura K, Yoo C, Kim W, Van Oosten M, Hyun Y, Somers D, Lee I (2007). The SUMO E3 ligase, *AtSIZ1*, regulates flowering by controlling a salicylic acid-mediated floral promotion pathway and through effects on *FLC* chromatin structure. The Plant Journal. **53**: 530-540

Johnson ES (2004). Protein modification by SUMO. Annual Review of Biochemistry. **73**: 355–82

Jones HD, Dohery A, Wu H (2005). Review of methodologies and a protocol for the *Agrobacterium*-mediated transformation of wheat. Plant Methods. **1**: 5

Jones HD (2005). Wheat transformation: current technology and applications to grain development and composition. Journal of Cereal Science. **41**: 137–147

Kasuga M, Miura S, Shinozaki K, Yamaguchi-Shinozaki K (2004). A combination of the *Arabidopsis DREB1A* gene and stress-inducible *rd29A* promoter improved drought and low-temperature stress tolerance in tobacco by gene transfer. Plant Cell Physiology. **45**: 346–350

Kavas M, Öktem HA, Yücel M (2008). Factors affecting plant regeneration from immature inflorescence of two winter wheat cultivars. Biologia Plantarum. **52**: 621–626

Kiba T, Kudo T, Kojima M, Sakakibara H (2010). Hormonal control of nitrogen acquisition: roles of auxin, abscisic acid, and cytokinin. Journal of Experimental Botany. **62**: 1399-1409

Kizis D, Pagès M (2002). Maize DRE-binding proteins *DBF1* and *DBF2* are involved in *rab17* regulation through the drought-responsive element in an ABA-dependent pathway. Plant Journal. **30**: 679–689

Kruger-lebus PI (1985). A simple and efficient method for direct gene transfer to petunia hybrida without electroporation. Plant Molecular Biology. **5**: 289–294

Kurepa J, Walker JM, Smalle J, Gosink MM, Davis SJ, Durham TL, Sung DY, Vierstra RD (2003). The small ubiquitin-like modifier (SUMO) protein modification system in *Arabidopsis*; Accumulation of SUMO1 and -2 conjugates is increased by stress. The Journal of Biological Chemistry. **278**: 6862–72

Lacock L, Botha AM (2000). Genotype variation in regeneration and transient expression efficiencies of 14 South African wheat cultivars. South African Journal of Plant and Soil. **17**: 4

Langridge P, Lopato S (2011). Improvement of stress tolerance of wheat and barley by modulation of expression of *DREB/CBF* factors. Plant Biotechnology Journal. **9**: 230– 249

Latchman DS (1997). Transcription factors: An overview. International Journal of Biochemistry and Cell Biology. **29**: 1305–1312

Law CN, Worland AJ (1997). Genetic analysis of some flowering time and adaptive traits in wheat. New Phytologist. **137**: 19–28

Liang C, Theo WP, van de Wiel CC, Kok EJ (2014). Safety aspects of genetically modified crops with abiotic stress tolerance. Trends in Food Science & Technology. **40**: 115–122

Linkies A, Leubner-Metzger G (2011). Beyond gibberellins and abscisic acid: how ethylene and jasmonates control seed germination. Plant Cell Report. **31**: 253-270

Li SJ, Hankey W, Hochstrasser M (2005). Preparation and characterization of yeast and human desumoylating enzymes. Methods Enzymology. **398**: 457–67

Li J, Ye X, An B, Du L, Xu H (2012). Genetic transformation of wheat: current status and future prospects. Plant Biotechnology Reports. **6**: 183-193

Lloyd G, Mccown B (1980). Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. International Plant Propagators Society. **30**: 421–427

Lopato S and Langridge P (2011). Engineering stress tolerance in cereals using *DREB/CBF* genes; outcome, problem, perspective. Information System for Biotechology News. **1**: 2–4

Loyola-vargas VM (2006). Wheat transformation. *In* F Loyola-Vargas, Victor M. II. Vázquez-Flota, ed, Plant Cell Culture. Protocol, Second edition. Humana Press Inc, Totowa, New Jersey, pp 273–283

Lupi R, Denery-Papini S, Rogniaux H, Lafiandra D, Rizzi C, De Carli M, Moneret VD, Masci S, Larré C (2013). How much does transgenesis affect wheat allergenicity? Assessment in two GM lines over-expressing endogenous genes. Journal of Proteomics. **80**: 281–91

Mahmood I, Razzaq A, Khan Z, Hafi IA, Kaleem S (2012). Evaluation of tissue culture responses of promising wheat (*Triticum aestivum* L.) cultivars and development of efficient regeneration system. The Pakistan Journal of Botany. **44**: 277-284

Martin J (1975). Comparison of agar media for counts of viable soil bacteria. Soil Biology and Biochemistry. **7**: 401-402

Mattoo A, Upadhyay R, Rudrabhatla S (2015). Abiotic stress in crops: Candidate genes, osmolytes, polyamines, and biotechnological intervention. *In* Pandey G, ed, Elucidation of Abiotic Stress Signaling in Plants, 1st ed. Springer, New York, pp 415-437

Mazzucotelli E, Mastrangelo AM, Crosatti C, Guerra D, Stanca AM, Cattivelli L (2008) Abiotic stress response in plants: When post-transcriptional and post-translational regulations control transcription. Plant Science. **174**: 420–431

Mertz O, Halsnaes K, Oelsen J, Rasmussen K (2009). Adaptation to climate change in developing countries. Environmental Management. **43**:743–752

Meyre D, Leonardi A, Brisson G, Vartanian N (2001). Drought-adaptive mechanisms involved   in   the escape / tolerance strategies of *Arabidopsis Landsberg erecta* and *Columbia* ecotypes and their F1 reciprocal progeny. Journal of Plant Physiology. **158**: 1145–1152

Miller G, Jen-Huang I, Welkie G, Pushnik J (1993). Function of iron in plants with special emphasis on chloroplasts and photosynthetic activity. *In* Abadía J, ed, Iron Nutrition in Soils and Plants, 1st ed. Springer Netherlands, Netherlands, pp 19–28

Miura K, Rus A, Sharkhuu A, Yokoi S, Karthikeyan AS, Raghothama KG, Baek D, Koo YD, Jin JB, Bressan R (2005). The *Arabidopsis* SUMO E3 ligase SIZ1 controls phosphate deficiency responses. Proceedings of the National Academy of Sciences. **102**: 7760–5

Miura K, Jin JB, Hasegawa PM (2007) Sumoylation, a post-translational regulatory process in plants. Current Opinion in Plant Biology. **10**: 495–502

Miura K, Jin JB, Lee J, Yoo CY, Stirm V, Miura T, Ashworth EN, Bressan R a, Yun DJ, Hasegawa PM (2007). SIZ1-mediated SUMOylation of *ICE1* controls *CBF3/DREB1A* expression and freezing tolerance in *Arabidopsis*. Plant Cell. **19**: 1403–14

Miura K, Lee J, Jin JB, Yoo CY, Miura T (2009). SUMOylation of *ABI5* by the *Arabidopsis* SUMO E3 ligase SIZ1 negatively regulates abscisic acid signaling. Proceedings of the National Academy of Sciences. **106**: 5418-5423

Miura K, Hasegawa PM (2010). SUMOylation and other ubiquitin-like post-translational modifications in plants. Trends in Cell Biology. **20**: 223–32

Miura K, Ohta M (2010). SIZ1, a small ubiquitin-related modifier ligase, controls cold signalling through regulation of salicylic acid accumulation. Journal of Plant Physiology. **167**: 555–60

Moghaieb R, El-Arabi N, Momtaz O, Youssef S, Soliman M (2010). Genetic transformation of mature embryos of bread (T. *aestivum*) and pasta (T. *durum*) wheat genotypes. Genetically Modified Crops. **1**: 87–93

Morran S, Eini O, Pyvovarenko T, Parent B, Singh R, Ismagul A, Eliby S, Shirley N, Murtas G, Reeves PH, Fu Y, Bancroft I, Dean C, Coupland G (2003). A nuclear protease required for flowering-time regulation in *Arabidopsis* reduces the abundance of small Ubiquitin-Related Modifier (SUMO) conjugates. The Plant Cell. **15**: 2308–2319

Morran S, Eini O, Pyvovarenko T, Parent B, Singh R, Ismagul A, Eliby S, Shirley N, Langridge P, Lopato S (2011). Improvement of stress tolerance of wheat and barley by modulation of expression of *DREB/CBF* factors. Plant Biotechnology Journal. **9**: 230–249

Murashige T, Shoog F (1962). A revise medium for rapid growth and bioassays with tabacco tissue caltures. Physiologia Plantarum. **15**: 473-497

Nerson N, Sibony N, Pinthus M (1980). A scale for the assessment of the developmental stages of the wheat (*Triticum aestivum* L.) spike. Annauls of Botany. **45**: 203-204

Noack AL, Pouw NR (2015). A blind spot in food and nutrition security: where culture and social change shape the local food plate. Agriculture and Human Values. **32**: 169–182

Normile D (2014). China pulls plug on genetically modified rice and corn. American association for the advancement of science news Magazine 1 (http://news.sciencemag.org/asiapacific/2014/08/china-pulls-plug-genetically-modified-rice-and-corn accessed on 15 September 2015)

Oerke E, Dehne H (1997). Global crop production and the efficacy of crop protection – current   situation and future trends. European Journal of Plant Pathology. **103**: 203-215

Ogawa Y, Sakurai N, Oikawa A, Kai K, Morishita Y, Mori K, Moriya K, Fujii F, Aoki K, Suzuki H (2012). High-throughput cryopreservation of plant cell cultures for functional genomics. Plant Cell Physiology. **53**: 943–52

Ponya Z, Barnabas B (2003). Regeneration of fertile wheat (*Triticum aestivum* L.) plants from isolated zygotes using wheat microspore culture as nurse cells. Plant Cell, Tissue and Organ Culture. **74**: 243–247

Popov S, Popova V, Nikishina V, Vysotskaya N (2006). Cryobank of plant genetic resources in Russian Academy of Sciences. International Journal of Refrigeration. **29**: 403–410

Porter R and Gawith (1999). Temperatures and the growth and development of wheat: a review. European Journal of Agronomy. **10**: 23–36

Quatrano R (1968). Freeze-preservation of cultured flax cells utilizing dimethyl sulfoxide. Plant Physiology. **43**: 2057–2061

Rasco-Gaunt S, Riley A, Cannell M, Barcelo P, Lazzeri PA (2001). Procedures allowing the transformation of a range of European elite wheat (*Triticum aestivum* L.) varieties via particle bombardment. Journal of Experimental Botany. **50**: 865–874

Reynolds MP, Hays D, Chapman S (2010). Breeding for adaptation to heat and drought stress. *In* M.P. Reynolds MP, ed, Climate Change and Crop Production. CAB Intl, Wallingford, UK. p. 71-91

Rice X (2010). Severe drought causes hunger for 10 million in West Africa. Eastern Sahel crisis is worst in Niger where 7.1 million are hungry as livestock and crops are lost and food prices surge. Guardian. (http://www.guardian.co.uk/environment/2010/jun/03/drought-hunger-west-africa, accessed on 3 August 2015).

Roberts-Oehlschlager SL, Dunwell JM, Faulks R (1990). Changes in sugar content of barley anthers during culture on different carbohydrates. Plant Cell, Tissue and Organ Culture. **22**: 77-85

Roden J, Eardley L, Hotson A, Cao Y, Mudgett MB (2004). Characterization of the *Xanthomonas* AvrXv4 effector, a SUMO protease translocated into plant cells. Molecular Plant-Microbe Interactions. **17**: 633–43

Rong W, Qi L, Wang J, Du L , Xu H , Wang A , Zhang Z (2013). Expression of a potato antimicrobial peptide SN1 increases resistance to take-all pathogen *Gaeumannomyces graminis var. tritici* in transgenic wheat. Functional and Integrative Genomics. **13**: 403–409

Saad A, Elshahed A (2015). Plant tissue culture media. *In* Leva A, Rinaldi L, ed, Recent Advances in Plant *In vitro* Culture, 1st ed. InTech, Croatia, pp 29-40

Sakai A, Hirai D, Niino T (2008). Development of PVS-based vitrification and encapsulation-vitrification protocols. *In* Reed BM, ed, Plant Cryopreservation: A practical guide, Springer, New York, pp 33-57

Sangwan R, Sangwan-Norreel B (1996). Cytological and biochemical aspects of *in vitro* adrogenesis in higher plants. *In* S Mohan-Jain, S Sopory, R Veilleux, ed, *In vitro* Haploid Production in Higher Plants; Fundamental Aspects and Methods, 1st ed. Springer, Netherlands, Dordrecht, pp 95-101

Saracco S a, Miller MJ, Kurepa J, Vierstra RD (2007). Genetic analysis of SUMOylation in *Arabidopsis*: conjugation of SUMO1 and SUMO2 to nuclear proteins is essential. Plant Physiology. **145**: 119–34

Schenk RU, Hildebrandt AC (1972). Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Canadian Journal of Botany. **50**: 199-204

Simmons SR and Crookston RK (1979). Rate and duration of growth of kernels formed at specific florets in spikelets of spring wheat. Crop Science. **19**: 690–693

She M, Yin G, Li J, Li X, Du L, Ma W, Ye X (2013). Efficient regeneration potential is closely related to auxin exposure time and catalase metabolism during the somatic embryogenesis of immature embryos in *Triticum aestivum* L. Molecular Biotechnology. **54**: 451–60

Shewry PR (2009). Wheat. Jouranal of Experimental Botony. **60**: 1537–53

Shungu D, Valiant M, Tutlane V, Weinberg E, Weissberger B, Koupal L, Gadebusch H, Stapley E (1983). GELRITE® as an agar substitute in bacteriological media. Applied and Environmental Microbiology. **46**: 840-845

Slafer G and Savin R (1991). Developmental base temperature in different phenological phases of wheat (*Triticum aestivum*). Journal of Experimental Betony. **42**: 1077–1082

Solov'eva AI, Dolgikh Y, Vysotskaya O, Popov A (2011). Patterns of ISSR and REMAP DNA markers after cryogenic preservation of spring wheat calli by dehydration method. Russian Jounral of Plant Physiology. **58**: 423–430

Southgate E, Davey MR, Power JB, Marchant R (1995). Factors affecting the genetic engineering of plants by microprojectile bombardment. Biotechlology Advances. **13**: 631–651

Sreenivasulu N, Schnurbusch T (2012). A genetic playground for enhancing grain number in cereals. Trends in Plant Science. **17**: 91-101

Sun XH and Chen MJ (2002). A brief account of promoter cloning. Acta Edulis Fungi. 9:57–62

Tang G, Qin J, Dolnikowski G, Russell R, Grusak M (2009). Golden Rice is an effective source of vitamin A. The American Journal of Clinical Nutrition. **89**: 1776–1783

The University of Nottingham (2015) Transfer of wild relative chromosome segments into wheat. (http://www.nottingham.ac.uk/wisp/wild-relative-gene-introgression/research.aspx, accessed on 12 October 2015)

Tinak-Ekom DC, Udupa SM, Benchekroun MN, Ennaji MM, Abdelwahd, Iraqi D (2014). Immature embryo-derived of two bread wheat (*Triticum aestivum* L.) Varieties transformation using particle bombardment method. Annual Research & Review in Biology. **4**: 3904–3914

Turhan H, Baser I (2004). Callus induction from mature embryo of winter wheat (*Triticum aestivum* L.). Asian Journal of Plant Sciences. **3**: 17–19

Uchendu E, Leonard S, Traber M, Reed B (2010). Vitamins C and E improve re-growth and reduce lipid peroxidation of blackberry shoot tips following cryopreservation. Plant Cell Reports. **29**: 25–35

Van der Vyver A (2013). The relative value between barley and wheat from a production point of view: Northern Cape irrigation areas. Department of Agricultural Economics, Extension and Rural Development, University of Pretoria.

Vandenelzen PJ, Townsend J, Lee KY, Bedbrook JR (1985). A chimaeric hygromycin resistance gene as a selectable marker in plant cells. Plant Molecular Biology. **5**: 299–302

Vanstraelen M, Benková E (2012) Hormonal Interactions in the Regulation of Plant Development. Annual Review of Cell and Developmental Biology. **28**: 463-487

Vasil IK, Vasil V (2006). Transformation of wheat via particle bombardment. *In* Loyola-Vargas VM and Vazquez-Flota F. Plant Cell Culture Protocols, Methods in Molecular Biology, Humana Press Inc. Totowa, New Jersey, pp 273-283,

Viertel K, Hess D (1996). Shoot tips of wheat as an alternative source for regenerable embryogenic callus cultures. Plant Cell, Tissue and Organ Culture. **44**: 183-188

Vilardell J, Goday A, Freire M, Torrent M, Martínez MC, Torné J, Pagès M (1990). Gene sequence, developmental expression, and protein phosphorylation of *Rab17* in maize. Plant Molocular Biology. **14**: 423–432

Waldron C, Murphy EB, Roberts JL, Gustafson GD, Armour SL, Malcolm SK (1985). Resistance to Hygromycin-B a new marker for plant transformation studies. Plant Molecular Biology. **5**: 103–108

Wageningen U (2015). Cryopreservation. (Available at https://www.wageningenur.nl/en.htm)

Wang B, Zhang Z, Yin Z, Feng C, Wang Q (2012). Novel and potential application of cryopreservation to plant genetic transformation. Biotechnology Advances. **30**: 604–12

Wowk B (2007). How cryoprotectants work. Cryonics. **28**: 3–7

Wei Z, Xin-min W, Rong F Gui-xiang F, Ke W, Li-pu D, Le-le X1, Xing-guo Y (2015). Effects of inter-culture, arabinogalactan proteins, and hydrogen peroxide on the plant regeneration of wheat immature embryos. Journal of Integrative Agriculture. **14**: 11–19

Wilhite A, and Glantz H (1985). Understanding the drought phenomenon: the role of definitions. Water International 10: 111–120

White PR (1963). The cultivation of animal and plant cells, 2nd edition, Ronald Press, New York

Wu Y, Zhou H, Que YX, Chen RK, Zhang MQ (2008). Cloning and identification of promoter *rd29A* and its application in sugarcane drought resistance. Sugar Technology. **10**: 36–41

Xia L, Ma Y, He Y, Jones HD (2012). Genetically modified wheat development in China: current status and challenges to commercialization. Journal of Experimental Botany. **63**: 1785–90

Xu F, Liu Z, Xie H, Zhu J, Zhang J, Kraus J, Blaschnig T, Nehls R, Wang H (2014). Increased drought tolerance through the suppression of *eskmo*1 gene and over expression of *CBF*-related genes in *Arabidopsis*. Public Library of Science. **9**: 1

Yaseen M, Ahmad T, Sablok G, Standardi A, Hafiz I (2012). Review: role of carbon sources for *in vitro* plant growth and development. Molecular Biology Reports. **40**: 2837-2849

Yin G, Wang Y, She M, Du L, Xu H, Ma J, Ye X (2011). Establishment of a highly efficient regeneration system for the mature embryo culture of wheat. Agricultural Science in China. **10**: 9–17

Yi H, Qiong W, Jian Z, Tao S, Guang-xiao Y, Guang-yuan H (2015). Current status and trends of wheat genetic transformation studies in China. Journal of Integrative Agriculture. **14**: 438–452

Yoo C, Miura K, Jin J, Lee J, Park H, Salt D, Yun D-J, Bressan R, Hasegawa P (2006). SIZ1 small ubiquitin-like modifier E3 ligase facilitates basal thermotolerance in *Arabidopsis* independent of salicylic acid. Plant Physiology. **142**: 1548–58

Yordanov I, Velikova V, Tsonev T (2003). Plant responses to drought and stress tolerance. Bulgarian Journal of Plant Physiology. **1**: 187–206

ZhaoBo L, JianKang Z (2015). *OST*1 phosphorylates *ICE1* to enhance plant cold tolerance. Science China Life Sciences. **58**: 317–318

Zhao Y (2012) Auxin Biosynthesis: A simple two-step pathway converts tryptophan to Indole-3-Acetic acid in plants. Molecular Plant **5**: 334-338

Zhang N, Si HJ, Wang D (2005). Cloning of *rd29A* gene promoter from *Arabidopsis* thaliana and its application in stress-resistance transgenic potato. Acta Agronomica Sinica. **31**: 159–164

Zhang L, Zhang G, Zhao X, Yang S (2013). Screening and analysis of proteins interacting with TaPDK from physiological male sterility induced by CHA in wheat. Journal of Integrative Agriculture. **12**: 941–950

CHAPTER 3

In vitro regeneration and cryopreservation by encapsulation-dehydration of southern African bread wheat cultivars (*Triticum aestivum* L.).

Submitted for publication

Ref: SAJB_2015_15

3.1 Introduction

Wheat (*Triticum aestivum* L.) is one of the main cereal crops grown worldwide providing essential proteins and carbohydrates to the human diet (Feuillet *et al*., 2007). Global wheat production measured 660 million tons for the 2012/13 growth season with major producers being China, India and the USA (FAO Stat, 2013). In sub-Saharan Africa, South Africa is the second largest wheat producing country after Ethiopia with production standing at 1.8 million tons in the 2014 season according to the Mundi index (www.indexmundi.com/agriculture/?country). However, the wheat industry has been declining in South Africa over the past two decades due to a number of environmental factors such as drought and heat, as well as biotic factors such as bollworms, aphids and mite infestation (Wheat Resource Center, 2009; van der Vyver, 2013).

The transfer of resistant genes into wheat cultivars and the elimination of undesired characteristics by backcrossing is time consuming in a hexaploid crop such as wheat, where multiple copies of genes can represent a challenge during conventional breeding (Akbari *et al*., 2006). Genetic transformation therefore provides an alternative to target and introduce specific resistance or trait genes into wheat for crop improvement. Transgenic wheat studies have been a challenging task and transformation of the Gramineae family of crops is not routine or widely established especially in Southern Africa. Gene transfer to higher plants requires robust tissue culture protocols from which viable and fertile transgenic plants can be regenerated. To date, wheat cultivars for which *in vitro* regeneration systems have been established mostly include European, Asian, North African and North American cultivars such as Bobwhite, Canon, Florida, Cadenza, Vesna, Veery-5, and Fielder, none of which are suitable for the climate of the Southern hemisphere (Jones *et al*., 2005; Wu *et al*., 2003; Zhao *et al*., 2006; Chugh *et al*., 2012). *in vitro* regeneration of these wheat cultivars was mostly achieved by using explant material such as immature and mature embryos, microspores, shoot tips, leaves, endosperm and seeds (Jones *et al*., 2005; Viertel and Hess, 1996; She *et al*., 2013; Yin *et al*., 2011; Ponya and Barnabas, 2003; Kavas *et al*., 2008). From all the explants tested, the immature embryo was proven to be the best for callus formation and somatic embryogenesis. However, the availability of immature embryos is limited to the cooler months of the year, excluding the peak summer months of December to April in Southern Africa, restricting the supply of explant material needed for *in vitro* propagation and subsequent transgenic studies.

Cryopreservation of explant material might be a viable option to supply unlimited fresh explant material throughout the year.

Cryopreservation is defined as the storage of biological material at sub-zero temperatures (Benson, 2008). These sub-zero temperatures create the platform for cellular and metabolic processes to enter their quiescent state and, in theory, plant material can be preserved at these temperatures for an indefinite period of time (Benson, 2008; Harding, 2007; Ogawa *et al*., 2012; Wang *et al*., 2011). In the context of wheat, cryopreservation is tissue dependant and a personalized cryopreservation protocol for each cultivar needs to be established (Engelmann, 2004). Expensive equipment (programmable freezers) and lengthy protocols outline the findings of cryopreservation for wheat, which often result in non-reproducible results. In recent years many attempt to simplify cryopreservation, by eluding the sophisticated and expensive equipment and applying the concept of dehydration and vitrification, ultimately addressing the intracellular water content of an explant.

The aim of this study was to investigate the *in vitro* regeneration capacity of six South African wheat cultivars, subsequently using the parameters for the establishment of a cryopreservation platform for immature wheat seeds (explants). These systems therefore create a platform for genetic transformation studies of wheat cultivars of importance to sub-Saharan Africa.

3.2 Materials and methods

3.2.1 *In vitro* regeneration

3.2.1.1 Plant material

To identify Southern African bread wheat (*Triticum aestivum* L.) cultivars with superior *in vitro* regeneration capabilities, six diverse cultivars namely Tugela-*Dn5,* Tugela-*Dn2;* Palmiet*;* Palmiet *Dn5 x Dn1;* Gamtoos R (*Dn7+*) and Gamtoos S *(Dn7-)*, were cultivated under glasshouse conditions in natural light conditions and humidity. Seedlings were grown to maturity (3 months) and spikelets collected 12-16 days post-anthesis. Immature seeds were surface sterilized for 2 minutes in 70% (w/v) ethanol followed by 15 minute in 20% (w/v) bleach (sodium hypochlorite), containing 0.01% Tween-20, and rinsed three times with sterilised distilled water.

3.2.1.2 Establishment of *in vitro* cultures

Immature embryos were extracted under sterile conditions with the aid of a binocular microscope. The scutella was removed from the immature embryo axis, and placed on four different callus induction media, as shown in Table 3.1 (Murashige and Skoog Supplemented A (MSS A) and Gamborg vitamins Supplemented B (MSS B), Induction A (Induc A) and Induction B (Induc B)). Cultures were maintained for 25 days at 26°C in the dark and sub-cultured every second week. At the end of this phase the explants were visually assessed and scored according to growth, colour and the presence of pro-embryogenic cell mass. Subsequently all callus material was transferred to one of four designed regeneration media, under a photoperiod of 16 h/8 h (day/night) for 5-7 weeks at 26°C, to evaluate the potential to induce shoots and roots as shown in Table 3.1 (MSS A- Regeneration (MSS A-Reg) and MSS B- Reg; Regeneration - zeatin A (RDZ A) and RDZ B. At the end of this culturing phase the embryogenicity of the calli and number of plantlets was recorded. Adventitious

root induction was done on MS (Murashige and Skoog 1962) or half-strength MS followed by *ex vitro* acclimatization in the greenhouse.

Acclimatization of *in vitro* generated plantlets was done in closed plastic containers in the greenhouse in a mix of potting soil, vermiculite and sand (ratio of 1:1:1) at 26°C under natural light conditions. Every 3rd day the containers were partially opened. Plantlets were then transferred to a greenhouse, where hardening off was completed, allowing the plants to acclimatize to the bigger, drier greenhouse environment. Plantlets were then regularly irrigated (3 times a day) via a dripper irrigation system containing nutrients (Multifeed TM, South Africa).

Table 3.1 Media composition for callus induction and shoot regeneration of wheat explants. Listed concentrations indicate amount per litre of growth medium

	MSS A	MSS B	Induc A	Induc B	MSS A-Reg	MSS B-Reg	RDZ A	RDZ B
*MS salts	4.4 g	4.4 g	4.4 g	4.4 g	4.4 g	4.4 g	4.4 g	4.4 g
*MS vit	+		+		+		+	
*B5 vit		+		+		+		+
Sucrose	20 g	20 g			20 g	20 g		
Maltose			40 g	40g			20 g	20 g
Casein	100 mg	100 mg	100 mg	100 mg	100 mg	100 mg	100 mg	100 mg
Glutamine	50 mg	50 mg	500 mg	500 mg	50 mg	50 mg	500 mg	500 mg
pH	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8
Gelrite	2.2 g	2.2 g	2.2 g	2.2 g	2.2 g	2.2 g	2.2 g	2.2 g
BAP	5 mg	5 mg			5 mg	5 mg		
Zeatin							5 mg	5 mg
2,4-D	2 mg	2 mg	0.5 mg	0.5 mg			0.1 mg	0.1 mg
Picloram			10 mg	10 mg				

All media constituents, with the exception of hormones and vitamins, were autoclaved at 121ºC/103 kPa. All stock solutions and growth regulators were filtered sterilized (Millipore, 0.22 µm, Merck, SA), and added to the media after autoclaving. *MS (Murashige and Skoog 1962; Highveld Biological, SA); B5 vitamins (Gamborg *et al*. 1968, Sigma-Aldrich, SA). All other chemicals and hormones were purchase from Sigma-Aldrich, SA. + = addition of vitamins

3.2.1.3 Evaluation of the effects of silver nitrate on *in vitro* regeneration

In addition, 60 Gamtoos S immature embryos were placed on callus induction media A (Table 3.1, Induc A) containing filter sterilized silver nitrate at concentrations between 10 and 80 μM. Callus production was visually assessed after incubation in the dark at 26°C for four weeks. After 4 weeks, callus was transferred to RDZ A media without any silver nitrate, under a photoperiod of 16/8 hours (day/night) for 5-6 weeks at 26ºC. Somatic embryogenesis and shoot formation were visually monitored.

3.2.1.4 Data analysis and statistics

Callus formation frequency was analysed per 30 explants (C), frequency of embryogenic callus formation per 10 calli (E), and number of shoots per isolated embryos (S). On the basis of these indices, calculation for the *in vitro* culture efficiency coefficient (Coef.) was done by the formula C/100 · E/100 · S. (Miroshnichenko *et al*., 2013). An ordinal scale (Appendix A, Fig. A1) was applied to translate qualitative description to quantitative data. All data were assessed for normality using XlStat (Addinsoft, 2014). Mean data are presented with their standard error of the mean (SEM) and analysed using parametric one way-ANOVA, following the post analysis Bonferroni, using Prism Graphpad software version 5.0 (Motulsky, 2015). Significant difference were calculated at $p < 0.05$ (n = 30 and repeated 3 times).

3.2.2 Cryopreservation

3.2.2.1 Plant material

Due to superior *in vitro* regeneration abilities, the spring wheat *(Triticum aestivum* L.*)* cultivar Gamtoos S *(Dn7-)* was chosen to establish the cryopreservation protocol. Plants were grown, immature seeds collected and immature embryos isolated as previously described.

3.2.2.2 Cryopreservation using Slow freeze / Non-dehydration technique

Pre-treatment and cryo-protectant solutions

Sterile immature embryos (250) were subjected to an antioxidant pre-treatment at 26ºC in the dark for 1 week. For pre-treatment, Induc A media was supplemented with either 0.3 M proline, 2 g/l charcoal, 2 µM abscisic acid (ABA), 15 g/l ascorbic acid, or cold treatment done at 4ºC, according to the method suggested by Fretz and Lörz (1995). After pre-treatment explants were placed in cryo tubes (Nunc® CryoTubes®, Sigma-Aldrich, SA) with the sterilised cryo-protectant solution, chilled 10% (w/v) DMSO (Engelmann 2011). A further 250 immature embryo explants were isolated and transferred into cryo tubes to test additional cryo-protectant solutions namely, 10% (w/v) DMSO, 0.5 M glycerol, 0.1 M proline, 0.5 M sorbitol or 0.5 M sucrose (Fretz and Lorz, 1995; Engelmann, 2011).

Freezing and thawing

Cryogenic tubes containing explants were placed within a controlled-cooling polycarbonate canister, called "Mr.Frosty" (Thermo Scientific, South Africa), containing 250 ml isopropyl alcohol. This non-mechanical device is placed within an ultra-freezer, and allows for a cooling rate of 1°C/min until the temperature reach -80°C. After a four week storage, the cryogenic tubes were plunged into a 40ºC water bath for 90-120 sec as suggested by Engelmann (2011). The contents of the cryo tubes were poured out onto a large autoclaved filter paper under sterile conditions, and rinsed twice with sterile water to remove cryo-protectants. Explants

underwent the entire *in vitro* regeneration process as previously described and visually assessed for differences in biomass and embryogenicity.

Cell viability assay

The cell viability of the cryopreserved tissue was determined by an Evan's Blue staining as described by Indra *et al*. (2006).

3.2.2.3 Cryopreservation using Encapsulation / Vitrification technique

Autoclaved calcium-free MS medium containing 4% (w/v) sodium alginate was prepared. From this, 1.5 ml media was repeatedly removed, using a sterile micropipette fitted with a modified 5 ml pipette tip, and dropped into a 400 mM $CaCl_2$ solution prepared in MS medium. Semi-hardened beads formed and immature seeds were meticulously inserted into these beads. The beads were then dipped in the MS alginate medium and placed back into 400 mM Calcium chloride ($CaCl_2$) for complete polymerization for 30-40 min. The beads were collected and osmo-protected in liquid MS medium containing 0.5 M sucrose for 20 hours at 25ºC on a rotary shaker at 130 rpm. The same process was repeated for isolated immature embryos, cultured 1-6 days on Induc A medium prior to encapsulation.

A total of 920 immature seeds and 500 immature embryos were encapsulated. The encapsulated immature seeds were organised according to two desiccation time points (22 and 74 hours; 460 samples per group), while the immature embryos was desiccated for 15 minute, 30 minute, 1 hour or 4 hours (125 immature embryos per group). Desiccation was done in a horizontal laminar flow cabinet with an average airflow velocity of 0.46 m/sec. The relative moisture content was determined from the average of 20 encapsulated seeds (n = 20) by dividing the average dry weight, at the different time points (12, 20, 24, 36, 48, 60 and 72 hours), by the original average fresh weight of the encapsulated immature seeds and converting to a percentage.

Cryo-protectant solutions for vitrification

Desiccated encapsulated immature seeds (EIS) and embryos (EIE) were placed in three different sterilized cryo-protectant solutions namely 80% (v/v) glycerol, 50% (v/v) glycerol or 10% (v/v) DMSO, each containing 160 EIS or 40 EIE per sub-group. A 500 µl cryo-protectant solution was placed in each cryo vial with the addition of 3-4 dehydrated explants per tube.

Freezing and thawing

The experimental sub groups were further divided into two freezing regimes, either frozen by placement in a "Mr.Frosty" at -80°C or flash frozen by direct placement into liquid nitrogen (-196°C). The samples were kept at the respective sub-zero temperature for 4 weeks. Rapid thawing of groups was done by plunging cryo tubes into a water bath at 40°C for 5 min. After thawing the explants were carefully removed from the beads, using fine tweezers. The seeds were allowed to rehydrate for 6-8 hours and embryos for 1 hour in MS medium (pH 5.8) at 26°C. Immature embryos and immature embryos extracted from the cryo-preserved immature seeds were *in vitro* regenerated as previously described.

3.2.2.4 Statistical analysis

Callus production after 4 weeks was quantified by measuring the callus coverage area (mm^2) produced from each immature embryo, using isometric paper. All data were assessed for normality using XlStat (Addinsoft, 2014). Mean data are presented with their standard error of the mean and analysed using Kruskall-Wallis non-parametric one way-ANOVA, following the multiple comparison test (Dunn, 1964; Siegel, 1956) using Prism Graphpad software. Significant difference were calculated at $p < 0.05$. Callus formation frequency (Cf%) were calculated per 80 explants using the formula Cf/80*100, and plantlet formation frequency (Pff%) using the formula Pff/80*100.

3.3 Results

3.3.1 *In vitro* regeneration

Plants from the six wheat (*Triticum aestivum* L.) cultivars were grown to maturity and immature embryos isolated 15 days post anthesis. Callus was induced from these explants on medium containing auxin growth regulators such as 2,4-dichlorophenoxyacetic acid (2,4-D) and picloram, which generally resulted in 50% to 90% callus formation frequencies across all the different wheat cultivars, with Gamtoos S performing the best. Reduced callus formation was observed for the two Tugela cultivars (50% to 70%; Table 3.2) when compared to the other cultivars tested. It was noted that the lower axis of the embryos needed to be removed to prevent embryo germination and induce callus formation. The total callus induction phase lasted 4 to 6 weeks. A prolonged callus induction phase resulted in severe necrosis of the callus tissue and poor embryo development

After callus formation, the calli were placed on media with reduced amounts of auxin, which resulted in the formation of somatic embryos. Calli derived from Induc A and MSS A medium displayed noticeable sites of embryogenic clusters with large somatic embryoids, while calli from Induc B and MSS B mostly resulted in non-embryogenic, soft, watery, creamish cell clusters. Overall, the spring wheat Gamtoos S performed the best *in vitro* with 70% shoot regeneration, ultimately resulting in a 34.3% *in vitro* co-efficiency rate (Figure 3.1, Table 3.2). The preferred medium for all the cultivars contained MS nutrients and vitamins for callus induction with a mix of auxins (2,4-D and picloram) and zeatin for shoot formation. Only the Tugela cultivars seemed to not prefer MS vitamins but rather Gamborg B5 vitamin mix. Furthermore, the Palmiet cultivars clearly preferred a cytokinin/auxin mix for callus induction (MSS A; 80%) in combination with 6-benzylaminopurine (BAP) for shoot formation (MSS AReg; 53-55%) resulting in a relatively high *in vitro* regeneration co-efficiency rate of 25-26% (Table 3.2). Overall, all the cultivars developed some shoots, via somatic embryogenesis, on the different tested media (Figure 3.1). However, the Tugela genotypes showed a limited ability to regenerate *in vitro* with a regeneration co-efficient ranging from 0 to 4.5% at most on the tested media (Table 3.2). Shoots from all cultivars developed roots on basal MS or half strength MS medium without

any additional hormone supplements. Well established plantlets with roots were transferred to the greenhouse, hardened off and displayed the normal phenotypic characteristics of wheat plants. Seeds were produced from these *in vitro* developed plants (Figure 3.2).
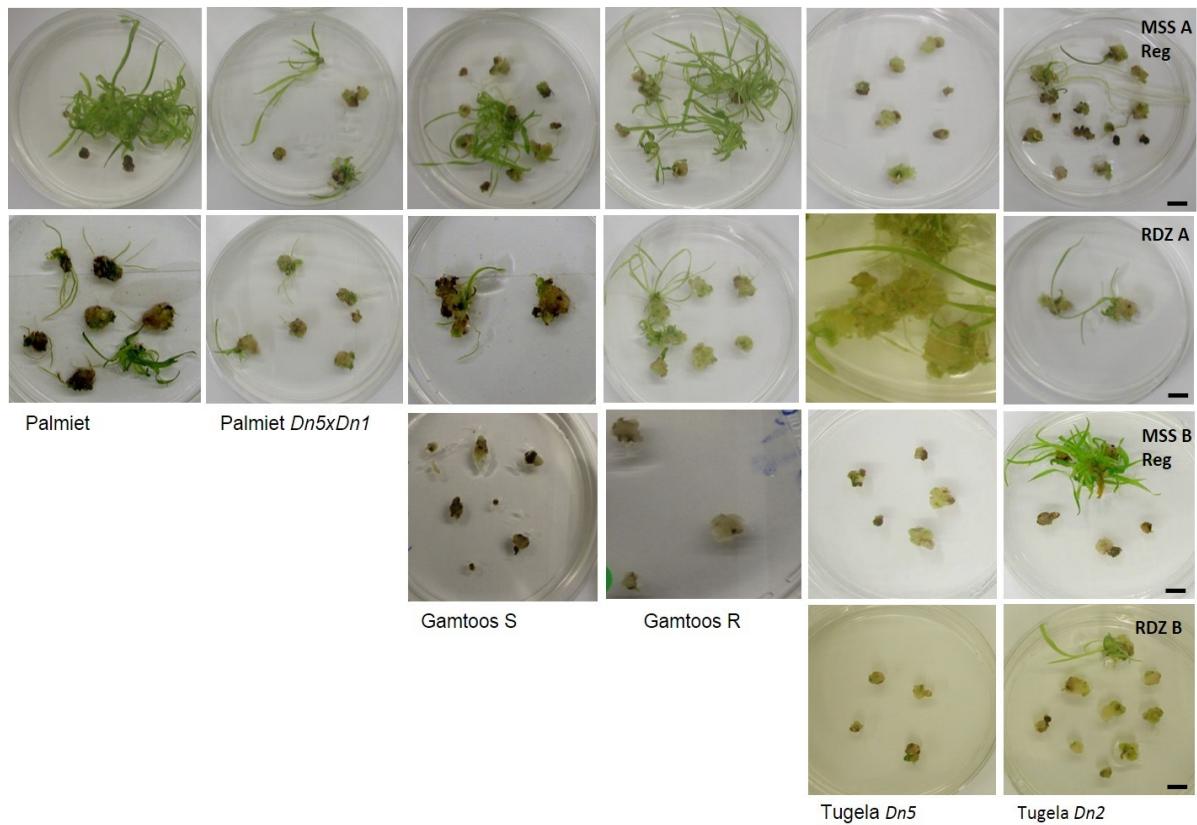
Figure 3.1 *In vitro* regeneration of six wheat cultivars, as indicated vertically from left to right, on four different shoot regeneration media, MSS A, MSS B, RDZ A and RDZ B (top to bottom), as represented by a sample set of induced immature embryos. Absent pictures planes indicated that no shoot regeneration occurred on that specific medium for that specific cultivar.

Figure 3.2 *In vitro* regenerated wheat plants. (A) Plantlets prior to e*x vitro*-acclimatization; (B) Plantlets are planted out in autoclaved potting soil, and covered with a dome ensuring a high humidity environment; (C) Plantlets with well-established roots and shoots; (D) Mature and fertile wheat plants.
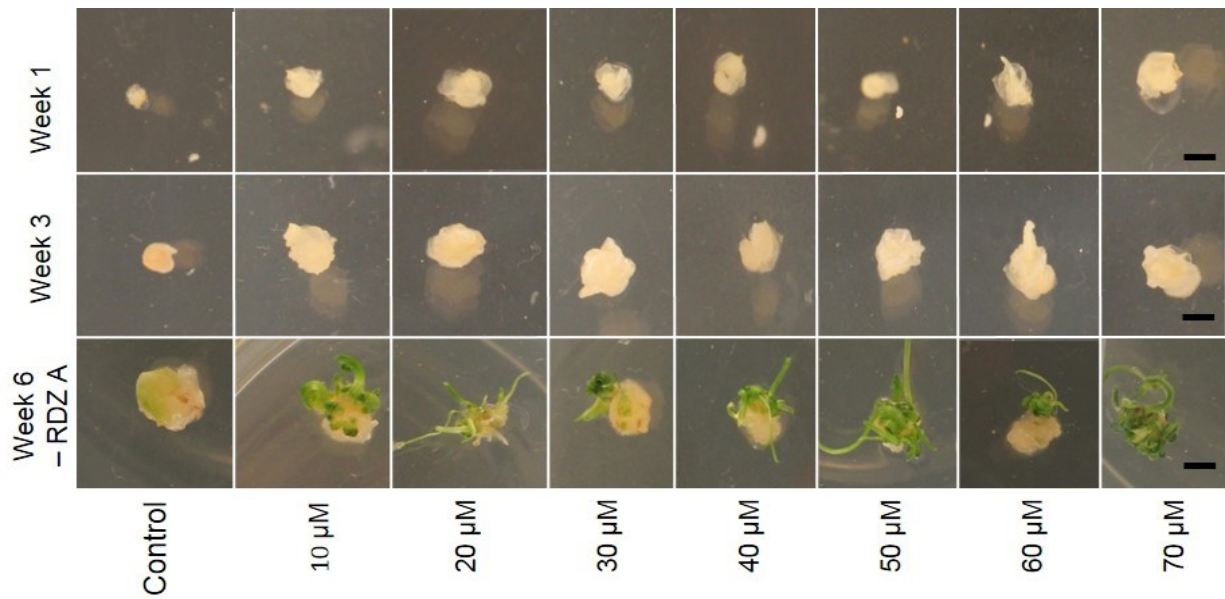
Figure 3.3 *In vitro* regeneration of immature embryos on callus induction (Induc A) medium containing various concentrations of silver nitrate for four weeks in the dark. Embryo regeneration was initiated after the callus induction phase on RDZ A medium without silver nitrate. Scale bar = 0.5 mm.

3.3.1.1 Silver nitrate exposure

Six day old immature Gamtoos S embryos were exposed to various concentration of silver nitrate induced on Induc A medium for the duration of the four week callus formation phase (Figure 3.3). Silver nitrate seems to increase the callus biomass compared to callus placed on Induc A without silver nitrate. Overall, silver nitrate enhanced shoot formation after 6 weeks of culture with an optimal concentration of 50 μM during the callus induction phase (Figure 3.3).

Table 3.2 *In vitro* callus formation, somatic embryogenesis and regeneration of shoots from immature embryos of six wheat cultivars placed on different growth mediums. Numerical values indicate the (C%) callus formation frequency based on callus biomass developed from one immature embryo; (E%) embryogenic callus frequency; (S%) shoot formation frequency based on the number of shoots per callus clump originating from one immature embryo; (Co) *In vitro* culture co-efficiency in percentage calculated from a starting 30 immature embryos (Miroshnichenko *et al*. 2013).

Genotype	Induc A		RDZ A		Induc B		RDZ B		Mss A		MSS A-Reg		Mss B		MSS B- Reg	
	C %	E %	S %	Co %	C %	E %	S %	Co %	C %	E %	S %	Co %	C %	E %	S %	Co %
Palmiet	50 ±0.33 [a]	25±0.72 [d]	13±0.30 [g]	1.63	90±0.34 [a]	45±0.83 [c]	0 [d]	0	80±0.49 [a]	60±0.92 [d]	55±0.68 [g]	26.4	60±0.33 [a]	55±0.88 [d]	30±0.65 [e]	9.9
*Palm Dn5xDn1	73 ±0.40 [a]	10±0.60 [d]	5±0.34 [g]	0.37	50±0.47 [b]	45±0.80 [c]	5±0.21 [e]	1.25	80±0.50 [a]	60±0.81 [d]	53±0.60 [g]	25.4	75±0.23 [a]	50±0.81 [d]	10±0.20 [f]	3.75
Gamtoos R	90 ±0.35 [b]	45±0.88 [e]	40±0.63 [h]	16.2	90±0.41 [a]	25±0.40 [c]	10±0.77 [f]	2.25	50 ±0.40 [b]	25±0.63 [e]	13±0.83 [h]	1.63	90±0.16 [b]	55±0.90 [d]	10±0.24 [f]	4.95
Gamtoos S	70 ±0.40 [b]	70±0.23 [e]	70±0.59 [h]	34.3	70±0.50 [a]	35±0.72 [c]	25±0.42 [f]	6.13	90±0.40 [b]	90±0.16 [f]	45±0.13 [i]	36.5	50±0.21 [c]	25±0.53 [e]	20±0.16 [e]	2.5
Tugela-*Dn5*	50 ±0.42 [c]	25±0.78 [f]	10±0.13 [g]	1.25	50±0.29 [b]	25±0.82 [c]	25±0.36 [g]	3.1	50±0.32 [c]	25±0.61 [e]	10±0.81 [h]	1.25	50±0.16 [c]	25±0.62 [d]	10±0.19 [f]	1.25
Tugela-*Dn2*	50 ±0.33 [c]	25±0.72 [f]	0 [i]	0	60±0.47 [b]	30±0.74 [c]	25±0.35 [g]	4.5	60±0.21 [c]	30±0.81 [f]	25±0.75 [h]	4.5	70±0.27 [a]	35±0.57 [d]	18±0.15 [e]	4.4

All frequencies with calculated ±SEM were scored according to an ordinal scale (Appendix A, Figure A1).

.

3.3.2 Cryopreservation

3.3.2.1 Slow-freeze / Non-dehydration

Immature embryos and callus tissue were pre-treated by exposure to cold (4°C) or in numerous different anti-oxidant solutions such as proline, charcoal, ABA, ascorbic acid, and placed in 10% (w/v) DMSO for cryopreservation. Different cryoprotectant solutions (DMSO, glycerol, proline, sorbitol or sucrose) were also tested for their ability to protect immature embryos and callus cells at sub-zero temperatures. However, lack of *in vitro* tissue regeneration post preservation was seen for all explants (Appendix A, Figures A2-A3). Cell damage of these tissues was suspected and indeed confirmed with a cell viability assay using Evan's Blue staining (Figure 3.4). Blue dye penetrated the compromised cell membranes, as opposed to viable cells which were not stained.

A.   **B.**   **C.**
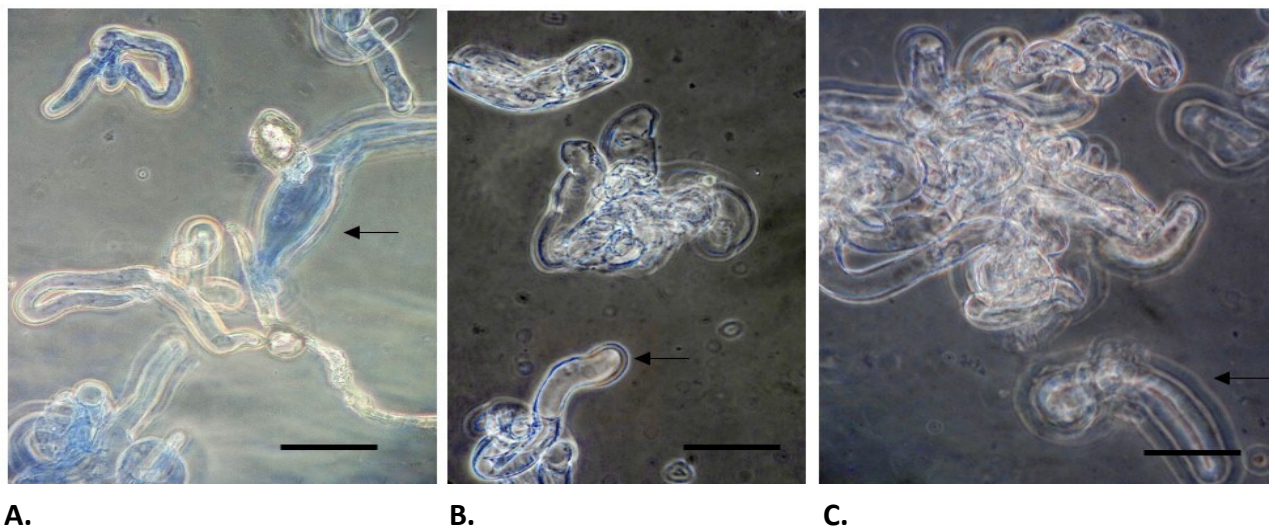
Figure 3.4 Evan's Blue staining of tissue post cryopreservation. (A) Damaged cells with blue dye penetrating the cell membrane from calli tissue pre-treated with ABA for 3 days prior to cryopreservation; (B) un-damaged cells from calli regenerated *in vitro* from cryopreserved encapsulated/dehydrated immature seeds and; (C) control non-preserved calli tissue showing staining blocked by cell membranes. Scale bar = 100 µm

3.3.2.2 Encapsulation / Vitrification: Dehydration prior to cryopreservation

Explants were encapsulated in alginate beads (Figure. 3.5) and dehydrated in a laminar flow cabinet for 15 minute to 4 hours (immature embryos) or 22 to 72 hours (immature seeds), respectively. During the total 72 hour dehydration period the encapsulated immature tissue lost approximately 74% of its relative moisture content (RMC) (Figure. 3.6). After sucrose treatment there was a substantial drop (66%) in RMC of the seeds up to 12 hours followed by small (2%) incremental loss of RMC from 22 to 72 hours (26 to 28%). There were no significant difference in the *in vitro* regeneration ability of cryopreserved immature seeds desiccated for 22 or 72 hours when cryoprotected in 10% DMSO or 50% glycerol (Figure. 3.7). However, the dehydration period seems to have a significant influence on the tissue protected in 80% (w/v) glycerol when desiccated either 22 or 72 hours, regardless of the small incremental loss (2%) in total moisture content between the two time points or the freezing method used (Figure. 3.7).

A.　　　　　　　　　　　　　**B.**　　　　　　　　　　　　　**C.**
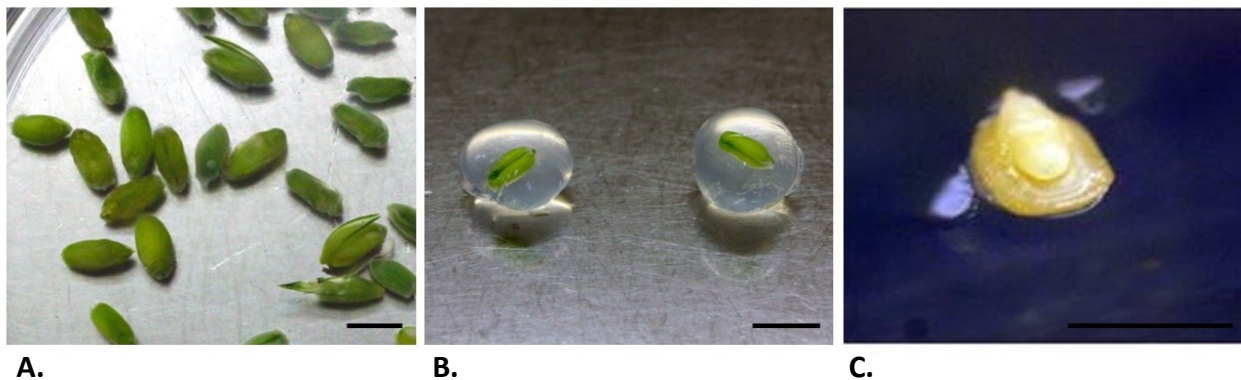
Figure 3.5 Steps during the Encapsulation / Vitrification cryopreservation of immature wheat seeds. (A) Immature seeds isolated 15 days after anthesis; (B) Immature seeds encapsulated in alginate beads; (C) Immature embryos isolated after cryopreservation from the immature seeds. Scale bar = 0.5 mm.
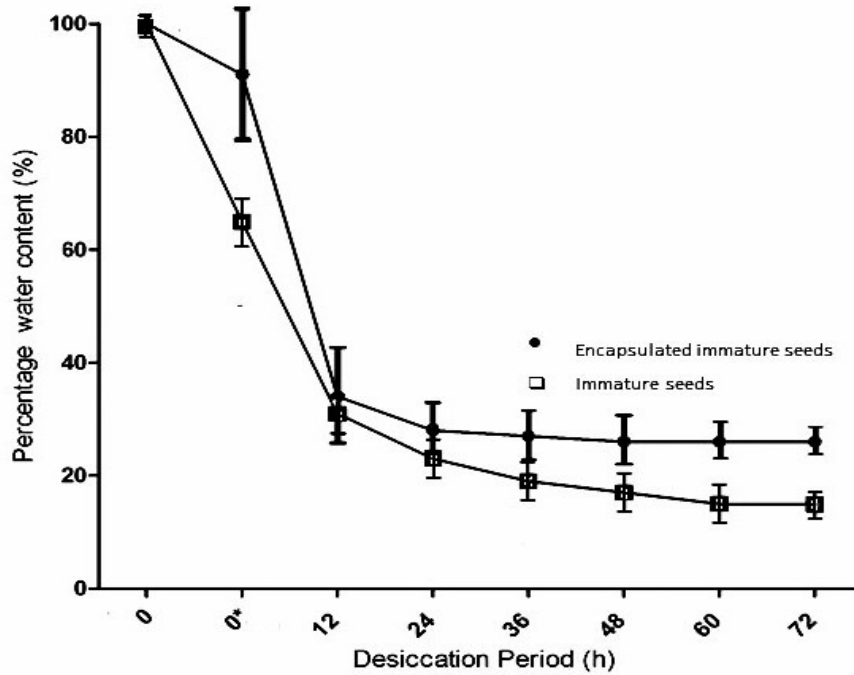
Figure 3.6 Relative moisture content of immature seeds and encapsulated immature seeds desiccated in a horizontal laminar flow cabinet over a 72 hour period. n = 20 per desiccation period. The time points 0 and 0* indicate the fresh weight of the tissue and moisture content after 20 hours of sucrose treatment prior to desiccation, respectively.
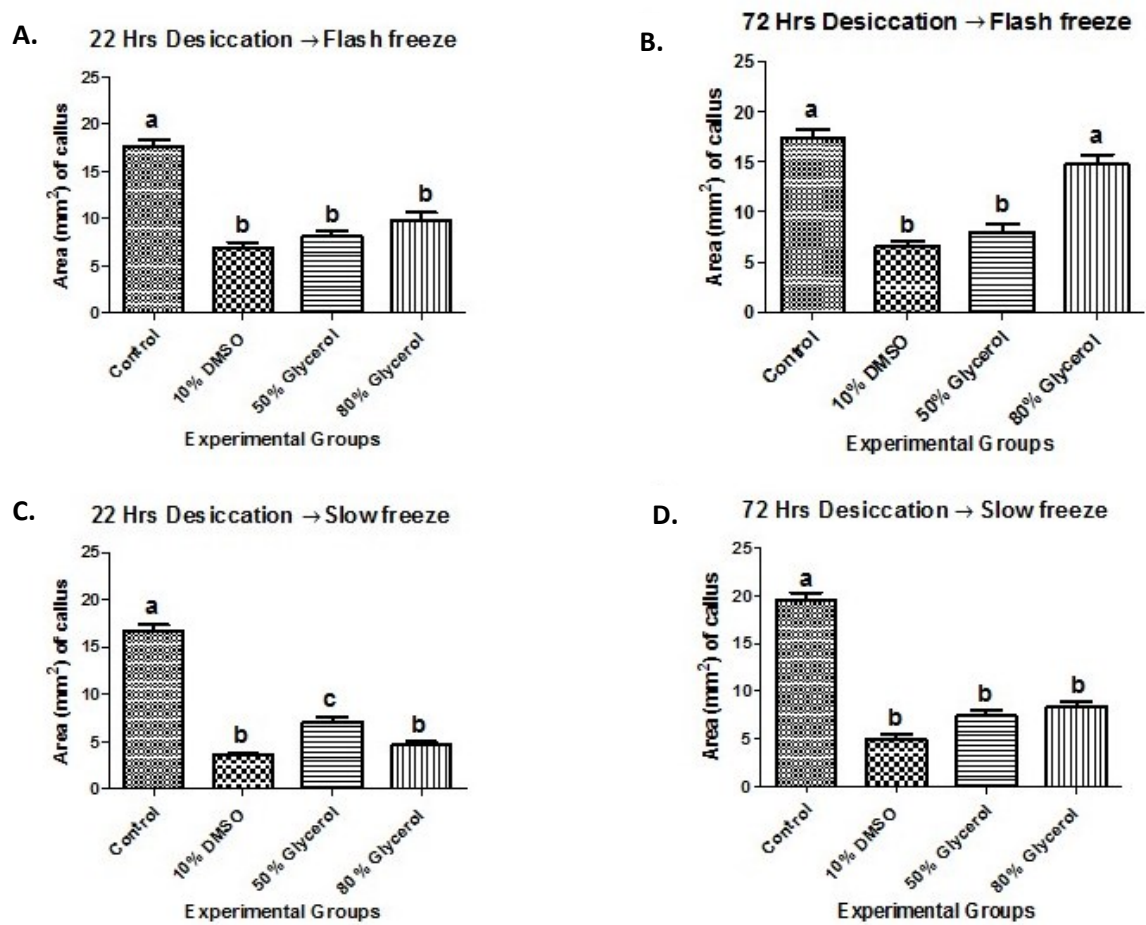
Figure 3.7 *In vitro* callus growth measurements, three weeks post-cryopreservation. Data are presented as the average (n = 160) with standard error of the mean. (A) 22 hour desiccation, following flash freeze; (B) 72 hour desiccation, following flash freeze; (C) 22 hour desiccation, following slow freeze; (D) 72 hour desiccation, following slow freeze. Significant difference were calculated at $p < 0.05$, and columns with the same letter ([a,b,c]) indicate no significant difference, whereas different letter indicate significance.

3.3.2.3 Encapsulation / Vitrification: Immature embryos *versus* immature seeds

Immature embryo survival post cryopreservation was unsuccessful as no tissue development *in vitro* was observed apart from initial rudimental cell differentiation (Appendix A, Fig. A3). For this reason attempts to cryopreserve this type of tissue was abandoned. However, immature embryos isolated from cryopreserved immature seeds were able to regenerate *in vitro* into plantlets irrespective of the different desiccation time points, cryoprotectants and freezing methods (Figure. 3.8).
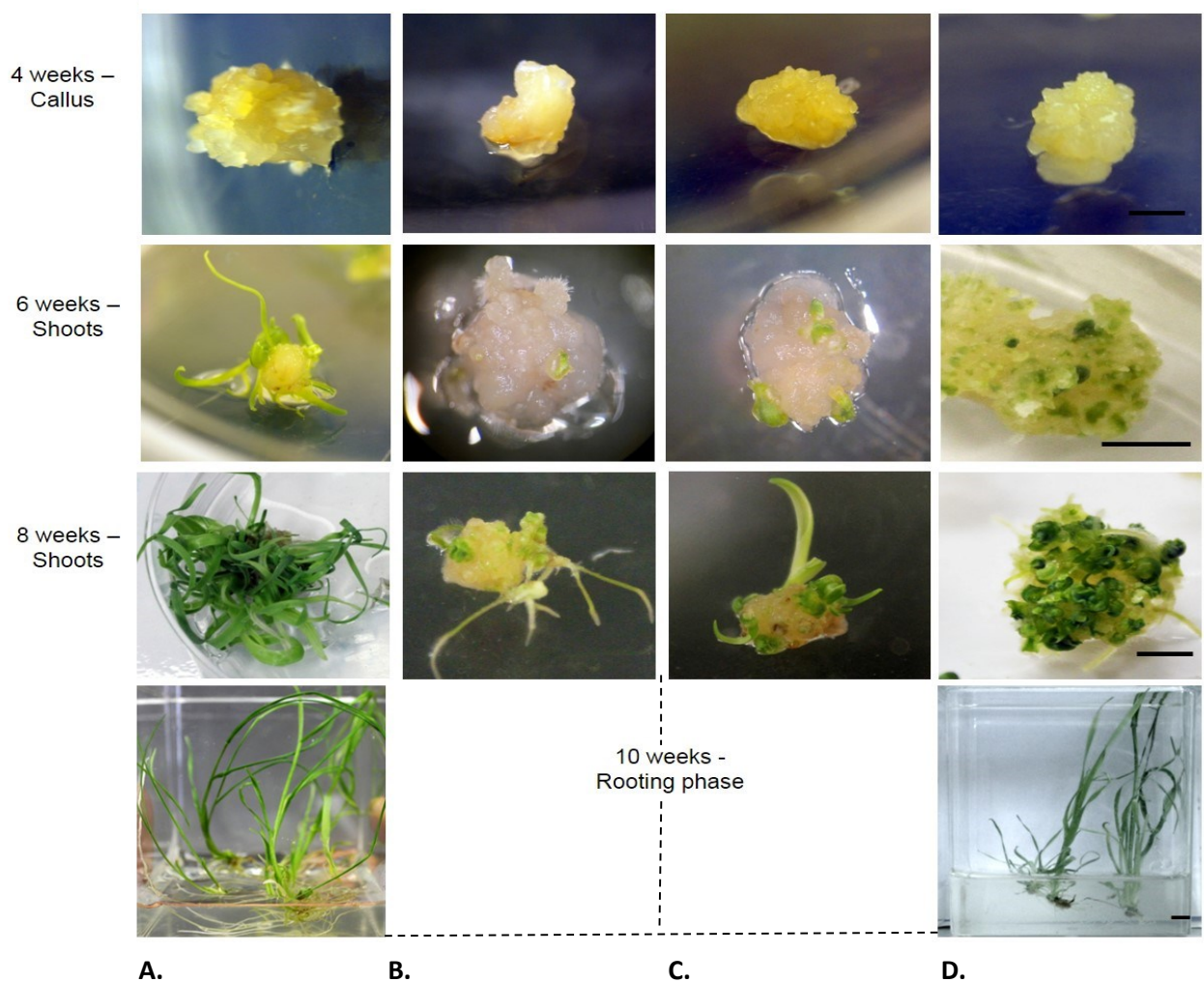
Figure 3.8 *In vitro* plant regeneration from immature embryos isolated from encapsulated / dehydrated cryopreserved immature seeds; (A) Control not frozen; (B) Cryoprotected in 10% DMSO; (C) 50% glycerol; (D) 80% glycerol across a 10 week period. Absent pictures planes indicated insufficient resulting in no rooting for that specific treatment. Scale bar = 0.5 mm.

3.3.2.4 Encapsulation / Vitrification: Influence of cryoprotectant on immature seeds

Calli growth from immature embryos isolated from cryopreserved immature seeds were assessed after four weeks in the dark on INDUC A media. Optimal *in vitro* callus formation was seen for immature seeds desiccated for 72 hours, vitrified in 80% glycerol and flash frozen (Figs. 3.7 and 3.8). Callus regeneration from these immature embryos was comparable to the non-cryopreserved control calli. Control batches were encapsulated, vitrified in sucrose, dehydrated and generated *in vitro*, but not frozen. Vitrification in 10% DMSO consistently resulted in the poorest calli formation frequency, regardless of freezing method or desiccation time (Figure 3.7).

Four weeks after cryopreservation all calli were transferred to cytokinin containing media (RDZ A). Shoots started to appear after two weeks and shoots clusters of about 0.5 cm high were transferred to MS media for adventitious rooting. Shooting of calli tissue for all treatments was delayed by around 2 weeks when compared to non-preserved control tissue. Plantlet formation varied considerably between the different experimental groups. Tissue that underwent a 22 hour desiccation followed by slow freeze showed the lowest plantlet formation, specifically DMSO with a plantlet frequency of only 6% (Table 3.3). Similarly as with the callus development, treatment in 80% glycerol and flash frozen resulted in the highest shooting frequency (68%).

Table 3.3 Plantlet formation nine weeks post cryopreservation. Immature seeds were cryopreserved and immature embryos isolated for *in vitro* regeneration. The average amount of shoots per embryogenic callus clump were recorded and the percentage plantlet formation frequency was calculated from the original n = 80 immature embryos introduced *In vitro* per treatment. Control batches = encapsulated, vitrified in sucrose, dehydrated and generated *in vitro*, but not frozen. Means followed by the same superscript within a column were not significantly different (p < 0.05).

	22 hours desiccation				72 hours desiccation			
	Slow Freeze		**Flash Freeze**		**Slow Freeze**		**Flash Freeze**	
	Shoots	Plantlets	Shoots	Plantlets	Shoots	Plantlets	Shoots	Plantlets
Control	4.13 ±0.26 [a]	73%	3.77 ± 0.24 [a]	69%	4.14 ±0.26 [a]	69%	3.95 ±0.23 [a]	70%
10% DMSO	0.21 ±0.09 [b]	6%	0.35 ±0.11 [b]	15%	0.24 ±0.07 [b]	11%	0.48 ±0.10 [c]	19%
50% Glycerol	1.81 ± 0.19 [d]	26%	1.63 ± 0.23 [d]	25%	2.1 ±0.21 [d]	31%	2.8 ± 0.20 [d]	41%
80% Glycerol	1.1 ±0.19 [e]	15%	1.15 ± 0.18 [e]	30%	2.5 ±0.1.9 [d]	35%	3.44 ± 0.23 [a]	68%

3.3.2.5 Freezing method comparisons

Flash freeze (liquid nitrogen) and slow freeze (Mr Frosty) were evaluated and compared. The results varied considerably for the different cryoprotectants containing the immature seeds which was subjected to cryopreservationds. No significant difference (p< 0.05) were found when the immature seeds, vitrified in 50% glycerol, were frozen either in liquid nitrogen or in the Mr Frosty container. However, the freezing method did make a significant difference for the tissue vitrified in 80% glycerol (Figure 3.7). When tissue were desiccated for 22 hours and preserved in 10% DMSO, a significant difference was also seen between the two freezing methods. This was not the case for the 10% DMSO and 72 hour desiccation treatment. Overall, the tissue flash frozen in liquid nitrogen resulted in greater calli regeneration, (10 to 15 mm^2) compared to calli slow frozen in the Mr Frosty container (3 to 7 mm^2) for all cryoprotectant solutions.

3.4 Discussion

3.4.1 *In vitro* regeneration

3.4.1.1 Cultivar choice and *in vitro* regeneration potential

Many plant species, including wheat, show cultivar variation in tissue culture potential, an essential phase in most genetic engineering protocols (Moghaieb *et al*., 2010; Popelka *et al*., 2006; Abdul *et al*., 2010). For genetic engineering to be really beneficial, worldwide varieties designed with desirable traits for specific countries or regions should be targeted for genetic engineering. Robust *in vitro* regeneration systems for Southern African wheat cultivars will form the base for future genetic transformation of cultivars compatible with the Southern hemisphere environment. We investigated the *in vitro* regeneration abilities of six bread wheat cultivars of Southern African originally, developed mainly for Russian wheat aphid (RWA; *Diuraphis noxia*) resistance. Levels of resistance to RWA have been achieved through the identification and consequent breeding of the *Dn* genes into various wheat genetic backgrounds worldwide (Jankielsohn, 2011). Wheat tolerant to RWA has been available in South Africa in the form of Tugela-*Dn1* (Du Toit, 1989). Since then the *Dn* genes were bred into a number of Southern African bread wheat cultivars, such as Gamtoos, Betta and Palmiet, which were used in this study (Marasas *et al*., 2005; van der Westhuizen *et al*., 1998).

Immature embryos are the choice explant for wheat tissue culture trials due to its high regeneration competency (Wang *et al*., 2014). The tested cultivars showed variation in their ability to form embryogenic callus and multiple shoots via somatic embryogenesis. Similar results and variation between cultivar regeneration was also found in a previous study conducted by Lacock and Botha (2000), which also included some Southern African cultivars. The cultivars exhibiting the vigorous growth in this study were Gamtoos S and R, and to a lesser extent, Palmiet and Palmiet *Dn5* x *Dn1*.

3.4.1.2 Culture media influences tissue culture potential

The culture media played an important role in the cultivars *in vitro* performances. Regeneration of embryogenic callus from immature embryos, with clear embryogenic sites and embryoids, was more efficient on 2,4-D than on picloram for the two Gamtoos cultivars. Similar callus formation efficiencies (85-95%) were found for wheat embryos on 2 mg/l 2,4-D in studies conducted by Barro *et al*. (1999) and recently Miroshnichenko *et al*. (2013). With the addition of BAP to the regeneration media, the callus produced on 2,4-D seemed to be highly embryogenic and resulted in more shoot formation, with the exception of Gamtoos R.

Carbohydrates in the media also influence the tissue development by acting as energy sources and regulating the osmo-potential in the media. Exchanging maltose with sucrose increase the embryogenic and regeneration ability of most cultivars, which was not supported by the results concluded in the Barro *et al.* (1999) study but similar to results reported by Miroshnichenko *et al.* (2013).

When plant tissue is cultured *in vitro*, limited amounts of vitamins are synthesised (Razdan, 2003). It is therefore important to supply additional organic supplements to the growth media. Callus embryogenesis was severely reduced when the Gamborg B5 vitamin mix were included in the media (Gamborg, 1968). The Gamborg vitamin mix contains significantly higher amounts of nicotinic acid, pyridoxine and thiamine than the MS mix (Murashige and Skoog, 1962). All cultivars, with the exception of the Tugela cultivars, which showed overall low tissue culture potential, preferred vitamins as described by Murashige and Skoog (1962) included in the growth media.

The addition of silver nitrate to culture media have been known to improve *in vitro* regeneration ability of cells by inhibiting ethylene synthesis, which is a hormone involved in plant cell responses and senescence (Hyde and Phillips, 1996; Bleecker and Kende, 2000; Kumar *et al*., 2009). In tissue culture, ethylene generally appears to inhibit somatic embryogenesis, callus development and shoot regeneration (Vain *et al*., 1989; Biddington, 1992). By the addition of silver nitrate to the wheat growth media, calli biomass and embryogenicity appears to increase relative to the control. This was similar to studies conducted on Chinese

wheat cultivars when exposed to comparable silver nitrate concentrations (Liao *et al*., 2005; Wu *et al*., 2006). It should however be noted that this particular investigation within the context of this study serves as proof of concept and requires statistical validation.

3.4.2 Cryopreservation

With the use of immature embryos as the explant of choice for wheat *in vitro* regeneration, the problem of limited and sporadic supply of this tissue, especially in the hot, dry climate of the Southern hemisphere, pose a problem. It has been suggested that large pools of immature embryos can be isolated and continuously sub-cultured for uninterrupted *in vitro* tissue supply (Fretz and Lorz, 1995; Lambardi *et al*., 2005). However, there is the risk of culture contamination, increased possible somaclonal variation and the loss of embryogenicity that make this an undesired strategy (Jahne *et al*., 1991; Fretz and Lörz, 1995; Wang *et al*., 2014). Cryopreservation of explant material is therefore be a viable option to supply unlimited explant material throughout the year.

3.4.2.1 Cryopreservation of different tissue types

Different types of explants were assessed for their ability to survive and regenerate *in vitro* after cryopreservation. These included immature wheat zygotic embryos, callus and immature seeds from the Gamtoos S genotype. Of the different tissue types only immature wheat embryos isolated from preserved immature seeds were able to de-differentiate and regenerate after preservation into functional plants. These results now extend the list of wheat explants, such as mature seeds, callus and zygotic embryos, that has been reported to be successfully cryopreserved in the past, to include immature seeds (Chen *et al*., 1985; Kendal *et al*., 1990; Kendall *et al*., 1993; Wang *et al*., 2011; Solov'eva *et al*., 2011). When naked immature embryos and callus were encapsulated and cryopreserved, only initial primary structures formed, this was regardless of any and all anti-oxidant pre-treatment or cryoprotectant solutions tested. This is in contrast to previous studies done on barley callus and wheat zygotic embryos where ABA treatment prior to cryopreservation significantly

71

increases tissue survival post cryopreservation (Fretz and Lorz, 1995; Kendal *et al*. 1993). In the past, studies showed that genetic variants might play a role in the success of cryopreservation of zygotic embryos. For example, Kendal *et al*. (1990) showed that only 15% of wheat immature embryos, which ultimately displayed an enhanced freezing tolerance phenotype, were able to survive cryopreservation. However, the lack of dehydration of tissue prior to cryopreservation also contributed to the initial unsuccessful attempts of tissue preservation.

3.4.2.2 Dehydration / Vitrification of tissue prior to cryopreservation

Dehydration was found to be important to significantly increase wheat zygotic embryo survival after cryopreservation (Solov'eva *et al*., 2010).   Plant cells contain free unbound water molecules. These water molecules are capable of forming ice crystals during freezing which can lead to cell damage and death (Samygin, 1994; Popov *et al*., 2006). Due to this high internal water content in both immature embryos and callus tissue, many cells were severely damaged, as was evident from the results generated from the cell viability assay, on tissue preserved without desiccation. Internal water content was addressed by means of desiccating the explants post encapsulation. When alginate beads containing plant tissue were desiccated on a laminar flow bench, a pronounced and rapid loss of relative moisture content was seen (66%). It is however important not to over-dehydrate tissue to prevent damage caused by osmotic stress, membrane structural changes and protein denaturing (Hoekstra *et al*., 2001). For example, multiple studies done on orchid determined the optimal water content for cryopreservation of explants to be between 11-38% (Bian *et al*., 2002; Jitsopakul *et al*., 2008; Khoddamzadeh *et al*., 2011). Solov'eva *et al*., (2010) reduced the water content to between 13-37% in wheat calli prior to cryopreservation. In this study, water content was reduced to a similar range of between 25-28% resulting in tissue survival post preservation, which correlated with the amount of un-freezable water in a plant cell, ranging from 20 to 40%.

In addition, sucrose pre-treatments prior to cryopreservation, were shown to be important and species-specific (Khoddamzadeh *et al*., 2011; Moran *et al*., 1999; Tsukazaki *et al*., 2000). Sucrose can further reduce the freezable water through osmosis, protecting the cytoplasm by entering a vitrified state. However, excessive

concentration of cryoprotectant solutions can lead to over dehydration of the cell, resulting in plasmolysis and membrane rupture. In this study, alginated beads containing explant material were osmo-protected, after desiccation, in 0.5 M sucrose for 20 hours followed by placement in 10% (w/v) DMSO or 50-80% (w/v) glycerol as cryoprotectant solutions. The combination of 0.5 M sucrose and 80% (w/v) glycerol resulted in the highest success rate (68%) of tissue regeneration after cryopreservation. It was previously suggested that DMSO might be toxic and cause mutagenic effects on plant cells, which might explain the significantly lower regeneration ability of tissue cryoprotected in DMSO (Vannini and Poli, 1983).

To conclude, cryopreservation of wheat immature seeds was successfully achieved by encapculation, vitrification and dehydration prior to preservation at -196°C for a period of four weeks. Immature embryos were capable of regenerating *in vitro* and developed into fully fertile seed producing plants in the greenhouse. *In vitro* regeneration of wheat explant material was cultivar dependant and customised growth media will have to be developed for each wheat genotype introduced into culture. Overall, cryopreservation seems to be a viable option to supply continuous stock of explant material for future genetic transformation of Southern African bread wheat cultivars.

3.5 References

Abdul R, Ma Z, Wang H (2010). Genetic transformation of wheat (*Titicum aestivum* L): A review. Triticeae Genomics and Genetics. **1**: 1-7

Addinsoft (2014). XLSTAT, Data analysis and statistics software for Microsoft Excel, France. (https://www.xlstat.com/en/, accessed on 10 July 2014)

Akbari M, Wenzl P, Caig V, Carling J, Xia L, Yang S, Uszynski G, Mohler V, Lehmensiek A, Kuchel H, Hayden MJ, Howes N, Sharp P, Vaughan P, Rathmell B, Huttner E, Kilian A (2006). Diversity arrays technology (DArT) for high-throughput profiling of the hexaploid wheat genome. Theoretically Applied Genetics. **113**: 1409-1420

Barro F, Martin A, Lazzeri PA, Barcelo P (1999). Medium optimization for efficient somatic embryogenesis and plant regeneration from immature inflorescences and immature scutella of elite cultivars of wheat, barley and tritordeum. Euphytica **108**: 161-167

Benson EE (2008). Cryopreservation of phytodiversity: a critical appraisal of theory and practice.Critical Reviews in Plant Sciences. **27**: 141–219

Bian HW, Wang JH, Lin WQ, Han N, Zhu MY (2002). Accumulation of soluble sugars, heat-stable proteins and dehydrins in cryopreservation of protocorm-like bodies of *Dendrobium candidum* by the air-drying method. Journal of Plant Physiology. **159**: 1139-1145

Biddington NL (1992). The influence of ethylene in plant tissue culture. Plant Growth Regulation. **11:** 173-178

Bleecker AB, Kende H (2000). Ethylene, A gaseous signal molecule in plants. Annual   Review of Cell and Developmental Biology. **16**: 1-18

Chen TH, Kartha KK, Gusta LV (1985). Cryopreservation of wheat suspension culture and regenerable callus. Plant Cell, Tissue and Organ Culture. **4**: 101-109

Chugh A, Mahalakshmi A, Khurana P (2012). A novel approach for *Agrobacterium*- mediate germ line transformation of Indian bread wheat (*Triticum aestivum* L.) and pasta wheat (*Triticum durum*). Journal of Phytology. **4**: 22-29

Dunn OJ (1964). Multiple comparisons using rank sums. Technometrics. **6**: 241-252

Du Toit F (1989). Inheritance of resitance in two *Triticum aestivum* lines to Russian wheat aphid (Hornoptera, Aphididae). Journal of Economic Entomology **82**: 1251-1253

Du Toit F, Walters MC (1984). Damage assessment and economic threshold values for the chemical control of the Russian wheat aphid, *Diuraphis noxia* (Moldvilko), on winter wheat. Technical Communication, Department of Agriculture, RSA. **191**: 58-62

Engelmann F (2004). Plant cryopreservation, Progress and prospects. *In vitro* Cellular and Developmental Biology Plant. **40**: 427-433

Engelmann F (2011). Cryopreservation of Embryos: An Overview. Methods in Molecular Biology. **710**: 155-184

Feuillet C, Langridge P, Waugh R (2007). Cereal breeding takes a walk on the wild side. Trends in Genetics. **24**: 24-32

Food and Agriculture Organization of the United Nations FAOSTAT (2013). (http://www.faostat.fao.org, accessed on 15 September 2015)

Fretz A, Lorz H (1995). Cryopreservation of *in vitro* cultures of barley (*Hordeum vulgare* L. and H. *murinum* L.) and transgenic cells of wheat (*Triticum aestivum* L.). Journal of Plant Physiology. **146**: 489-496

Gamborg OL, Miller RA, Ojima K (1968). Nutrient requirements of suspension culture of soybean root cells. Experimental Cell Research **50**: 151-158

Harding K (2007). Genetic integrity of cryopreserved plant cells, the review. Cryo Letters. **25**: 3-22

He Y, Jones HD, Chen S, Chen XM, Wang WD, Li KX, Wang DS, Xia LQ (2010). *Agrobacterium*-mediated transformation of durum wheat (*Triticum turgidum* L. var. *durum* cv *Stewart*) with improved efficiency. Journal of Experimental Botany. **61**: 1567-1581

Hoekstra AF, Golovina AE, Buitink J (2001). Mechanisms of plant desiccation tolerance. Trends in Plant Science. **6**: 431-438

Hyde CL, Phillips GC (1996). Silver nitrate promotes shoot development and plant regeneration of chile pepper (*Capsicum annuum* L.) via organogenesis. *In Vitro* Cell and Developmental Biology. **32**: 72-80

Indra K, Vasil V, Vimla V (2006). Transformation of wheat via particle bombardment. *In*, Loyola-Vargas F, Victor M, Vázquez-Flota II, (eds) Plant Cell Culture Protocols, Methods in Molecular Biology, Humana Press Inc, Totowa, New Jersey, pp 273-283

Jahne A, Lazzeri PA, Jager-Gussen M, Lorz H (1991). Plant regeneration from embryogenic cell suspensions derived from anther cultures of barley (*Hordeum vulgare* L.). Theoretical and Applied Genetics. **82**: 74-80

Jankielsohn A (2011). Distribution and diversity of Russian wheat aphid (*Hemiptera, Aphididae*) biotypes in South Africa and Lesotho. Journal of Economic Entomology.**104**: 1736-1741

Jitsopakul N, Thammasiri K, Ishikawa K (2008) Cryopreservation of *Bletilla striata* mature seeds, 3-day germinating seeds and protocorms by droplet-vitrification. Cryoletters. **29**: 517-526

Jones HD, Dohery A, Wu H (2005). Review of methodologies and a protocol for the *Agrobacterium*-mediated transformation of wheat. Plant Methods. **1**: 5

Kavas M, Öktem HA, Yücel M (2008). Factors affecting plant regeneration from immature inflorescence of two winter wheat cultivars. Biologia Plantarum. **52**: 621–626

Kendal EJ, Kartha KK, Qureshi JA, Chermak P (1993). Cryopreservation of immature spring wheat zygotic embryos using an abscisic acid pretreatment. Plant Cell Reports. **12**: 88-94

Kendal EJ, Qureshi JA, Kartha KK, Leung N, Chevrier N, Caswell K, Chen THH (1990). Regeneration of freezing-tolerant spring wheat (*Triticum aestivum* L.) plants from cryoselected callus. Plant Physiology. **94**: 1756-1762

Khoddamzadeh AA, Sinnah UR, Lynch P, Kadir MA, Kadzimin SB, Mahmood M (2011). Cryopreservation of protocorm-like bodies (PLBs) of *Phalaenopsis bellina* (Rchb.f.) christenson by encapsulation dehydration. Plant Cell, Tissue and Organ Culture. **107**: 471-481

Kumar V, Parvatam G, Ravishankar GA (2009). AgNO3 a potential regulator of ethylene activity and plant growth modulator. Electronic Journal of Biotechnology. **12**: 1-15

Lacock L, Botha A-M (2000). Genotype variation in regeneration and transient expression efficiencies of 14 South African wheat cultivars. South African Journal of Plant and Soil. **17**: 170-174

Lambardi M, Benelli C, De Carlo A (2005). Cryopreservation as a tool for the long-term conservation of woody plant germplasm, Development of the technology at the *CNR/IVALSA* Institute of Florence. **1**: 181–182.

Liao X, Tang, L, Zhang F (2005). Effect of silver nitrate on callus regeneration and activity of some enzymes during tissue culture of wheat immature inflorescence. Science Paper Online. **1**: 1-11

Motulsky H (2015). GraphPad Prism. GraphPad Software, Inc, San Diego, California

(http://www.graphpad.com/scientific-software/prism/, accessed on 10 July 2014)

Marasas CN, Anandajayasekeram P, Millard S, van Rooyen CJ (2005). Farm-level adoption and impact of agricultural technology, the case of Russian wheat aphid resistant cultivars in South Africa. South African Journal of Agricultural Extension. **34**: 318-333

Miroshnichenko DN, Filippov MV, Dolgov SV (2013). Medium optimization for efficient somatic embryogenesis and *in vitro* plant regeneration of spring common wheat varieties. Russian Agricultural Sciences **39**: 24–28

Moghaieb REA, El-Arabi NI, Momtaz OA, Youssef SS, Soliman MH (2010). Genetic transformation of mature embryos of bread (*T. aestivum*) and pasta (*T. durum*) wheat genotypes. GM Crops **1**: 87-93

Moran M, Cacho M, Fernandez-Tarrago J, Corchete P (1999). A protocol for the cryopreservation of *Digitalis thapsi* L. cell cultures. Cryo Letters. **20**: 193-198

Mundi Index (2014). Agricultural production, supply, and distribution. (http://www.indexmundi.com/agriculture/?country, accessed on 1 October 2014)

Murashige T, Skoog F (1962). A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Plant Physiology. **15**: 473-497

Ogawa Y, Sakurai N, Oikawa A, Kai K, Morishita Y, Mori K, Moriya K, Fujii F, Aoki K, Suzuki H, Ohta D, Saito K, Shibata D (2012). High-throughput cryopreservation of plant cell cultures for functional genomics. Plant Cell Physiology. **53**: 943-952

Ponya Z, Barnabas B (2003). Regeneration of fertile wheat (*Triticum aestivum*) plants from isolated zygotes using wheat microspore culture as nurse cells. Plant Cell, Tissue and Organ Culture. **74**: 243–247

Popelka JC, Gollasch S, Moore A, Molvig L, Higgins TJV (2006). Genetic transformation of cowpea (*Vigna unguiculata L.)* and stable transmission of the transgenes to progeny. Plant Cell Report. **25**: 304-312

Popov SA, Popova VE, Nikishina VT, Vysotskaya NO (2006). Cryobank of plant genetic resources in Russian Academy of Sciences. International Journal of Refrigeration. **29**: 403-410

Rasco-Gaunt S, Riley A, Cannell M, Barcelo P, Lazzeri PA (2001). Procedures allowing the transformation of a range of European elite wheat (*Triticum aestivum* L.) varieties via particle bombardment. Journal of Experimental Botany. **50**: 865-874

Razdan MK (2003). Introduction to plant tissue culture. Science Publishers Inc, New Hampshire, USA, pp 26

Sakai A, Hirai D, Niino T (2008). Development of PVS-based vitrification and encapsulation-vitrification protocols. *In*, Reed BM (ed) Plant Cryopreservation, A Practical Guide, Springer, New York, pp 33-57

Samygin GA (1994). Causes of damage of plant cells by extracellular ice. Russian Journal of Plant Physiology. **41**: 614-625

She M, Yin G, Li J, Li X, Du L, Ma W, Ye X (2013). Efficient regeneration potential is closely related to auxin exposure time and catalase metabolism during the somatic embryogenesis of immature embryos in *Triticum aestivum* L. Molecular Biotechnology. **54**: 451–60

Siegel S (1956). Non-parametric statistics for behavioural science. The American Statistician. **3**: 13-19

Solov'eva AI, Vysotskaya ON, Popov AS, Dolgikh YI (2010). Freezing of dehydrated calli of spring wheat (*Triticum aestivum* L.) in liquid nitrogen and their morphogenetic potential. Biology Bulletin. **37**: 489-495

Tsukazaki H, Mii M, Tokuhara K, Ishikawa K (2000). Cryopreservation of *Doritaenopsis* suspension culture by vitrification. Plant Cell Reports **19**: 1160-1164

Vain P, Flament P, Soudain P (1989). Role of ethylene in embryogenic callus initiation and regeneration in *Zea mays* L. Journal of Plant Physiology. **146**: 537-540

Van der Vyver A (2013). The relative value between barley and wheat from a production point of view, Northern Cape irrigation areas. Department of Agricultural Economics, Extension and Rural Development, University of Pretoria.

Van der Westhuizen AJ, Qian XM, Botha A-M (1998). Differential induction of apoplastic peroxidase and chitinase activities in susceptible and resistant wheat cultivars by Russian wheat aphid infestation. Plant Cell Reports. **18**: 132-137

Vannini GL, Poli F (1983). Binucleation and abnormal chromosome distribution in *Euglena gracilis* cells treated with dimethyl sulfoxide. Protoplasma. **114**: 62-66

Vasil IK, Vasil V (2006). Transformation of wheat via particle bombardment. *In*, Loyola-Vargas VM and Vazquez-Flota F. Plant Cell Culture Protocols, Methods in Molecular Biology, Humana Press Inc. Totowa, New Jersey, pp 273-283

Viertel K, Hess D (1996) Shoot tips of wheat as an alternative source for regenerable embryogenic callus cultures. Plant Cell, Tissue and Organ Culture. **44**: 183-188

Wang G, Wang Q, Zhang W, Yao D, Zheng W, Fan H, Wang H, Wang F (2011) Effect of cryopreservation in liquid nitrogen on germination rate and vigor of wheat seeds. Chinese Seed. **2**: 1-4

Wang X, Xian R, Gui-Xiang Y, Wang K, Li J, Du L, Xu H, Ye X (2014). Effects of environmental temperature on the regeneration frequency of the immature embryos of wheat (*Triticum aestivum* L.). Journal of Integrative Agriculture. **13**: 722-723

Wheat Resource Centre (2009). Department of Agriculture, Forestry and Fisheries, Pretoria, South Africa. (www.nda.agric.za/docs/Brochures/prodGuideWheat.pdf, accessed on 12 September 2015)

Wu H, Sparks C, Amoah B, Jones HD (2003). Factors influencing successful *Agrobacterium*-mediated genetic transformation of wheat. Plant Cell Reports. **21**: 659-668

Wu LM, Wei YM, Zheng YL (2006). Effects of silver nitrate on the tissue culture of immature wheat embryos. Russian Journal of Plant Physiology. **53**: 530-534

Yin G, Wang Y, She M, Du L, Xu H, Ma J, Ye X (2011). Establishment of a highly efficient regeneration system for the mature embryo culture of wheat. Agricultural Sciences in China. **10**: 9–17

Zhao TJ, Zhao SY, Chen HM, Zhao QZ, Hu ZM, Hou BK, Xia GM (2006). Transgenic wheat progeny resistant to powdery mildew generated by *Agrobacterium* inoculum to the basal portion of wheat seedling. Plant Cell Reports. **25**: 1199-1204

CHAPTER 4

Engineering wheat (*Triticum aestivum* L.) for abiotic stress tolerance.

4.1 Introduction

Wheat, together with rice and maize, is one of the primary food crops that are crucial to the everyday lives of both humans and animal. It is evident worldwide that drought is becoming more prevalent, and it comes as no surprise that the small grain industry is becoming increasingly more concerned about this problem (Braun, 2015). Thus biotechnologists, together with plant breeders, are continuously in search of a means to address the drought problem in relation to the plant. Genetic engineering of wheat currently holds great potential as a means of improving this crop. However genetic transformation of wheat is still regarded as a major obstacle due to its inherent nature of recalcitrance to tissue culture and transformation procedures (Przetakiewicz *et al*., 2004; Orgihara *et al*., 2015). Despite of the aforementioned, research has made progress with particular genes that activate or deactivate suites of drought-responsive regulons. Many of the genes involved in drought response encode for a diverse array of proteins such as enzymes involved in metabolic rearrangement, proteasomal degradation and post translation modification (Seo and Lee, 2003; Sanghera *et al*., 2011; Hassan *et al*., 2013).

Among the various transcription factor (TF) with associative roles in abiotic stress are the well characterized C-repeat (CRT) binding factors (CBFs) or drought-responsive element (DRE) binding (DREB) proteins (Morran *et al*., 2011). Drought response, in particular, is polygenic, however many of the genes involved in drought response have a core motif known as (A/G) CCGAC. This facilitates the binding of the CBF/DREB TF, hereby activating the subsequent gene (Thomashow, 1998; Morran *et al*., 2011). The *CBF/DREB* gene requires activation, and Gilmour *et al.* (1998) discovered the activator and named it Inducer of CBF expression 1 (*ICE1*). Thus ICE1 is often regarded as a master TF as its availability influences all regulons associated with water stress. Constitutive over expression of *CBF/DREB* has been explored in *Arabidopsis thaliana*, maize, tobacco, sugarcane, wheat and barley and the results show improved stress tolerance. However the plants suffer major metabolic overload which translates to unwanted phenotypic attributes, i.e. stunted growth, mild or strong dwarfism, slower development and a delay in flowering time (Sakuma *et al*., 2006; Qin *et al*., 2007; Bilhani *et al*., 2011; Chen *at al*., 2008; Morran *et al*., 2011). Using an inducible promoter with all the necessary *cis*-elements (i.e. DRE/CRT, ABRE, etc.) holds the potential to overcome the subsequent effects of constitutive

expression, and the ideal promoter is *Rab17*, as it has been shown to be non-leaky in wheat and barley, as opposed to the well characterized *Rd29a* promoter (Lopato and Langride, 2011; Morran *et al*., 2011).

The most important characteristics associated with activation of stress-inducible genes are the strength and duration of expression. Gene families associated with abiotic stress responses are generally divided into two categories, those that can sense drought, and those involved in stress management after the plant perceives the abiotic stress (Mahajan and Tuteja, 2005; Kovalchuk *et al*., 2013). Furthermore expression of associative genes are driven by promoters that can either be dependent or independent of abscisic acid (ABA) signalling (Shinozaki and Yamaguchi-Shinozaki, 2000; Yamaguchi-Shinozaki *et al*., 2006). Often with constitutive expression, successful transcription of a gene does not necessarily translate to a functional protein. Whether or not the end product is a functional protein is dependent on post-translational modification (Sakuma *et al*., 2003). The above factors all need consideration, but more importantly it requires validation in a hexaploid crop such as wheat.

Post translational modifications (PTM) are influenced by protein geometry and regulated by environmental factors, consequently bringing about changes at multiple levels, i.e. transcriptome, proteome, metabolome, etc., within the plant. SUMOylation is a PTM mechanism and refers to the conjugation of SUMO onto protein substrates to bring about changes in protein function or tagging for proteasomal degradation. The attachment of SUMO is reversible through the action of SUMO proteases, which acts as isopeptidase that specifically cleave the SUMO substrate linkages to recycle free SUMO (Johnson, 2004; Saracco *et al*., 2007). *Arabidopsis thaliana* deficient in SUMO proteases have elevated levels of SUMO and several phenotypic abnormalities i.e. small stature and inflorescence development defects (Conti *et al*., 2008). SUMO–proteases have also been investigated in the context of plant-pathogen interaction. The study in question demonstrated that increases in SUMO proteases can lead to decreased SUMO conjugates on targeted proteins, hereby subverting defence in host cells (Roden *et al*., 2003). Despite the vital role of SUMOylation and the key enzyme SUMO protease, there is no physiological information for SUMO proteases within the context of bread wheat and their presumed roles in the management of abiotic (and to an extent biotic) stressors. As a result of this, it was decided to investigate the application of two SUMO proteases, Overly Tolerance to Salt 1 (*OTS1*) and *OTS2*

as key genes for over expression in wheat, essentially expanding on what has been described in *A. thaliana*. Coti *et al*. (2008) investigated *OTS1* in *A. thaliana* in the context of salinity stress, and found that over expression of this gene lead to a reduction in SUMO conjugates with minimal phenotypic variation. Therefore SUMOylation in its entirety is an influential mechanism for targeted protein management, as SUMO proteases play a vital role in regulating pathway flux and are therefore ideal targets for manipulating stress-responsive regulons.

In this study, plant expression vectors were designed and validated for *ICE1*, *OTS1* and *OTS2* over expression using an ubiquitin promoter for constitutive expression, with the aim of agronomical and molecular assessment. Furthermore a drought inducible vector was designed for *ICE1* with the aim of showing differences in constitutive *verses* inducible expression.

4.2 Material and Methods

4.2.1 General molecular procedures

All reagents and chemicals used were obtained from Sigma-Aldrich (St Louis, MO, USA), Fermentas (Pretoria, SA), Promega (Madison, WI, USA), Bio-Rad (Hercules, CA, USA), Merck Chemicals (Gauteng, RSA) and Whatman (Maidstone, United Kingdom).

All enzyme and kits used were obtained from Thermo Scientific (Waltham, MA, USA), Separations (Johannesburg, RSA), England Biolabs (Ipswich, MA, USA), Fermentas (Hanover, MD, USA), Qiagen (Dusseldorf, Germany), Invitrogen (Carlsbad, CA, USA), Sigma-Aldrich (St. Louis, MO, USA) and Zymo Research (Orange, CA, USA).

4.2.1.1 Quantification of DNA and RNA

NanoDrop 2000c UV-Vis spectrophotometer (Thermo Scientific) was used for all quantification, according to manufacturer instructions.

4.2.1.2 Separation of DNA and restriction digest reactions by gel electrophoresis

PCR amplicons and restriction digests in all instances (unless otherwise stated) were separated on a 1 % (w/v) agarose gel containing 5% (v/v) pronosafe (Separations, Johannesburg, RSA). Gels were submerged in 1X TE buffer containing 2.75 g/l boric acid, 4 g/l Tris base, 0.465 g/l EDTA (pH 8.0) and separated by running the gel at 90 mV for 80 minutes (Bio-Rad Power Pac 1000). The Multi Genius Bio Imaging system (SynGene, South Africa) and ultraviolet (UV) light was used for visualisation of the DNA fragments on the gel. Corresponding bands were excised under UV light, and purified using the GeneJet PCR purification kit or GeneJet Gel extraction Kit as described by the manufacturer (Thermo Fisher Scientific, MA, USA)

4.2.1.3 Bacterial culture

For all routine bacterial growth, Luria Broth liquid (LBL) media was used, containing 10 g/l peptone, 5 g/l yeast extract, 5 g/l NaCl incubated at 37°C, while shaking at 200-250 revolutions per minute (RPM) until cultures were turbid. For bacterial colony formation LBL media was used with 15 g/l agar (LBA). All bacterial culture media, whether liquid or solid, was supplemented with filter sterilized ampicillin at 80 mg/L (using a 0.22 µm syringe filter). All media constituents, with the exception of ampicillin, were dissolved in sterile distilled water, and autoclaved at 121°C and pressure of 103 kPa. Ampicillin was only added to media after cooling to approximately 45°C.

4.2.1.4 Sequencing of plasmids

All plasmids were sequenced at the Central Analytical Facilities (University of Stellenbosch, South Africa) with a PRISM® 3730X1 DNA analyser (Applied Biosystems Inc.), with either forward or reverse primers. Raw sequence data (nucleotide sequences) were subjected to BLAST and alignment analysis on NCBI or TAIR.

4.2.1.5 Colony polymerase chain reaction (PCR)

Colony PCR reaction essentially used 1 μl of liquid bacterial cultured as DNA template, with each reaction volume at 10-15 μl with the following constituents; 5 U/μl GoTaq DNA Polymerase (Promega; USA), with GoTaq 5X Green Reaction Buffer, 10 mM of each Dntp and 10 μM primer. The general cycle parameters were as follows: initial denaturation at 95°C for 3 min 30 amplification cycles consisting of denaturation at 94°C for 1 min, annealing temperature at 60°C for 30 s extension at 72°C for 2 min and final extension at 72°C for 10 min.

4.2.2 Targeted gene isolation from *Arabidopsis thaliana*

4.2.2.1 Gene-specific primer design

All gene ontology information was obtained from *The Arabidopsis Information Resource (TAIR)* (https://www.arabidopsis.org/) and all sequence information was cross checked with available peer reviewed literature. To avoid potential silencing due to high similarity of genes, *Arabidopsis thaliana* (ecotype Columbia-0) was used as a genetic resource to isolate *ICE1*, *OTS1* and *OTS2*. FASTA files of the following genes, *OTS1*: (AT1G60220), *OTS*2: (AT1G10570) and *ICE1*: (AT3G26744.4), were imported into Oligo Explore 1.2 (http://genelink.com) for primer selection and analyzed using Oligo Analyzer 1.2 (http://genelink.com). All primers were synthesized by Inqaba Biotech® (Pretoria, South Africa) and duly reconstituted and diluted according to manufacturer's specifications.

4.2.2.2 Plant material

Arabidopsis thaliana seeds were planted into Jiffy Peat Pallets® (www.jiffygroup.com), grown in a growth room (14 hours night (15°C) and 10 hours light (21°C)) and watered every second day under controlled humidity and light for a period of 4 weeks.

4.2.2.3 RNA extraction and cDNA synthesis

At 4 weeks old, leaf material was harvested and total RNA isolated using RNeasy Plant Mini Kit (QIAGEN, Germany), according to manufacturer instructions, followed by quantification with the Nanodrop (Section 4.2.1.1). RNA (10 pg - 0.5 µg) was then used as template for cDNA synthesis using the RevertAid First-strand cDNA Synthesis Kit (Thermo Scientific, USA) according to manufacturer instructions, and using Oligo (dT)18 primers.

cDNA (template at 50-100 ng/µl) was used in high fidelity amplification of *ICE1*, *OTS1* and *OTS2* using proof-reading DNA Polymerase Phusion® (Thermo Scientific, USA). Master mixes were assembled for 50 µl reactions according to the manufactures instructions with respective primer pairs listed in Table 4.1. The parameters for each gene are outlined in Table 4.2. PCR products were separated, visualized, purified and quantified as outlined previously in section 4.2.1.1 and 4.2.1.2.

Table 4.1 All primers were designed to have similar T_m and a maximum length of 20-25bp, with low GC content.

Primer Name	Sequence	GC % Min/Max	T_m
ICE1-Reverse	5'-GCTCTGCTTCAA CAG ATG CTT-3'	50/50	60.6°C
ICE- Forward	5'-AGGTGTCAACTTTGGCGATG-3'	47.6/47.6	60.4°C
OTS1- Forward	5'-AGATGACGAAGAGGAAGAAGGAAGT-3'	43.8/43.8	60.9°C
OTS1- Reverse	5'-CTGGTTTACTCTGTCTGGTCACT-3'	47.8/48.8	62.7°C
OTS2- Forward	5'-AGAAGAGATGAAGAGACAAAGAGCA-3'	40/40	61.3°C
OTS2- Reverse	5'-TTTGTTCTGATTATGTCATGCTCCT-3'	36/36	59.6°C

87

Table 4.2 Optimized parameters that allow for sufficient amplification of the respective genes, given that Phusion® High-Fidelity DNA Polymerase (Thermo Scientific, USA) is used.

Steps	ICE1		OTS1		OTS2	
Initial denature	98°C	30 s	98°C	30 s	98°C	30 s
Denature	98°C	6 s	98°C	6 s	98°C	35 s
Annealing	65°C	35 s	65°C	30 s	65°C	35 s
Extension	72°C	50 s	72°C	50 s	72°C	57 s
Final extension	72°C	10 min	72°C	10 min	72°C	10 min
Hold	4°C	-	4°C	-	4°C	-

4.2.2.4 Gene(s) preparation for blunt-end cloning

Phusion® High-Fidelity DNA Polymerase generates non-polyadenylated products, thus blunting was not required. However phosphorylation was necessary. Briefly, 20 pmol of DNA of each gene was prepared by incubating with 10X reaction buffer A containing 10 mM adenosine triphosphate (ATP) and 10 U T4 DNA polynucleotide kinase at 37°C for 20 min. Termination of reactions is recommended at 75°C for 10 min. This was followed by PCR cleanup with the GeneJet PCR purification kit (Thermo Scientific, USA) as described by the manufacturer.

4.2.2.5 Gene(s) cloning into pJET1.2/blunt and sequencing

The purified genes were each respectively cloned into the pJET1.2/blunt vector using the CloneJET PCR Cloning Kit (Thermo Scientific, USA) according to manufacturer instructions. Each construct housing each respective gene, was transformed into MAX Efficiency® DH5α™ Competent Cells according to manufacturer instructions. Colony PCR outlined in section 4.2.1.5 was done to confirm insertion of gene using pJET1.2 reverse primer (provide by kit; 5'-AAGAACATCGATTTTCCATGGCAG-3') and gene specific forward primers (Table 4.1). Amplified product was separated by gel electrophoresis and visualized under UV light as

outlined in section 4.2.1.2. Positive clones were inoculated into LBL media and purified using GeneJET Plasmid Miniprep Kit (Thermo Scientific, USA). Purified plasmids were sequenced at CAF (Stellenbosch University, South Africa).

4.2.3 Designing constructs for constitutive over expression in wheat

4.2.3.1 Vector manipulation

pUBI-510 and pEmuKN (co-bombardment vector) (Figure 4.1) were independently streaked out from -80°C glycerol stocks onto LBA plates containing ampicillin (100 mg/ml). These vectors were provided by the Institute for Plant Biotechnology (Stellenbosch University, South Africa). Single colonies were selected for inoculation in 100 ml LBL and incubated overnigt at 37°C, while shaking at 220 RPM. Large scale purification of both vectors was conducted, using GenElute HP Endotoxin-Free Plasmid Maxiprep Kit (Sigma Aldrich, USA) according to manufacturer's instructions. pUBI-510 was linearized with restriction enzyme *Sma*I , followed by dephosphorylation with alkaline phosphatase (FastAP). Briefly the reaction was incubated at 37°C for 20 minutes and then 75°C for 5 minutes to terminate the reaction, after which the plasmid was purified using the GeneJet PCR purification kit (Thermo Scientific, USA).

A.

B.
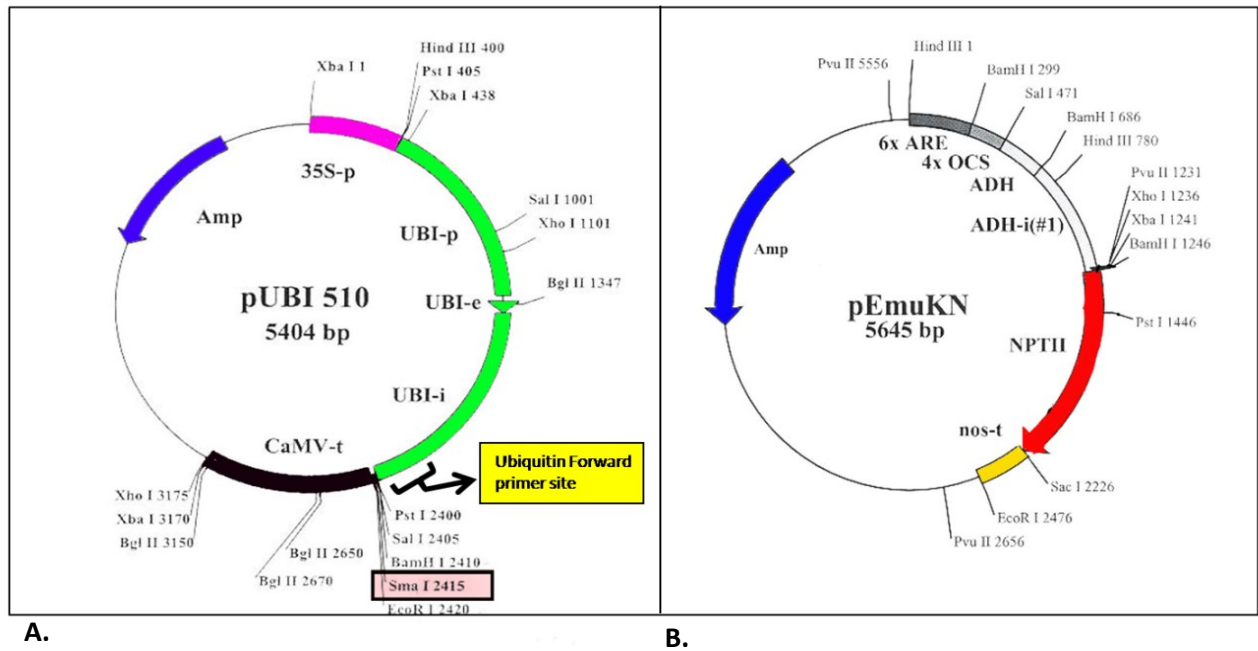
Figure 4.1 Schematics of plant expression vectors. (A) pUBI-510 with annotated ubiquitin primer site and the *Sma*I site highlighted in pink; (B) pEmuKN co-bombardment vector which house the selection gene *npt*II conferring resistant to geneticin.

4.2.3.2 Final ligation of gene(s) into linear pUBI-510

Each respective phosphorylated gene (prepared in section 4.2.2.4) was independently ligated into linearized and dephosphorylated 50 ng/µl pUBI-510 using a 1:3 vector to gene insert ligation ration. The three individual ligation reactions were prepared as follows; 10X T4 ligase buffer, T4 DNA ligase (5 U), pUBI-510 (50 ng), 50% (w/v) polyethylene glycol (PEG) 4000 and 49 ng/µl (*OTS1*) or 47 ng/µl (*OTS2*) or 57 ng/µl (*ICE1*). Each ligation reaction was incubated overnight at room temperature. Post incubation, 3 µl of the ligation reaction(s) was used for bacterial transformation using MAX Efficiency® DH5α™ Competent Cells (50 µl) according to manufacturer instructions.

Colony PCR was done to confirm gene presence and orientation simultaneously. Individual colonies were selected, and suspended in 10 µl PCR reaction mix, (Section 4.2.1.5). The primer combination used consisted of 10 µM each of a reverse gene-specific primer (Table 4.1) and a forward ubiquitin primer (5'-

90

AATTTGATATCCTGCAGTGCAGCGTG-3') using the previously described PCR cycling parameters. PCR products were subjected to electrophoresis (1% agarose) and visualisation under UV light. Single colonies were selected for inoculation in 100 ml LBL and incubated overnight as described (Section 4.2.1.3), following large scale purification of pUBI:*ICE1*, pUBI:*OTS1*, pUBI:*OTS2*, using GeneElute HP Endotoxin-Free Plasmid Maxiprep Kit (Sigma Aldrich, USA) according to manufacturer instructions, followed by quantification by Nanodrop to ensure a DNA concentration of 1 µg/µl for particle bombardment. Each transgene plasmid were sent for sequencing to confirm transgene integrity.

4.2.4 Vector constructs for drought inducible expression in wheat

4.2.4.1 *In silico* analysis of *Rab17* and primers.

Gene ontology was obtained from National Centre for Biotechnology Information (NCBI) and sequence information cross checked with available peer reviewed literature. *Rab17* with accession: X15994.1, was analyzed with the aid of the *cis*-acting regulatory elements databases PLACE (Higo *et al*., 1999) and PlantCARE (Lescot *et al*., 2002). Primers were designed based on genomic DNA sequence to include all necessary *cis*-elements using Oligo Explore 1.2 (http://genelink.com) for primer selection and analyzed using Oligo Analyzer 1.2 (http://genelink.com).

Table 4.3 *Rab17* promoter primer pair covering the region upstream from the start codon of the *Rab17* gene. The total size of amplified fragment is 650 bp

Name	Sequence	GC % Min/Max	T_m
Rab17-Forward	5'-TAACAAACATGTCCTAATTGGTACTCC-3'	37.4/37.04	61.6°C
Rab17- Reverse	5'-GTTGCCGTACTGGTCGACATG-3'	57.14/57/14	64.5°C

4.2.4.2 Promoter amplification, purification and sequencing

gDNA was isolated from four week old maize callus (callus material was provided by Institute for Plant Biotechnogy, Stellenbosch University, South Africa) followed by high-fidelity amplification of the *Rab17* promoter region using Phusion® High-Fidelity DNA Polymerase (Thermo Scientific, USA). Master mixes were prepared for 50 µl reactions according to the manufacturer instructions with 150 ng/ul template DNA and primer pairs listed in Table 4.3. The PCR cycle parameters for *Rab17* promoter region are outlined in Table 4.4. The PCR product was separated by gel electrophoresis (1.6% agarose gel), visualized under a UV light, purified using GeneJet gel purification kit (Thermo Scientific, USA) and quantified as outlined in sections 4.2.1.1 and 4.2.1.2.

Table 4.4 *Rab17* promoter PCR cycle parameters

Steps	Temperatures	Time
Initial denature	98°C	35 seconds
Denature	98°C	35 seconds
Annealing	65°C	35 seconds
Extension	72°C	35 seconds
Final extension	72°C	7 minutes
Hold	4°C	Hold

4.2.4.3 Construction of pJet:*Rab17:ICE1* vector

The non-phosphorylated *Rab17* promoter region was ligated into the pJET1.2/blunt vector using the CloneJET PCR Cloning Kit (Thermo Scientific, USA) and transformed into MAX Efficiency® DH5α™ Competent Cells according to manufacturer instructions. Colony PCR was done to confirm insertion of *Rab17* using pJET1.2 reverse primer (5'-AAGAACATCGATTTTCCATGGCAG-3'*)* and *Rab17* forward- primers (Table 4.3). Amplified products were electrophoresed (1.6% agarose) and visualized under UV light.

The positive clones were used to inoculate LBL media and allowed to grow overnight at 37°C. Cultures underwent vector extraction by means of using the GeneJET Plasmid Miniprep Kit *(*Thermo Scientific, USA), followed by sequencing at the CAF (University of Stellenbosch, South Africa). The orientation of *Rab17* in pJET1.2/blunt vector was determined by raw sequenced data that was imported into *CLC* sequence viewer (http://www.clcbio.com). Once orientation was confirmed the pJet:*Rab17* plasmid was linearized by restriction digest using *Xho*I (Figure 4.2a-b), after which a PCR purification was done using the GeneJet PCR purification kit (Thermo Fisher Scientific, USA). The linearized vector ends were blunted with T4 DNA polymerase and dephosphorylated with T4 Kinase.

Phosphorylated *ICE1* (prepared in section 4.2.2.4) was ligated into dephosphorylated pJet:*Rab17* (Figure 4.2b-c) at a ratio of 1:3. Ligation reactions were prepared as follows; 50 ng/µl linear pJet:*Rab17* vector (Figure 4.2c), 10X T4 ligase buffer, T4 DNA ligase (5 U), 50% (w/v) polyethylene glycol (PEG) 4000 and 50 ng/µl (*ICE1*). The reaction was incubated for 15 min at room temperature. Post incubation 4 µl of the ligation reaction was used for bacterial transformation using MAX Efficiency® DH5α™ Competent Cells (50 µl*)* and allowed to grow in LBL media with ampicillin (80 mg/ml) according to manufacturer instructions.
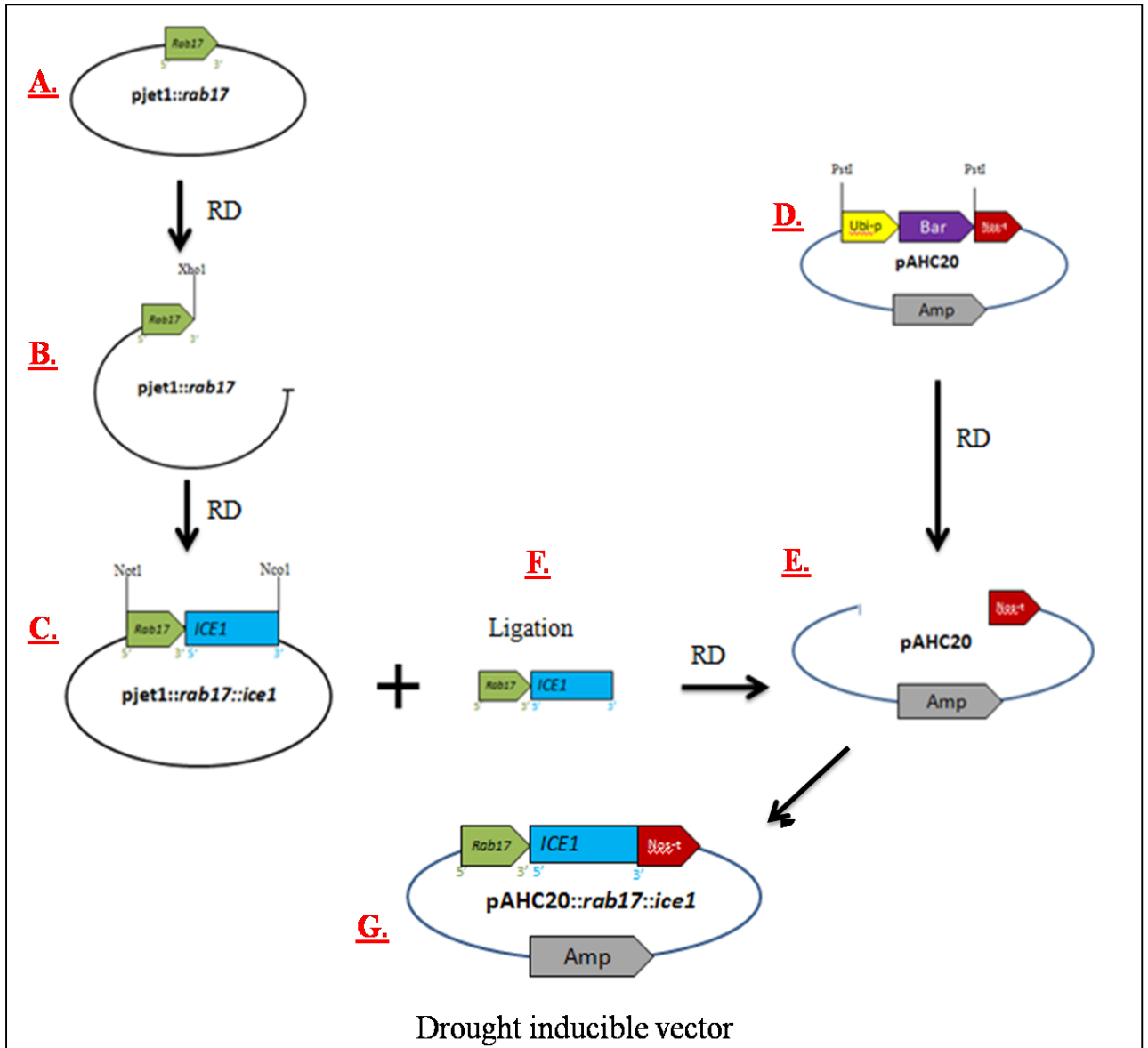
Figure 4.2 Sequential steps during the design of the stress inducible vector. (A-B) Manipulation of pJET1.2/blunt cloning vector for inclusion of *Rab17* and subsequently *ICE1*; (D-E) Removing the ubiquitin promoter and *bar* gene from vector pAHC20; (F) Ligation reaction of *Rab17:ICE1* into pAHC20; (G) Drought inducible vector. RD refers to restriction digest

Colony PCR was done to confirm gene presence but also allows for determining gene orientation. Individual colonies were selected, and suspended in 10 µl PCR reaction, which was prepared as outlined in section 4.2.2.5, using 10 µM each from the reverse *ICE1* primer (Table 4.1) and forward *Rab17* primer (Table 4.3) following the colony PCR parameters outlined in section 4.2.1.5. PCR products were subjected to electrophoresis (1% agarose) and visualisation under UV light (Section 4.2.1.2). Single colonies were selected for inoculation in LBL and overnight incubated (Section 4.2.1.3), followed by plasmid purification. Purified plasmid pJet:*Rab17:ICE1* were sequenced at CAF (Stellenbosch University, South Africa) (Section 4.2.1.4).

Rab17:ICE1 was excised as a single unit, thus the pJET1.2 vector housing *Rab17:ICE1* was double digested with *Not*I and *Nco*I (Figure 4.2c). These enzymes were chosen due to the fact that their endonuclease recognition sequences do not lie within the *Rab17:ICE1* gene itself. The reaction was subjected to electrophoresis on a 1% (w/v) agarose and corresponding bands were excised under UV light, and purified with GeneJet PCR purification kit as described by the manufacturers (Thermo Scientific, USA). The concentration and purity of samples were determined with the Nanodrop ND-2000 spectrophotometer and phosphorylation was done as described in section 4.2.2.4.

4.2.4.4 Final destination vector (pAHC20) manipulation

The plant expression vector pAHC20, contains a selectable marker phosphinotricin acetyltransferase (*bar*), under the transcriptional control of a separate Ubi-1 promoter and a nopaline synthase terminator (nos-t) (Figure 4.2d) (Provided by the Institute for Plant Biotechnology-Stellenbosch University, South Africa) was streaked out from -80°C glycerol stocks, cultured overnight in LBL media and purified. Vector was then reconstructed by removing the entire promoter and selection gene (*bar*) (Figure 4.2e) simultaneously. This was achieved by enzymatically digesting pAHC20 with *Pst*I, and incubating the reaction at 37°C for 2-3 hours. Thus all that remained of the construct was the backbone containing a bacterial selection gene (Ampicillin) and nos-t. The digest reaction was electrophoresed (1% agrose) and vector backbone was excised, purified, quantified and dephosphorylated as previously described.

95

Phosphorylated *Rab17:ICE1* prepared in section 4.2.4.3 was ligated into dephosphorylated pAH20 backbone (Figure 4.2f-g) following bacterial transformation using MAX Efficiency® DH5α™ Competent cells (50 µl*)* and plated on LBA media contain ampicillin (80 mg/ml). Gene presence and orientation was confirm by colony PCR, as outlined in section 4.2.1.5 with the exception of 10 µM Rab17 forward primer (Table 4.3) and 10 µM reverse nos-t primer (5'-AAT TTGATATCCTGCAGTGCAGCGTG-3'). The following cycling parameters were used: initial denaturation at 95°C for 3 min; 35 amplification cycles consisting of denaturation at 94°C for 1 min, annealing temperature at 62°C for 30 s, extension at 72°C for 2.20 min; final extension at 72°C for 10 min. Positive colonies were selected for inoculation of a 100 ml of liquid LB media for large scale purification using GenElute HP Endotoxin-Free Plasmid Maxiprep Kit (Sigma-Aldrich; USA). The construct was sent for sequencing at CAF (Stellenbosch University, South Africa).

4.2.5 Tissue Culture

4.2.5.1 Donor plants

Due to its superior *in vitro* regeneration potential, all transformations were conducted on the Gamtoos R (*Dn7+*) cultivar, containing the *Dn7* resistance gene for RWA (Marais & du Toit, 1993). Donor plants were grown under conditions described in Chapter 3 (Section 3.2.1.1). A total of 5756 embryos were isolated.

4.2.5.2 Particle bombardment

Bombardment was done as described by Indra *et al.* (2006) with modifications. Briefly, explants 2-3 days post initiation on Induc A media (Chapter 3, Table 3.1) were transferred and clustered together on osmotic media (Induc A with 0.2 *M* sorbitol, 0.2 *M* mannitol) for 4-6 hours prior to bombardment. All DNA deliveries were done via a locally engineered particle inflow gun. Tungsten particles (M10 Bio-Rad, South Africa) were sterilized with absolute ethanol and washed 3 times and re-suspended in sterile water. To each aliquot of tungsten suspension 10 µl DNA (1 µg/ul) was added, followed by the simultaneous addition of 2.4 M $CaCl_2$

and 0.1 M spermidine (Sigma-Aldrich; USA), vortexed and placed on ice. Incubation occurred at 4ºC, followed by the removal of 100 μl of the aqueous layer prior to bombardment. For the bombardment, explants were clustered together and placed 13 cm below the particle expelling tip, covering a 2 cm diameter circle and enclosed with a metal grid. The particle tip containing 1 mm^2 metal grid was loaded with 5 μl of the DNA suspension. Air within the chamber was relinquished, until 80 kPa and 90 kPa and the suspensions (DNA) were expelled when helium, 1000 kPa, was released by a timer relay, 0.05 seconds. 16 hours after bombardment, the current media (turgidity treatment) was replaced with Induc A media and maintained at 24ºC in the dark for 3-4 weeks.

4.2.5.3 Regeneration and selection

Due to the efficacious nature of RDZA (Chapter 3, Table 3.1), the media were used in all subsequent tissue induction experiments. Therefore, 3-4 weeks post bombardment embryogenic calli were all transferred to regeneration RDZA media for shoot formation, under a photoperiod of 16h/8h (day/night) at 24ºC. A delayed selection phase was initiated 2 weeks after initial transfer to regeneration media when 35 mg/l geneticin was included in the RDZA media as selection agent. The selection phase lasted for 4-5 weeks, after which the plantlets that survived were transferred to RDZA without selection. Plantlets that did not have adventitious roots, were transferred to either half (1.1 g/l) or full strength MS (2.2 g/l) on solid media until roots formed. Note that the carbon source was substituted with 1% (w/v) fructose when plants presented symptoms of chlorosis (Jain *et al.,* 1997).

4.2.5.4 Statistical analysis

Callogenesis, embryogenesis and shoot formation frequency were statically validated in the same manner as indicated in Chapter 3 (Section 3.2.1.4).

4.2.5.5 *Ex vitro*-acclimatization and phenotyping

Acclimatization *in vitro* generated plantlets was done in closed glass or plastic containers in the laboratory with at temperatures of 23°C - 27ºC and natural light. For a period of 2 weeks the plants were not watered (as the humidity in the containers is usually high). Every 3rd day the containers were partially opened. Plantlets were then transferred to a greenhouse, where Acclimatization was completed, allowing the plants to acclimatize to the bigger, drier greenhouse environment. Plantlets were then regularly irrigated (3 times a day) via a dripper irrigation system containing nutrients (Multifeed TM, South Africa). Acclimatization date was recorded, following the date of spikelet formation and anthesis (flowering). All plants were visually assessed every 2nd day and any phenotypic alteration was compared to the control population.

4.2.5.6 Trans-gene confirmation

T_0 generation plants were analysed for the presence of the transgenes (*ICE1, OTS1*, and *OTS2*). Genomic DNA was isolated from 5 week old putative transgenic plants and non-transformed (NT) control plants using Gene JET Plant Genomic DNA Purification Mini Kit (Fermentas, Pretoria, SA) according to the manufacturer's instructions. PCR was conducted as follows; gDNA as template (250 ng/ul), 10 mM of dNTP, 10 µl of 5xGreen GoTaq buffer reaction buffer, GoTaq® DNA Polymerase (5 U), 10 µM reverse gene-specific primer (Table 4.1) and 10 µM forward ubiquitin primer (5'- AATTTGATATCCTGCAGTGC AGCGTG -3') (Figure 4.1a). PCR was initiated by denaturation at 95°C, for 3 min followed by 35 cycles of 1 min at 95°C , 30 sec at 60°C (annealing for all transgene primers) and 72°C for 2 min , ending off with 1 cycles of 72°C for 10 min. Post PCR all sample were electrophoresed with the following controls; 1) non-tranformed wheat plant (DNA) that has completed *in vitro* regeneration; 2) bombardment plasmid DNA; and 3) no template. PCR was repeated for all positive plants that showed transgene integration.

4.3 Results

4.3.1 Gene(s) amplification, isolation and purification

The genes *ICE1* (1540 bp), *OTS1* (1760 bp) and *OTS2* (1713 bp) (Appendix B; Figure B1-B3) were isolated using primer pairs covering the start and stop codon of each gene. Total RNA was extracted from plants and was used as template for cDNA synthesis which was eventually used in all subsequent amplification reaction for amplification of *ICE1, OTS1* and *OTS2*. All genes were amplified with Phusion High-Fidelity (HF) polymerase and products were visualized as shown in Figure 4.3.
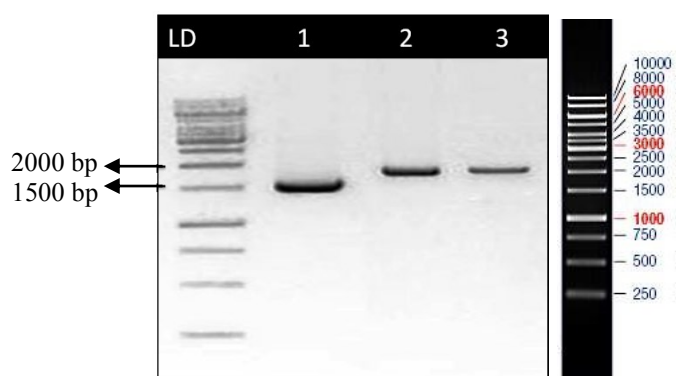
Figure 4.3 Amplification of genes from *A. thaliana*. LD refers to O'gene 1 kb rule; (lane 1) *ICE1* gene of 1500 bp; (lane 2) *OTS1* of 1700 bp and; (lane 3) as *OTS2* of 1700 bp.

4.3.2 Constructs for constitutive over expression in wheat

The full length genes (amplicons) were individually cloned into the cloning vector pJET1.2/blunt. To confirm gene insertion, colony PCR was performed with gene specific forward primer (Table 4.1) and reverse primer of the vector, the resulted fragments were 1500 bp (pJet:*ICE1*), 1700 bp (pJet:*OTS1*) and 1700 bp (pJet:*OTS2*) (Figure 4.4). The purified positive vectors housing the respective genes, were then sequenced. The raw sequence data was BLAST on TAIR which confirmed 99.5% - 100% identity with E- value of 0.0.
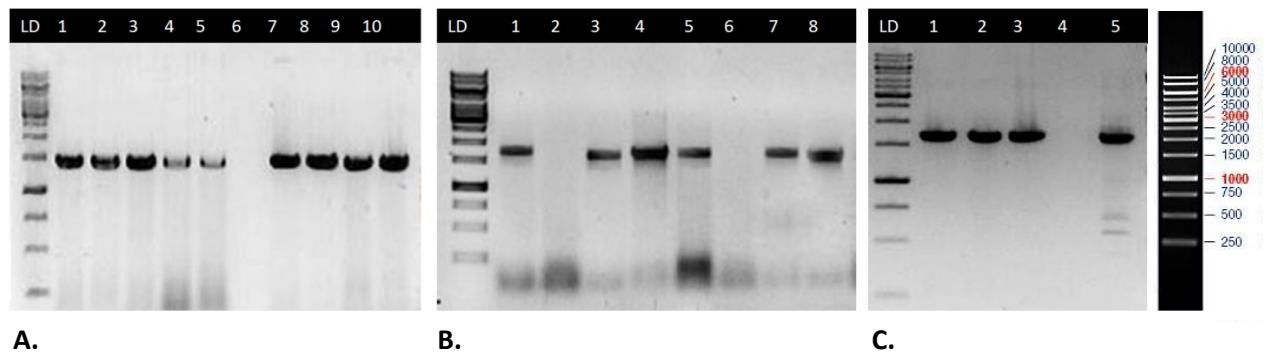
Figure 4.4 Colony PCR confirming gene insert using gene specific forward primers (Table 4.1) and pJET /1.2blunt reverse primer. Lanes with bands indicate presence of genes in pJET/1.2blunt vector. (A) *ICE1* at 1500 bp; (B) *OTS1* at 1700 bp; (C) *OTS2* at 1700 bp.

The initial PCR reaction containing the amplicon was phosphorylated then cloned into a final linear and dephosphorylated pUBI-510 expression vector. This was confirmed with colony PCR, by using gene specific reverse primers (Table 4.1) and ubiquitin promoter forward primer (Figure 4.1a), resulting fragments 1500 bp (pUBI:*ICE1*); 1700 bp (pUBI:*OTS1*); and 1700 bp (pUBI:*OTS2*). Many positive colonies were confirmed, however one was selected for sequencing to re-confirm the orientation and full length of genes (Figure 4.5)
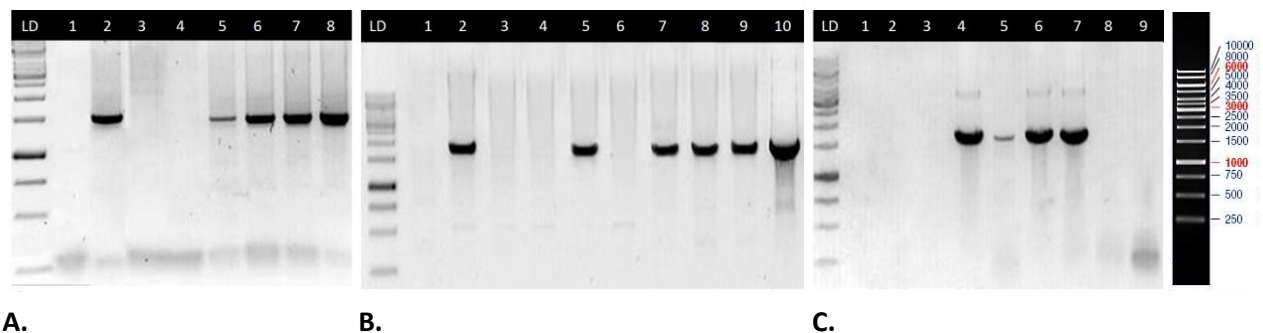
Figure 4.5 PCR analysis of transgenes cloned into the plant expression vector pUBI-510, using the ubiquitin promoter forward primer and gene-specific reverse primer for PCR. (A) pUBI:*ICE1*; (B) pUBI:*OTS1*; and (C) pUBI:*OTS2*.

4.3.3 Construct for drought inducible expression in wheat

4.3.3.1 *In silico* characterization of *Rab17* promoter

To confirm its functionality for drought inducible expression of *ICE1*, basic *in silico* analysis was required prior to primer design and subsequent cloning of *Rab17*. The complete 560 bp 5' DNA-flanking region upstream of the first codon was presumably the promoter of *Rab17* (not indicated on NCBI) (Figure 4.6). Several abiotic stress-related *cis*-elements were identified in the regulatory sequences, shown in Figure 4.6. These elements collectively allow up regulation of the promoter when the plant is being water stressed. The *DRE1* (ACCGAGA) and *DRE2* (ACCGAC) are representatives of common drought responsive element (TACCGACAT) also known as the C-repeat/DRE (Thomashow, 1998; Morran *et al*., 2011). To further ensure promoter functionality a core sequence known as *cis*-acting ABA responsive elements (ABRE) with sequence GTACGTGGCGC was also identified together with the Sph and GC rich Rab Activator (GRA). Finally primers were designed to amplify the entire promoter (650 bp).
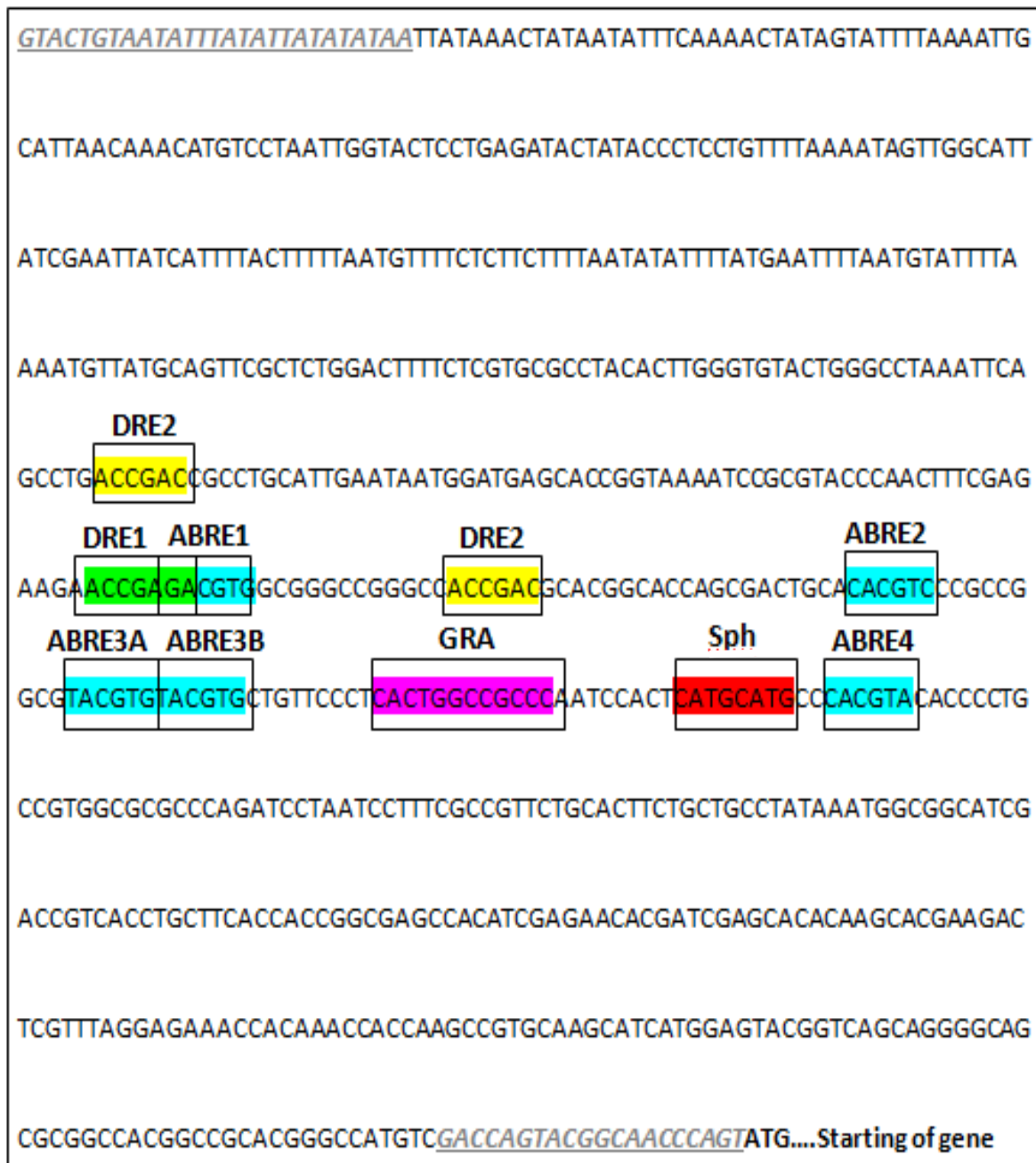
Figure 4.6 The complete promoter sequence 1-650 bp denoted on gDNA (maize) with the *cis*-elements indicated in enclosed colour boxes. Primer binding sites are underlined in grey.

Construction of pJet*:Rab17:ICE1 vector*

The gDNA was isolated from maize calli and used as template in the PCR reaction. The amplification products were visualized on a 1.5% agarose gel as indicated (Figure 4.7). A fragment of approximately 650 bp were purified out of the gel and subsequently ligated into pJET1.2/BLUNT vector and sequenced. The nucleotide sequence were BLAST on NCBI, which confirm 100% identity with E-value of 0, corresponding to the region upstream of the *Rab17* gene from maize (X15994.1). *ICE1* were then cloned downstream of the *Rab17* promoter region a in pJet*:Rab17*. This was validated by PCR using *Rab17* forward and *ICE1* reverse primers. Finally the *Rab17:ICE1* was excised as a single unit using restriction enzymes *Not*I and *Nco*I (Figure 4.8a).
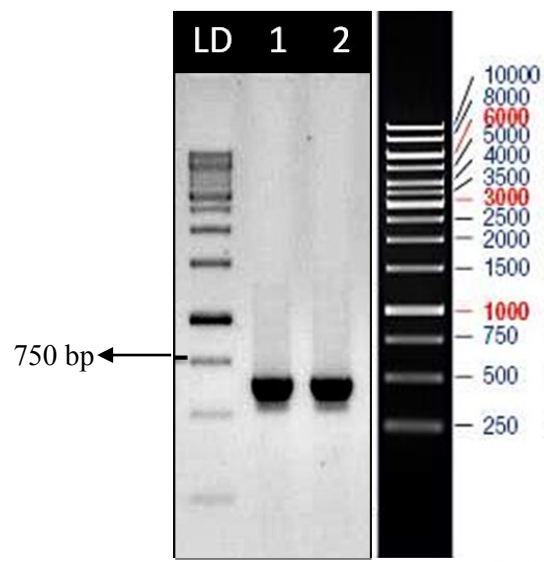
Figure 4.7 The amplification of the *Rab17* promoter region (650 bp) using HF polymerase.

4.3.3.2 Final destination vector (pAHC20) manipulation

Both the promoter (ubiquitin) and selectable marker (*bar*) were removed from pAHC20 plant expression vector using restriction enzyme *Pst*I (Figure 4.2a). *RAB17:ICE1* was then ligated into the remaining backbone of pAHC20. For confirmation of *RAB17:ICE1* position in pAHC20, the sequence was amplified by PCR using Rab17 forward primer and reverse primer of nos-t, resulting in a 2.7 kb fragment as seen in Figure 4.8b and Figure 4.9.
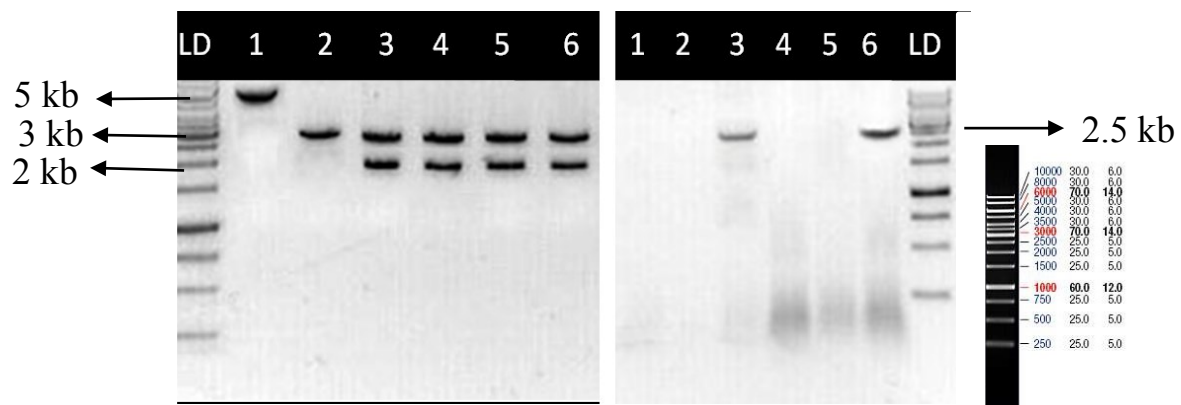
Figure 4.8 Cloning of the final drought inducible expression vector. (A) Restriction digestion of (lane 1) pJet:*Rab17:ICE1* (5 kb); (lane 2) empty pJET/1.2 blunt vector at 2.9 kb; (lane 3-6) *Rab17:ICE1* (2150 bp) from pJET/1.2 blunt vector; (B) Colony PCR confirming presence and orientation of two clones containing *Rab17:ICE1* (2.7 kb) in pAHC20.
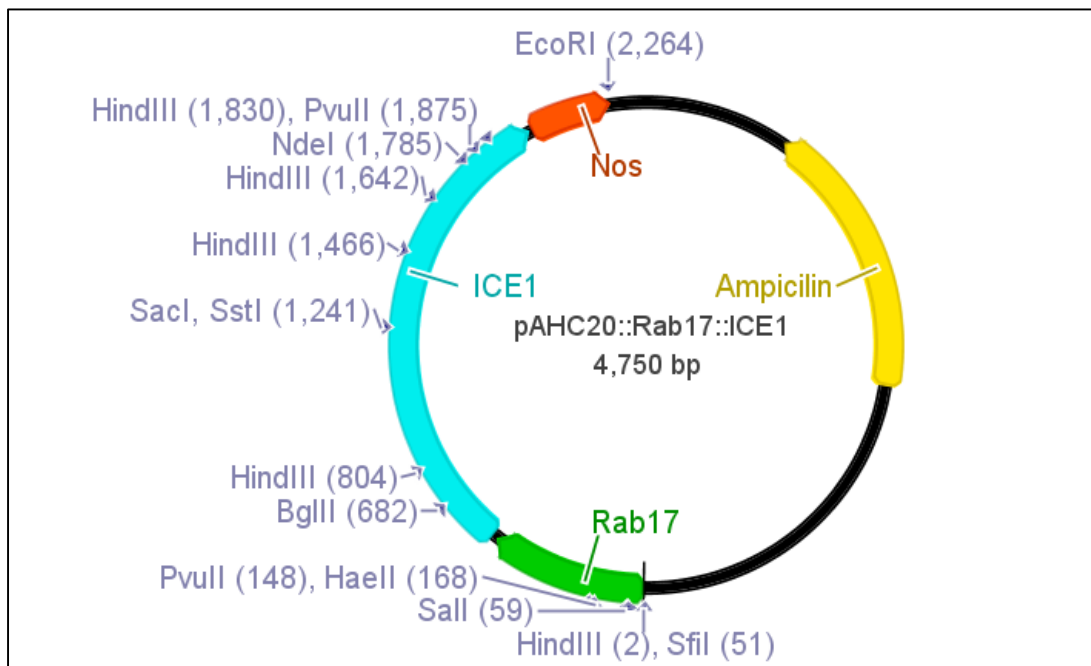
Figure 4.9 Final vector (pAHC20*:Rab17: ICE1*) for drought inducible expression in wheat with all annotated genes and restriction sites.

4.3.4 Transformation

In total, three constitutive vectors housing the respective genes, including empty vector control, were bombarded with co-bombardment vector pEmuKN into 2-3 day old immature embryos that were cultured on Induc A media. All embryos survived the bombardment, however not all underwent somatic embryogenesis, and only some formed calli after 4 weeks. Despite this, all calli and embrygenic tissue were transferred to RDZA without selection for two weeks. During this time nearly all embryogenic tissue had formed multiple shoots (59%-74%), however the majority stop developing and suffered necrosis (Table 4.5; Figure 4.10). At week 3, individual shoots intact with callus were transferred to selection RDZA containing 35 mg/L geneticin. The selection was only kept for a maximum of 4 weeks. At the end, a total of 1-2% plantlets survived the selection medi (Figure 4.11; Table 4.6). The remaining plantlets were transferred to a medium, in which the sucrose was replaced with filter sterilized fructose consisting of 0.5 g/l MS, 1% fructose and 1 g/l Gelrite. Roots formed within 3 days.

Table 4.5 *In vitro* regeneration of bombarded immature wheat embryos up to placement on selection medium. Numerical values indicate the formation frequency of callus (C%), embryogenicity (E%) and shoots (S%). Statistical methodology are outline in Chapter 3 (Section 3.2.1.4). All frequencies with calculated ±SEM were scored according to an ordinal scale (Appendix A, Figure A1).

Construct	Isolated/Bombarded	C%	E%	*S%
pUBI-*ICE1*	1159	59.4±0.45	40.6±0.81	36.2±0.16
pUBI-*OTS1*	1077	56.8±0.41	43.2±0.47	37.0±0.24
pUBI-*OTS2*	1150	61.0±0.30	39.0±0.73	37.4±0.24
pUBI-empty	1101	74.4±0.40	25.6±0.74	21.9±0.45
pAHC20:*Rab17:ICE1*	959	64.2±0.47	35.8±0.82	21.9±0.42

*S: refers to clumps of embryogenic tissue that often result in 5-8 shoots (Figure 4.11). These were regarded as isogenic and thus calculated as a single shoot.
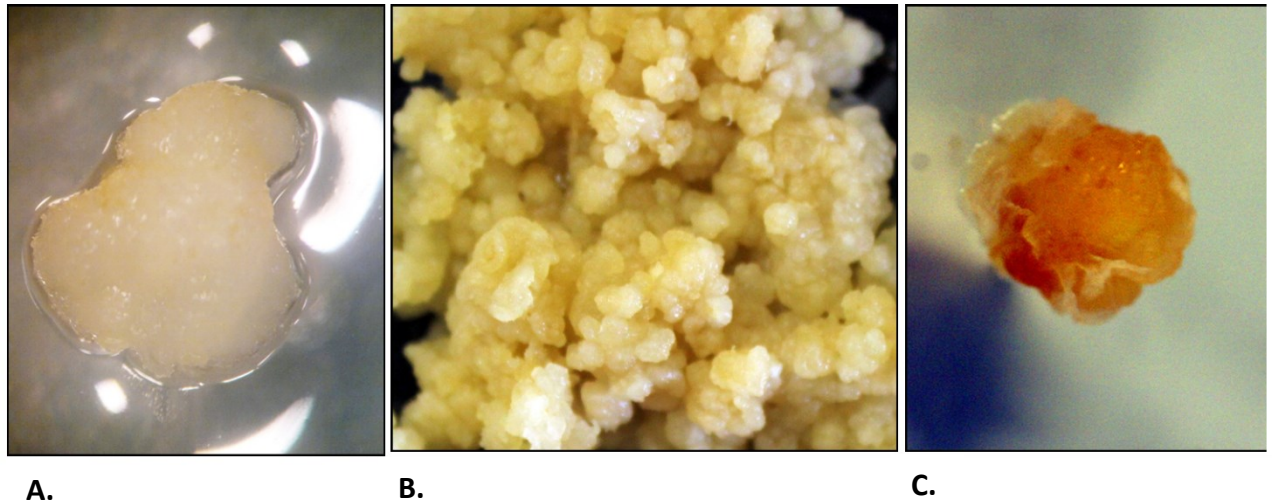
Figure 4.10 *In vitro* tissue development 4 weeks post-bombardment of immature embryos. (A) Embryo that only formed calli; (B) Somatic embryoids; (C) Calli to be transferred to RDZA media showing necrosis and no further development.
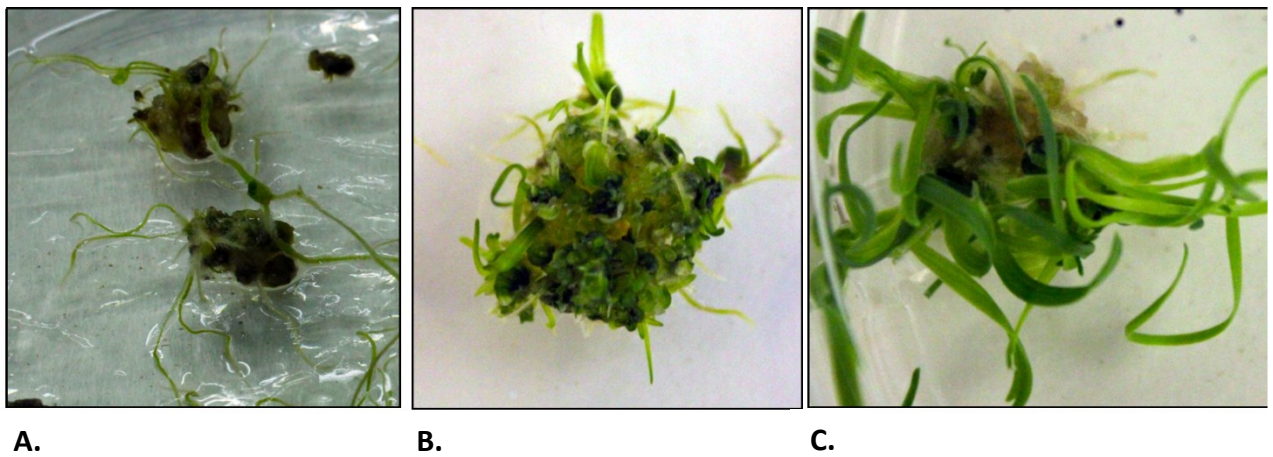
Figure 4.11 Selection on 30 mg/L geneticin after 4 weeks. (A) Embryogenic tissue suffering severe necrosis and ultimately death; (B) Clusters of shoots with some shoot starting to die due to selection; (D) Plantlets (isogenic) surviving selection period.

Table 4.6 *In vitro* tissue survival after the selection period. Indicated is the amount of shoots that survived the geneticin treatment and the eventual plantlets that form from the shoot with functional roots just prior to the hardened off phase

Construct	Survived Selection	Positives
pUBI-*ICE1*	2.6%	2
pUBI-*OTS1*	2.3%	4
pUBI-*OTS2*	2.3%	1
pUBI-empty	1.7%	0
pAHC20:*rab17:ICE1*	0	0

In vitro plantlets were successfully hardened off with a total of 35 plants surviving this process. Eventually one of the plants did not survive the greenhouse conditions. All remaining plants matured and started anthesis, although clear difference in anthesis (flowering) time was noticeable. The total duration from initial isolation, bombardment and Acclimatization, until seeding stage were 7-9 months (Figure 4.12).

A. **B.** **C.**
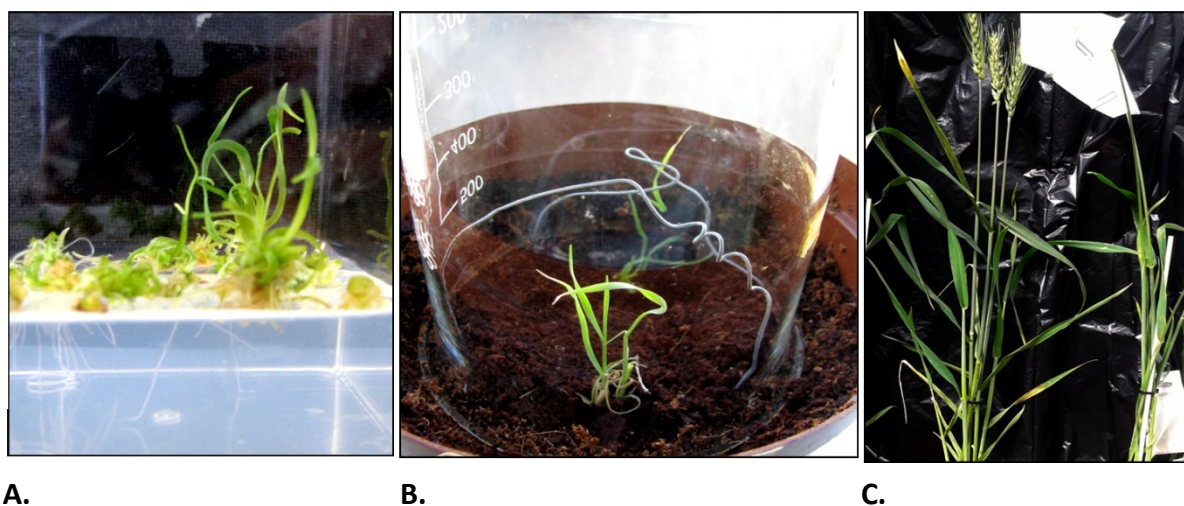
Figure 4.12 Post selection phase till *ex vitro* seed formation. (A) Plants on solid media for induction of root formation; (B) hardening off; (C) mature plant with spikelets. White bags covering ears prevented cross pollination

4.3.4.1 Trans-gene confirmation

DNA was extracted from T_1 generation plants and genomic DNA used as template to confirm transgene insertion in the putative transgenic plants. Five plants tested positive for pUBI:*OTS1* fragment size of 1700 bp, two plants for pUBI:*OTS2* with fragment size of 1700 bp, and one for pUBI:*ICE1* with fragment size of 1500 bp (one pUBI:*OTS1* plant died)(Figure 4.13).
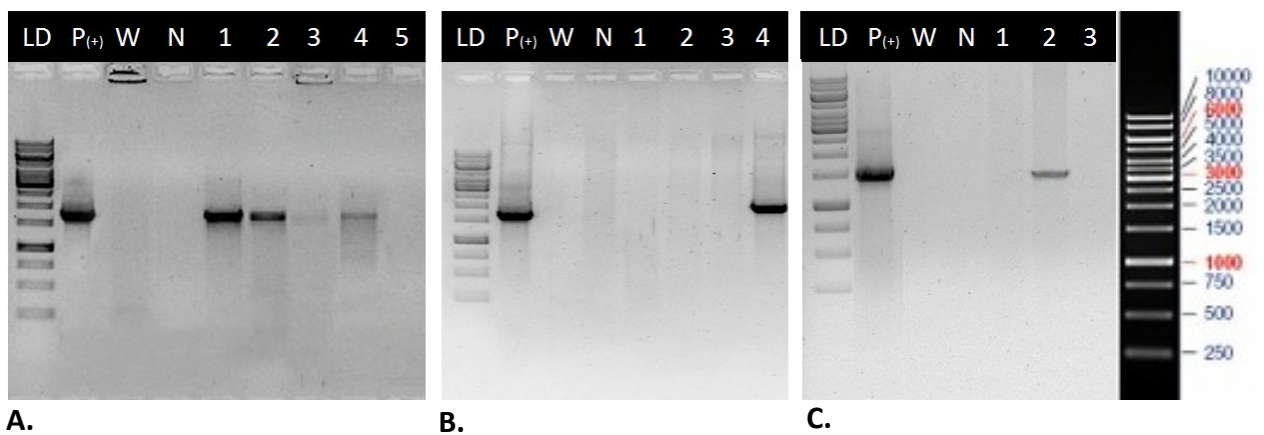
Figure 4.13 Four *OTS1* transgenics (1-4); B) two *OTS2* transgenics and; (C) one *ICE1* transgenic. *P (+)* refers to plasmid DNA used for bombardment; *W* refers to non-transformed wheat, *N* negative water control.

4.3.4.2 Basic phenotyping of T_0 generation.

All plants plant including escapes and transgenics, were closely assessed for the start of the first spikelet development, following annotating the day each plant started anthesis (Table 4.7). It was clear the total amount of days required to form spikelets for *OTS1* and *OTS2* was similar to that of the control group however *ICE1* required 20 additional days from the formation of a single spikelet. Furthermore *OTS1* plants took much longer then the control to start anthesis where as *ICE1* and *OTS2* was similar to that of the control (Table 4.7). Finally phenotypically *OTS1* and *OTS2* were indistinguishable from the control plant (Figure 4.14.). While the *ICE1* plant suffered severe growth impairment with abnormal spikelet development (Figure 4.14a)

Table 4.7 Basic assessment of transgenic plants.

Vector- plant	Total days of spikelet formation since hardened off	Starting date of anthesis post spikelet formation
Control	130-140 days	12-14 days
pUBI-*OTS1*	136-143 days	24-27 days
pUBI-*OTS2*	130-139 days	16-19 days
pUBI-*ICE1*	164 days	15 days

Figure 4.13 Phenotypes of spikelet and plants. (A) Spikelet of (pUBI-*ICE1*); (B) Control spikelet; (C) transgenic plants. Note a single plant was chosen as a representative for this photo. However there is only one transgenic plant for pUBI-*ICE1*

109

4.4 Discussion

Abiotic stress, such as drought has ripped through many countries, affecting both national and international productivity and yield of wheat harvests. Conventional breeding methods have made substantial progress in the past, however due to lack of genetic diversity, the progress of drought tolerance in wheat has been slow and less prevalent (Yi *et al*., 2015). The application of GM technologies to the enhancement of wheat has proven to be successful time and time again, and therefore could serve as a potential solution to the current crisis in the wheat industry. Vector construction plays an integral part in successful genetic transformation of plants, as many factors, i.e. vector size and type of promoter used, can influence the compatibility of the construct with the plant innate system, and genome size and complexity. In the context of wheat genetic transformation, the fact that this cereal crop is a hexaploid complicates matters even further. The recalcitrant nature of wheat to genetic modification is primarily attributed to the aforementioned (Bhalla *et al*., 2006; Harwood, 2012). However, many research groups have achieved stable expressing of selected genes, but timing and rate of expression has major impact on the outcome of the experiments. Constitutive over-expression of a gene often leads to an improvement in plant survival under stress, however many reports frequent pleiotropic effects in the transgenics under both stress and non-stress conditions. A method of overcoming this is the use of stress inducible promoters (Yi *et al*., 2010; Kovalchuk *et al*., 2013). The core focus of this study was the design and construction of three vectors for the constitutive expression of candidate transgenes, and an additional vector for drought inducible transgene expression. These constructs were then transformed into immature wheat embryos, which subsequently morphed into fully developed plants. PCR confirmed stable transgene integration (T_0), and basic phenotyping alluded to potential differences in gene function.

4.4.1 Constitutive over expression of transgenes in wheat

Gene ontology indicated that *OTS1* and *OTS2* have nearly identical nucleotide lengths and share many conserved sequences. Amino acid sequence alignment clearly shows that the target SUMO for *OTS1* and *OTS2* SUMO protease is very highly conserved, indicating functional conservation in downstream signalling. SUMO1 or SUMO2 are ubiquitin-like proteins that can be covalently attached to proteins as a monomer or a lysine-linked polymer. With the attachment the targeted protein function can be changed or poly-ubiquitinated for proteosomal degradation. Conti *et al*. (2008) demonstrated that over expressing *OTS1* in *Arabidopsis* leads to deconjugation of SUMO1/2 from hitherto unknown target proteins resulting in high salt tolerance. Furthermore the researchers also found the lines were phenotypically indistinguishable from the wild type *Arabidopsis* in non-stressed conditions. However, *OTS1* and *OTS2* are yet to be explored in wheat *in planta*, thus this research chapter / study explored the potential of overexpression the respective genes in wheat.

These constructs were successfully transformed into wheat. By over expressing the genes using a 35S promoter, the compatibility of the constructs were validated in wheat Gamtoos R (*Dn+*) for transgene integration. T_0 plants showed no undesirable pleiotropic effects, i.e. stunted growth, except that many of the *OTS1* population plants started athesis 12-14 days later than the control group. Generally for spring wheat e.g. Gamtoos R (*Dn+*) anthesis starts between 10-15 days post spike development (Screenivasulu and Schnurbusch, 2012). Furthermore, the average duration of anthesis is between 10-18 days (Whingwiri and Kemp, 1980; Screenivasulu and Schnurbusch, 2012). The marked delay in anthesis is in contrast to what has been reported by Reevas *et al*. (2002), who investigated the only other annotated SUMO protease *early in short days 4 (*esd4*)* in *Arabidopsis*. These authors illustrated that *Arabidopsis esd4* mutant shows extreme early flowering and alterations in shoot development. Moreover, Murtas *et al*. (2003), worked on the same mutant population (*esd4*) and demonstrated that SUMO protease also extends to developmental attributes of the plant for example broadening of the silique. Thus, both Reeva *et al*. (2002) and Murtas *et al*. (2003) suggested that SUMO proteases regulate the SUMO conjugates, hereby hypothesizing that SUMO has a direct influence on flowering time, also referred to as anthesis.

Transgenic *ICE1* plants constitutively expressed the transgene with the same promoter used for the *OTS1* and *OTS2*. However, T$_0$ clearly shows several growth defects. The *ICE1−* plant is nearly half the size of the control plants and alterations in phyllotaxy was obvious. This phenotype suggest the possibility of a high metabolic burden imposed by extreme accumulation levels of protein. This effect is often associated with constitutive expression of *ICE1* and or high copy number of the gene (Chinnusamy *et al*., 2003; Miura *et al*., 2007; Badawi *et al*., 2008; Fursova *et al*., 2009). *ICE1* is a master regulator which binds and activates the well characterized *DREB* gene promoters. However, with an increase in *ICE1,* there will be a concomitant increase in *DREB* levels, leading to complete metabolic overload, subsequently resulting in severe growth retardation. The aforementioned has been demonstrated in transgenic *Arabidopsi*, rapeseed, tomato, potato and rice (Jaglo-Ottosen *et al.,* 1998; Kasuga 1999; Jaglo *et al*., 2001; Hsieh *et al*., 2002; Gilmour *et al*., 2004; Pino *et al*., 2007; Welling and Palva, 2008). The over expression of *ICE1* was necessary to illustrated the effect in the hexaploid wheat since this has not yet been shown, thereby necessitating the need for inducible expression of *ICE1*.

4.4.2 Drought inducible expression of *ICE1* in wheat

A novel drought inducible vector was designed for over expression of *ICE1* in wheat. Basic promoter analysis needed to be conducted to validate the promoter, ensuring the presence of all necessary *cis*-elements. By using PLACE *cis*-element database (Higo *et al*., 1999), *cis*-element locations were identified, thus supporting the use of a functional drought stress promoter. Five abscisic acid-responsive (ABRE) and two DRE/CRT elements are present, which implies high functionality of the promoter in drought conditions, since DRE/CRT is involved in gene expression responsive to water stress (Shinozaki and Yamaguchi-Shinozaki, 2000; Yamaguchi-Shinozaki *et al*., 2006). ABRE-like sequence was also present which is often required for etiolation-induced expression of *erd1* (early responsive to dehydration) (Simpson *et al*., 2003; Nakashima *et al*., 2006). However, ABRE motifs are often closely linked to other unique motif such as Sph (RY), which is known to be a key *cis*-element for expression of many seed-specific genes. Suzuki *et al.* (2005) showed a short range combinational relationship between ABRE and Sph (RY), essentially indicating genes that lack sph (RY)

sequences may still be able to interact with ABRE, especially under ABA influx, which is synonymous with drought stress. Finally, the *rab17* promoter also has W-boxes, with the database specifically indicating binding sites for the WRKY71 TF (Xie *et al*., 2005). The latter, in particular, is a transcriptional repressor of the gibberellin signalling pathway, which implies possible pathogen-responsiveness (Zhang *et al*., 2004). With promoter analysis confirming the presence of *cis*-elements, the construction of the vector was conducted. The vector is comprised of the *Rab17* promoter (650 bp), *ICE1* and nopaline synthase terminator (nos-t). Unfortunately, all the explants bombarded with the designed construct died during the selection phase and thus the transgenic plants are not yet available.

This research chapter demonstrates the vector constructions and transformation of wheat with key stress genes, with end results of GM-wheat. Next will be to advance the current GM-wheat generations (T_0) by self-pollination, where the traits should be stably inherited. T1 seed will be harvested and germinated to identify homozygous lines by means of qPCR.

4.5 References

Awad J, Stotz H, Fekete A, Krischke M, Engert C, Havaux M, Berger S, Mueller M (2015). 2-cysteine peroxiredoxins and thylakoid ascorbate peroxidase create a water-water cycle that is essential to protect the photosynthetic apparatus under high light stress conditions. Plant Physiology. **167**: 1592–1603

Badawi M, Reddy YV, Agharbaoui Z, Tominaga Y, Danyluk J, Sarhan F, Houde M (2008). Structure and functional analysis of wheat *ICE* (inducer of CBF expression) genes. Plant Cell Physiology. **49**: 1237–1249

Barbara W, Richard IS, Fioa RM, Elizabeth SD (1998). Comparison of three selectable marker genes for transformation of wheat by microprojectile bombardment. Australian Journal of Plant Physiology. **25**: 39-44

Bhalla PL, Ottenhof HH, Singh MB (2006). Wheat transformation – an update of recent progress. Euphytica. **149**: 353–366

Bilhani P, Char B, Bhargava S (2011). Transgenic expression of sorghum *DREB2* in rice improves tolerance and yield under water limitation. Journal of Agricultural Science. **149**: 95–101

Braun H (2015). How wheat improvement research impacts the world. *In* 9th International Wheat Conference. IWC conference Secretariat, Sydney Australia, pp 6-7

Busk PK, Jensen AB, Pages M (1997). Regulatory elements *in vivo* in the promoter of the abscisic acid responsive gene *rab17* from maize. **11**: 1285-1295

Chen JQ, Meng XP, Zhang Y, Xia M, Wang XP (2008). Over expression of *OsDREB* genes lead to enhanced drought tolerance in rice. Biotechnology Letters. **30**: 2191–2198

Chinnusamy V, Ohta M, Kanrar S, Lee BH, Hong X, Agarwal M, Zhu JK (2003). *ICE1*: A regulator of cold-induced transcriptome and freezing tolerance in arabidopsis. Genes Development. **17**: 1043–1054

Conti L, Price G, O'Donnell E, Schwessinger B, Dominy P, Sadanandom A (2008). Small ubiquitin-like modifier proteases *OVERLY TOLERANT TO SALT* 1 and -2 regulate salt stress responses in *Arabidopsis*. Plant Cell. **20**: 2894–908

Finnegan J and McElroy D (1995). Transgene inactivation: plants fight back. Biotechnology. **12**: 883–888

Fursova OV, Pogorelko GV, Tarasov VA (2009). Identification of *ICE2*, a gene involved in cold acclimation which determines freezing tolerance in *Arabidopsis thaliana*. Gene. **429**: 98–103

Gilmour SJ, Zarka DG, Stockinger EJ, Salazar MP, Houghton JM, Thomashow MF (1998). Low temperature regulation of the *Arabidopsis CBF* family of AP2 transcriptional activators as an early step in cold-induced *COR* gene expression. Plant Journal. **16**: 433–442

Gilmour SJ, Fowler SG, Thomashow MF (2004). Arabidopsis transcriptional activators CBF1, CBF2, and CBF3 have matching functional activities. Plant Molecular Biology. **54**: 767-81

Hassan NM, El-Bastawisy ZM, El-Sayed AK, Ebeed HT, Nemat-Alla MM (2015). Roles of dehydrin genes in wheat tolerance to drought stress. Journal of Advanced Research. **6**: 179-188

Harwood WA (2012). Advances and remaining challenges in the transformation of barley and wheat. Journal of Experimental Botany. **63**: 1791-1789

Higo K, Ugawa Y, Iwamoto M, Korenaga T (1999). Plant *cis*-acting regulatory DNA elements (PLACE) database. Nucleic Acids Research. **27**: 297-300

Hsieh TH, Lee JT, Charng YY, Chan MT (2002). Tomato plants ectopically expressing *Arabidopsis CBF1* show enhanced resistance to water deficit stress. Plant Physiology. **130**: 618–626

Indra K, Vasil V, Vimla V (2006) Transformation of wheat via particle bombardment. *In:* Loyola-Vargas F, Victor M, Vázquez-Flota II, (eds) Plant Cell Culture Protocols, Methods in Molecular Biology, Humana Press Inc. Totowa, New Jersey, pp 273-283

Jaglo-Ottosen KR1, Gilmour SJ, Zarka DG, Schabenberger O, Thomashow MF (1998). *Arabidopsis CBF1* overexpression induces *COR* genes and enhances freezing tolerance. Science. **280**: 104-106

Jaglo KR, Kleff S, Amundsen KL, Zhang X, Haake V, Zhang JZ, Deits T, Thomashow, MF (2001). Components of the Arabidopsis C-repeat/dehydration-responsive element binding factor cold-response pathway are conserved in *Brassica napus* and other plant species. Plant Physiology. **127**: 910-917

Johnson ES (2004). Protein modification by SUMO. Annual Review of Biochemistry. **73**: 355–82

Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1999). Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nature Biotechnology. **17**: 287-91

Kikker J, Vidal J, Reisch B (2004). Stable Transformation of plant cells by particle bombardment. *In* L Peña, ed, Transgenic Plants: Methods and Protocols, 2nd ed. Humana Press, Totowa, New Jersey, pp 61-78

Kovalchuk N, Jia W, Eini O, Morran S, Pyvovarenko T, Fletcher S, Bazanova N, Harris J, Beck-Oldach K, Shavrukov Y, Langridge P. and Lopato S   (2013). Optimization of *TaDREB3* gene expression in transgenic barley using cold-inducible promoters. Plant Biotechnology Journal. **11**: 659–670

Lescot M, Déhais P, ThijsG, Marchal K, MoreauY, Van de Peer Y, Rouzé P, Rombauts S (2002). PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for *in silico* analysis of promoter sequences. Nucleic Acids Research **30**: 325–327

Lopato S, Langridge P (2011). Engineering Stress Tolerance in Cereals Using. ISB news Rep 2–4

Mahajan S, Tuteja N (2005). Cold, salinity and drought stresses: an overview. Archives of Biochemistry and Biophysics. **444**: 139–158

Miura K, Jin JB, Lee J, Yoo CY, Stirm V, Miura T, Ashworth EN, Bressan RA, Yun D-J, Hasegawa PM (2007). SIZ1-mediated sumoylation of *ICE1* controls *CBF3/DREB1A* expression and freezing tolerance in Arabidopsis. Plant Cell **19**: 1403–14

Morran S, Eini O, Pyvovarenko T, Parent B, Singh R, Ismagul A, Eliby S, Shirley N, Langridge P, Lopato S   (2011). Improvement of stress tolerance of wheat and barley by modulation of expression of DREB/CBF factors. Plant Biotechnology Journal. **9**: 230–249

Murtas G, Reeves PH, Fu YF, Bancroft I, Dean C, and Coupland G (2003). A nuclear protease required for flowering-time regulation in *Arabidopsis* reduces the abundance of Small Ubiquitin-Related Modifier (SUMO) conjugates. Plant Cell. **15**: 2308-2319

Nakashima K, Fujita Y, Katsura K, Maruyama K, Narusaka Y, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2006). Transcriptional regulation of *ABI3*- and *ABA*- responsive genes including *RD29B* and *RD29A* in seeds, germinating embryos, and seedlings of *Arabidopsis*. Plant Molecular Biology. **60**: 51-68

Ogihara Y, Takumi S, Handa H (2015). High efficiency wheat transformation mediated by *Agrobacterium tumefaciens*. *In* Y Ishida, Y Hiei, T Komari, edition, Advances in Wheat Genetics: From Genome to Field, 1st ed. Springer Japan, Japan, pp 167-173

Pino MT, Skinner JS, Park EJ, Jeknić Z, Hayes PM, Thomashow MF, Chen TH (2007). Use of a stress inducible promoter to drive ectopic *AtCBF* expression improves potato freezing tolerance while minimizing negative effects on tuber yield. Plant Biotechnology. **5**: 591-604

Przetakiewicz A, Karaś A, Orczyk W, Nadolska-Orczyk A (2004). *Agrobacterium* mediated transformation of polyploid cereals. The efficiency of selection and transgene expression in wheat. Cell Molecular Biology Letters. **9**: 903–917

Qin F, Kakimoto M, Sakuma Y, Maruyama K, Osakabe, Tran YP, Shinozaki K, Yamaguchi-Shinozaki K (2007). Regulation and functional analysis of *ZmDREB2A* in response to drought and heat stress in Zea *mays L*. Plant Journal. **50**: 54–69

Reeves PH, Murtas G, Dash S, and Coupland G. (2002). Early in short days 4, a mutation in *Arabidopsis* that causes early flowering and reduces the mRNA abundance of the floral repressor *FLC*. Development. **129**: 5349-5361

Roden J, Eardley L, Hotson A, Cao Y, Mudgett MB (2004). Characterization of the *Xanthomonas AvrXv4* effector, a SUMO protease translocated into plant cells. Molecular Plant-Microbe Interactions. **17**: 633–43

Simpson SD, Nakashima K, Narusaka Y, Seki M, Shinozaki K,Yamaguchi-Shinozaki K (2003).Two different novel *cis*-acting elements of *erd1*, a *clpA* homologous *Arabidopsis* gene function in induction by dehydration stress and dark-induced senescence. Plant Journal. **33**: 259-270

Sakuma Y, Maruyama K, Qin F, Osakabe Y, Sek M, Shinozaki K, Yamaguchi-Shinozaki K (2006). Dual function of an *Arabidopsis* transcription factor DREB2A in water stress responsive and heat-stress-responsive gene expression, Proceedings of the National Academy of Sciences. **18**: 828–18833

Sanghera GS, Wani SH, Hussain W, Singh N (2008). Engineering cold stress tolerance in crop plants. Current Genomics. **12**: 30-43

Saracco SA, Miller MJ, Kurepa J, Vierstra RD (2007). Genetic analysis of SUMOylation in *Arabidopsis*: conjugation of SUMO1 and SUMO2 to nuclear proteins is essential. Plant Physiology. **145**: 119–34

Seo JW and Lee KJ (2003). Post-translational modifications and their biological functions: proteomic analysis and systematic approaches. Journal of Biochemistry and Molecular Biology. **37**: 35-44

Shinozaki K, Yamaguchi-Shinozaki K (2000). Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signalling pathways. Current Opinion in Plant Biology. **3**: 217–223

Sreenivasulu N, Schnurbusch T (2012). A genetic playground for enhancing grain number in Cereals. Trends in Plant Science. **17**: 91-101

Suzuki M, Ketterling MG, McCarty DR (2005) quantitative statistical analysis of *cis-* regulatory sequences in *ABA/VP1-* and *CBF/DREB1*-regulated genes of Arabidopsis. Plant Physiology. **139**: 437–447

Thomashow MF (1998). Role of Cold-Responsive Genes in Plant Freezing Tolerance. Plant Physiology. **118**: 1–8

Vasil IK, Vasil V (2006). Transformation of wheat via particle bombardment. *In*: Loyola-Vargas VM and Vazquez-Flota F. Plant Cell Culture Protocols, Methods in Molecular Biology, Humana Press Inc. Totowa, New Jersey, pp 273-283

Vilardell J, Goday A, Freire M, Torrent M, Martínez MC, Torné J, Pagès M (1990). Gene sequence, developmental expression, and protein phosphorylation of *Rab-17* in maize. Plant Molocular Biology. **14**: 423–432

Welling A, Palva ET (2008). Involvement of CBF transcription factors in winter hardiness in birch. Plant Physiology. **147**: 1199-211

Witrzens B, Brettell RS, Murray FR, McElroy D, Li Z, Dennis ES (1998). Comparison of three selectable marker genes for transformation of wheat by microprojectile bombardment. Australian Journal of Plant Physiology. **25**: 3–44

Xie Z, Zhang ZL, Zou X, Huang J, Ruas P, Thompson D, Shen QJ (2005). Annotations and functional analyses of the rice *WRKY* gene superfamily reveal positive and negative regulators of abscisic acid signaling in aleurone cells. Plant Physiology. **137**: 176–189

Yamaguchi-Shinozaki K, Sakuma Y, Ito Y and Shinozaki K (2006). The DRE/DREB regulon of gene expression in *Arabidopsis* and rice in response to drought and cold stress. *In* Ribaut, JM, ed.Drought Adaptation in Cereals, Binghamton, NY: The Haworth Press, Inc, pp. 583–598.

Yi N, Kim YS, Jeong HM, Oh SJ, Jeong JS, Park SH, Jung H, Choi YD, Kim JK (2010). Functional analysis of six drought-inducible promoters in transgenic rice plants throughout all stages of plant growth. Planta. **232**: 743-754

Yi H, Qiong W, Jian Z, Tao S, Guang-xiao Y, Guang-yuan H (2015).Current status and trends of wheat genetic transformation studies in China. Journal of Integrative Agriculture. **14**: 438–452

Zhang ZL, Xie Z, Zou X, Casaretto J, Ho TH, Shen QJ (2004). Gibberellin signaling pathway in aleurone cells. Plant Physiology. **134**: 1500-1513

CHAPTER 5

Synthesis and Future Prospects

It is apparent that abiotic stress, i.e. drought, is a major concern worldwide as it is not only associated with high temperature but also with biotic stress, both of which lead to financial losses, ultimately affecting the consumer. Evidently GM technology has the potential to improve this crop in the goal of improving wheat, however the application of genetic transformation may not necessarily lead to commercialization of the GM-wheat (at this point in time). But the applications are valuable in the process of unravelling wheat genomes by conducting functional analysis of genes *in planta*. This adds additional value to conventional breeding practices.

The long term aim of this study is to improve drought tolerance in South African wheat by manipulating and altering the SUMOylation pathway. For the first objective, six South African wheat cultivars were tested for superior *in vitro* regeneration capacity. Briefly the explant of choice was the immature embryo owing to its high regeneration capacity. All cultivar explants were exposed to four different media for induction of callus, with subsequent induction of regeneration for plantlet formation by testing four different media. The tested cultivars showed variation in their *in vitro* co-efficiency abilities, with efficiencies ranging from 0 to 36.5%. Gamtoos and Tugela cultivars displayed the highest and lowest regeneration efficiencies, respectively on the tested growth media. The data indicated that immature embryos, isolated 12 days post anthesis, resulted in embryogenic calli formation 4 to 6 days after initiation in the presence of picloram and/or 2,4-dichlorophenoxyacetic acid (2,4-D). Adventitious shoots emerged from 3-4 week old calli in the presence of 6-benzylaminopurine (BAP) or zeatin. Maltose as carbon source and MS vitamin mix and the addition of 50 µM silver nitrate also increased the *in vitro* regeneration abilities of the wheat explant material. Rooting of emerged *in vitro* shoots was established on MS or half strength MS without the addition of any phytohormones.

The aforementioned objective demonstrated the importance and obvious difference between spring wheat *versus* winter wheat for *in vitro* regeneration. This objective thus identified a cultivar with superior *in vitro* regeneration capabilities. This platform, in totality, forms the basis of all subsequent genetic transformation in this study. In future the *in vitro* regeneration capacity of explants from more elite cultivars, from a broader genetic background, should be assessed, hereby allowing the application of genetically transforming a wider range of cultivars with relevance to Africa.

Often transformation is strictly limited to periods when immature embryos are available which is basically limited to the cooler months in Southern Africa. Thus, the second objective was to validate all available cryopreservation methodologies for cryopreserving explants to ensure the constant supply of immature embryos throughout the year. None of the available methods developed previously for wheat and other plants species proved successful within the context of this study. Thus, a novel cryopreservation protocol was successfully developed by Encapsulation / Vitrification and dehydration of immature wheat seeds. Immature seeds encapsulated in 4% (w/v) alginate beads, vitrified in 0.5 M sucrose, desiccated for 72 hours, cryoprotected in 80% glycerol and flash frozen in liquid nitrogen resulted in the highest survival rate of immature embryos isolated after cryopreservation and regenerated *in vitro*. Desiccation of tissue prior to cryopreservation seems to be the singularly most important step to ensure successful preservation of wheat tissue. The preserved explants were subjected to the *in vitro* regeneration platform that was previously designed, and finally immature embryos were able to morph into fully functional plants. The objective thus illustrates that cryopreservation of South African Spring wheat cultivars (Gamtoos S and R) is possible. Aside from the statistical validation of the cryopreservation methodology, perhaps it would be wise to assess if any genetic alteration, during or after the cryopreservation process, has occurred. This includes the assessment of metabolites by using mass spectrometry and chromatography-based methods, as cryopreservation could influence the metabolome as described by Orgawa *et al*. (2012). Further analysis such as gene expression (microarray analysis) could further substantiate the results, thereby reaffirming the genetic stability of the explant for subsequent transformation.

With all the aforementioned platform established, genetic transformation could be implemented. Prior to the transformation, transgene containing vectors were construction and validated. Note sequencing analysis was conducted after each step thoughout the entire cloning process on constructs housing the specific genes, to ensure that error free full-length genes are obtained. Key drought associative genes (*ICE1*, *OTS1*, and *OTS2*) were isolated from the dicotyledonous plant *Arabidopsis thaliana*. The dicotyledonous plant was used as the primary source of genetic material, to eliminate the possibility of silencing by over expression of the endogenous wheat genes. Finally, three constitutive vectors were constructed, housing *ICE1, OTS1* and *OTS2* respectively. The fourth objective entailed the construction of a drought inducible vector housing the *Rab17* promoter to drive the expression of *ICE1* since literature showed that constitutive expression of *ICE1* can lead to stunted growth and other unwanted attributes, however the concept still required validation in a hexaploid such as wheat.

With objective five, the drought inducible vector and empty vector control was bombarded into wheat embryos but all plantlets died during the selection phase, in spite of this, transformation attempts with the two aforementioned construct will be repeated in the near future. However, the constitutive constructs were successfully transformed into immature wheat embryos. *OTS1* and *OTS2* plants were not distinguishable from the control, however *OTS1* plants required considerably more time to initiate anthesis when compared to *ICE1*, *OTS2* and control plants. Differences in flowering time possibly allude to the fact that *OTS1* and *OTS2* do not have the same function as stated on NCBI and by Conti *et al* (2008). Amino acid sequence alignments clearly show that the target SUMO for *OTS1* and *OTS2* SUMO proteases are very highly conserved, indicating functional conservation in downstream signalling. However, post-translation modification (PTM) might be at play, thus resulting in changes in the protein of *OTS1* and *OTS2*. Furthermore, the *ICE1* transgenic suffered severe growth retardation and delay in spikelet formation, however the transgenic population requires further validation both molecularly and phenotypically, which should extend across T1 to T4 generations. Future studies will involve the analysis of the respective transgenics with abiotic stress trials. Factors such as; 1) identification of homozygous lines; 2) SUMOylation patterns and accumulation; 3) gene copy number; 4) efficacy of overexpression genes by measuring gene transcript levels, will all be validated. This should lead to more conclusive results regarding gene function and influences on PTM, ultimately affecting the phenotype.

Finally, the transformation portion of this study was faced with some difficulties after the selection period. Many of the surviving plantlets suffered chlorosis, presenting with an off-white phenotype, despite no longer being on the selection media. The reason for chlorosis are still unknown but might have been influence by the lighting conditions of the available growth room. The addition of the monomer fructose to the subsequent growth media resulted in the conversion of some of the plantlets back to the vibrant green healthy state. However many plantlets could not be revived, and thus ceased any further development. Due to the aforementioned, the transformation frequency was substantially low and need to be improved in the future.

In summary, contributions to the field from this study are a fully established *in vitro* regeneration and cryopreservation protocol, and transgenic populations over expressing key drought associative genes. These plants hold the potential to unravel the effect of SUMOylation in crops, more specially wheat (*Triticum aestivum* L.), as this is the first report to overexpress *OTS1, OTS2* and *ICE1* in a hexaploid crop such as wheat.

5.1 References

Ogawa Y, Sakurai N, Oikawa A, Kai K, Morishita Y, Mori K, Moriya K, Fujii F, Aoki-K, Suzuki H (2012). High-throughput cryopreservation of plant cell cultures for functional genomics. Plant Cell Physiology **53**: 943–52

Conti L, Price G, O'Donnell E, Schwessinger B, Dominy P, Sadanandom A (2008). Small ubiquitin-like modifier proteases overly tolerant to salt 1 and -2 regulate salt stress responses in Arabidopsis. Plant Cell. **20**: 2894–908

Appendices

Appendix A

1. Alive < 20 % surface formed callus

2. 100% surface covered by callus < 5mm

3. 100% surface = 5mm

4. 100% surface > 5mm

5. 100% surface > 5mm and show signs of embryonic tissue
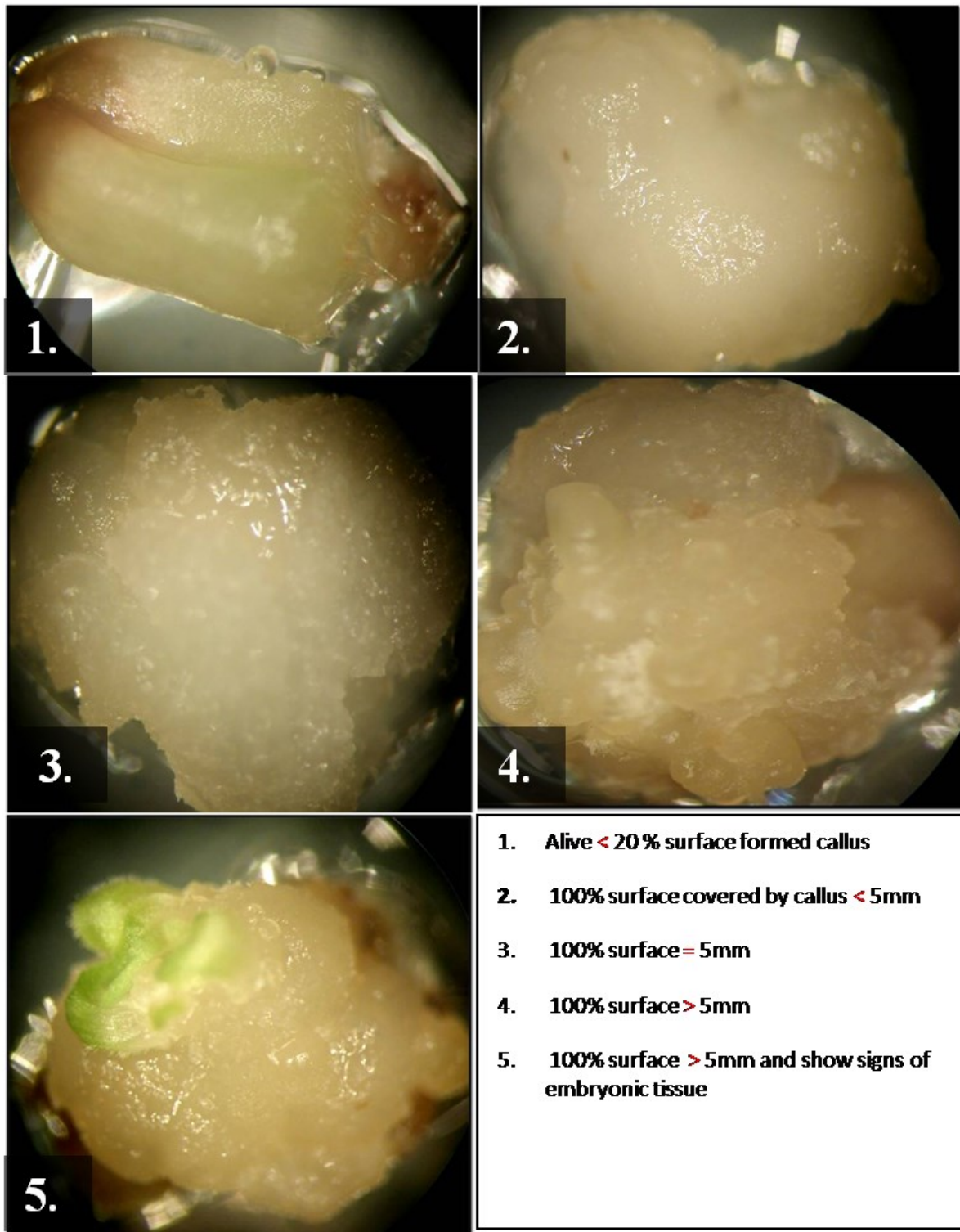
Figure A.1 Ordinal scale for *in vitro* analysis: 1-5, based on callus size, colour and embryogenecity Callus was scored after 3 weeks from initial embryo isolation.
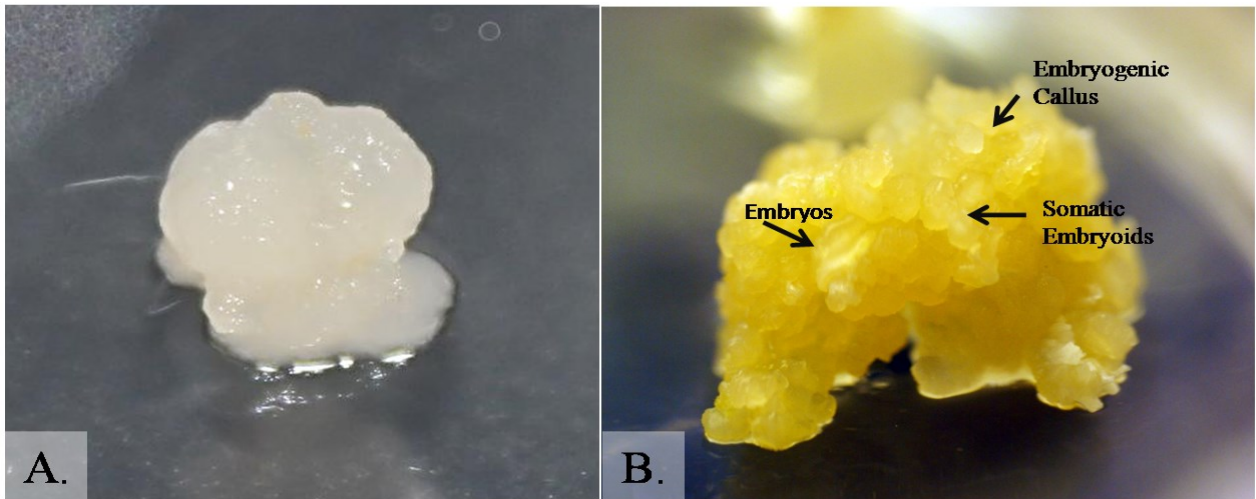
Figure A.2 Induced callus from immature embryo explants. (A) no embryogenicity; (B) High embryogenicity
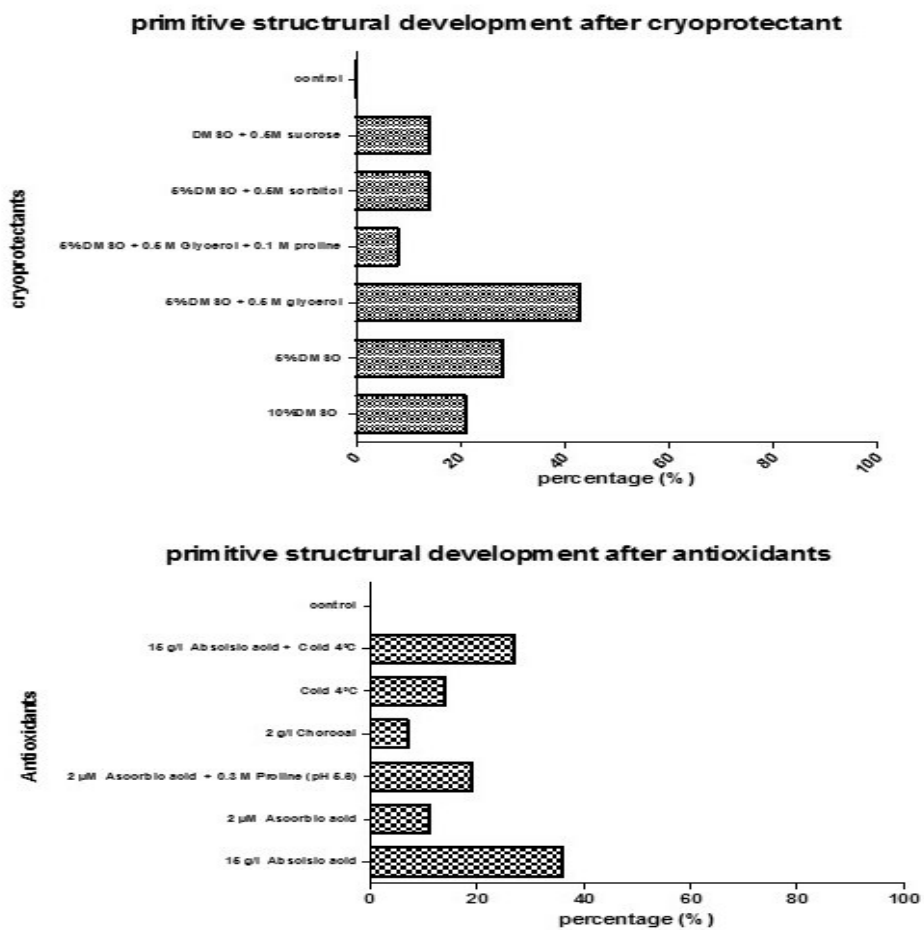
Figure A.3 Rudimental structural development post cryopreservation of; (A) immature embryos due to cryoprotectants or; (B) anti-oxidant pre-treatment
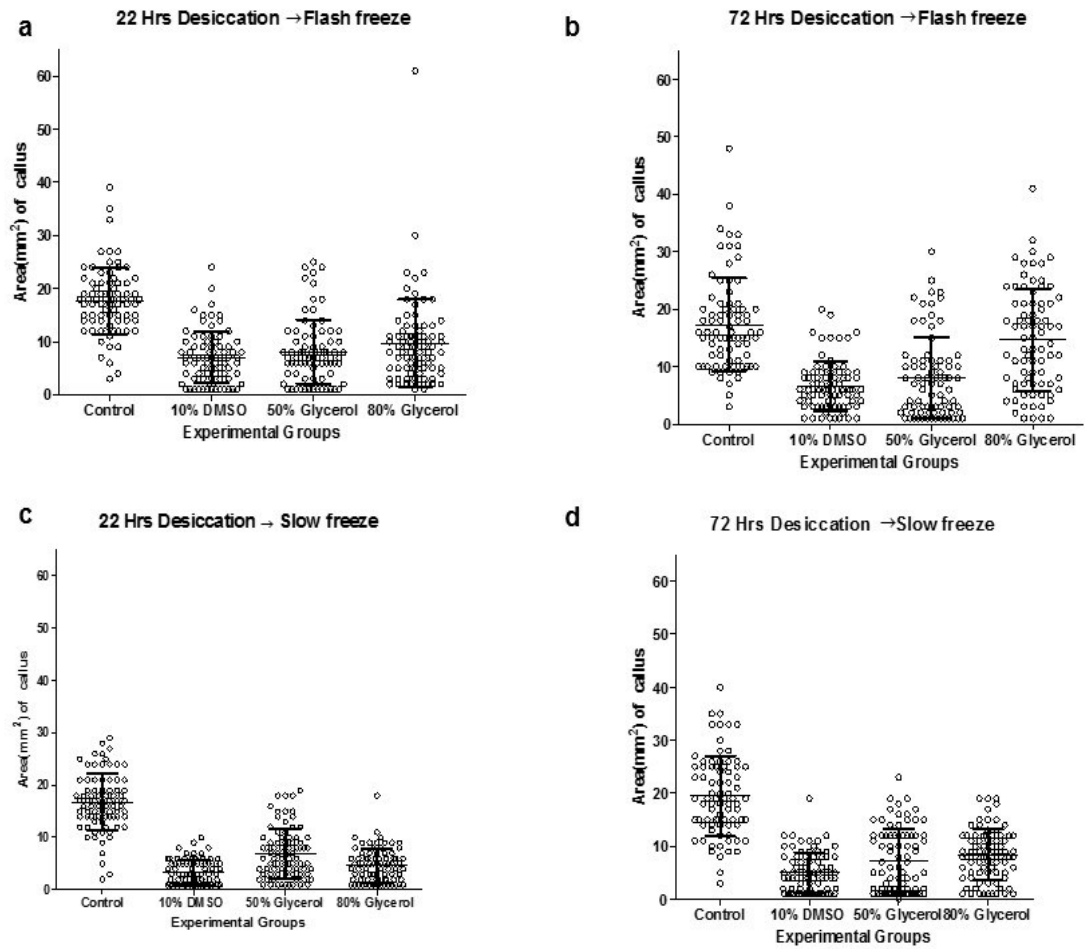
Figure A.4 *In vitro* callus growth measurements, three weeks post-cryopreservation. Data are presented as the average (n = 160) with standard error of the mean. (A) 22 hour desiccation, flash frozen; B) 72 hour desiccation, following flash freeze; (C) 22 hour desiccation, following slow freeze; (D) 72 hour desiccation, following slow freeze. Significant difference were calculated at $p < 0.05$

127

Appendix B

AGGTGTCAACTTTGGCG<mark style="background:green">ATG</mark>GGTCTTGACGGAAACAATGGTGGAGGGGTTTGGTTAAACGGTGGTGGTGGA
GAAAGGGAAGAGAACGAGGAAGGTTCATGGGGAAGGAATCAAGAAGATGGTTCTTCTCAGTTTAAGCCTA
TGCTTGAAGGTGATTGGTTTAGTAGTAACCAACCACATCCACAAGATCTTCAGATGTTACAGAATCAGCCAG
ATTTCAGATACTTTGGTGGTTTTCCTTTTAACCCTAATGATAATCTTCTTCTTCAACACTCTATTGATTCTTCT
TCTTCTTGTTCTCCTTCTCAAGCTTTTAGTCTTGACCCTTCTCAGCAAAATCAGTTCTTGTCAACTAACAACA
ACAAGGGTTGTCTTCTCAATGTTCCTTCTTCTGCAAACCCTTTTGATAATGCTTTTGAGTTTGGCTCTGAATC
TGGTTTTCTTAACCAAATCCATGCTCCTATTTCGATGGGGTTTGGTTCTTTGACACAATTGGGGAACAGGGA
TTTGAGTTCTGTTCCTGATTTCTTGTCTGCTCGGTCACTTCTTGCGCCGGAAAGCAACAACAACAACACAAT
GTTGTGTGGTGGTTTCACAGCTCCGTTGGAGTTGGAAGGTTTTGGTAGTCCTGCTAATGGTGGTTTTGTTGGG
AACAGAGCGAAAGTTCTGAAGCCTTTAGAGGTGTTAGCATCGTCTGGTGCACAGCCTACTCTGTTCCAGAA
ACGTGCAGCTATGCGTCAGAGCTCTGGAAGCAAAATGGGAAATTCGGAGAGTTCGGGAATGAGGAGGTTTA
GTGATGATGGAGATATGGATGAGACTGGGATTGAGGTTTCTGGGTTGAACTATGAGTCTGATGAGATAAAT
GAGAGCGGTAAAGCGGCTGAGAGTGTTCAGATTGGAGGAGGAGGAAAGGGTAAGAAGAAAGGTATGCCTG
CTAAGAATCTGATGGCTGAGAGGAGAAGGAGGAAGAAGCTTAATGATAGGCTTTATATGCTTAGATCAGTT
GTCCCCAAGATCAGCAAAATGGATAGAGCATCAATACTTGGAGATGCAATTGATTATCTGAAGGAACTTCT
ACAAAGGATCAATGATCTTCACAATGAACTTGAGTCAACTCCTCCTGGATCTTTGCCTCCAACTTCATCAAG
CTTCCATCCGTTGACACCTACACCGCAAACTCTTTCTTGTCGTGTCAAGGAAGAGTTGTGTCCCTCTTCTTTA
CCAAGTCCTAAAGGCCAGCAAGCTAGAGTTGAGGTTAGATTAAGGGAAGGAAGAGCAGTGAACATTCATA
TGTTCTGTGGTCGTAGACCGGGTCTGTTGCTCGCTACCATGAAAGCTTTGGATAATCTTGGATTGGATGTTC
AGCAAGCTGTGATCAGCTGTTTTAATGGGTTTGCCTTGGATGTTTTCCGCGCTGAGCAATGCCAAGAAGGAC
AAGAGATACTGCCTGATCAAATCAAAGCAGTGCTTTTCGATACAGCAGGGTATGCTGGTATGATC<mark style="background:yellow">TGA</mark>TCTG
ATCCTGACTTCGAGTCCATT<u>AAGCATCTGTTGAAGCAGAGC</u>

Figure B.1 Nucleotide sequence of *ICE1* in the pUBI-510 vector. The start and stop codons is highlighted in green and yellow respectively. Underlined areas indicated location of primers.

<u>AG<mark style="background-color: green">ATG</mark>ACGAAGAGGAAGAAGGAAGT</u>AATAGATGTCGATTGCTCCGAGAAGAAAGATTTTGTGATTGATT
GGTCTTCCGCTATGGATAAGGAAGACGAAGTTCCCGAGCTCGAGATTGTTAATACCACCAAACCTACTCC
TCCGCCACCGCCAACGTTTTTCTCCGACGATCAAACCGATTCTCCGAAACTCCTAACCGATCGTGACCTCG
ACGAGCAGCTAGAGCGTAAAAAAGCGATCCTGACATTAGGTCCGGGCTTACCCGACAAGGGTGAGAAAA
TTCGACTCAAAATCGCTGATCTCGAAGAGGAGAAGCAGCGTAGAGTTTTAGAAGGCTCGAAAATGGAGG
TGGACAGAAGTTCTAAGGTCGTGTCTTCGACTAGCTCAGGTTCAGATGTTTTACCGCAAGGAAATGCAGT
TTCAAAAGATACCTCTAGAGGGAATGCAGACTCAAAAGACACCTCTAGACAAGGGAATGCAGATTCAAA
AGAAGTCTCACGGTCAACATTTTCTGCGGTTTTCAGTAAACCAAAAACGGATTCTCAGTCAAAGAAAGCC
TTTGGTAAAGAACTAGAAGATCTGGGATGTGAAAGGAGGAAACACAAGGCTGGTAGAAAGCCTGTAACA
AGGCTGAGCAACGGGTGGCGGTTGTTGCCAGATGTAGGGAAAGCTGAGCACAGTGCAAAGCAGTTTGAT
TCTGGACTTAAAGAATCAAAAGGGAATAAGAAATCCAAGGAACCTTATGGAAAGAAAAGGCCCATGGA
ATCTTCGACTTATTCTCTGATTGATGATGATGATGATGATGATGATGATGATGACAACGACACCAGTGGC
CATGAAACTCCTAGGGAGTGGTCTTGGGAAAAATCTCCATCACAAAGTTCAAGGCGCCGTAAGAAATCA
GAGGACACAGTGATAAATGTGGATGAAGAAGAAGCTCAGCCTTCAACAGTGGCGGAGCAAGCGGCTGA
ACTGCCTGAAGGCTTACAGGAAGATATATGCTACCCAACAAGGGATGATCCTCACTTTGTTCAAGTTTGT
CTTAAAGATCTTGAATGCCTTGCACCTCGAGAATATCTGACATCGCCGGTTATGAATTTCTACATGAGGTT
CTTGCAGCAGCAGATATCATCATCGAATCAAATCTCTGCTGATTGTCACTTCTTTAATACCTATTTCTACA
AGAAGCTCAGTGACGCTGTTACGTACAAGGGGAATGACAAGGATGCCTTCTTTGTGAGGTTCAGGCGGT
GGTGGAAGGGTATTGATCTATTTCGTAAGGCTTATATTTTCATACCAATACATGAAGATCTCCACTGGAG
CCTTGTGATAGTTTGCATCCCTGATAAGAAAGATGAATCGGGGTTGACTATACTTCACCTTGATTCTCTAG
GACTTCACTCGAGAAAATCAATTGTTGAAAATGTAAAAAGGTTTCTAAAAGACGAATGGAATTATTTGAA
TCAAGATGACTATTCCTTGGATCTGCCTATCTCAGAAAAAGTATGGAAAAACCTCCCTCGTAGGATCAGC
GAAGCTGTTGTTCAGGTTCCGCAGCAGAAAAACGATTTTGATTGTGGTCCGTTTGTGCTCTTCTTCATTAA
ACGGTTCATTGAAGAGGCGCCTCAAAGGCTGAAAAGGAAAGACCTGGGAATGTTCGACAAGAAGTGGTT
TAGACCCGATGAAGCCTCTGCTCTGAGAATCAAAATCCGAAACACGCTCATCGAGCTATTCCGTGTC<u>AGT
GACCAGACAGAGTAAAC<mark style="background-color: yellow">CAG</mark></u>TACAGA

Figure B.2 Nucleotide sequence of *OTS1* in the pUBI -510 vector. The start and stop codon is highlighted in green and yellow respectively. Underlined areas indicate the location of primers designed for amplification of the full length gene

AGAAGAGATGAAGAGACAAAGAGCAATCGAGTTAGATCGAGTGAAGAAGACAATGTTGAACATAGA
TTGGGACGATGCTTTGGGCGATGAGGAAGTTCCCGAGCTCGAGATCATCGCTACTGACAAAATTCCG
CCGCGTGAACCTACTCTTTCCGGCTATGAACCCGCCGTCTCTGTGCGATCCCTCAGGGATAACGAACT
CGACGATCATCTGAAGCGTCAGAGATCACTTCTTACTCGTCTCGGTGATAAGTTGGCAGACAAGGGC
GAGAAAATCCGAAACAGAATCGGAGAGCTCGAGTACGAGAAGCAGCGAAGGATGTTCCAACAACGG
ACCAAAATGGATGCAGACAACGGATGTCAGATTCTCGAGAAACCAAAAAGCTCAGATGTGTTTATGC
GAGCGAGTACAGCCTCAAAAGACACCTCTGGACAAGGGACTTCGGGATCGAAAGACGTGTCTCGGTC
AACATTTGCTGCTCATTTTAGTGACAATCTCAAAATGGGACCTCAACCAGTGAAACTAGTTAATGACA
AACTACAAGATTTGGGACGTGGAAGCTGGATAAGCAAAGCTAATAGAGACTCAATAATAGAGAAAA
ATAATGTGTGGCGGTCGCTGCCAAGATTAAGCAAGTGTAAGGTCAGTTTAAAGAACTTTTATTCTGAG
TCTAAGGATCCTAAAGGGGATCGAAGACCCAATGAAGCTTATGGTAAGGGAAAGCCAAATGAATCTT
CCCCTTATTTACTTGTCGACGATGATGATGGCGATGACGACAAAGTCATTGGTTATGAAACTCCCAGG
CATTGGAGTTTAAAAGCATCGCCACTACAGAGTTCAAGCTGCAGGAAGAAATCAGATGATAAAGTGA
TTAATTTGGATGAAGATGAACCTCTGTCTCCAATGGTAGTAGAGGAAGCATGTGAACTTCCTGAAGG
GTTACCAGAAGATATTTACTACCCATCAAGTGATCAAAGTGATGGGCGAGACCTTGTTCAAGTATCTC
TTAAAGATCTGAAATGCCTTTCACCTGGGGAATATCTTACATCGCCAGTTATAAATTTCTACATCAGG
TACGTGCAACATCACGTGTTTTCAGCTGATAAGACTGCTGCTAATTGTCATTTCTTCAATACGTTTTTC
TACAAGAAGCTCACAGAAGCTGTTTCATACAAGGGTAATGACAGGGATGCATATTTTGTGAAGTTCA
GGCGGTGGTGGAAGGGTTTTGATCTATTCTGTAAATCATATATATTTATACCAATACATGAAGATCTT
CACTGGAGCTTAGTCATAATTTGCATCCCAGACAAGGAGGACGAATCGGGATTGACTATAATTCACT
TGGATTCATTGGGACTTCACCCAAGAAATTTGATTTTCAATAATGTCAAAAGATTTCTGAGAGAGGAA
TGGAACTATCTAAATCAAGATGCTCCATTGGATTTACCAATTTCAGCAAAAGTATGGAGAGACCTTCC
CAATATGATCAACGAAGCTGAAGTGCAGGTTCCACAACAGAAGAACGATTTCGACTGTGGTCTGTTT
CTGCTCTTCTTCATAAGACGTTTCATCGAAGAGGCTCCTCAAAGGCTGACATTGCAGGATTTGAAAAT
GATTCACAAGAAGTGGTTTAAACCGGAAGAAGCTTCCGCTTTGAGGATCAAAATCTGGAACATACTC
GTTGATCTATTCCGCAAGGGTAACCAAACAGATTAAAGCTGTATAGAACAGTGTCAAGTTTGGTCAC
TCTAATTAAGGAATGAAATGAAAGATACGTTTTTGTTAACAATCATTTTGTTCAAGTCCTGTAATAAA
CTAAGGAGCATGACATAATCAGAACAAATCAACAAGGTAATAATAACGTATCCGAATAAAAAGCCA
ACATTGCTGC

Figure B.3 Nucleotide sequence of *OTS2* in the pUBI-510 vector. The start and stop codon is highlighted in green and yellow respectively. Underlining indicated location of primers, which was chosen to have similar T$_m$.

GTTGCCGTACTGGTCGACATGGCCCGTGGCGCCGTGGCCGCGCTGCCCCTGCTGACCGTACTCCATGATGCTT
GCACGGCTTGGTGGTTTGTGGTTTCTCCTAAACGAGTCTTCGTGCTTGTGTGCTCGATCGTGTTCTCGATGTG
GCTCGCCGGTGGTGAAGCAGGTGACGGTCGATGCCGCCATTTATAGGCAGCAGAAGTGCAGAACGGCGAAA
GGATTAGGATCTGGGCGCGCCACGGCAGGGGTGTACGTGGGCATGCATGAGTGGATTGGGCGGCCAGTGAG
GGAACAGCACGTACACGTACGCCGGCGGGACGTGTGCAGTCGCTGGTGCCGTGCGTCGGTGGCCCGGCCCG
CCACGTCTCGGTTCTTCTCGAAAGTTGGGTACGCGGATTTTACCGGTGCTCATCCATTATTCAATGCAGGCGG
TCGGTCAGGCTGAATTTAGGCCCAGTACACCCAAGTGTAGGCGCAGCAGAAAAGTCCAGAGCGAACTGCAT
AACATTTTAAAATACATTAAAATTCATAAAATATATTAAAAGAAGAGAAAACATTAAAAAGTAAAATGATA
ATTCGATAATGCCAACTATTTTAAAACAGGATGGTATAGTATCTCAGGAGTACCAATTAGGACATGTTTGTT
AACTTTGGCG**ATG**GGTCTTGACGGAAACAATGGTGGAGGGGTTTGGTTAAACGGTGGTGGTGGAGAAAGGG
AAGAGAACGAGGAAGGTTCATGGGGAAGGAATCAAGAAGATGGTTCTTCTCAGTTTAAGCCTATGCTTGAA
GGTGATTGGTTTAGTAGTAACCAACCACATCCACAAGATCTTCAGATGTTACAGAATCAGCCAGATTTCAGA
TACTTGGTGGTTTTCCTTTTAACCCTAATGATAATCTTCTTCTTCAACACTCTATTGATTCTTCTTCTTCTTGTT
CTCCTTCTCAAGCTTTTAGTCTTGACCCTTCTCAGCAAAATCAGTTCTTGTCAACTAACAACAACAAGGGTTG
TCTTCTCAATGTTCCTTCTTCTGCAAACCCTTTTGATAATGCTTTTGAGTTTGGCTCTGAATCTGGTTTTCTTAA
CCAAATCCATGCTCCTATTTCGATGGGGTTTGGTTCTTTGACACAATTGGGGAACAGGGATTTGAGTTCTGTT
CCTGATTTCTTGTCTGCTCGGTCACTTCTTGCGCCGGAAAGCAACAACAACAACACAATGTTGTGTGGTGGTT
TCACAGCTCCGTTGGAGTTGGAAGGTTTTGGTAGTCCTGCTAATGGTGGTTTTGTTGGGAACAGAGCGAAAG
TTCTGAAGCCTTTAGAGGTGTTAGCATCGTCTGGTGCACAGCCTACTCTGTTCCAGAAACGTGCAGCTATGCG
TCAGAGCTCTGGAAGCAAAATGGGAAATTCGGAGAGTTCGGGAATGAGGAGGTTTAGTGATGATGGAGATA
TGGATGAGACTGGGATTGAGGTTTCTGGGTTGAACTATGAGTCTGATGAGATAAATGAGAGCGGTAAAGCG
GCTGAGAGTGTTCAGATTGGAGGAGGAGGAAAGGGTAAGAAGAAAGGTATGCCTGCTAAGAATCTGATGGC
TGAGAGGAGAAGGAGGAAGAAGCTTAATGATAGGCTTTATATGCTTAGATCAGTTGTCCCCAAGATCAGCA
AAATGGATAGAGCATCAATACTTGGAGATGCAATTGATTATCTGAAGGAACTTCTACAAAGGATCAATGATC
TTCACAATGAACTTGAGTCAACTCCTCCTGGATCTTTGCCTCCAACTTCATCAAGCTTCCATCCGTTGACACC
TACACCGCAAACTCTTTCTTGTCGTGTCAAGGAAGAGTTGTGTCCCTCTTCTTTACCAAGTCCTAAAGGCCAG
CAAGCTAGAGTTGAGGTTAGATTAAGGGAAGGAAGAGCAGTGAACATTCATATGTTCTGTGGTCGTAGACC
GGGTCTGTTGCTCGCTACCATGAAAGCTTTGGATAATCTTGGATTGGATGTTCAGCAAGCTGTGATCAGCTGT
TTTAATGGGTTTGCCTTGGATGTTTTCCGCGCTGAGCAATGCCAAGAAGGACAAGAGATACTGCC**TGA**TCAA
ATCAAAGCAGTGCTTTTCGATACAGCAGGGTATGCTGGTATGATCTGATGA

Figure B.4 Nucleotide sequence of *Rab*:*ICE1* in the pAHC20 vector. Red indicated promoter region and the start codon of *ICE1* highlighted in green followed by its stop codon in yellow.

Table B.1 The presence of all *cis-* elements in the Rab17 promoter (650 bp) generated by using the PLACE database. Highlighted in green represents the most crucial *cis*-element for drought response. Yellow indicated involvement with biotic stress.

Factor or Site Name	Loc.(Str.)	Signal Sequence	SITE #
CURECORECR	1 (-)	GTAC	S000493
CURECORECR	1 (+)	GTAC	S000493
CACTFTPPCA1	2 (+)	YACT	S000449
ROOTMOTIFTAPOX1	8 (-)	ATATT	S000098
SEF1MOTIF	9 (+)	ATATTTAWW	S000006
ROOTMOTIFTAPOX1	9 (+)	ATATT	S000098
TATABOX2	11 (-)	TATAAAT	S000109
ROOTMOTIFTAPOX1	15 (+)	ATATT	S000098
TATABOX4	18 (-)	TATATAA	S000111
TATABOX4	21 (+)	TATATAA	S000111
ROOTMOTIFTAPOX1	39 (-)	ATATT	S000098
ROOTMOTIFTAPOX1	40 (+)	ATATT	S000098
ERELEE4	42 (+)	AWTTCAAA	S000037
CACTFTPPCA1	55 (-)	YACT	S000449
CAATBOX1	66 (-)	CAAT	S000028
AMYBOX1	73 (+)	TAACARA	S000020
MYBGAHV	73 (+)	TAACAAA	S000181
GAREAT	73 (+)	TAACAAR	S000439
AACACOREOSGLUB1	74 (+)	AACAAAC	S000353
CAATBOX1	89 (-)	CAAT	S000028
CCAATBOX1	89 (-)	CCAAT	S000030
CURECORECR	93 (-)	GTAC	S000493
CURECORECR	93 (+)	GTAC	S000493
CACTFTPPCA1	94 (+)	YACT	S000449
GATABOX	103 (+)	GATA	S000039
CACTFTPPCA1	105 (+)	YACT	S000449
GT1CONSENSUS	136 (-)	GRWAAW	S000198
IBOXCORE	137 (-)	GATAA	S000199
GATABOX	138 (-)	GATA	S000039
GT1CONSENSUS	144 (-)	GRWAAW	S000198
IBOXCORE	145 (-)	GATAA	S000199
GATABOX	146 (-)	GATA	S000039
CACTFTPPCA1	154 (+)	YACT	S000449
DOFCOREZM	156 (-)	AAAG	S000265
CARGCW8GAT	156 (-)	CWWWWWWWWG	S000431
CARGCW8GAT	156 (+)	CWWWWWWWWG	S000431
POLLEN1LELAT52	167 (-)	AGAAA	S000245
NODCON2GM	170 (+)	CTCTT	S000462
OSE2ROOTNODULE	170 (+)	CTCTT	S000468
DOFCOREZM	175 (-)	AAAG	S000265
ROOTMOTIFTAPOX1	180 (-)	ATATT	S000098
LECPLEACS2	183 (-)	TAAAATAT	S000465
ROOTMOTIFTAPOX1	183 (+)	ATATT	S000098
DOFCOREZM	234 (-)	AAAG	S000265
POLLEN1LELAT52	236 (-)	AGAAA	S000245
RHERPATEXPA7	240 (-)	KCACGW	S000512
DPBFCOREDCDC3	250 (+)	ACACNNG	S000292
EBOXBNNAPA	251 (-)	CANNTG	S000144
MYCCONSENSUSAT	251 (-)	CANNTG	S000407
EBOXBNNAPA	251 (+)	CANNTG	S000144
MYCCONSENSUSAT	251 (+)	CANNTG	S000407
CACTFTPPCA1	251 (+)	YACT	S000449

```
CURECORECR          260  (-)  GTAC                          S000493
CURECORECR          260  (+)  GTAC                          S000493
CACTFTPPCA1         261  (+)  YACT                          S000449
SITEIIATCYTC        264  (+)  TGGGCY                        S000474
SORLIP2AT           265  (+)  GGGCC                         S000483
WBOXNTCHN48         280  (+)  CTGACY                        S000508
WRKY71OS            281  (+)  TGAC                          S000447
WBOXNTERF3          281  (+)  TGACY                         S000457
DRE2COREZMRAB17     283  (+)  ACCGAC                        S000402
DRECRTCOREAT        283  (+)  RCCGAC                        S000418
CBFHV               283  (+)  RYCGAC                        S000497
LTRECOREATCOR15     284  (+)  CCGAC                         S000153
INTRONLOWER         291  (-)  TGCAGG                        S000086
CAATBOX1            296  (-)  CAAT                          S000028
POLASIG3            300  (+)  AATAAT                        S000088
GT1CONSENSUS        317  (+)  GRWAAW                        S000198
ARR1AT              322  (-)  NGATT                         S000454
CGCGBOXAT           325  (-)  VCGCGB                        S000501
CGCGBOXAT           325  (+)  VCGCGB                        S000501
CURECORECR          329  (-)  GTAC                          S000493
CURECORECR          329  (+)  GTAC                          S000493
DOFCOREZM           337  (-)  AAAG                          S000265
DRE1COREZMRAB17     349  (+)  ACCGAGA                       S000401
SURECOREATSULTR11   352  (+)  GAGAC                         S000499
ACGTATERD1          355  (-)  ACGT                          S000415
BOXIIPCCHS          355  (+)  ACGTGGC                       S000229
ACGTABREMOTIFA2OSEM 355  (+)  ACGTGKC                       S000394
ABRELATERD1         355  (+)  ACGTG                         S000414
ACGTATERD1          355  (+)  ACGT                          S000415
SORLIP1AT           357  (-)  GCCAC                         S000482
SORLIP2AT           362  (+)  GGGCC                         S000483
SORLIP2AT           367  (+)  GGGCC                         S000483
SORLIP1AT           369  (+)  GCCAC                         S000482
DRE2COREZMRAB17     372  (+)  ACCGAC                        S000402
DRECRTCOREAT        372  (+)  RCCGAC                        S000418
CBFHV               372  (+)  RYCGAC                        S000497
LTRECOREATCOR15     373  (+)  CCGAC                         S000153
CGACGOSAMY3         374  (+)  CGACG                         S000205
PRECONSCRHSP70A     389  (+)  SCGAYNRNNNNNNNNNNNNNNNNHD      S000506
ABRELATERD1         398  (-)  ACGTG                         S000414
ACGTATERD1          399  (-)  ACGT                          S000415
ACGTATERD1          399  (+)  ACGT                          S000415
CURECORECR          412  (-)  GTAC                          S000493
ACGTOSGLUB1         412  (+)  GTACGTG                       S000278
CURECORECR          412  (+)  GTAC                          S000493
ACGTATERD1          414  (-)  ACGT                          S000415
ABRELATERD1         414  (+)  ACGTG                         S000414
ACGTATERD1          414  (+)  ACGT                          S000415
CURECORECR          418  (-)  GTAC                          S000493
ACGTOSGLUB1         418  (+)  GTACGTG                       S000278
CURECORECR          418  (+)  GTAC                          S000493
ACGTATERD1          420  (-)  ACGT                          S000415
RHERPATEXPA7        420  (-)  KCACGW                        S000512
ABRELATERD1         420  (+)  ACGTG                         S000414
ACGTATERD1          420  (+)  ACGT                          S000415
GTGANTG10           433  (-)  GTGA                          S000378
GRAZMRAB17          434  (+)  CACTGGCCGCCC                  S000150
CACTFTPPCA1         434  (+)  YACT                          S000449
GCCCORE             439  (+)  GCCGCC                        S000430
CCAATBOX1           444  (+)  CCAAT                         S000030
SV40COREENHAN       445  (-)  GTGGWWHG                      S000123
```

133

```
CAATBOX1             445 (+) CAAT                     S000028
ARR1AT               446 (-) NGATT                    S000454
UPRMOTIFIIAT         449 (+) CCNNNNNNNNNNNNNCCACG      S000426
CACTFTPPCA1          450 (+) YACT                     S000449
PREATPRODH           451 (+) ACTCAT                   S000450
RYREPEATVFLEB4       454 (-) CATGCATG                 S000102
RYREPEATLEGUMINBOX   454 (+) CATGCAY                  S000100
RYREPEATVFLEB4       454 (+) CATGCATG                 S000102
RYREPEATGMGY2        454 (+) CATGCAT                  S000105
RYREPEATBNNAPA       454 (+) CATGCA                   S000264
RYREPEATLEGUMINBOX   455 (-) CATGCAY                  S000100
RYREPEATGMGY2        455 (-) CATGCAT                  S000105
RYREPEATBNNAPA       456 (-) CATGCA                   S000264
ABREOSRAB21          461 (-) ACGTSSSC                 S000012
ACGTOSGLUB1          464 (-) GTACGTG                  S000278
ABRELATERD1          464 (-) ACGTG                    S000414
ACGTATERD1           465 (-) ACGT                     S000415
ACGTATERD1           465 (+) ACGT                     S000415
CURECORECR           467 (-) GTAC                     S000493
CURECORECR           467 (+) GTAC                     S000493
SORLIP1AT            480 (-) GCCAC                    S000482
CGCGBOXAT            483 (-) VCGCGB                   S000501
CGCGBOXAT            483 (+) VCGCGB                   S000501
ARR1AT               498 (-) NGATT                    S000454
DOFCOREZM            502 (-) AAAG                     S000265
E2FCONSENSUS         503 (+) WTTSSCSS                 S000476
MYBCOREATCYCB1       508 (-) AACGG                    S000502
CACTFTPPCA1          516 (+) YACT                     S000449
TATABOX2             529 (+) TATAAAT                  S000109
GCCCORE              536 (-) GCCGCC                   S000430
CBFHV                542 (+) RYCGAC                   S000497
ASF1MOTIFCAMV        548 (-) TGACG                    S000024
WRKY71OS             549 (-) TGAC                     S000447
GTGANTG10            550 (-) GTGA                     S000378
EBOXBNNAPA           551 (-) CANNTG                   S000144
MYCCONSENSUSAT       551 (-) CANNTG                   S000407
EBOXBNNAPA           551 (+) CANNTG                   S000144
RAV1BAT              551 (+) CACCTG                   S000315
MYCCONSENSUSAT       551 (+) CANNTG                   S000407
GTGANTG10            559 (-) GTGA                     S000378
SORLIP1AT            572 (+) GCCAC                    S000482
DPBFCOREDCDC3        596 (+) ACACNNG                  S000292
RHERPATEXPA7         602 (+) KCACGW                   S000512
POLLEN1LELAT52       621 (+) AGAAA                    S000245
MYB1AT               623 (+) WAACCA                   S000408
SV40COREENHAN        629 (-) GTGGWWHG                 S000123
MYB1AT               630 (+) WAACCA                   S000408
CACTFTPPCA1          658 (-) YACT                     S000449
CURECORECR           659 (-) GTAC                     S000493
CURECORECR           659 (+) GTAC                     S000493
WBOXNTERF3           663 (-) TGACY                    S000457
WBOXNTCHN48          663 (-) CTGACY                   S000508
WRKY71OS             664 (-) TGAC                     S000447
CGCGBOXAT            677 (-) VCGCGB                   S000501
CGCGBOXAT            677 (+) VCGCGB                   S000501
SORLIP1AT            682 (+) GCCAC                    S000482
SORLIP2AT            695 (+) GGGCC                    S000483
CRTDREHVCBF2         702 (-) GTCGAC                   S000411
CBFHV                702 (-) RYCGAC                   S000497
CRTDREHVCBF2         702 (+) GTCGAC                   S000411
CBFHV                702 (+) RYCGAC                   S000497
```

134

```
CACTFTPPCA1              709 (-) YACT                    S000449
CURECORECR               710 (-) GTAC                    S000493
CURECORECR               710 (+) GTAC                    S000493
SEF3MOTIFGM              717 (+) AACCCA                  S000115
```