Molecular characterization and expression analysis of *ABA 8'- hydroxylase 1* and *abscisic acid insensitive 5* genes with respect to seed dormancy in wheat (*Triticum aestivum* L.)

by

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A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

Department of Plant Science

University of Manitoba

Winnipeg

ACKNOWLEDGEMENT

I would like to express my special appreciation and thanks to my supervisor Dr. Belay T. Ayele, Department of Plant Science for his for valuable guidance, scholarly inputs and consistent encouragement I received throughout the research work.

My cordial and sincere thanks are due to members of my advisory committee members, Dr. Gavin Humphreys, AAFC, Ottawa Research & Development Center, Dr. Douglas J. Cattani, Department of Plant Science, and Dr. Gavin Chen, Department of Biological Sciences for their advice, valuable suggestions and encouragement during the tenure of this study.

Finally, I would like to thank my mother Mrs. Kuldeep Kaur, father Mr. Jarnail Singh and brothers Gurinderjeet and Armaan for their selfless persuasion and sacrifice. I express my heartfelt sentiments for their moral and emotional support for enabling me to carry on my academic pursuits. Their blessings and affection are not to be reciprocated in words but are to be felt in the deepest corner of my heart.

With due reverence and gratitude, I owe my special thanks to the members of Dr. Ayele's lab (Parneet, Tran Nguyen, Dr. Rohit, Rupinder, Luc Ouellete, Dr. Pham and Dr. Aihua Liu) for their constant help, valuable guidance and kind cooperation throughout my research work.

Pleasant company, ever willing help, regular encouragement and sweet memories of my lovely friends Gurwinder, Jaspinder, Harsimran, Raman, Kawal, Preeni, Navu and Berry and will always remain as a precious asset with me.

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ABSTRACT

Rehal K. Pawanpuneet. MSc. The University of Manitoba, Nov 23, 2018. Molecular characterization and expression analysis of *ABA 8'- hydroxylase 1* and *abscisic acid insensitive 5* genes with respect to seed dormancy in wheat (*Triticum aestivum* L.) M.Sc. supervisor: Dr. Belay T. Ayele.

In cereal crops such as wheat, seed dormancy is closely associated with the occurrence of preharvest sprouting (PHS), germination of the grain prior to harvest under moist conditions. While higher level of seed dormancy results in a delay in germination, low level of seed dormancy causes preharvest sprouting which negatively affects yield and end use quality of wheat grains. Therefore, it is necessary for wheat grains to have certain level of dormancy to prevent preharvest sprouting. Abscisic acid (ABA) is one of the plant hormones that predominantly regulates seed dormancy and germination, and molecular elements involved in its metabolic and signalling pathways play crucial roles in this regard. The ABA catabolic gene *ABA 8'-hydroxylase 1* (*ABA8'OH1*) has been known as a key regulator of the amount of ABA in the seeds of several plant species while the transcription factor *abscisic acid insensitive 5* (*ABI5*) plays pivotal role to determine ABA responsiveness of the seed, which in turn influences the degree of dormancy. To better understand the roles of *ABA8'OH1* and *ABI5* in wheat seed dormancy and germination, the present study performed molecular characterization and expression analysis of the genes in different wheat genotypes that showed a range of dormancy phenotypes.

FORWARD

This thesis is written in manuscript style. A general introduction about the research project and a literature review precedes the manuscript. An abstract, introduction, materials and methods, results and discussion form a complete manuscript. The manuscript is followed by a general discussion and conclusions, a list of references and appendices.

1.0 GENERAL INTRODUCTION

Wheat is cultivated in almost every part of the world. It is an important part of global food and nutritional security as it serves as the main food source and supplies essentials nutrients to the human diet (CIMMYT, 2018). In addition, it serves as the main source of income for millions of smallholder farmers, and therefore is grown as a cash crop. Wheat is adaptable to wide range environmental conditions; however, its production for example in colder and humid regions can be negatively affected by preharvest sprouting (PHS), an incidence in which mature seeds in the spikes germinate prior to harvesting. In addition to wheat, PHS also has an adverse effect on the production of other cereal crops such as barley (*Hordeum vulgare*), millet (*Parso millet*), maize (*Zea mays*) and sorghum (*Sorghum bicolor*) (Shu *et al.*, 2015). Canada is well known for producing high-quality wheat and is the fourth largest exporter of wheat worldwide (FAO, 2017). However, because of humid weather conditions, the problem of PHS causes serious damage to wheat farmers and food processors by reducing seed quality and crop yield.

Preharvest sprouting is a complex trait regulated by the level of seed dormancy, an inherited trait which does not allow the viable seeds to germinate under favourable environmental conditions. Seed dormancy is influenced by several internal and external/environmental factors (Imtiaz *et al.*, 2008). Among the internal factors affecting seed dormancy plant hormones. Abscisic acid (ABA) is one of the major hormones that act as key regulators of seed dormancy and both its metabolism and signaling play important roles (Nambara *et al.*, 2010). ABA metabolism involves its biosynthesis and catabolism, and both processes are important to control ABA level and dormancy (Tuan *et al.*, 2018). ABA biosynthesis is mainly regulated by 9-cis epoxycarotenoid dioxygenases (NCED), which is encoded in several plant species by multigene families, and these genes play crucial roles in the induction and maintenance of seed dormancy (Finkelstein, 2013).

On the other hand, catabolism of ABA is carried out by ABA-8′ hydroxylase (also known as *CYP707A*), which is also encoded by multigene family. Similar to the *NCED* genes, the *ABA8'OH* genes also regulate ABA level and dormancy (Finkelstein, 2013). Studies in Arabidopsis seeds have shown that mutants of *CYP707A* genes accumulate more ABA than the overexpression of ABA biosynthetic genes (Finkelstein, 2013), suggesting the importance of ABA catabolism in controlling ABA levels and therefore seed germination and dormancy. In addition, the level of *CYP707A1* expression has been shown to determine ABA level in dry Arabidopsis seeds (Okamoto *et al.,* 2006). Several studies have also shown the function of ABA catabolic genes of cereals in seed dormancy. For example, previous studies in barley and wheat have demonstrated the role of *ABA8'OH1* and its homeologues in influencing seed ABA level and dormancy (Chono *et al.,* 2013; Son *et al.,* 2016).

Reverse genetic studies in Arabidopsis explained the role of ABA signaling components in determining ABA response in seeds. The ABA signalling pathway involves several positive and negative mediators that affect the expression of ABA responsive genes (Nambara *et al.,* 2010). The downstream ABA signaling transcriptions factors (ABI3, ABI4 and ABI5) are among the ABA signaling components that positively influence ABA signaling/response, and seed dormancy in both dicot and monocot species (Finketstein *et al.,* 2013). Several studies in Arabidopsis have indicated the role of *ABI5* in enhancing seed dormancy by inhibiting germination (Finkelstein and Lynch, 2000). *TaABF1*, a homolog of wheat *ABI5*, has been shown to have a higher expression level in dormant seeds as compared to non-dormant seeds during imbibition, suggesting the seed-specific role of *ABI5* in wheat seed germination and dormancy (Johnson *et al.,* 2002). Several other studies have also reported the correlation between the expression pattern of *TaABI5* and seed dormancy (Rikiishi *et al.,* 2010; Liu *et al.,* 2013; Zhou *et al.,* 2017).

Given their importance in regulating the degree of seed dormancy, the overall goal of the thesis project is to investigate the roles of *ABA8'OH1* and *ABI5* genes in regulating PHS by comparing wheat genotypes with diverse backgrounds. The objectives of the project are to 1) Examine the dormancy/germination phenotype of the seeds of the different wheat genotypes; 2) Analyze variations in the genomic nucleotide sequence and expression of *ABA8'OH1*; 3) Examine differences in the genomic nucleotide sequence of *ABI5* homeologues and their expression.

2.0 LITERATURE REVIEW

2.1 Wheat

2.1.1 Evolution of hexaploid wheat

Wheat (*Triticum aestivum*) is the world's most important cereal crop after maize (*Zea mays*) and rice (*Oryza sativa*), in terms of both area cultivated and amount of grain produced. It belongs to grass family *Poaceae*, tribe *Triitcae*, genus *Triticum*. Domestication of wheat occurred about 10,000 years ago in the Middle East, during ′Neolithic revolution′ when a shift from the hunting and collecting food to settled agriculture happened (Dubcovsky and Dvorak, 2007; Shewry, 2009). The present alloheaxaploid wheat *′Triticum aestivum′* is the result of two ancient hybridizations that took place among the ancestral genomes of the wheat (Eversole *et al.,* 2014). The first polyploidization occurred between *Triticum urartu* (AA; 2n=2x=14) and *Agilopes speltoides ′*goat grass′ (BB; 2n=14) around 300,000-500,000 years ago and this event resulted in the formation of an allotetraploid wheat, *Triticum turgidum* (2n=4x=28; AABB), an ancestor of wild emmer wheat. The earliest evidence that man used wild emmer and wild barley for processing and baking of flour was found at called Ohallo in Israel, which was a permanent site for hunter-gatherers 19,000 years before present (BP).

Around 9,000 BP, a second hybridization of *Triticum turgidum* with a diploid grass species, *Aegilops tauschii* (DD) produces an early spelt (*Triticum spelta;* 2n=6x=42; AABBDD). About 8,500BP, natural mutations in spelt wheat resulted in the free-threshing hexaploid *T. aestivum* (2n=6x=42; AABBDD) (Peng *et al.*, 2011). The timing for the origin of hexaploid wheat and its absence in wild populations is entirely based on archeological evidence. Although certain evidence demonstrate the relatedness of bread wheat sub genomes with diploid wheat species, the phylogenetic and divergence history among the A, B and D genomes is still unclear (Eversole *et*

al., 2014). With respect to production, hexaploid wheat dominates wheat production globally (Eversole *et al.,* 2014).

2.1.2 Domestication of wheat

Domestication is a process by which adaptable plants or animals are cultivated or reared by humans (Brown, 2010). It assembles all the cultivars to become human dependent and able to persist particularly under cultivation in agricultural environments to meet human demands (Peng *et al.,* 2011). Modern agriculture and wheat domestication in the Fertile Crescent occurred about 10,000 years ago and played a critical role in the advancement of civilization (Peng *et al.,* 2011). At the beginning of agriculture, only diploid wheats (*Aegilops spp. and Triticum spp.*) were used, and these were later substituted with domesticated tetraploid and polyploid wheat species. Wild emmer and wild einkorn wheats were the first to be subjected to domestication selection. The wild einkorn wheat was first found in the Middle East where it was growing as weed and it was later domesticated in the Karacadag mountain range in southeast Turkey. At present, einkorn wheat is a relic crop grown in parts of Mediterranean countries for animal feed (Peng *et al.,* 2011).

On the other hand, discovery of wild emmer played important role in understanding the domestication of hexaploid wheat because it is the only true wild polyploid wheat of the lineage; it is the ancestor of today′s durum and modern wheat cultivars. The wild emmer wheat (*Triticum dicoccoides*) was naturally growing in Fertile Crescent and in 1906, it was discovered by Aaron Aaronsohn in Israel. The domesticated form of wild emmer is known as *Triticum dicccocum* (emmer, AABB), which was reportedly domesticated in southeast Turkey (Peng *et al.,* 2011). In ancient Egypt, emmer wheat was once the major crop for bread making but its cultivation declined afterwards. Tetraploid *durum* wheat, which is derived from *T. dicoccoides,* is now widely used for

pasta production (Peng *et al.*, 2011) and is mostly adapted to a dry the Mediterranean climate. Hexaploid wheat, also known as modern wheat or bread wheat, now represents 95% of the wheat grown in the world, and much of the remaining is comprised of durum wheat. At present, wheat species such as einkorn, emmer and spelt or hulled are cultivated in some parts of Spain, Turkey, the Balkans, and the Indian subcontinent (Peng *et al.*, 2011).

2.1.3 Food and alternative uses of wheat

Modern wheat is a type of grass cultivated worldwide and serves as a staple food for 40% of the human population (Shewry, 2009). Wheat grain is well known for its nutritional value and multipurpose food uses. Due to its exceptional dough forming properties, protein content, viscoelasticity, cohesiveness and milling properties, wheat is used to produce a greater variety of food products as compared to other cereal crops (Shewry, 2009). Wheat endosperm is used to make all-purpose flour which is used to make yeast breads, cakes, cookies, pastries and noodles (Kumar *et al.,* 2011). It provides more nutrients to the human body than any other single food source. It accounts for ~20% of calories in the daily human diet and supplies protein, carbohydrates, fats, dietary fibre and vitamins B and E. Wheat also provides micronutrient elements such as magnesium, phosphorus, potassium, and zinc.

Based on the grain hardness, common wheat may also be classified as hard or soft wheat. Hard wheat requires more milling time and energy as compared to soft wheat. Durum wheat is one of the hard wheat species that are used for making pasta products and to produce semolina (coarse flour) (FAO, 2013). Depending on the gluten content of the wheat classes, hard red wheats are known for their high protein content and are used to make breads, while soft white wheats have lower amounts of protein, and are utilized to produce a wide variety of bakery products and even

used as raw material to produce malt and beer. Wheat grain also has medicinal properties that can cure a number of diseases (Kumar *et al.,* 2011). Wheat germ prevents heart diseases whereas whole wheat with bran and wheat germ is able to mitigate diseases such as constipation, ischaemic, heart disease, disease of the colon called diverticulum, appendicitis, obesity and diabetes (Kumar *et al.*, 2011).

2.1.4 Wheat production and its economic importance

Wheat has became an important cereal crop because of its cultivation and consumption throughout the world. Wheat is found in more crop production areas than any other commercial crop and is considered as the main food source for humans (Enghiad *et al.,* 2017). The major reason behind the success of wheat as an important crop is its adequate genetic diversity and adaptability to a wide range of climatic conditions, which has allowed wheat breeders to produce over 25,000 wheat varieties (Shewry, 2009). Wheat is grown between the latitudes of 30°N to 60°N and 27°S to 40°S and at altitudes of up to 3000 meters above sea level. The temperature conditions for wheat range from 3° and 32° Celsius (Enghiad *et al.*, 2017).

The Green Revolution increased productivity of wheat, which occurred in developing countries in the1960s through the introduction of high yielding varieties along with the use of fertilizer, pesticides and herbicides. World wheat production was doubled between 1965 and 1970 (Curtis and Halford, 2014). Since then, wheat has been the most widely grown crop in the world (Curtis and Halford, 2014). In 2017 alone, ~760 million tonnes of wheat was produced, making its global production third after maize (1054 million tonnes) and rice (506 million tonnes) (FAO, 2017). Global trading of wheat is higher than any other crop; for example, the amount of wheat traded in 2017 was estimated to be 171 million tonnes followed by maize (137 million tonnes) and

rice (44 million tonnes) (FAO, 2017). Wheat demand is expected to increase in developing countries by 2050 by up to 60% (Dixon *et al.,* 2009). Therefore, availability of wheat needs to be doubled without increasing total cultivated area. However, the increase in wheat yield has slowed due to a number of reasons including extreme changes in climatic conditions (Alston *et al.*, 2010). For example, wheat production for the year 2017 is estimated to be ~760 million tonnes, showing a significant decrease of 2.7% (20.3 millions of tonnes) from what was produced in 2016 (FAO, 2016). This along with an increase in world population, which is expected to exceed 9 billion by 2050, calls for the need of a "Second Green Revolution" to boost wheat yield, and this is now one of the top priorities for agricultural research (Davies *et al.*, 2009).

Wheat has a largest and complex genome, and this has been one of the major obstacles for the wheat scientists to expand their understanding of genetic components controlling its grain quality and yield. The recent publication of the wheat genome sequence along with a better understanding of its organisation, function and evolution of its large and complex genome paves the way for the development of genomic tools that can support the effort of increasing wheat yield and quality (Feuillet *et al.,* 2005).

2.1.5 Wheat production in Canada

Wheat cultivation made its debut in western Canada in 19th century and it was first grown by Selkirk settlers from Scotland. In 1812, a group of 22 settlers started to plant the winter wheat they brought from Scotland in the area where the Red River meets the Assiniboine River (Buller, 1919). The same source of wheat seed was used during the spring season, but both attempts failed to produce a harvest due to lack of knowledge, severe winter, and repeated attack by pests (Buller, 1919). Until 1820 famers achieved a decent harvest from new seeds transported from Wisconsin,

United States. The modern history of wheat in Canada began in 1882, when a Ukrainian wheat variety ′Red Fife′ was introduced, and this variety was well known for its traits such as higher yield, early maturity and good milling properties. Because of this, certain crosses were made with these traits to produce new wheat varieties. The cultivar 'Marquis' cultivar was developed in 1903 and raised the production of wheat in Canada from 2 to 7.7 million tonnes (Campbell, 2015). Marquis and Red fife made Canada famous for its high quality hard red spring wheat. The Prairie Provinces (Alberta, Saskatchewan and Manitoba) produce most of the Canadian wheat. Spring wheat is more widely grown as compared to winter and durum wheat. According to the current statistics, Saskatchewan produces 41% of the spring wheat followed by Alberta 39% and Manitoba 18% (Statistics Canada, 2018) and it contributes $11 billion annually to the Canada′s economy (National Research Council of Canada, 2013). In 2018, wheat covers over 24.3 million acres nationally, making it the largest crop cultivated in Canada, and its production is estimated to be 30 million tonnes (Statistics Canada, 2018). Today, Canada is the sixth largest producer of the wheat in the world and most of the western Canadian wheat is exported and marketed in a highly regulated fashion (National Research Council of Canada, 2013).

However, wheat production in Canada is affected negatively by different biotic and abiotic factors. Biotic factors include different kind of diseases and pests (National Research Council of Canada, 2013). Abiotic factors such as drought, heat, cold and reduced nitrogen fertilizer requirements cause wheat losses. One of the major abiotic stress related problems is early sprouting or germination of grains while still on the ear, also known as known as PHS, due to wet or humid harvest conditions. PHS leads to increased alpha amylase activity in seeds that ultimately causes losses in yield, test weight, grain functionality and viability of the seed. In 2000, 2002 and 2010, Canadian wheat production witnessed serious damage due to PHS (Singh *et al.*, 2014), and PHS

in spring wheat alone is reported to cost ~$100 million a year (DePauw *et al.*, 2012). PHS is a major problem in Prairie Provinces of Canada that affects wheat producers due to these economic losses. Therefore, effective and reliable methods of introducing improved PHS tolerance in wheat cultivars are required (Humphreys and Noll, 2002).

2.1.6 Preharvest sprouting

PHS is the germination of physiologically matured seeds prior to harvesting (Figure 2.1) (McCaig and DePauw, 1992). It occurs in cereal crops such as wheat, barley, rice, millet and sorghum in cool or rainy conditions and results in yield loss and reduced seed quality (Gubler *et al.*, 2005). PHS is strongly influenced by seed dormancy, a trait that blocks the germination of seeds under optimal condition. Thus, dormancy at the stage of harvest is desirable to prevent early sprouting of grains in the head under higher moisture conditions (Gubler *et al.*, 2005).

Rapid and uniform germination in cereals crops has been achieved through selection for low levels of seed dormancy. This increases the risk of germination of physiologically mature seeds before harvest (Shu *et al.*, 2015). On the other hand, high level of seed dormancy results in delayed germination and shorter growing seasons by delaying the time for sowing for next season (Skubacz and Golec, 2017). In modern wheat cultivars, low levels of seed dormancy results in PHS and the annual global financial losses associated with PHS of cereal crops is estimated to be $1 billion (Black *et al.*, 2006). Therefore, moderate levels of dormancy in the seed is considered as an enticing prospect to prevent PHS (Gao and Ayele, 2014).

Plant hormones especially ABA and gibberellins (GAs) have primary roles in controlling seed dormancy and has been well demonstrated in seeds of dicot species (Rodríguez *et al.*, 2015). Seed dormancy is regulated by endogenous ABA levels and signalling (Nambara *et al.*, 2010).

Mutational analysis in Arabidopsis revealed that the seeds of ABA biosynthesis *nced* mutants failed to induce seed dormancy and germinated rapidly as compared to the wild type (Frey *et al.,* 2012). Other studies in cereals such as wheat and barley also indicated the role of ABA biosynthesis genes in influencing ABA level and seed dormancy (Chono et al., 2006; Son et al., 2016). On the other hand, ABA catabolic mutants of Arabidopsis, barley and wheat accumulated high ABA levels, and therefore exhibit increased seed dormancy (Okamoto *et al.,* 2006; Gubler et al., 2008; Chono *et al.,* 2013). Previous studies in Arabidopsis and cereal crops such as wheat, barley oat, rice and sorghum have shown the importance of ABA signalling or seed sensitivity to ABA for the regulation of seed dormancy (Walker-Simmons, 1987; Nambara *et al.,* 2010; Rodríguez *et al.,* 2015). These findings show that ABA metabolic and signalling genes play an important role in controlling seed dormancy, and therefore a further understanding of their roles in wheat and other cereals will be critical in improving seed dormancy to prevent PHS.

Figure 2.1. Sprouting of wheat spike before harvest
(Source: http://www.arc.agric.za/arc-sgi/Crop physiology.aspx)

2.2 Seed

2.2.1 Seed development and maturation

Seed development is a basic and critical step in plant life. In angiosperms, seed formation starts with pollination, in which male gametophytes or pollen grains from the anthers are transferred to the female organ, stigma of the flower, followed by the development of pollen into pollen tube that elongates through the style towards ovule. After the pollen tube finds its entrance into the ovary and through the micropyle of the ovule, the sperm nuclei are ultimately released into the embryo sac. In the embryo sac, the megaspore maternal cells undergo cell division through meiosis, which results into eight haploid nuclei. The important nuclei for seed formation are the haploid egg cells and the two polar nuclei. For successful seed formation, double fertilization occurs in which one sperm cell nucleus combines with the egg cell nucleus to form the diploid zygote, and subsequently develop into the embryo, and the other sperm cell fuses with the two polar nuclei to form a triploid nucleus from which the endosperm develops (Bewley *et al.*, 2013).

After double fertilization, early morphogenesis starts in the embryo sac where fertilized egg divides to form an apical and a basal cell. The embryo originates from the apical cell whereas basal cell forms the suspensor. During seed development, the suspensor transports nutrient and growth regulating hormones to the embryo. Endosperm development consists of the syncytial, cellularization and differentiation phases. During the initial stages of seed development, the endosperm is called syncytium, and the syncytial phase involves rapid proliferation and expansion of the endosperm. After this, cellularization, which divides the syncytium into individual cells, occurs and then the endosperm enters into a differentiation phase. After the embryo and endosperm complete morphogenesis and patterning stages, seeds enter into maturation phase where the embryo develops into highly specialized organ along with accumulation of the storage reserves in

the endosperm. During the maturation phase of seed development, water loss/desiccation, cessation of embryo growth, accumulation of storage products, induction of dormancy and acquisition of desiccation tolerance occur (Bewley and Black, 1994).

2.2.2 Seed dormancy

Failure of the intact viable seed to germinate under favourable conditions is termed as seed dormancy (Gao and Ayele, 2014). It is an inherent property of the seed that ensures the fitness of a plant under different environments (Graeber *et al.,* 2012). Ability of seed to germinate is determined by a number of physiological and biochemical processes which are regulated by the endogenous and exogenous factors (Finkelstein *et al.,* 2008; Shu *et al.,* 2016).

Based on the time of induction of dormancy, seed dormancy is categorized as primary or secondary. Primary dormancy is inborn dormancy which is inherited by the seed when they disperse from mother plant while secondary dormancy is acquired by the seed after harvest due to unfavorable germination conditions (Baskin and Baskin, 2004). Primary dormancy is further divided into two categories; embryonic dormancy and coat-imposed dormancy. Dormancy caused by embryos is termed as embryonic dormancy in which the embryos themselves are unable to germinate. In case of seed coat-imposed dormancy, the germination of the embryo is limited by the surrounding structure (Bewley, 1997). Coat-imposed dormancy can be released by removing the tissues that surrounds the embryo, which including the endosperm, pericarp or extra floral organs (Bewley, 1997). Depending on the genotype and environment, dormancy can be induced during seed development and is referred to as primary dormancy while the dormancy induced after harvesting due to some environmental cues is known as secondary dormancy (Bewley, 1997). Both environment and internal factors regulate the induction, maintenance, and release of seeds from

primary dormancy. External factors experienced by the mother plant such as temperature, light, and nitrates affect the level of seed dormancy (Nambara *et al*., 2010). At the genetic level, different regulatory pathways control seed dormancy most of which are well studied in the model plant species *Arabidopsis thaliana* (Nee *et al.,* 2017). In wheat, the loss and maintenance of seed dormancy have been reported to be related with the physiological changes that are associated with alterations in gene expression and protein expression, oxidative modification of gene transcripts and proteins, and epigenetic alterations, and these alterations are regulated mainly by two plant hormones ABA and GA (Tuan *et al.,* 2018). ABA is known to induce dormancy while GA enhances the germination of seeds (Shu *et al*., 2016). Consequently, the ABA/GA balance establishes a central regulatory mechanism underlying the maintenance and release of seed dormancy (Tuan *et al.,* 2018). Other hormones such as auxin, brassinosteroid and ethylene influence the interaction between GA and ABA, and thereby influence seed dormancy (Shu *et al.,* 2016). Apart from this, intrinsic signalling factors such as reactive oxygen species (ROS) and external factors such as temperature and light can also affect ABA and GA balance (Tuan *et al.,* 2018).

2.2.3 Release of seed dormancy

Seed dormancy can be released by a number of processes such as after-ripening (AR), cold stratification and warm stratification. In addition, exogenous application of plant hormones such as GA, smoke and nitrogenous compounds (nitric oxide and nitrate) promote seed germination (Bethke *et al.,* 2006). The transition of seeds from dormant state to non-dormant state during dry storage at room temperature is termed as AR (Iglesias-Ferna´ndez *et al.,* 2011). AR of dormant seeds in barley, wheat and Brachypodium leads to changes in the expression of ABA metabolic

and signaling genes (Gubler *et al.,* 2008; Barrero *et al.,* 2013; Liu *et al.,* 2013). Imbibition of dormant seeds at low temperature, which is also called cold stratification, causes dormancy release in many plant species (Bewley *et al.,* 2013). In cereal crops, changes in the environmental factors such as temperature, light and oxygen affect seed dormancy (Benech-Arnold *et al.,* 2006). For example, blue light increases the ABA content and therefore enhances seed dormancy in barley seeds (Gubler *et al.,* 2008) while high temperature inhibits the germination of wheat seeds by inducing the expression of ABA biosynthetic and signaling genes and repressing GA synthesis and signalling (Izydorczyk *et al.,* 2018).

2.2.4 Seed germination

Seed germination is an important checkpoint for the survival of plants. It starts with the uptake of water by mature dry seed and terminates with emergence of radicle through the seed coat (Bewley and Black, 1994). Due to its considerable susceptibility to biotic and abiotic stress, germination is considered as the most crucial step of crop yield. Seed germination involves three phases of water uptake. In the first phase, the quiescent dry seed absorbs water and as a result swelling of the seed along with changes in size and shape occurs. During first phase, structural changes on the membranes of imbibed seeds lead to an immediate and rapid leakage of solutes and low molecular weight metabolites into the surrounding imbibition solution. Upon imbibition, dry seeds rapidly resume energy metabolism and the respiration process. In addition, other processes such as repair of DNA and mitochondria damaged due to maturation drying, and synthesis of protein from existing mRNA occurs. After the water content in the seeds becomes constant, the second phase seeds of water uptake begin which is also called the plateau phase. During this phase protein synthesis from newly synthesized transcripts and synthesis of new mitochondria is initiated. Due

to expansion of the embryo axis, seed experience a phase change to germination during which the radicle emerges through the surrounding structures, leading to the completion of germination. At this point the seed enters to the third phase in which mobilization of storage reserves, and elongation of radicle cells, cell division and the synthesis of new DNA occur. During phase three, there is an increase in the water uptake by the young seedling which is utilized for the cell divisions and expansion of the growing radicle (Nonogaki *et al.,* 2010).

2.3 Regulation of seed germination and dormancy by ABA

ABA is one of the important plant hormones present in all vascular plants (Yan and Chen, 2017). It regulates different plant growth and developmental processes including major events occurring during seed formation such as deposition of storage reserves, acquisition of desiccation tolerance and induction of primary dormancy (Kermode, 2005). During seed development phases, ABA has a key role in maintaining embryo development until seeds are fully mature and the accumulation of adequate reserves to allow germination and subsequent seedling growth (Kermode, 2005). Several reports using ABA biosynthetic mutants have demonstrated that ABA produced in embryos of developing seeds is necessary for the induction of seed dormancy, and therefore it acts as a regulator of primary seed dormancy (Gubler *et al.,* 2005).

ABA content is low during the initial stages of seed development and peaks around the mid maturation phase and declines afterwards. This pattern of seed ABA content is closely associated with the expression patterns of genes encoding the ABA biosynthetic enzymes zeaxanthin epoxidase (ZEP) and NCED (Finkelstein, 2013). Studies on ABA metabolism in Arabidopsis have identified several ABA biosynthetic mutants such as *aba1, aba2, aba3, nced6* and *nced9* that display low level of ABA and reduced or no dormancy while, ABA catabolic

mutants such as *cyp707a1* and *cyp707a2* show enhanced ABA level and seed dormancy, implying a positive correlation between dormancy and ABA (Finkelstein *et al.,* 2008). Defects in the ABA response pathway also leads to variation in the levels of seed dormancy (Yan and Chen, 2017). Several ABA insensitive mutants that control seed response to ABA and dormancy have been isolated in Arabidopsis and in monocot plants (Finkelstein, 2013).

Apart from its metabolic and signaling components, the interaction of ABA with other phytohormones also influences seed dormancy and germination. One typical examples of such interactions is the antagonistic relationship of ABA with GA (Gubler *et al.,* 2005). In Arabidopsis, ABA deficient mutants displayed higher expression of GA synthesis genes, suggesting that ABA suppresses GA synthesis in wild type seeds. In agreement, downregulation of GA synthesis genes by ABA positively promotes seed dormancy (Seo *et al.,* 2006). Additionally, ABA plays a role in the transcriptional activation of GA catabolic genes (Bewley *et al.,* 2013). This double regulation of GA metabolism by ABA results in decreased GA levels and seed germination, and therefore an increased level of seed dormancy. Germination of non-dormant seeds is associated with a decrease in ABA levels, which is stimulated by increased activity of ABA catabolism and a decreased rate of ABA biosynthesis, and an increase in GA level, which is regulated by increased GA biosynthesis (Al-Rachedi *et al.,* 2004). A recent study on spatiotemporal balance between ABA and GA with respect to temperature showed the role of ABA and GA metabolic and signalling genes in controlling seed germination/dormancy in wheat seeds (Izydorczyk *et al.,* 2018).

2.3.1 ABA metabolism and signaling pathways

2.3.1.1 ABA Biosynthesis

The *de novo* biosynthesis of ABA takes place both in the plastid and cytosol and involves many enzymatic reactions (Figure 2.2) (Nambara and Marion-Poll, 2005). Synthesis of isopentenyl diphosphate (IPP) in the plastid leads to the production of GGPP (a C_{20} compound) which is converted into phytoene by phytoene synthase. Further lycopene, produced from phytoene undergoes cyclization to form beta carotene. In the first step of ABA biosynthesis, beta carotene is converted into zeaxanthin by beta carotene hydroxylase, which in turn is converted into violaxanthin by ZEP (Figure 2.2). Synthesis of neoxanthin from violaxanthin requires two enzymes, one is neoxanthin synthase (NSY) and the other is an unknown isomerase. The second step involves xanthophyll cleavage in which the NCED enzyme cleaves the cis isomers of violaxanthin and neoxanthin to C_{15} xanthoxin and a C_{25} metabolite. The third step of ABA biosynthesis involves the production of biologically active ABA from *cis*-xanthoxin through two enzymatic reactions. In the first reaction short-chain dehydrogenase/reductase (SDR) converts *cis*-xanthoxin to abscisic acid aldehyde, which is further converted into ABA by abscisic acid aldehyde oxidase (AAO3) (Nambara and Marion-Poll, 2005).

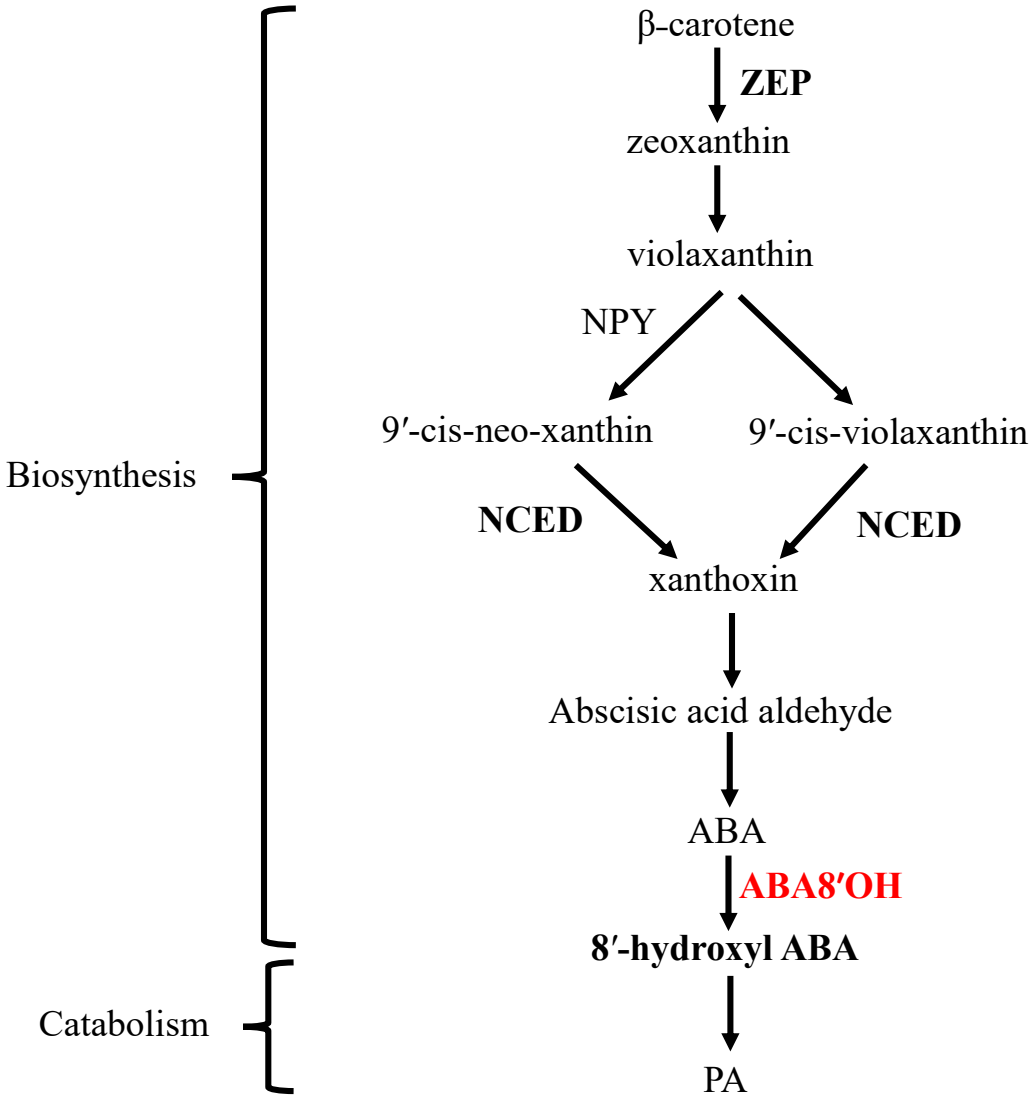

Figure 2.2. Simplified ABA metabolic pathway in plants. ABA is synthesized from carotenoids, and this process involves many enzymatic reactions including those catalyzed by zeaxanthin epoxidase (ZEP) and 9-cis epoxycarotenoid dioxygenases (NCED). ABA inactivation is mainly catalyzed by ABA 8′ hydrolase (ABA8′OH), which converts ABA to phaseic acid (PA).

2.3.1.2 ABA catabolism

The ABA catabolic reactions play an important role in the control of ABA level (Nambara and Marion-Poll, 2005). The ABA catabolic pathway includes two main reactions, hydroxylation

(Figure 2.2) and conjugation. The hydroxylation of ABA takes places at three different methyl groups located at C-7, C-8 and C-9. However, hydroxylation at C-8 position, catalyzed by ABA 8′ hydrolases (ABA8′OH), is the most important ABA catabolic reaction, and this reaction produces 8′-hydroxy ABA. Spontaneous cyclization of 8′hydroxy ABA forms phaseic acid (PA), which in turn is catabolised to form dihydrophaseic acid (DPA) by a soluble reductase (Figure 2.2) (Nambara and Marion-Poll, 2005). The ABA8′OH enzyme is encoded by *CYP707A* genes and Arabidopsis has four *CYP707A* gene family members designated as *CYP707A1*, *CYP707A2*, *CYP707A3* and *CYP707A4* (Kushiro *et al.*, 2004). These genes show distinct spatial and temporal patterns of expression and thereby influence ABA levels and various physiological or developmental mechanisms (Kushiro *et al.,* 2004; Okamoto *et al.,* 2006).

2.4 Role of ABA inactivation by *CYP707A1* in seed germination and dormancy

Functional and expression analysis of the CYP707A genes in Arabidopsis has shown that *CYP707A1* and *CYP707A2* play important roles in regulating seed germination and dormancy. The amount of endogenous ABA in dry seeds appears to be determined by the expression level of *CYP707A1*. In agreement with this, *cyp707a1* mutant seeds contain a higher level of ABA than the wild type seeds, resulting in increased dormancy and a decrease in germination percentage (Okamoto *et al.,* 2006). The expression of *CYP707A1* in immature siliques of Arabidopsis has been found to be higher until 12 days after flowering (DAF) but declined afterwards. Similarly, siliques of *cyp707a1* mutant Arabidopsis showed high ABA levels at 12 DAF, suggesting the predominant role of *CYP707A1* in regulating ABA level at mid-maturation stages (Okamoto *et al.,* 2006). Among c*yp707a* double mutants, seeds of the *cyp707a1* and *cyp707a2* exhibit higher ABA levels and a strong dormancy phenotype as compared to the wild type during mid-maturation stage

of seed development (Okamoto *et al.,* 2006). These results indicate the role of *CYP707A1* in influencing ABA level and seed dormancy. In the cereal crop barley two genes encode ABA8′OH (Millar *et al.,* 2006). Expression of *HvABA8'OH1* (also known as *HvCYP707A1)* in the embryo have been reported to be higher during seed imbibition as compared to *HvABA8'OH2,* and this leads to reduced ABA content that accounts for high germination rates (Chono *et al.,* 2006). Furthermore, AR of dormant barley seeds causes an increase in the expression of *HvABA8'OH1* during imbibition (Gubler *et al.,* 2008). The same authors also indicated that reducing the expression of *HvABA8OH1* using RNA interference (RNAi) technology resulted in ABA accumulation and low seed germinability. These results indicate that *ABA8'OH1* plays crucial role in the release of seed dormancy.

Studies on ABA catabolism in rice (*Oryza sativa*) have identified three genes encoding ABA8′OH that are designated as *OsABA8ox1*, *OsABA8ox2* and *OsABA8ox3* (Ye *et al.,* 2012). Induction in the expression of *OsABA8ox2 and OsABA8ox3* is reported to occur following seed imbibition, suggesting their role in lowering seed ABA content during seed germination (Ye *et al.,* 2012). Five ABA catabolic genes have been reported in maize (*Zea mays*) including *ZmABA8Ox1a*, *ZmABA8ox1b*, *ZmABA8ox2*, *ZmABA8ox3a* and *ZmABA8ox3b* and these genes share homology to the Arabidopsis *CYP707A* gene family members. As observed in Arabidopsis and other species, the expression pattern of maize *ABA8ox* genes is tissue-specific. *ABA8ox3a* is abundantly expressed in leaves and while the expression of *ABA8ox1a* occurs in roots. With respect to seed tissues, *ABA8ox2* and *ABA8ox1a* are expressed in endosperm while the expression of *ABA8ox2*, *ABA8ox1a* and *ABA8ox1b* occurs in the embryo (Vallabhaneni and Wurtzel, 2010).

2.4.1 Functions of *ABA8'OH1* in wheat

Advances in gene mutational research have made important contributions for investigating gene functions (Rikiishi *et al.,* 2010). However only a few mutants related to seed dormancy have been isolated in wheat because of its complex genome, which makes it difficult to simultaneously mutate a gene at two or three loci to create mutants (Chono *et al.,* 2013). In common wheat, *TaABA8'OH1* located on group chromosome 6, is expressed highly in seeds as compared to the other *ABA8'OH* member (Nakamura *et al.,* 2009). A study on *ABA8'OH1* in Japanese wheat cultivars showed the effect of mutations in *ABA8'OH1* on seed germination (Chono *et al.,* 2013). Single mutants having an insertional mutation in the D genome copy of *TaABA8'OH1* did not show any difference in seed germination as compared to the wild type, whereas double mutants carrying an additional deletion mutation on the A genome copy of *Ta ABA8'OH1* exhibited a decrease in ABA8′OH1 activity and accumulation of high amounts of ABA, and therefore a decrease in germination as compared to the wild type (Chono *et al.,* 2013). Ectopic expression of the B genome copy *TaABA8'OH1* in Arabidopsis has demonstrated the function of this homeologue with respect to regulation seed ABA level and dormancy (Son *et al.,* 2016). In addition, this study showed that only the A and B genomes contribute transcripts to the expression of *TaABA8'OH1* in mature wheat tissues (embryo and endosperm), indicating the significance of these specific sub-genomes in regulating the activity of seed localized *TaABA8'OH1* (Son *et al.,* 2016).

2.5 ABA signaling pathway

The signal of ABA in plants is perceived by its receptors PYRABACTIN RESISTANCE/PYRABACTINLIKE/REGULATORY (PYR/PYL/RCAR). Identification of PYR/PYL/RCAR proteins played an important role in the unfolding of the ABA signalling

mechanism (Ng *et al.,* 2014). Previous studies in Arabidopsis shown that ABA signalling involves at least three core components: PYR/PYL/RCAR components of ABA receptors, protein phosphatase 2cs (PP2cs), and SNF1-related protein kinase 2s (SnRKs) (Fig. 2.3) (Gao and Ayele, 2014). The signaling of ABA is initiated by the binding of ABA to the receptor proteins PYR/PYL/RCAR, which in turn bind to and inactivates PP2Cs, which negatively regulates ABA signaling through repressing SnRKs, which acts as a positive regulator of the downstream ABA signaling components. Inhibition of PP2Cs leads to the activation of SnRKs through auto phosphorylation, which further activates downstream transcription factors such as the basic leucine zipper (bZIP) transcription factor abscisic acid insensitive 5 (ABI5), the AP2-type transcription factor ABI4, and the B3-type protein ABI3, which are key to regulating the expression of ABA responsive genes in seeds (Nambara *et al.,* 2010). In the absence of ABA, PP2Cs dephosphorylate and deactivate SnRK2s. These components of ABA signalling have been reported to be conserved in the seeds of both dicot and monocot plants (Kim *et al.,* 2012).

Promoter regions of the ABA regulated genes consist of a number of cis-acting DNA elements designated as ABA-responsive elements (ABREs). The ABREs have PyACGTGG/TC consensus sequence that consists of a core ACGT sequence and belongs to the G-box family (CACGTG), and this consensus sequence is important for several gene expression mechanisms in plants (Ng *et al.,* 2014). In Arabidopsis, nine homologs of ABRE-binding proteins (AREB) or ABRE-binding factors (ABFs) encoding the basic domain leucine zipper (bZIP) transcription factors have been identified and they appear to share a highly conserved C-terminal bZIP domain and three additional N-terminal conserved regions labelled as C1, C2, C3 and C4 (Ng *et al.,* 2014). It has been reported that 13 and 11 members of ABF/AREB/ABI5 family are present in Arabidopsis and rice, respectively (Nijhawan *et al.,* 2008). On the other hand, only a few members

of ABF/AREB/ABI5 have been identified in wheat and barley as whole genome sequence data of these two crops are still being analyzed (Zhou *et al.*, 2017).

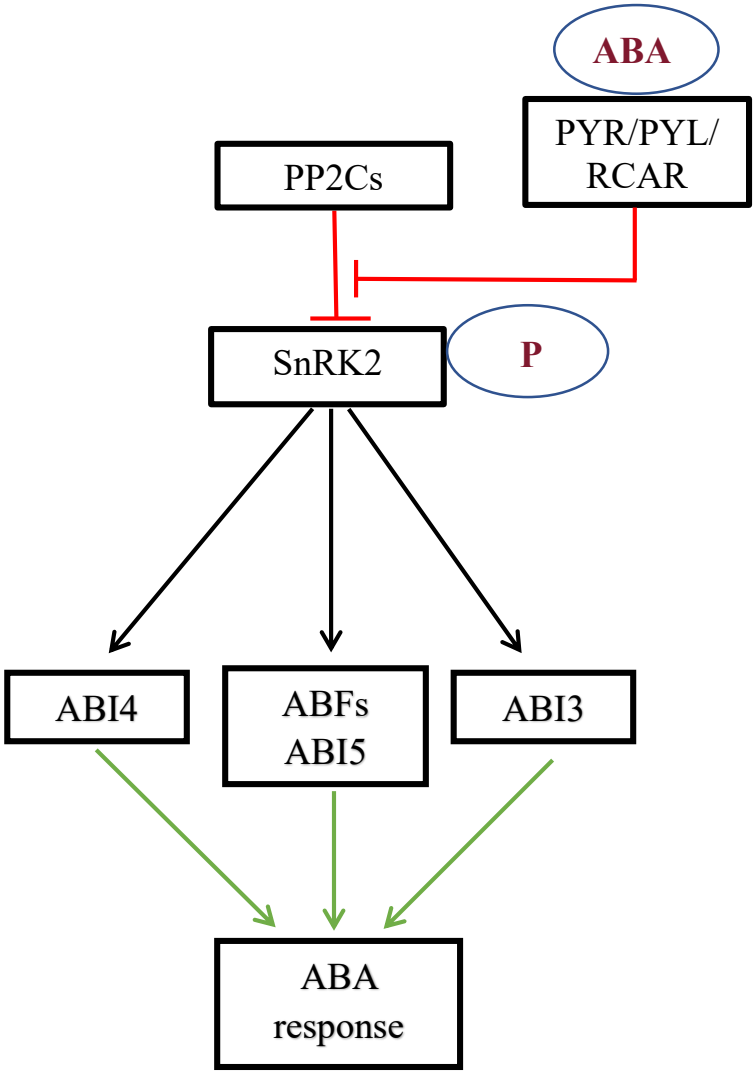

Figure 2.3. Schematic diagram of ABA signalling pathway. ABA binds to the PYR/PYL/RCAR receptor and this leads to inhibition of PP2Cs that negatively regulate ABA signalling by supressing SnRKs, positive regulator of ABA signaling. The activation of SnRKs via phosphorylation (P) activates the down stream transcription factors, ABI3, ABI4 and ABI5, and therefore ABA responsive genes.

2.5.1 Abscisic acid insensitive mutants

Studying the defects in ABA synthesis and response mutants reveals the role of ABA in the regulation of seed dormancy and germination (Nambara *et al.*, 2010). Arabidopsis mutants of ABA response have been isolated by screening a mutagenized population for ABA hypersensitivity or insensitivity phenotypes. The ABA insensitive mutants revealed six ABA insensitive genes; *ABI1*, *ABI2, ABI3, ABI4, ABI5* and *ABI8* that play role in the ABA signaling pathway (Finkelstein *et al.*, 2002). Genetic studies in Arabidopsis have shown that *ABI1* and *ABI2* genes encodes PP2Cs. Loss-of-function mutations in *ABI1* and *ABI2* display ABA hypersensitivity, indicating that *ABI1* and *ABI2* are negative regulators of ABA signalling (Nishimura *et al.*, 2007; Nambara *et al.*, 2010). However, SnRK regulated downstream transcription factors ABI3, ABI4 and ABI5 are important for ABA responsive genes and this indicates that the SnRKs act as positive mediators of the ABA signalling pathway (Nambara *et al.*, 2010).

In Arabidopsis, *ABI3* is involved in regulating seed maturation and plays a significant role in inhibiting precocious germination and inducing seed dormancy (Raz *et al.*, 2001). Furthermore, it mediates ABA signaling positively by supressing the expression of *ABI1* and *ABI2* (Suzuki *et al*., 2003). A study in wild oat (*Avena fatua*) has shown a strong correlation between the expression of *vp1*, an orthologue of *ABI3*, and seed dormancy levels (Jones *et al.*, 1997). In maize, Vp1 (viviparous -1) is a major regulator of late embryo development and seed dormancy (McKibbin *et al.*, 2002). Mutants of Vp1 showed altered embryo maturation and germination of the embryos while still attached to the mother plant (Vivipary). In wheat, ectopic expression of the maize *Vp1* gene displayed enhanced seed dormancy and tolerance to PHS (Huang *et al.*, 2012). Additionally, the dormancy pattern in wheat seeds has been shown to be correlated with expression of *Vp1* (Nakamura and Toyama, 2001).

Defects in ABA signalling in sorghum lead to reduced sensitivity of the embryo to ABA and enhanced germination (Gualano *et al.,* 2007). Mutational studies of *ABI4* have also shown the role of *ABI4* in regulating the expression of ABA catabolic genes and thus seed dormancy (Shu *et al.,* 2013). On the other hand, the bZIP transcription factor *ABI5* have been shown to positively mediate ABA signalling and seed dormancy (Shu *et al.,* 2016).

2.6. Role of *ABI5* in enhancing seed dormancy

Members of the abscisic acid responsive element (ABRE) transcription factor (ABF) family are important components in ABA signalling. These transcription factors bind to specific CACGTGGC core sequence elements of ABRE to activate the transcription of many ABA responsive genes and regulate ABA sensitivity in seeds (Choi *et al.,* 2000). One of these ABFs is ABI5 transcription factor that functions as a core component of ABA signalling (Finkelstein and Lynch, 2000). It has been shown that *ABI5* plays a crucial role in regulating seed-specific processes such as seed maturation and seed germination (Nakamura *et al.,* 2007).

The *ABI5* gene was first mapped on chromosome 2 of Arabidopsis and an insertional mutant designated as *abi*5 was described as ABA insensitive during seed germination (Finkelstein and Lynch, 2000). Earlier reports suggested that *ABI5* does not have a role in inducing seed dormancy because seeds of the *abi5* mutant show a level of dormancy similar to that observed in the wild type (Finkelstein, 1994). However, further studies showed that accumulation of *ABI5* due to exogenously applied ABA causes abortion of seed germination and inhibits early seedling growth, suggesting its role in the inhibition of seed germination. In germinating seeds, the occurrence of drought and salt stress activates the expression of *ABI5* which in turn causes inhibition of seed germination and early seedling growth (Lopez-Molina *et al.,* 2002). It has been reported that *ABI5*

acts downstream of *ABI3* during germination and early seedling growth to revive genes involved in late embryogenesis (Lopez-Molina *et al.,* 2002).

Sequence analysis of the Arabidopsis *ABI5*, which encodes a bZIP-type protein, has shown that *ABI5* has three N-terminal (C1, C2 and C3) and one C terminal (C4), although the importance of these regions except for bZIP is still unclear (Tezuka *et al.,* 2013). The interaction between *ABI3* and *ABI5* is mapped to the C2 and C3 domain of *ABI5* (Nakamura *et al.,* 2001). A novel abi5 recessive allele (*abi5-9*) has been identified in the C3 domain of ABI5 due to single amino acid substitution at 214 residue, and the *abi5-9* plants displayed reduced ABA sensitivity during germination and low expression of genes encoding late embryogenesis abundant (LEA) proteins, indicating the effect of allelic variation to alter interaction between *ABI5* and *ABI3* (Tezuka *et al.,* 2013).

It is well established that an antagonistic relationship between ABA and GA controls seed germination and dormancy (Gubler *et al.,* 2005). Previous studies in Arabidopsis showed that decreased GA synthesis leads to an increase in ABA level and inhibition of germination through over accumulation of RGL2, a key DELLA protein that represses germination, which results in activation of ABI5 through phosphorylation by SnRK (Piskurewicz *et al.,* 2008). Studies in sorghum showed that *ABI4* and *ABI5* have the ability to bind with SbGA2-oxidase promoter that contains an ABA responsive complex and compete for the *cis*-acting regulatory sequences (Cantoro *et al.,* 2013). Gene expression studies indicated that *ABI4* and *ABI5* promote the expression of GA catabolic genes, which results in the degradation of bioactive gibberellin to maintain seed dormancy (Cantoro *et al.,* 2013). In another study, *ABI5*, under negative regulation by peroxisome defective 3 (PED), targets polygalacturonate inhibiting proteins to repress seed coat rupture and germination by inhibiting the activity of polygalacturonates (Kanai *et al*., 2010).

Genes encoding ABI5 have also been identified from other crop species. For example, the *ABI5* in rice acts in an equivalent way as *ABI5* of Arabidopsis and it possess the ability to bind to G-box elements of ABA responsive elements. Overexpression of *ABI5* of rice in an *abi5* mutant of Arabidopsis reversed an ABA insensitive phenotype to wild type during seed germination, suggesting *ABI5* of rice has a similar function as *ABI5* of Arabidopsis in the ABA signalling pathway (Zou *et al.,* 2008). The ABI5 of barley has been shown to have high amino acid sequence homology with ABI5 of Arabidopsis and is involved in activating the expression of ABA-induced genes (Casaretto and Ho, 2003). It has become clear from these studies that *ABI5* has a akey role in ABA-mediated inhibition of seed germination and enhancement of seed dormancy (Piskurewicz *et al.,* 2008; Cantoro *et al.,* 2013).

2.6.1 Functions of *ABI5* in wheat

The importance of seed ABA sensitivity in the control of seed dormancy in wheat has been reported previously (Walker-Simmons, 1987). A study on hard red wheat has shown that ABA insensitive mutants exhibit a decreased level of grain dormancy and enhanced seed germination, suggesting that ABA sensitivity is an important aspect of dormancy control in wheat seeds (Schramm *et al.,* 2012).

Mutational and QTL analyses in wheat revealed the role of seed ABA sensitivity in modulating dormancy (Gao and Ayele, 2014). For example, *TmABF*, a homologue of *ABI5* in diploid wheat, *Trticum monococcum,* has been mapped to a seed dormancy QTL located on chromosome 3A (Nakamura *et al.,* 2007). Consequently, this gene is considered as a candidate gene for controlling dormancy (Johnson *et al.,* 2008). The homologue of *ABI5* in hexaploid wheat, designated as *TaABF* is highly expressed during seed development and imbibition, suggesting that

its expression is seed specific (Johnson *et al.,* 2002). In agreement with this, no transcript of *TaABI5* has been detected in the leaf, root and stem tissues (Zhou *et al.,* 2017). Furthermore, *TaABF* is reported to show higher expression in imbibed dormant wheat seeds as compare to the corresponding AR seeds, suggesting the role of *TaABF* in regulating seed dormancy and germination in wheat (Johnson *et al.,* 2008). Comparative transcriptome analysis between dormant and AR seeds also showed downregulation of *TaABF* in the AR seeds, suggesting it may participate in regulating an AR mediated decline in seed ABA sensitivity and dormancy (Liu *et al.,* 2013). Analysis of the expression of *TaABF1* showed a close association between its expression pattern and seed ABA sensitiveness and dormancy level (Rikkishi *et al.,* 2010). Reduced seed dormancy (RSD32) wheat mutants exhibited lower expression of *TaABF1* as compare to wild type, while, no changes in the expression of other signalling components such as *TaPKAB1, TaAFP, TaVP1* has been observed, suggesting *TaABF1* is an important factor to control wheat seed dormancy (Rikkishi *et al.,* 2010).

The role of *TaABI5* and its homologs in controlling seed dormancy is mediated not only by prompting the expression of ABA-induced genes but also by repressing the expression of GA-related genes. It has been shown previously that *TaABF1* interacts with the PKABA1 (ABA-inducible protein kinase) in wheat seeds and thereby downregulates the expression of GA-inducible genes (Johnson *et al.,* 2002, 2008). Recent seed transcriptome analysis revealed that specific ABRE motifs, which serve as binding sites for *ABI5*, are enriched in the tissues of dormant seed, indicating the presence of distinct mechanisms underling the role of *ABI5* in the establishment of seed dormancy (Yamasaki *et al.,* 2017)

Environmental factors play important roles in regulating ABA signaling during imbibition of wheat seed through modulating the expression of ABA signalling genes (Izydorczyk *et al.,*

2018). For example, suppression of *ABI5* during imbibition at optimal temperature (22°C) leads to reduced seed sensitivity to ABA and enhanced germination as compared to imbibition at suboptimal and supraoptimal temperatures, which caused increased expression of *ABI5* and inhibition of seed germination (Izydorczyk *et al.*, 2018). Gene expression analysis between PHS resistant and susceptible wheat cultivars indicated the accumulation of more transcripts of *ABI5* in developing seeds at 25 days after pollination of the PHS resistant cultivar, suggesting the role of *TaABI5* in enhancing PHS tolerance (Zhou *et al.*, 2017). The occurrence of PHS is associated with increased activity of α-amylase during seed maturation, and the expression of a gene encoding this enzyme, designated as *TaAmyl*, has been reported to be higher in the PHS susceptible cultivar than the resistance one (Yang *et al.*, 2014). The role of ABI5 in PHS tolerance is associated with its repression of the transcription of α-amylase genes (Zhou *et al.*, 2017). Furthermore, *TaABF1* downregulates GAMyB, a transcription factor that is involved in GA signaling which promotes α-amylase transcription in the aleurone cells of imbibed wheat grains (Yang *et al.*, 2014).

3.0 Molecular characterization and expression analysis of *ABA 8'- hydroxylase 1* and *abscisic acid insensitive 5* genes with respect to seed dormancy in wheat (*Triticum aestivum* L.)

Abstract

Seed dormancy is a trait that prevents the germination of seeds under favourable conditions, and it regulates preharvest sprouting of seeds. The plant hormone abscisic acid (ABA) plays an important role in regulating the level of dormancy, and this role of ABA is mediated by its level and signaling. This study reports comparative analysis of the genomic nucleotide sequences and total expression levels of the ABA metabolic gene ABA *8'-hydroxylase 1* (*ABA8'OH1*) and ABA signaling gene *abscisic acid insensitive 5* (*ABI5*) in wheat (*Triticum aestivum* L.) genotypes that exhibit a range of variation in the level of seed dormancy. Analysis of the genomic sequences of *TaABA8'OH1B* from the different genotypes showed no allelic variation, and overall clear correlation was found between the expression pattern of *TaABA8'OH1* and the level of seed dormancy in the wheat genotypes although a correlation was evident between the two factors in selected genotypes. Sequence analysis of *TaABI5* homeologues, however, showed four allelic variations in the A genome copy of *ABI5*, and one of these base substitutions, which occurred in the first exonic region, appears to affect the expression of *TaABI5* and dormancy in selected wheat genotypes. Dormant wheat genotypes displayed high expression of *TaABI5* as compared to the intermediate and non-dormant genotypes; and strong correlation was observed between the levels of *TaABI5* expression and seed dormancy, suggesting the important role of *ABI5* in regulating seed dormancy and therefore preharvest sprouting in wheat.

3.1 Introduction

Common wheat (*Triticum aestivum* L.) is an integral part of the world economy with yearly production of over 700 million tonnes (FAO, 2017). Wheat production however is affected by number of biotic and abiotic factors. Preharvest sprouting (PHS), germination of the grain prior to harvest under high humidity and wet environment is one of the serious problems of wheat production resulting in reduction of grain quality and yield (Tuan *et al.,* 2018). Seed dormancy, which refers to an adaptive trait that inhibits seed germination under favourable conditions, is central to the control of PHS (Gao and Ayele, 2014). The level of dormancy in cereal crops such as wheat is closely associated with PHS. Higher levels of seed dormancy result in delayed germination while low seed dormancy can result in preharvest sprouting. Therefore, it is necessary for wheat grains to have a certain level of dormancy to prevent sprouting damage (Gao and Ayele, 2014).

Dormancy is a complex trait regulated by intrinsic factors such as plant hormones as well as environmental factors (Finkelstein *et al.,* 2008). To maintain dormancy in cereal crops, ABA levels and sensitivity, which are regulated by the expression of genes encoding ABA metabolic enzymes and ABA signalling receptor components, play an important role (Tuan *et al.,* 2018). Although the ABA metabolic pathway involves several reactions, the reactions catalyzed by NCED and CYP707A1 play key roles in the induction and maintenance of seed dormancy (Nambara *et al.,* 2010). Similarly, the ABA signaling pathway consist of PYL, PP2C and SnRK as central components with downstream transcription factors including *ABI3*, *ABI4* and *ABI5* (Nambara *et al.,* 2010). Genetic studies in Arabidopsis revealed that these components play important roles in regulating ABA response and thereby seed germination and dormancy (Nambara *et al.,* 2010). Preharvest sprouting of wheat grains has always been an important trait

for wheat breeding programs since PHS can result in losses to grain yield and quality. It is well established that PHS tolerance in wheat is associated with high seed dormancy (Gao and Ayele, 2014), and wheat mutants with enhanced seed dormancy and PHS tolerance have been isolated (Schramm *et al.,* 2010). This implies that the manipulation of seed dormancy is an effective way to prevent PHS, the initiation and maintenance of which is regulated partly by ABA level and seed response to ABA response (Yokota *et al.,* 2016).

The balance between ABA synthesis and catabolism regulates the amount of ABA in the seed (Nambara *et al.,* 2010). Mutations in genes that influence ABA biosynthesis such as the NCED genes causes ABA deficiency and this leads to increased germination while mutations in ABA catabolic genes such as *CYP707A1* cause ABA accumulation and increased dormancy (Kushiro *et al.,* 2004; Lefebvre *et al.,* 2006). Dormancy breaking treatments such as AR, affect ABA levels in Arabidopsis and barley seeds through transcriptional activation of ABA catabolic genes encoded by the *CYP707A* family (Millar *et al.,* 2006). Similarly, silencing of the *ABA8'OH1* of barley results in higher ABA levels in isolated embryos of dry and imbibed grains, causing dormancy (Gubler *et al.,* 2008). Imbibition of AR wheat embryos is also associated with significant increase in the expression of *ABA8'OH1* (Jacobson *et al.,* 2013), and change in the expression of *ABA8'OH1* has been shown to be associated with seed dormancy level or germination (Izydorczyk *et al.,* 2018). Mutations in the A and D genome homologues of *TaABA8'OH1* caused a decrease in ABA catabolism and thereby an increase in ABA level and inhibition of germination (Chono *et al.,* 2013). Ectopic expression of the B genome copy of *TaABA8'OH1* in Arabidopsis indicates the importance of this homologue of *TaABA8'OH1* in controlling ABA level and seed dormancy (Son *et al.,* 2016). Overall these reports indicate that the modulation of *TaABA80H1* expression plays

important role in regulating ABA level and seed dormancy/PHS in cereal crops (Fidler *et al.*, 2015).

Several studies in cereals have indicated the role of seed ABA response in controlling dormancy and germination (Tuan *et al.*, 2018). For example, overexpression of the ABA receptor (*PYL/RCAR5*) leads to hypersensitivity to ABA and reduced germination in rice seeds (Kim *et al.*, 2012). Mutations in the downstream components of ABA signaling including ABI3, ABI4 and ABI5 strongly influence the expression of ABA responsive genes by binding to their promoter (Nambara *et al.*, 2002), and genes encoding these downstream components are important for regulating seed response to ABA and thereby dormancy (Nambara *et al.*, 2010).

ABI5 is an important member of ABI5/AREB/ABF family that belongs to the bZIP type of transcription factors. This gene family was identified because of its participation in ABA signalling, for example, during seed development and germination (Jakoby *et al.*, 2002). Studies in Arabidopsis have indicated that a conserved region in ABI5 with potential phosphorylation sites is important to influence seed germination and dormancy (Nakamura *et al.*, 2001). Studies that involve mutations or allelic variations in these domains indicated their importance in influencing seed germination, for example, under stress conditions (Tezuka *et al.*, 2013). Studies in sorghum showed that dormant seeds of sorghum display a higher expression of *ABI5* as compared to non-dormant seeds (Rodriguez *et al.*, 2009). Studies in wheat also showed that the expression of *TaABF1*, an ortholog of *ABI5* in wheat, is higher in dormant/PHS resistant than non-dormant/PHS susceptible wheat seeds or embryos during imbibition (Johnson *et al.*, 2002; Zhou *et al.*, 2017). In addition, a study that involved different cultivars of wheat indicated a close association between the expression level of *TaABF1* and the level of seed dormancy (Rikiishi *et al.*, 2010). Inhibition of wheat seed germination due to suboptimal or supraoptimal temperatures have also been shown

to be associated with increased expression of *ABI5* and other signalling genes (*ABI3*, *SnRK2*, *PYL5*), suggesting increased seed sensitivity to ABA (Izydorczyk *et al.*, 2018). All these results suggest the potential role of *TaABF1* in regulating wheat seed dormancy and thereby PHS resistance. The genetic mechanism underlying the association between the expression patterns of ABA metabolic and signaling genes and seed dormancy, however, is not well studied. This study investigates natural allelic variation in *TaABA8'OH1* and *TaABI5* genes and the effects of the allelic variations on the expression of the respective genes and seed dormancy.

3.2 Material and methods

3.2.1 Plant material and growth conditions

A set of 25 spring wheat cultivars with red and white seed coat colour and varying with degree of resistance to preharvest sprouting were obtained from Dr. Gavin Humphreys' lab at the Ottawa Research and Development Centre of Agriculture and Agri-Food Canada (Table 3.1). These cultivars were collected from different geographical locations including Canada, Australia, Japan, United States, Mexico and New Zealand. For seed multiplication, seeds from each cultivar were imbibed in Petri dishes with 7 mL of water. Germinated seeds were transplanted to a 1 gallon-pot (1 seed per pot at a depth of approximately 2.5 cm) containing LA4 sunshine mix (LA4; Sungro Horticulture, Bellevue, WA, USA) and Cornell mixture (1 bag: 100 g calcium carbonate, 150 g osmocote, 120 g superphosphate, 2 g fritted trace elements, 15 g chelated iron [13.2%], 0.7 g chelated zinc [14%]).Plants were grown in a growth chamber at 18-22°C/14-18°C (day/night) in a 16/8 h photoperiod with high-pressure sodium light (270 $\mu E/m^{-2}sec^{-1}$). Plants were watered every other day and fertilized with N-P-K (20:20:20) mix once every two weeks.

Table 3.1 List of the wheat cultivars used in this study and their seed coat colour

Cultivar	Seed coat colour
AC Domain	Red
AC Majestic	Red
AC Intrepid	Red
OS38	Red
Grandin	Red
Harvest	Red
HR 5603	Red
Otane	Red
Red RL4137	Red
Roblin	Red
RL4452	Red
Mckenzie	Red
CDC Teal	Red
AUS 1408	White
AC Karma	White
Janz	White
Kenya	White
SC8019-R1	White
SC8021-V2	White
Sunstate	White
White RL4137	White
Snowbird	White
Kanata	White
ES34	White
CIMMYT-11	White

3.2.2 Tissue harvesting

Leaf tissues were harvested from each cultivar by the time of flowering and immediately frozen in liquid nitrogen and then stored at -80°C until DNA extraction. Spikes were harvested from each cultivar at maturity and the seeds were threshed by hand. Seeds were after-ripened for two weeks at room temperature and then stored at -80°C until needed for further use.

3.2.3 Germination test

For examining the dormancy level of each cultivar, seeds were first surface sterilised with 5% sodium hypochlorite and 70% ethanol. Seeds were then rinsed five-six times with sterile water. Seed were then imbibed between layers of two Whatman #1 filter papers (GE Healthcare UK Limited, UK) and moistened with 7 mL autoclaved water in a 9-cm Petri-dish. The Petri-dishes were sealed with parafilm and incubated in darkness at 22°C. Completion of germination was marked by emergence of the coleorhiza through the seed coat. Germination was monitored daily for 21 days.

3.2.4 Germination index

Germination index (GI) of each cultivar was calculated based on 15 days of imbibition using the following formula.

$$GI= [(15\times g1) + (14\times g2) + (13\times g3) \ldots\ldots.1\times g15)]/ (15\times n)$$

Where g is the number of seeds germinated on the first, second, ……, and 15^{th} day of imbibition and n is the total number of seeds imbibed.

3.2.5 Genomic DNA extraction

Leaf samples of each cultivar were used for isolation the genomic DNA (gDNA) using modified CTAB method as described previously (Doyle and Doyle, 1990). Leaf samples (50-100 mg) were ground to a fine powder using pestle and mortar with liquid nitrogen and mixed with 400 µL of 2X CTAB buffer (100 mM Tris-HCL, pH:8; 1.4M NaCl; 20 mM EDTA; 2% CTAB; 0.2% β-mercaptoethanol). After mixing, the samples were incubated at 65°C for 1 h and then 200 µL of saturated phenol and chloroform: isoamyl alcohol (24:1) was added. Samples were centrifuged at 9000*g* for 10 min and the supernatant was transferred to fresh tubes. To remove RNA from the samples, 1 µL of 10 mM RNase was added to each sample and then incubated at 37°C for 30 min. Following this, 400 µL of chloroform: isoamyl alcohol (24:1) was added to each sample and centrifuged at 10,000*g* for 10 min. The upper aqueous phase containing DNA was transferred to a fresh tube, and the DNA was precipitated with 800 µL of chilled absolute alcohol by inverting the tubes several times and then centrifuged at 16,000*g* for 2 min. After decanting the supernatant, impurities were removed from the DNA by washing with 500 µL of 70% ethanol. After the wash, centrifugation was carried out at 16,000*g* for 1 min. The supernatant was then discarded, and pellet was allowed to dry at room temperature (approximately 15 min). The DNA was then resuspended with 50 µL of sterile DNase free water. The purity and quantity of DNA obtained was determined using Epoch microplate spectrophotometer using 2 µL of the DNA sample while the integrity of the DNA samples was determined using agarose gel electrophoresis

3.2.6 Primer Design

The full-length genomic sequences of the three homeologues of *TaABA8'OH* and *TaABI5* were identified by BLAST searching the sequences of *TaABA8'OH* (GeneBank ID: AB714574.1) and

ABI5 (GeneBank ID: AB238932) against URGI (https://wheat-urgi.versailles.inra.fr/) and ENSEMBL PLANTS (http://uswest.ensembl.org/index.htmL) databases. After obtaining the genomic sequences of the three homologues of each gene, the sequences were multi aligned and polymorphic regions in 5' and 3' UTRs were chosen for designing genome specific primers. Primers were designed using the NCBI primer designing tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 3.2).

Table 3.2 Sequence analysis of genome specific primers for amplifying *TaABA8'0H1* and *TaABI5* genes

Primer Name	Type	Primer sequence (5'to 3')	Amplicon
TaABA8'0H1A	Forward	AGCTGACGGAACCGACGAAAG	2081bp
	Reverse	GGCGTCGCCTCTATCGTGCCG	
TaABA8'0H1B	Forward	CCTCGTCTTCCTCGGACT	2085bp
	Reverse	GGAGCCGATCCAGCTCGCATG	
TaABA8'0H1D	Forward	GCTAACGGAACCGAAGAAAGC	2015bp
	Reverse	TCGCCTCTATCGCGCTGTTGA	
TaABI5A	Forward	GGAGAACGCTCGTCTGAAAG	1795bp
	Reverse	TGACGTTCACGTTCTCCTTG	
TaABI5B	Forward	TACCCACAAGCAAGAGAGTT	1676bp
	Reverse	GGAAGCTGAATGGCAGGTCT	
TaABI5D	Forward	GTAGAGCGAGACCAACCACA	1737bp
	Reverse	CCGGAAAGATAATGTTACTGCTATAAA	

3.2.7 PCR assay

The A, B and D genome copies of *TaABA8'OH1* and *TaABI5* were amplified using PCR using gene specific primers (Table 3.1). The amplification was performed with 30-50 ng of gDNA as a template, 2 µL of 10X Buffer, 0.2 µL of 10 mM dNTP's, 0.6 µL of 10 µM forward primer, 0.6 µL

of 10 µM reverse primer, 0.1 µL of Dream Taq polymerase (5 units/µL) and 15.8 µL sterile water with a total reaction volume of 20 µL. Amplification was performed by using of the following thermocycling condition: initial denaturation at 95°C for 3 min followed by 35 cycles of 95°C for 30 secs, annealing at 59.5°C for 30 secs (*TaABI5A*), 58°C for 30 sec (*TaABI5B*), 57°C for 30 sec (*TaABI5D*), and 61°C for (*TaABA8'0H1A, TaABA8'0H1B, TaABA8OH1D*), and initial extension at 72°C for 2 min and final extension at 72°C for 10 min. For size determination, the amplified products were mixed with 2 µL of 6X loading dye and 20 µL of the sample was loaded into 1.5% agarose gel prepared in 0.5X TBE buffer. DNA marker (1µg; Invitrogen, Carlsbad, CA, USA) was also loaded to the gel and used to determine the sizes of the fragments. After running the gel, it was visualized under UV light and photographed using Gel documentation system (Bio-Rad).

3.2.8 Purification of PCR products

The gel consisting of the target DNA fragments was excised with a clean and sharp scalpel blade and transferred to a 2 mL microcentrifuge tube. The excised DNA fragments were purified using Thermo Scientific GeneJET PCR Purification Kit (Thermo Scientific, MA, USA) following the manufacturer's protocol**.** The gel piece was placed into 2 mL microcentrifuge tube after which 100 µL of Binding Solution was added for every 100 mg of gel and incubated at 50-60°C until the gel piece was completely dissolved. After thoroughly mixing the contents, 800 µL of the mixture was transferred into GeneJET purification column placed onto a collection tube, and centrifuged at 16,000*g* for 1 min. After discarding the flow through, and the column was placed again onto the same collection tube. To remove impurities, 700 µL of Wash Solution (diluted with ethanol) was added and centrifuged at 16,000*g* for 1 min. After discarding the flow through, the column was placed back onto the same collection tube and centrifuged at 16,000*g* for 1 min. The column was

then transferred to a clean microcentrifuge tube and then 12 µL of sterile water was added to the column and incubated at room temperature for 5 min followed by centrifugation at 16,000*g* for 1 min to elute the DNA. The size of the eluted DNA was determined by gel electrophoresis using a 1 kb DNA marker.

3.2.9 Ligation and transformation

Cloning of the target genes was performed by using pGEM-T Easy vector systems (Promega, Madison, WI, USA). The PCR fragment with ligated to the vector using a reaction mixture containing 5 µL of 2X rapid ligation buffer, 1 µL of pGEM-T Easy vector (50 ng/µL), 2 µL of purified PCR product, 1 µL T4 DNA ligase (3 U/µL) and 1 µL sterile water with a total reaction volume of 10 µL. The reaction mixture was incubated at 16°C overnight using a thermocycler. The ligated product was transformed with *E. coli* DH5α competent cells (Invitrogen) plated on LB agar Petri dishes containing 20 µL of 100 mg/mL carbenicillin, 10 µL isopropyl β-D-1-thiogalactopyranoside (IPTG) and 25 µL of X-gal. The plates were incubated at 37°C overnight. Transformed colonies were used to inoculate 4 mL of LB media with carbenicillin. The inoculated LB media was incubated at 37°C overnight on a shaker at 225 rpm.

3.2.10 Plasmid isolation and restriction enzyme digestion

Plasmid extraction was carried out by using GeneJET Plasmid Miniprep Kit (Thermo Scientific) following the manufacturers protocol. The liquid culture was transferred to 2 mL tubes and centrifuged at 9,600*g* for 3 min. The pellet was mixed with 250 µL of resuspension solution and then 250 µL of lysis solution was added. Subsequently, 350 µL of neutralization solution was added followed by centrifugation at 12,000*g* for 5 min. The supernatant was transferred to

GeneJET spin column and centrifuged at 12,000*g* for 1 min. After discarding the flow through, 500 µL of wash solution (diluted with ethanol) was added to the column and centrifuged at 12,000 *g* for 1 min. This step was repeated to remove impurities. The GeneJET spin column was transferred to clean 1.5 mL tube, and then 30 µL of sterile water was added to elute the DNA followed by incubation for 2 min and centrifugation at 14,000*g* for 1 min. The resulting plasmid DNA was digested with EcoRI (Thermo Scientific) using a reaction mixture consisting of 2 µL of 10X buffer, 2 µL of plasmid DNA, 1 µL of ECORI and 15 µL of sterile water with total reaction volume of 20 µL. The reaction mixture was incubated at 37°C for 60 min and subjected to gel electrophoresis to verify the size of the cloned product.

3.2.11 DNA sequencing and phylogenetic analysis

The purified plasmid was sent out for DNA sequencing (Macrogen, USA). Sequencing of the *TaABI5* homeologues (*TaABI5A, TaABI5B* and *TaABI5D*) was performed with genome specific primers (Table 3.2) and the universal M-13 forward and M-13 reverse primers (Table 3.3). Sequencing of *TaABA8'OH1* homeologs (*TaABA8'OH1A, TaABA8'OH1B* and *TaABA8'OH1D*) was performed using the universal M13 forward and M13 reverse primers (Table 3.3) and internal primers (Table 3.3).

Table 3.3 Sequence of primers for DNA sequencing of *TaABA8'OH1* and *TaABI5*

Primer Name	Type	Primer sequence (5'to 3')
M13	Forward	GTAAAACGACGGCCAGT
M13	Reverse	GCGGATAACAATTTCACACAGG
CYP_B_INT-1	Forward	TCCCCGCCATCGAGGCTATCG
CYP_B_INT-2	Reverse	TCGGCGACAACCCCGCCG

The resulting sequences were BLAST searched against GeneBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm their identity with the annotated *ABI5* sequence. Fragments obtained by sequencing with each primer were assembled into one contiguous sequence using CAP3 sequence assembly tool (http://doua.prabi.fr/software/cap3). Alignments of the sequences from different lines were performed by using MultAlign (http://multalin.toulouse.inra.fr/multalin). After analyzing the sequence, the nucleotide sequences of *TaABI5* were translated into amino acid sequences by using ExPASy-Translate tool (https://web.expasy.org/translate/).

For conserved domains analysis, available ABI5 protein sequences of wheat and Arabidopsis were downloaded from NCBI database and aligned by using DNAMAN.10 software (https://www.lynnon.com/). Phylogenetic analysis was carried out to show the similarity between *TaABI5* and other bZIP transcription factors of ABF/AREB/ABI5 subfamily of Arabidopsis and other cereal crops. To this end, 15 amino acid sequences of Arabidopsis and other cereals were downloaded from NCBI database and neighbor joining phylogenetic tree was created by using MEGA 7.0 software (https://www.megasoftware.net/).

3.2.12 RNA isolation

Extraction of RNA samples was conducted from imbibed seeds. For imbibition, mature seeds were first surface sterilised with 70% ethanol (1 min) and 5% sodium chloride (for 20 min on a shaker) and rinsed with sterile water five times. The seeds were then imbibed for 24 h between two layers of Whatman #1 filter papers in Petri-plate at 22^{o}C under darkness. Following 24 h imbibition, seeds were separated into embryo and endosperm tissues, and RNA isolation was performed as described previously with minor modification (Mornkham *et al.*, 2013). Briefly, the embryos tissues (~100 mg) were ground into fine powder in liquid nitrogen. After this, 950 µL of Buffer 1

(8 M LiCl and 2% PVP) was added along with 50 µL of β-mercaptoethanol into the fine powder followed by incubation at room temperature for 5 min. Subsequently, 150 µL of chloroform was added into the mixture followed by centrifugation at 2400*g* for 5 min. The supernatant was then discarded, and 588 µL of Buffer 2 (1.4% SDS w/v, 0.075 M NaCl, 0.025 M EDTA), 12 µL of β-mercaptoethanol and 600 µL chloroform were added to resuspend the pellet. After centrifugation at 2400*g* for 5 min, 500 µL of the supernatant was transferred to a new tube, and an equal volume of isopropanol was added. The mixture was then centrifuged at 12,000*g* for 10 min to form the pellet. The pellet was washed with 1 mL of 75% ethanol and air dried for 10-15 min at room temperature, and then dissolved with 50 µL of DEPC water followed by addition of 700 µL of TRIZOL reagent (Thermo scientific) and 140 µL of chloroform. Following centrifugation, the clear supernatant containing the RNA was transferred to a fresh tube and mixed with isopropanol. The mixture was incubated for 20 min at room temperature and then centrifuged 12,000*g* for 10 min. After removing the supernatant, the pellet was washed with 75% ethanol and then dissolved with 50 µL DEPC water. The RNA samples were immediately stored at -80°C until future use.

3.2.13 DNA digestion and cDNA synthesis

To remove the genomic DNA contaminants, the RNA samples were digested with DNA-Free Kit (Ambion, Austin, TX, USA). The reaction mixture contained up to 10 µg of RNA, 5 µL of 10X DNase 1 Buffer, 1 µL of DNase (2 U) and sterile water to make total volume of 50 µL. After incubating the mixture at 37°C for 30 min, 5 µL DNase inactivation reagent was added. The mixture was then incubated at room temperature for 2 min followed by centrifugation at 10,000*g* for 2 min. After digestion, cDNA was synthesized by using iScript Reverse transcriptase supermix (Bio-Rad, Hercules, CA, USA). The cDNA synthesis reaction mixture contained 4 µL of iScript,

1 µg RNA and sterile water to make a total reaction volume of 20 µL. The reaction mixture was incubated at 25°C for 5 min followed by reverse transcription at 46°C for 20 min and inactivation of the reverse transcriptase at 95°C for 1 min. The cDNA samples were diluted 20X before use for quantitative PCR (qPCR) assay.

3.2.14 qPCR assay

qPCR assays were performed with gene specific primers which are designed to pick the total gene expression of the target (*TaABI5* and *Ta ABA8'OH1*) and the reference *Taβ-actin* (Table 3.4) genes. The reaction mixture contains 5 µL of diluted cDNA as template,10 µL of SsoFast EvaGreen Supermix (Bio-Rad, USA), 1.2 µL of 5 µM forward primer (300 nM final concentration), 1.2 µL of 5 µM reverse primer (300 nM final concentration) and 2.6 µL of nuclease free water with a total reaction volume of 20 µL. The thermocycling condition was carried out in 96-well plates using CFX96 Real-Time PCR with temperature conditions; 95°C for 3 min followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 secs and extension at 72°C for 30 sec. The relative expression levels of the target genes was calculated according to Livak and Schmittgen (2001) after normalization with *Taβ-actin*.

Table 3.4 Sequence of gene specific primers for qPCR analysis of *TaABA8'OH1* and *TaABI5*

Primer Name	Type	Primer sequence (5'to 3')
TaABA8'OH1	Forward	GCCAGGAAGCGGAACAAG
	Reverse	AAGAGGTGCGCCTGAGTA
TaABI5	Forward	GGAGAACGCTCGTCTGAAAG
	Reverse	TGACGTTCACGTTCTCCTTG
Taβ-actin	Forward	GCTGGAAGGTGCTGAGGGA
	Reverse	GCATCGCCGACAGGATGAG

3.2.15 Statistical analysis

Significant differences in germination index and relative gene expression levels of *TaABI5* and *TaABA8'OH1* among the different wheat genotypes was tested using one-way ANOVA. Fisher's least significant difference (LSD) test at the 5% level of significance was used to compare the mean values. Correlation between seed dormancy and expression of *TaABI5* and *TaABA8'OH1* in different the different genotypes was analyzed by using Pearson's coefficient of correlation (r), which was calculated by using the mean values.

3.3 Results

3.3.1 Seed germination

Seed dormancy level of the different wheat genotypes was evaluated using the germination index (GI), which is calculated based on monitoring of germination daily for a period of 1, 2 and 3 weeks. Based on the GI data for 1 week (7 days), “Janz” has the highest GI value of 0.98 and “AUS 1408”, “OS38”, “HR5603”, “Red RL4137” , “Snowbird”, “SC8019-R1”, “AC Domain”, “AC Majestic” and “White RL4137” had the lowest GI of 0.0 (Appendix 1). Germination index values were also generated based on the monitoring germination for an additional week (a total of 2 weeks), and based on these GI values, genotype “Janz” again exhibited the highest GI value of 0.99 while genotype “AUS 1408”, “OS38”, “HR5603”, “Red RL4137” and “Snowbird” showed the lowest GI value of 0.0 (Figure 3.1). A similar pattern of germination index was observed following seed imbibition for 3 weeks (Appendix 3). Overall, based on the 2 weeks GI data, seed dormancy level of the different genotypes was categorized into three classes: genotypes with high level of dormancy (GI < 0.25), intermediate level of dormancy (0.25-0.75), and low level of dormancy (non-dormant) (> 0.75) (Figure 3.1).

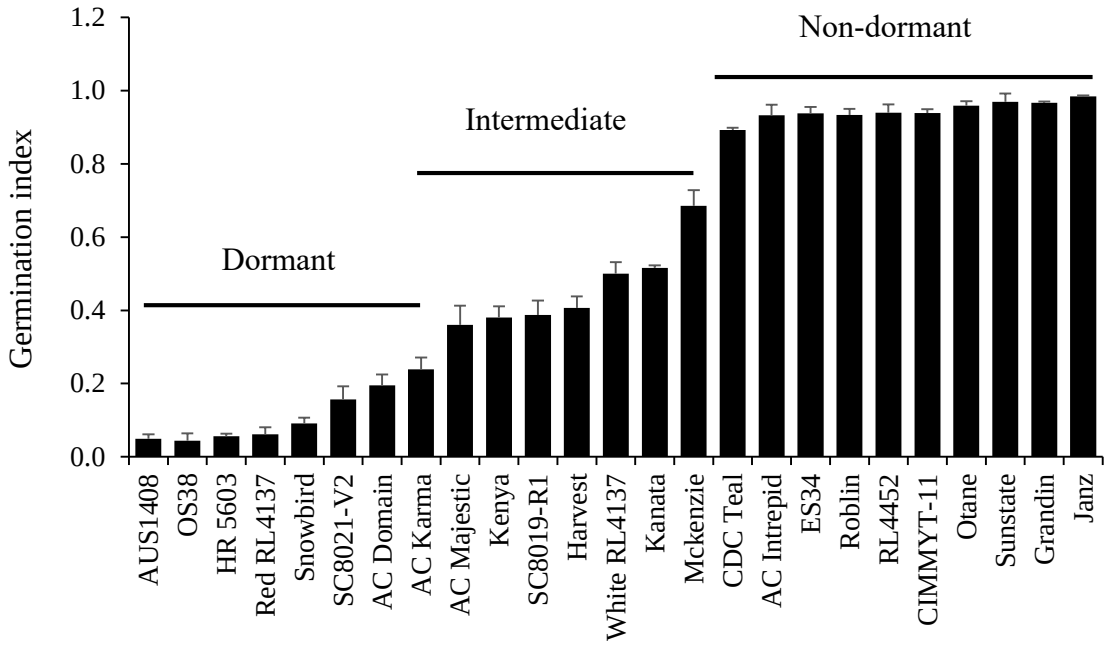

Figure 3.1 Germination index of the different wheat genotypes after imbibition for a period of two weeks. Data are means of three biological replicates ± SE. Significant differences between the dormancy levels was calculated using Fisher LSD method ($P < 0.05$; Appendix 2).

3.3.2 Molecular cloning of *TaABA8'OH1* in different wheat genotypes

Amplification of the genomic sequences of the three homeologs of *TaABA8'OH1* was attempted using homeolog/genome specific primers, however, only the primers that are specific to the B genome copy of *TaABA8'OH1B* were able to produce DNA fragment of expected size (2085 bp). (Figure 3.2)

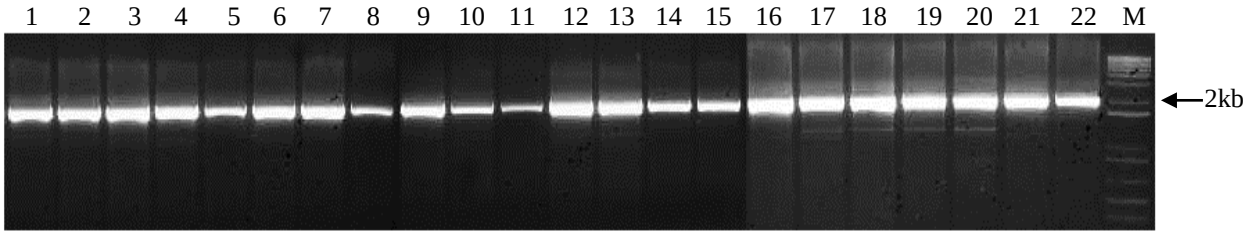

Figure 3.2 Amplification products of the B genome copy of *TaABA8'OH1* from different wheat genotypes. Lane 1, AC Domain; Lane 2, AC Karma; Lane 3, AC Majestic; Lane 4, AUS1408; Lane 5, Kanata; Lane 6, Snowbird; Lane 7, ES34; Lane 8, Grandin; Lane 9, Janz; Lane 10, Kenya; Lane 1, Otane; Lane 12, Red RL4137; Lane 13, Roblin; Lane 14, SC8019-R1; Lane 15, SC8021-V2; Lane 16, Sunstate; Lane 17, White RL4137; Lane 18, OS38; Lane 19, Mckenzie; Lane 20, CIMMYT-11; Lane 2, Harvest; Lane 22, HR5603; Lane, 23 AC Intrepid; Lane 24, CDC Teal; Lane 25, RL4452. Lane M is for 1Kb plus DNA ladder.

3.3.2.1. Analysis of the *TaABA8'OH1B* genomic sequences

Sequencing of the purified PCR fragments obtained from the different genotypes and BLAST searching of the resulting sequences against the GenBank database displayed that B genome copy of *TaABA8'OH1* exhibited 99% homology to annotated *TaABA8'OH1B* (GenBank ID: AB714575.1) and *TaABA8'OH1* (EU430344.1) gene sequences (Table 3.5).

Table 3.5. Identity of the genomic nucleotide sequence of *TaABA8'OH1B* homeolog with other *TaABA8'OH1* from wheat in GenBank database

Accession	Gene	Max Score[a]	E-value[b]	Max Identity[c]
AB714575.1	*TaABA8'OH1B*	3831	0.0	99%
EU430344.1	*TaABA8'OH1*	3749	0.0	99%

[a]Maximum score indicates the amount of sequence homology between *TaABA8OH1* and *ABA8OH1*s from other species.
[b]E-Value determines the significance of the alignment score.
[c]Maximum identity refers to percentage of identical nucleotides between *TaABA8OH1* and the respective *ABA8OH1*s from other species.

3.3.2.2 Allelic variations in *TaABA8'OH1B* sequences

Analysis of the full-length genomic sequences of *TaABA8'OH1B* derived from the 22 different wheat genotypes for allelic variation using the Multalin sequence alignment tools showed that no nucleotide substitution occurred in the *TaABA8'OH1B* (Figure 3.3).

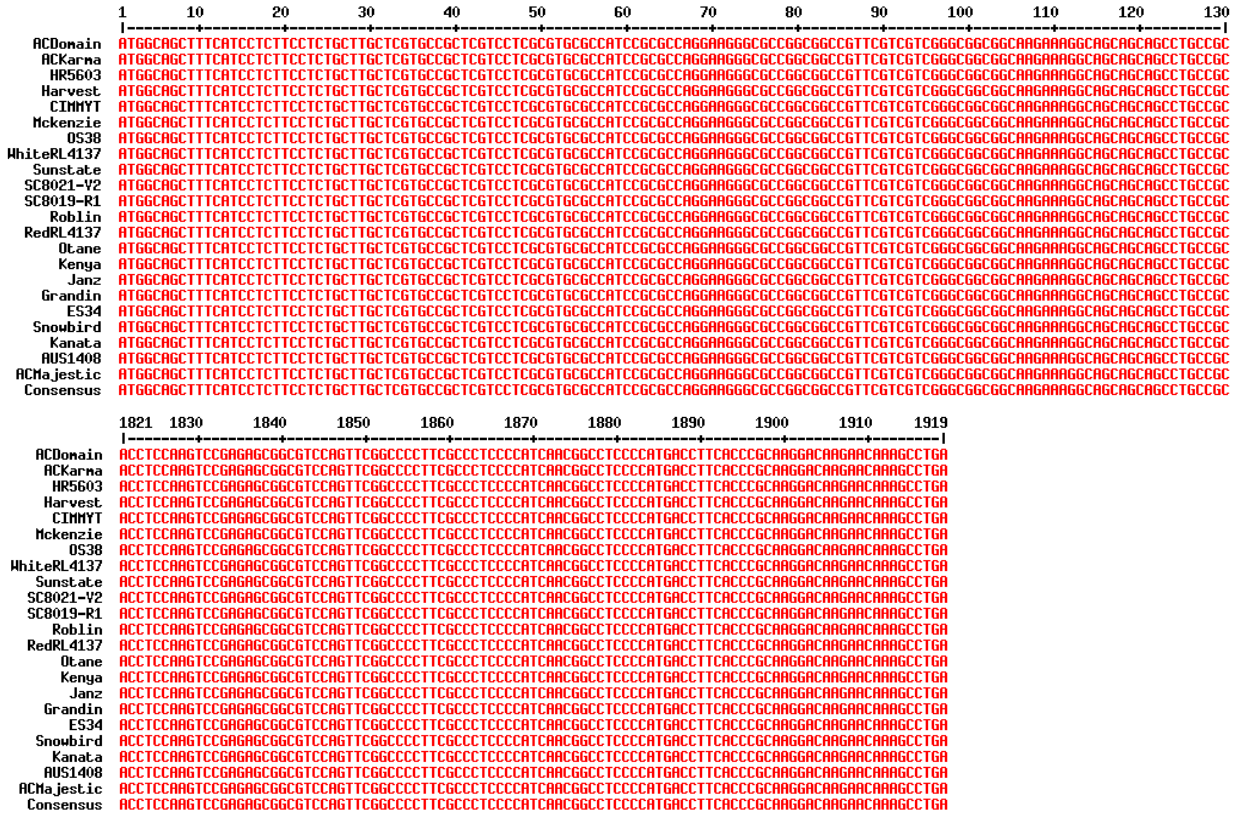

Figure 3.3 Multiple sequence alignment of the genomic nucleotide sequences of *TaABA8'OH1B* originated from different wheat genotypes.

3.3.2.3 Exonic and Intronic regions of *TaABA8'OH1B*

BLAST searching of putative full-length genomic sequences of *TaABA8'OH1B* obtained from the different wheat genotypes against Ensembl Plants database (https://plants.ensembl.org/index.html) showed that the DNA fragment contains five exons (coding region) and four introns (non-coding

region). The coding region of *TaABA8'OH1B* originated from all the genotypes studied appeared to have a size of 1431 bp (Figure 3.4).

ATGGCAGCTTTCATCCTCTTCCTCTGCTTGCTCGTGCCGCTCGTCCTCGCGTGCGCCATCCGCG
CCAGGAAGGGCGCCGGCGGCCGTTCGTCGTCGGGCGGCGGCAAGAAAGGCAGCAGCAGCCTGCC
GCTGCCGCCCGGGTCGATGGGGTGGCCGTACGTGGGCGAGACCACGCAGCTGTACTCCTCCAAG
AACCCCAACGTCTTCTTCGCCAGGAAGCGGAACAAGTACGGGCCCATCTTCAAGACGCATATCC
TCGGGTGCCCCTGCGTCATGGTGTCCAGCCCCGAGGCCGCGAAGTTCGTGCTTGTTACTCAGGC
GCACCTCTTCAAGCCTACCTTCCCGGCCAGCAAGGAGCGGATGCTGGGTCCCCAGGCCATCTTC
TTCCAGCAGGGGGACTACCACGCTCACCTCCGCCGTCTGGTCTCCCGCGCCTTCTCCCCCGAGG
CCATCCGCGGCTCCGTCCCCGCCATCGAGGCTATCGCCCTCCGCTCCCTCGGCTCCTGGGAAGA
CCTTCAAGTCAACACCTTCCAAGAGATGAAGACTGTGAGTGCTTCTTCCTCTTCCATTCCCACT
TGCTCTGCTTCCCTCTGCTCTGCTCTACTGCTAAATAATTGGAGCTTGAGACTGATCCTTCTGT
TGGTGTCGTGGCGCAGTACGCTCTGAATGTGGCATTGCTGTCCATCTTCGGGGAGGAGGAGATG
CAGTACATCGAGGAGCTGAAGCAGTGCTACCTGACGCTGGAGAAGGGGTATAACTCGATGCCGG
TGAACCTGCCGGGCACGCTGTTCCACAAGGCCATGAAGGCCCGGAAGCGGCTGGGCGCCATTGT
GGCCCACATCATCTCGGCCCGGCGCGAGCGTGAGCGCGGGAGCGACCTCCTGGGCTCCTTCATG
GACGGCCGCGAGGCGCTCACCGACGACCAGATCGCCGACAACGCCATCGGCGTCATCTTCGCCG
CGCGCGACACCACCGCCAGCGTGCTCACGTGGATGGTCAAGTTCCTCGGCGACAACCCCGCCGT
GCTCAAAGCCGTCACCGTAAGTCGCCATCAACCAGCTGACCCGCTTGGTACCCGATCGAAAAGC
AGCGACTGACCCGTGCATCCAACAATTAACAGGAAGAGCATGCCGAGATCGCGAGGGAGAAGGC
GTTGTCCGGCGAGCCGCTGTCGTGGGCCGACACGCGGCGGATGCGGATGACGGGCCGGGTGATC
CAGGAGACGATGCGGGTGGCGTCCATCCTCTCCTTCACCTTCAGGGAGGCCGTGGAGGACGTGG
AGTACCAAGGTGAGACATCAATCAACTTCGCGCGCGCGCGCTTTGGTCGTTTGCGGCAGCGC
AGCGCCGTACTGTGCTGTCCCTCTCGGAGTACAGCAGTGCGCTGCCTGCCTGCCTGCGCATGAA
CTGGCTCGGAAAGGACGTGCTCCTAACCGAACGGGAATAGACCAACTCGAACTCGCAACTCACC
TCGACTCGCTCTCTTCTGTGCGTGCAGGGTACCTGATTCCCAAGGGCTGGAAAGTGCTTCCCCT
GTTCCGGAACATCCACCACAACCCCGACCACTTCCCCTCCCCCGAAAAGTTCGATCCTTCACGA
TTCGAGGTCAGCATCATAACAACCCTCTATTTGACGAGCCTGCTTCGATTCGATTGATCATTAT
CTGATTATACGTTTTGGTTCGTGACTGCAGGTGGCCCCCAAGCCCAACACGTTCATGCCGTTCG
GGAACGGGACCCACTCGTGCCCCGGCAACGAGCTGGCCAAGCTGGAGATGCTCGTCCTCTGCCA
CCACCTCGCCACCAAGTACAGATGGTCCACCTCCAAGTCCGAGAGCGGCGTCCAGTTCGGCCCC
TTCGCCCTCCCCATCAACGGCCTCCCCATGACCTTCACCCGCAAGGACAAGAACAAAGCCTGA

Figure 3.4 Genomic nucleotide sequence of *TaABA8'OH1B*. Highlighted regions in grey represent exons while the regions that are not highlighted represent introns.

3.3.2.4 Amino acid sequence analysis of *TaABA8′OH1B*

Translation of the putative coding sequence of the *TaABA8′OH1B* from the different genotypes into amino acid sequence using ExPASy-Translate tool (https://web.expasy.org/translate) produced a protein with 476 amino acids (Figure 3.5). Since there was no allelic variation in the coding sequence of *TaABA8′OH1B* derived from the different lines, no change or substitution in in amino acid was evident.

```
MAAFILFLCLLVPLVLACAIRARKGAGGRSSSGGGKKGSSSLPLPPGSMGWPYVGETTQLYSSK
NPNVFFARKRNKYGPIFKTHILGCPCVMVSSPEAAKFVLVTQAHLFKPTFPASKERMLGPQAIF
FQQGDYHAHLRRLVSRAFSPEAIRGSVPAIEAIALRSLGSWEDLQVNTFQEMKTYALNVALLSI
FGEEEMQYIEELKQCYLTLEKGYNSMPVNLPGTLFHKAMKARKRLGAIVAHIISARRERERGSD
LLGSFMDGREALTDDQIADNAIGVIFAARDTTASVLTWMVKFLGDNPAVLKAVTEEHAEIAREK
ALSGEPLSWADTRRMRMTGRVIQETMRVASILSFTFREAVEDVEYQGYLIPKGWKVLPLFRNIH
HNPDHFPSPEKFDPSRFEVAPKPNTFMPFGNGTHSCPGNELAKLEMLVLCHHLATKYRWSTSKS
ESGVQFGPFALPINGLPMTFTRKDKNKA
```

Figure 3.5 Amino acid sequence translated from the coding region of TaABA8′OH1B.

3.3.2.5 Phylogenetic analysis of TaABA8′OH1B and its orthologs in other crops

Phylogenetic analysis of TaABA8′OH1B and its orthologs in other cereal and dicot species including Arabidopsis indicated that the B genome copy of TaABA8′OH1 is highly related with ABA8′OH1 of barley and the diploid progenitor of wheat (Figure 3.6).

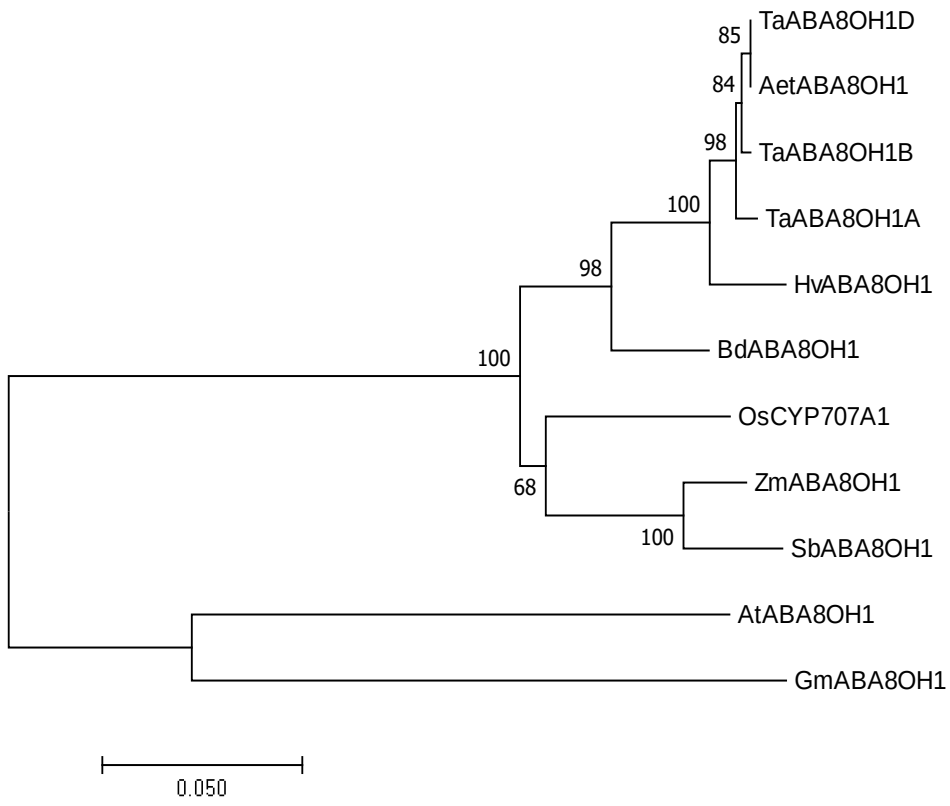

Figure 3.6 Phylogenetic analysis of the TaABA8′OH1 proteins derived from the different genotypes of wheat and other species. This evolutionary history of 11 amino acid sequences was constructed by the neighbour-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The evolutionary distance was computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site (0.05). The ABA8′OH1 protein sequences with their accession numbers are *Triticum aestivum*, TaABA8′OH1A, AB714574.1; TaABA8′OH1B, AB714575.1; TaABA8′OH1D, AB714576.1; *Aegilops taushii*, AetABA8′OH1, F775_16028; *Hordeum vulgare*, HvABA8′OH1, ABB71585.1; *Brachypodium distachyon*, BdABA8′OH1, BRADI3G52660.1; *Orzya sativa*, OsABA8′OH1, Os02g0703700; *Zea mays*, ZmABA8′OH1, Zm00001d017762_T002; *Sorghum bicolor*, SbABA8′OH1, SORBI_3004G268700; *Arabidopsis thaliana*, AtABA8′OH1, AT4G19230.1; and *Glycine max*, GmABA8′OH1, ABQ65856.1. The amino acid sequences TaABA8′OH1B derived from the different wheat genotypes studied here is identical to that obtained from the GenBank database (GenBank ID: AB714575.1).

3.3.2.6 Expression of *TaABA8'OH1* in imbibed seeds of different genotypes

Analysis of the total expression level of *TaABA8'OH1* in the embryos of imbibing seeds of the different wheat genotypes showed higher *TaABA8'OH1* expression level (over 3-foldwhen compared to the expression level detected in the non-dormant genotype "Janz", which is considered as a control) in genotypes that are characterized by a high dormancy level/lower GI values including "AUS1408", "HR5603", "Red RL4137", "Snowbird", "SC8021-V2", and "AC Karma" except "OS38" and "AC Domain" which displayed only slightly higher or a similar level of expression to the control (Figure 3.7). Genotypes that exhibit an intermediate level of dormancy/intermediate GI values including "Kenya", "AC Majestic", "SC8019-R1", "Kanata", "White RL4137" and "Mckenzie" showed relatively higher level of *TaABA8'OH1* expression (over 2.5- fold as compared to that detected in the non-dormant "Janz") except "Harvest", which showed a similar level of expression as observed for "Janz". Non-dormant genotypes that exhibit high GI values including "AC Intrepid", "Roblin", "Otane" and "Sunstate", showed similar or lower level of *TaABA8'OH1* expression as compared to the "Janz", while the other non-dormant genotypes including "CDC Teal", "ES34", "RL4452" and "Grandin" showed relatively higher levels of expression (≥ 2-fold) except "CIMMYT" that displayed over 5-fold expression level (Figure 3.7). The expression level of *TaABA8'OH1* in the embryos of imbibing seeds of the different wheat genotypes showed no significant difference among the genotypes studied ($P < 0.05$).

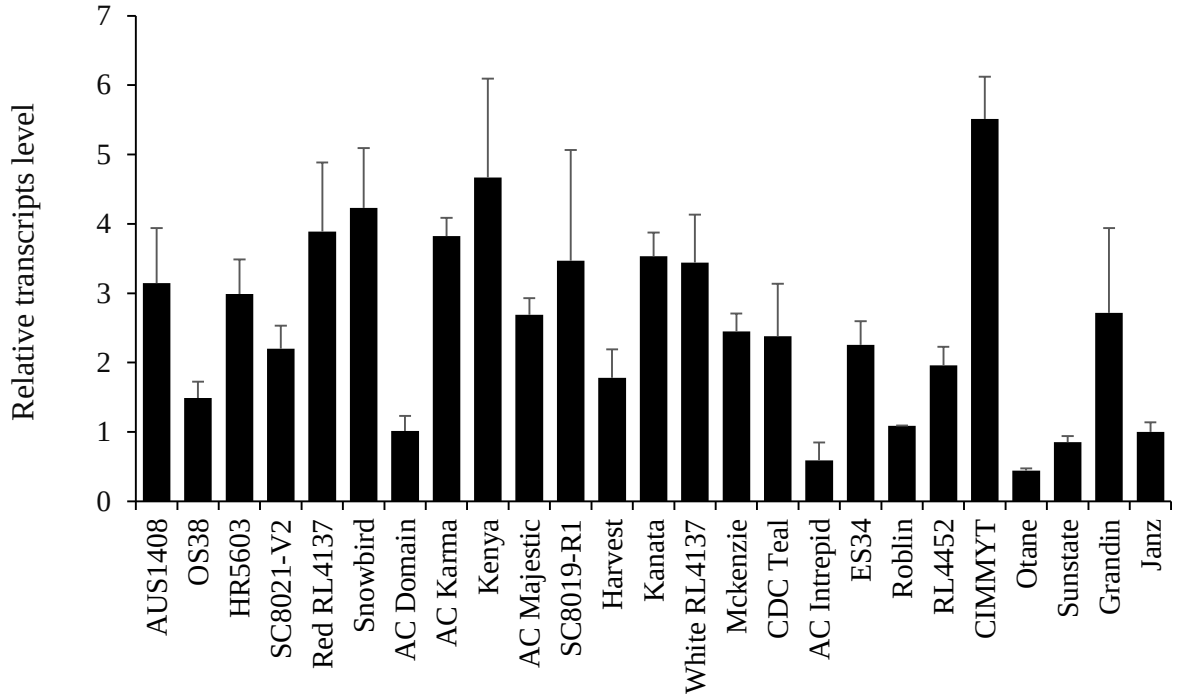

Figure 3.7 Relative transcript levels of *TaABA8'OH1* in the embryo wheat genotypes. Transcripts levels were compared using the mean transcript level of Janz, which was arbitrarily set to a value of 1. Data are means of three biological replicates ± SE. Significant difference in the relative transcript level *TaABA8'OH1* among the different genotypes was tested using Fisher LSD method ($P < 0.05$; Appendix 4).

3.3.2.7 Correlation between *TaABA8'OH1* expression and seed dormancy levels

The correlation between *TaABA8'OH1* expression and seed dormancy levels was determined using coefficient of co-relation (r) values between mean GI values and relative transcripts level of *TaABA8'OH1* in the different wheat genotypes. The correlation analysis revealed an r value of 0.31 (Figure 3.8). Similarly, correlation between the relative expression of *TaABA8'OH1* and seed dormancy level was evaluated using GI values obtained from 1 week and 3 week periods of imbibition (Appendix 5; Appendix 6).

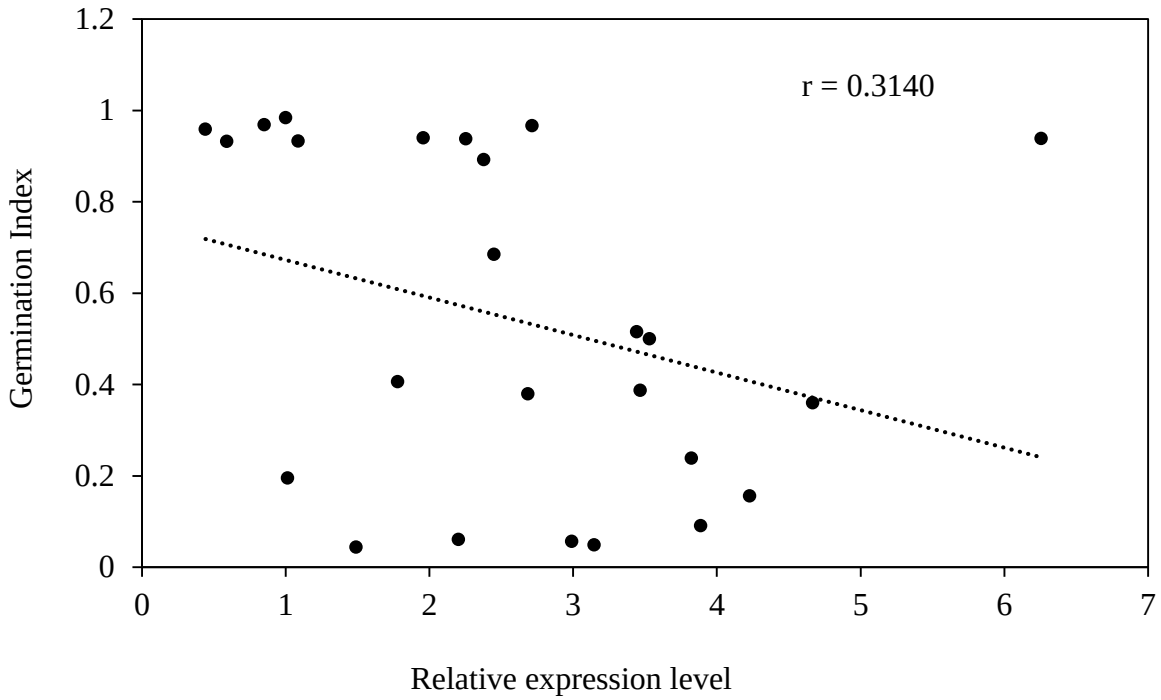

Figure 3.8 Correlation between the expression pattern of *TaABA8'OH1* and seed dormancy level of the different wheat genotypes.

3.3.3 Molecular cloning of *TaABI5* in different wheat genotypes

Amplification of the genomic sequences of the three homeologs of *TaABI5* from the 25 different wheat genotypes using homeolog/genome specific primers produced three different PCR fragments of expected size corresponding to *TaABI5A* (1795 bp), *TaABI5B* (1676 bp) and *TaABI5D* (1737 bp) (Figure 3.9).

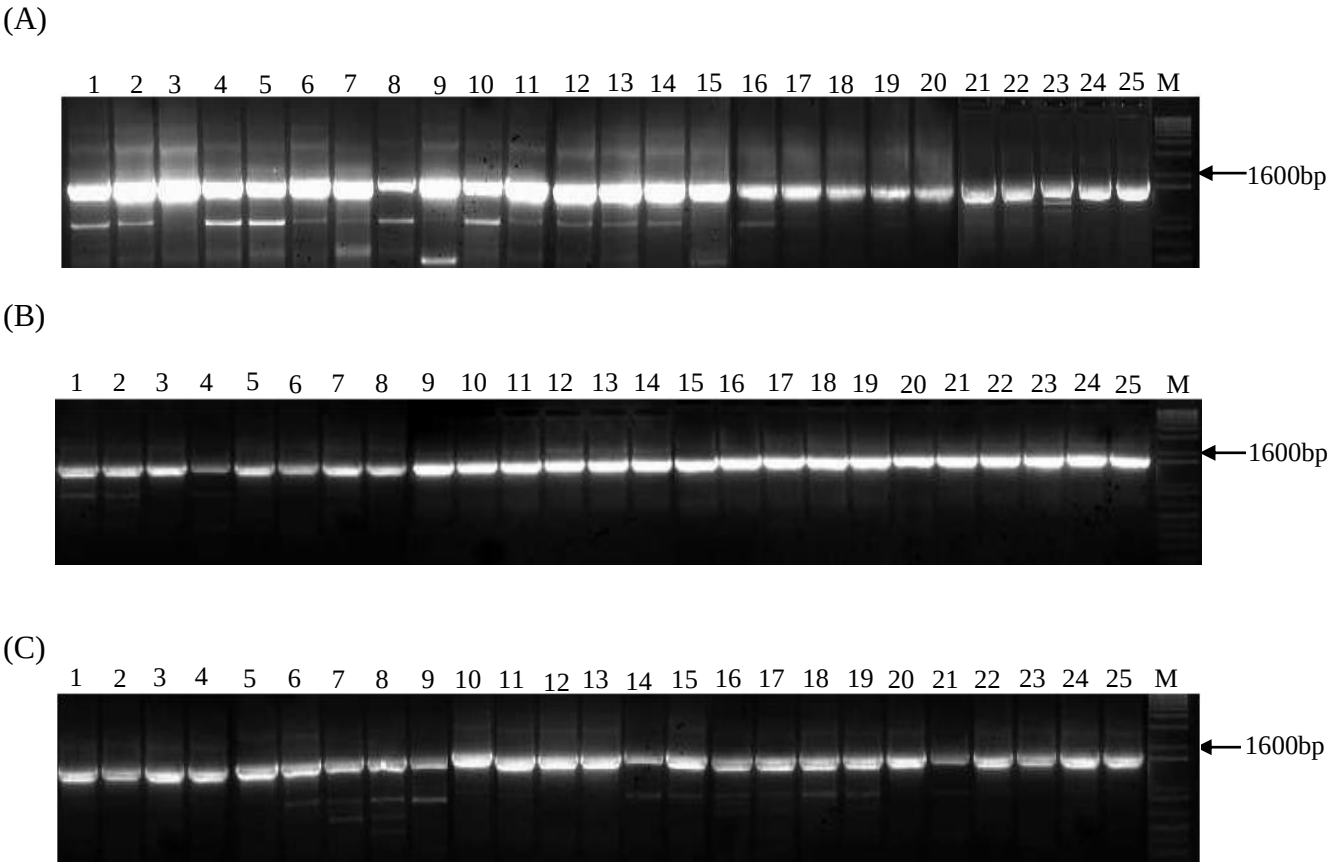

Figure 3.9 Amplification products of *TaABI5* homeologs from different wheat genotypes. Amplifications of *TaABI5A* (A), *TaABI5B* (B) and *TaABI5D* (C). Lane 1, AC Domain; Lane 2, AC Karma; Lane 3, AC Majestic; Lane 4, AUS1408; Lane 5, Kanata; Lane 6, Snowbird; Lane 7, ES34; Lane 8, Grandin; Lane 9, Janz; Lane 10, Kenya; Lane 1, Otane; Lane 12, Red RL4137; Lane 13, Roblin; Lane 14, SC8019-R1; Lane 15, SC8021-V2; Lane 16, Sunstate; Lane 17, White RL4137; Lane 18, OS38; Lane 19, Mckenzie; Lane 20, CIMMYT-11; Lane 2, Harvest; Lane 22, HR5603; Lane, 23 AC Intrepid; Lane 24, CDC Teal; Lane 25, RL4452. Lane M is for 1Kb plus DNA ladder.

3.3.3.1. Analysis of the *TaABI5* genomic sequences

Sequencing of the purified PCR fragments and BLAST searching of the resulting sequences against the GenBank database showed that the A genome copy of *ABI5* exhibited 98% homology with *TmABI5* (GenBank ID: AB238933.1) and 94% homology with *TaABI5* (GenBank ID: AB238934.1) (Table 3.6). On the other hand, the B genome copy of *ABI5* showed 92% homology

with *TaABI5* (GenBank ID: AB238933.1) and 91% homology with *TmABI5* (GenBank ID: AB238933.1) while the D genome copy displayed 100% homology with the gene sequence annotated as *TaABI5* (GenBank ID: AB238934.1), 95% homology with *TmABI5* (GenBank ID: AB238933.1) and 94% homology with *T. aestivum* 3B scaffold.

Table 3.6. Identity of the genomic nucleotide sequence of *TaABI5* homeologs with other *ABI5s* from wheat in GenBank database

Nucleotide sequence identity of TaABI5A with other gene sequences				
Accession	Gene	Max Score[a]	E-value[b]	Max Identity[c]
AB238933.1	*TmABI5*	2669	0.0	98%
AB238934.1	*TaABI5*	2318	0.0	94%
Nucleotide sequence identity of TaABI5B with other gene sequences				
Accession	Gene	Max Score[a]	E-value[b]	Max Identity[c]
AB238934.1	*TaABI5*	2126	0.0	92%
AB238933.1	*TmABI5*[d]	2017	0.0	91%
Nucleotide sequence identity of TaABI5D with other gene sequences				
Accession	Gene	Max Score[a]	E-value[b]	Max Identity[c]
AB238934.1	*TaABI5*	2833	0.0	100%
AB238933.1	*TmABI5*	2390	0.0	95%
HG670306.1	*T. aestivum* 3B scaffold	2327	0.0	94%

[a]Maximum score indicates the amount of sequence homology between *TaABI5* and *ABI5*s from other species.
[b]E-Value determines the significance of the alignment score.
[c]Maximum identity refers to percentage of identical nucleotides between *TaABI5* and *ABI5*s from other species.
[d]*Tm: Triticum monococcum*

3.3.3.2 Allelic variations in target gene sequences

The full-length genomic sequences of *TaABI5A, TaABI5B* and *TaABI5D* derived from the 25 different wheat genotypes were analyzed for allelic variation using the Multalin sequence

alignment tools. Comparison of the genomic sequences of *TaABI5A* originated from the 25 genotypes showed four nucleotide substitutions, and these include the substitution of guanine (G) by thymine (T) at 365th position in AC Domain, Harvest, Roblin, Sunstate, Janz, CIMMYT-11, Kanata and CDC Teal. In addition, substitutions of thymine (T) by guanine (G) at 378th position in AC Domain, Harvest, Roblin, Sunstate, Janz, CIMMYT-11, Kanata, AC Karma, AC Majestic, Snowbird, HR5603, Red RL4137, Kenya, Mckenzie, OS38, Otane, SC8021-V2 and SC8019-R1, cytosine (C) by adenine (A) at 408th position, and guanine (G) by adenine (A) at 765th position were observed in the genomic sequences of *TaABI5A* in AC Domain, Harvest, Roblin, Sunstate, Janz, CIMMYT-11, AC Karma, AC Majestic, Snowbird, HR5603, Red RL4137, Kenya, Mckenzie, OS38, Otane, SC8021-V2, SC8019-R1 and CDC Teal genotypes (Figure 3.10). No nucleotide variation was found in in the genomic nucleotide sequences of *TaABI5B* and *TaABI5D* (Appendix 7; Appendix 8).

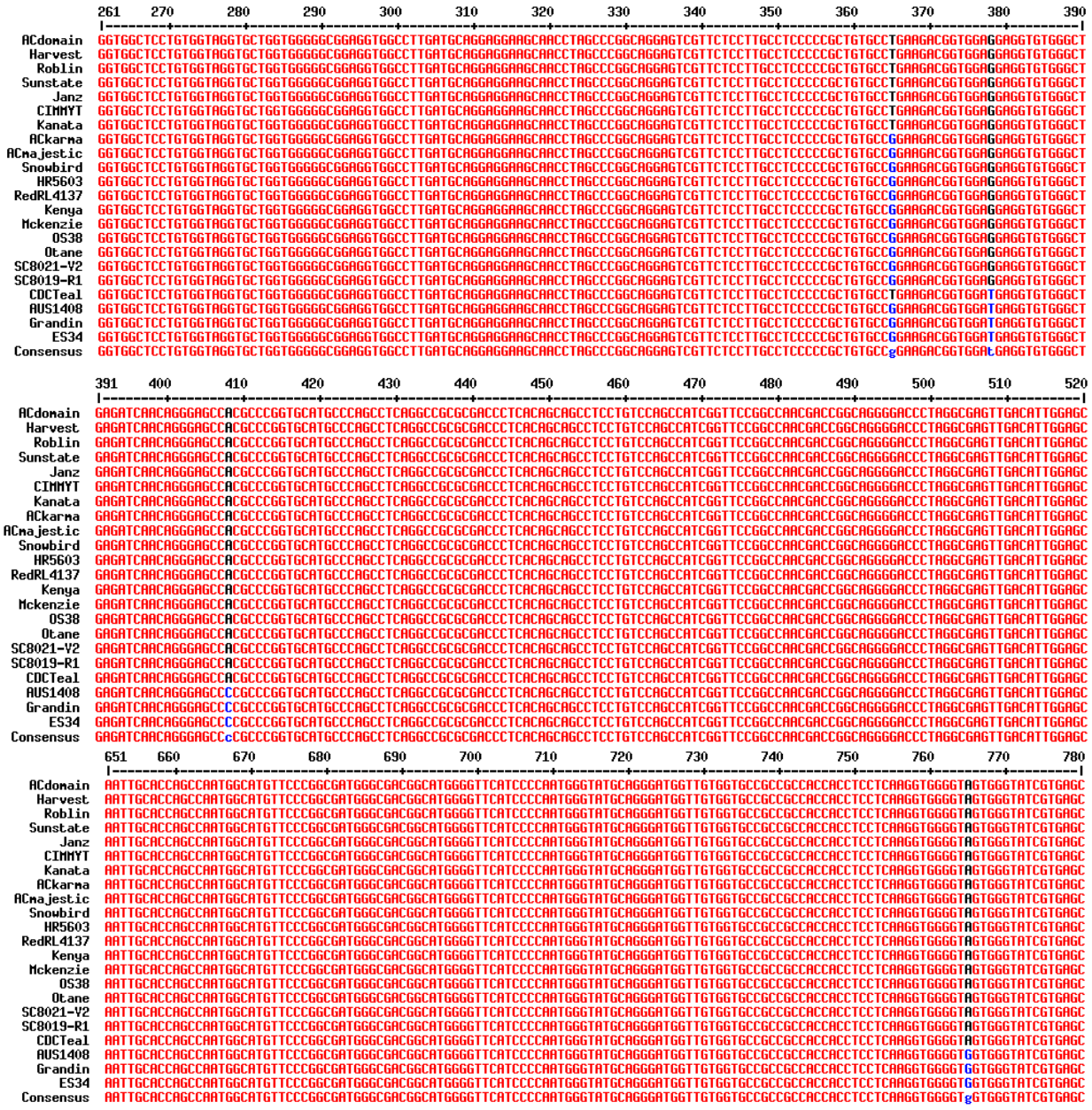

Figure 3.10 Multiple sequence alignment of the genomic nucleotide sequences of *TaABI5A* originated from the 22 different wheat genotypes.

3.3.3.3 Exonic and Intronic regions of *TaABI5*

Analysis of the putative genomic nucleotide sequences of *TaABI5* homeologs against Ensembl Plants database (https://plants.ensembl.org/index.html) showed that the DNA fragments of

TaABI5A, TaABI5B and TaABI5D contains four exons (coding region) and three introns (non-coding region). The allelic variations observed in the sequences of *TaABI5A* occur in the first exon of *TaABI5A* (Figure 3.11). The coding region of *TaABI5A*, *TaABI5B* and *TaABI5D* originated from all the genotypes studied appeared to have coding sequences of 1176 bp, 1164 bp, and 1173 bp, respectively (Figure 3.11, Appendix 9, Appendix 10).

ATGGCGTCGGAGATGAGCAAGGATGTGAAGTTCTCCGAGGAGGAAGTCACCTCACACCCGCGCG
TTCTCGAAGGTGAGGAGCAGGCGGTTGCGCCGGCGCGGCAGTCTTCCATCTTCGCGCTGACGCT
GGACGAGCTGCAATACTCGGTGTGCGAGGCAGGGCGCAATTTTGGGTCCATGAACATGGACGAG
TTCATGAGCAACATATGGAATGCCGATGAGTTCCAAGCAGCGACCGGCGGTGGCTTGGTGGGCA
TGGAGGTGGCTCCTGTGGTAGGTGCTGGTGGGGGCGGAGGTGGCCTTGATGCAGGAGGAAGCAA
CCTAGCCCGGCAGGAGTCGTTCTCCTTGCCTCCCCCGCTGTGCC**T**GAAGACGGTGGA**G**GAGGTG
TGGGCTGAGATCAACAGGGAGCC**A**CGCCCGGTGCATGCCCAGCCTCAGGCCGCGCGACCCTCAC
AGCAGCCTCCTGTCCAGCCATCGGTTCCGGCCAACGACCGGCAGGGGACCCTAGGCGAGTTGAC
ATTGGAGCAGTTCCTTGTCAAGGCCGGCGTGGTCCGGGGATCTGGCGCCGGCGGCCAGGCGCCT
GTGCCGGTCGGCATGGTCCATGGACAGATGAACCCCGCACAACAGGGGCAGCAGCCTGGCCCAA
TGATGTACCCAATTGCACCAGCCAATGGCATGTTCCCGGCGATGGGCGACGGCATGGGGTTCAT
CCCCAATGGGTATGCAGGGATGGTTGTGGTGCCGCCGCCACCACCTCCTCAAGGTGGGGT**A**GTG
GGTATCGTGAGCCCCGGGTCGTCGGACGGGAGGAGCGCCATGACGCAGGCTGACATGATGAACT
GCATGGGCGAAGGAGCGATGATGGAGAATGGCGGCACCCGGAAACGCGGTGCCCCAGAGGATCA
GTCTTGCGAGAGAAGCATCGAGCGCCGCCACCGCCGCATGATCAAGAACCGTGAGTCAGCGGCA
CGATCGCGTGCTAGGAAGCAGGTACTTACCTTCCTGCCAAAATTGTTGGTTTTTATGCAATGCT
ATTTCTGTAACGGTGGTAACAATGTTGGTGACACCTACACTACCTGTCTTTCTAAATCCTACAG
GCTTATACCGTGGAGCTTGAAGCTGAACTGAACCACCTCAAGGAGGAGAACGCTCGTCTGAAAG
CCGAGGAGGTACAGAGCTGCTGACCAGTGAAAACCATGAGAAAATTCAGTTTTCATATGCTTTT
CAGATCTTACCATTACTAAATTGTTTCATTGTTTTGTTGCAGAAGACAATTCTGCTGACCAAGA
AACAAATGGTATGTACAGTTTCCAACCATCACCTGGCAATCCTCCTACACTCGCCCCACTTGCC
AATTGCTTGTAACAAATCCACTATTATTGCTAAGTCTTAAAATAGTCAATGTGTGCATGCTGAT
GGAAATGCCTTGTGGCGGCGATCTTTTTTTACAGCTGGTGGAGAAAATGATAGAACAGTCCAAA
GAGAACGTGAACGCCAAGAAGGGTGCCCCCCTCTCGCGGCACTGCGGCAGCTGCATCTGGTGA

Figure 3.11 Genomic nucleotide sequence of *TaABI5A*. Regions highlighted in grey represent exons while regions that are not highlighted indicate introns. Nucleotides in bold and highlighted in yellow represent the positions at which allelic variation occurred.

3.3.3.4 Amino acid sequence analysis of *TaABI5*

Translation of the putative coding sequences of the three homeologs of *TaABI5* from the different genotypes into amino acid sequences using ExPASy-Translate tool (https://web.expasy.org/translate/) produced a protein with 391 amino acids for TaABI5A, 387 amino acids for TaABI5B and 390 amino acids for TaABI5D (Figure 3.12, Appendix 9, Appendix 10). Since there was no allelic variation in the coding sequence of *TaABI5B* and *TaABI5D* derived from the different lines, no change in amino acid sequence was evident (Appendix 11, Appendix 12).

```
MASEMSKDVKFSEEEVTSHPRVLEGEEQAVAPARQSSIFALTLDELQYSVCEAGRNFGSMNMDE
FMSNIWNADEFQAATGGGLVGMEVAPVVGAGGGGGGLDAGGSNLARQESFSLPPPLCRKTVDEV
WAEINREPRPVHAQPQAARPSQQPPVQPSVPANDRQGTLGELTLEQFLVKAGVVRGSGAGGQAP
VPVGMVHGQMNPAQQGQQPGPMMYPIAPANGMFPAMGDGMGFIPNGYAGMVVVPPPPPPQGGVV
GIVSPGSSDGRSAMTQADMMNCMGEGAMMENGGTRKRGAPEDQSCERSIERRHRRMIKNRESAA
RSRARKQAYTVELEAELNHLKEENARLKAEEKTILLTKKQMLVEKMIEQSKENVNAKKGAPLSR
HCGSCIW
```

Figure 3.12 Amino acid sequence translated from the coding region of TaABI5A.

Alignment of the putative amino acid sequences of TaABI5A from genotypes AC Karma, AC Majestic, Snowbird, HR5603, Red RL4137, Kenya, Mckenzie, OS38, Otane, SC8021-V2 and SC8019-R1 (designated as Group 1) while amino acid sequences of AC Domain, Harvest, Roblin, Sunstate, Janz, CIMMYT-11, Kanata and CDC Teal (designated as Group 2) using CLUSTALW alignment tool (https://www.ebi.ac.uk/Tools/msa/clustalw2/) revealed two of the nucleotide/base substitutions resulted in change of amino acid from arginine (R) to leucine (L) at 122th position and from aspartate (D) to glutamate (E) at 126th position. In addition, two substitutions coding for the same amino acid were observed at 136th and 255th amino acid positions. These types of base substitutions that result no amino acid change are also termed as silent substitutions (Figure 3.13).

```
Group 1       MASEMSKDVKFSEEEVTSHPRVLEGEEQAVAPARQSSIFALTLDELQYSVCEAGRNFGSM
Group 2       MASEMSKDVKFSEEEVTSHPRVLEGEEQAVAPARQSSIFALTLDELQYSVCEAGRNFGSM
              ************************************************************

Group 1       NMDEFMSNIWNADEFQAATGGGLVGMEVAPVVGAGGGGGGLDAGGSNLARQESFSLPPPL
Group 2       NMDEFMSNIWNADEFQAATGGGLVGMEVAPVVGAGGGGGGLDAGGSNLARQESFSLPPPL
              ************************************************************

Group 1       CRKTVDEVWAEINREPRPVHAQPQAARPSQQPPVQPSVPANDRQGTLGELTLEQFLVKAG
Group 2       CLKTVEEVWAEINREPRPVHAQPQAARPSQQPPVQPSVPANDRQGTLGELTLEQFLVKAG
              * ***:********************************************************

Group 1       VVRGSGAGGQAPVPVGMVHGQMNPAQQGQQPGPMMYPIAPANGMFPAMGDGMGFIPNGYA
Group 2       VVRGSGAGGQAPVPVGMVHGQMNPAQQGQQPGPMMYPIAPANGMFPAMGDGMGFIPNGYA
              ************************************************************

Group 1       GMVVVPPPPPPQGGVVGIVSPGSSDGRSAMTQADMMNCMGEGAMMENGGTRKRGAPEDQS
Group 2       GMVVVPPPPPPQGGVVGIVSPGSSDGRSAMTQADMMNCMGEGAMMENGGTRKRGAPEDQS
              ************************************************************

Group 1       CERSIERRHRRMIKNRESAARSRARKQAYTVELEAELNHLKEENARLKAEEKTILLTKKQ
Group 2       CERSIERRHRRMIKNRESAARSRARKQAYTVELEAELNHLKEENARLKAEEKTILLTKKQ
              ************************************************************

Group 1       MLVEKMIEQSKENVNAKKGAPLSRHCGSCIW 391
Group 2       MLVEKMIEQSKENVNAKKGAPLSRHCGSCIW 391
              ******************************
```

Figure 3.13 Amino acid sequence alignment of TaABI5As from the different wheat genotypes. Amino acids in bold, and yellow and blue highlights represent amino acid changes and silent substitutions, respectively. Group 1: AC Karma, AC Majestic, Snowbird, HR5603, Red RL4137, Kenya, Mckenzie, OS38, Otane, SC8021-V2 and SC8019-R1; Group 2: AC Domain, Harvest, Roblin, Sunstate, Janz, CIMMYT-11, Kanata and CDC Teal.

3.3.3.5 Analysis of the conserved domains of TaABI5

Amino acid sequence alignment of TaABI5 with other ABI5/ABF sub families derived from Arabidopsis showed the presence of three N-terminal conserved domains C1, C2 and C3, and one C-terminal conserved region, C4, and AB15/AREB/ABF bZIP domain. The amino acid substitutions, which occurred at 112^{th} and 126^{th} positions, are found to lie in the C2 conserved domain of TaABI5 (Figure 3.14).

Figure 3.14 Alignment of the amino acid sequence of TaABI5 with other proteins that belong to the Arabidopsis ABI5/ABF family members. The amino acid sequences compared include those derived from *Triticum aestivum*, TaABI5, AB238932; TaABF, AF519804; and those derived from *Arabidopsis thaliana*, AtABF1, AF093544; AtABF2, AF27180; AtABF3, AF093546; AtABF4, AF093547; AtABI5, AT2G36270; and TaABI5A, TaABI5B and TaABI5D (from this study). Black shading indicates conserved regions of C1, C2, C3, C4 and bZIP domains. The positions of conserved domains C1 to C4 and the bZIP domain are marked by solid orange line. Asterisks indicate phosphorylation sites. Blue circles represent the positions of substituted amino acids (R and E) in the amino acid sequence of TaABI5A in C2 domain.

3.3.3.6 Phylogenetic relationship between *TaABI5* and its orthologs in cereal crops

Phylogenetic analysis of TaABI5 and its orthologs in other cereal crops and Arabidopsis showed that TaABI5A is highly related with ABF of *Triticum monococum* and *Triticum aestivum* (Figure 3.15).

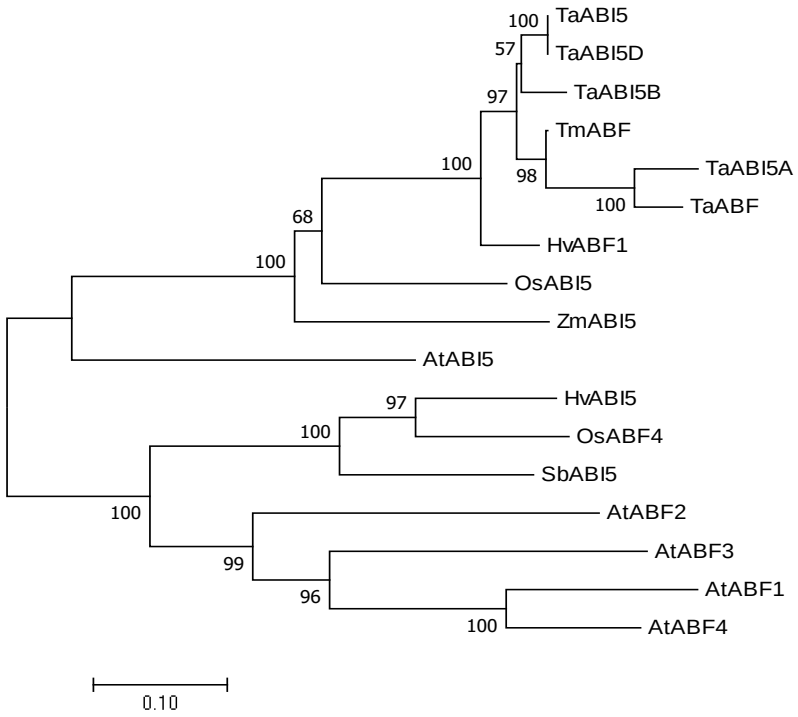

Figure 3.15 Phylogenetic tree of bZIP transcription factors of ABI5/ABF/AREB subfamilies of Arabidopsis and cereal crops including wheat, rice, sorghum, and maize. The 17 amino acids of ABI5/ABF/AREB subfamilies were aligned using Neighbour joining method to generate evolutionary history. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The aligned protein sequences with their accession numbers are *Triticum aestivum*, TaABI5, AB238932; TaABI5A, TaABI5B and TaABI5D (from this study); TaABF, AF519804*; Triticum monococum*, TmABF, BAF62441*; Horduem vulgare*, HvABF1, CAJW10244364*;* HvABI5, AAO06115*; Oryza sativa*, OsABI5, ABM90394*;* OsABF4, BAF625276*; Sorghum bicolor*, SbABI5, XM_002442802*; Arabidopsis thaliana*, AtABF1, AF093544; AtABF2, AF27180; AtABF3, AF093546; AtABF4, AF093547; AtABI5, AtABI5, AT2G36270.

3.3.3.7 Expression of *TaABI5* in wheat cultivars

Analysis of the total expression level of *TaABI5* in imbibing embryos of the seeds of the different wheat genotypes showed the prevalence of high expression level of *TaABI5* (over 10-fold as compared to the expression level detected in the non-dormant genotype Janz, which is considered as a control) in most genotypes that are characterized by high level of dormancy (lower GI values) including "AUS1408", "OS38", "Red RL4137", "SC8021-V2", "AC Domain" and "AC Karma" (Figure 3.16). Two genotypes, "HR5603" and "Snowbird" that exhibit high level of seed dormancy showed only 6- to 8-fold higher expression level of *TaABI5*, which is similar to that shown by genotypes with intermediate level of dormancy. Genotypes that are characterized by intermediate level of dormancy (intermediate GI values) including "Kenya", "SC8019-R1", "Harvest" and "White RL4137" and "Kanata" showed relatively intermediate level *TaABI5* expression (6- to 8-fold) except that "AC Majestic" showed an expression level similar to that shown by genotypes with high level of dormancy (~ 12-fold) and "McKenzie" showed an expression level similar to that shown by genotypes with low level of dormancy.

All the non-dormant cultivars except CIMMYT-11 showed low level of *TaABI5* expression (slightly higher or lower and similar level of expression detected in the non-dormant genotype Janz, which is considered as a control). The genotype "CIMMYT-11" exhibited an expression level similar to that shown by genotypes with intermediate level of dormancy (~6-fold higher expression than the control). Analysis of the expression of *TaABI5* in the embryos of imbibing seeds of the different wheat genotypes showed significant difference in expression level of *TaABI5* among the genotypes tested ($P < 0.05$).

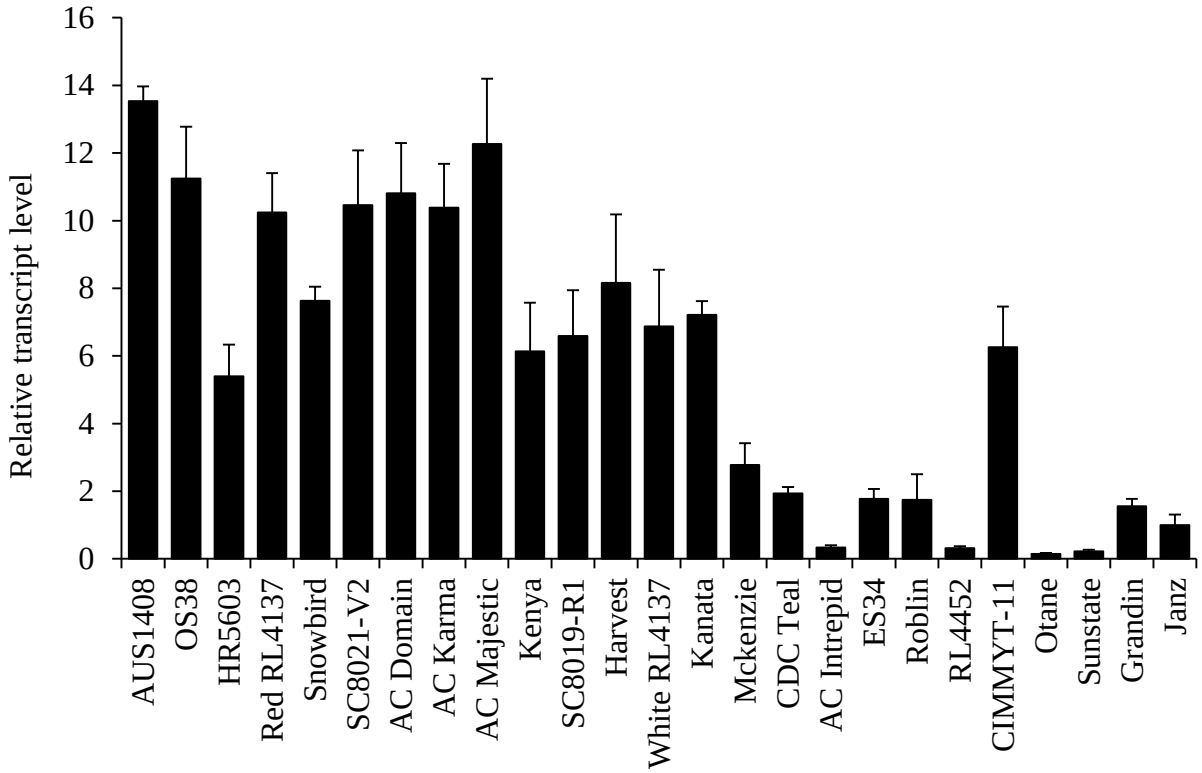

Figure 3.16 Relative transcript levels of *TaABI5* in the embryos of wheat genotypes. Transcripts levels were compared using the mean transcript level of Janz, which was arbitrarily set to a value of 1. Data are means of three biological replicates ± SE. Significant difference in the relative transcript level *TaABI5* among the different genotypes was tested using Fisher LSD method ($P < 0.05$; Appendix 15).

3.3.3.8 Correlation between *TaABI5* expression and seed dormancy levels

The correlation between *TaABI5* expression and seed dormancy levels was determined using coefficient of co-relation (r) values between mean GI values and relative transcripts level of *TaABI5* in the different wheat genotypes. The correlation analysis revealed an r value of 0.87 for the 25 genotypes studies. Correlation analysis that excluded "CIMMYT-11" and "HR5603" by considering their *TaABI5* expression level as outliers produced an r value of 0.94 (Figure 3.17). The similar pattern of correlation between the relative expression of *TaABI5* and seed dormancy level was evaluated 1 week/7days and 3 weeks of germination index (Appendix 16, Appendix 17).

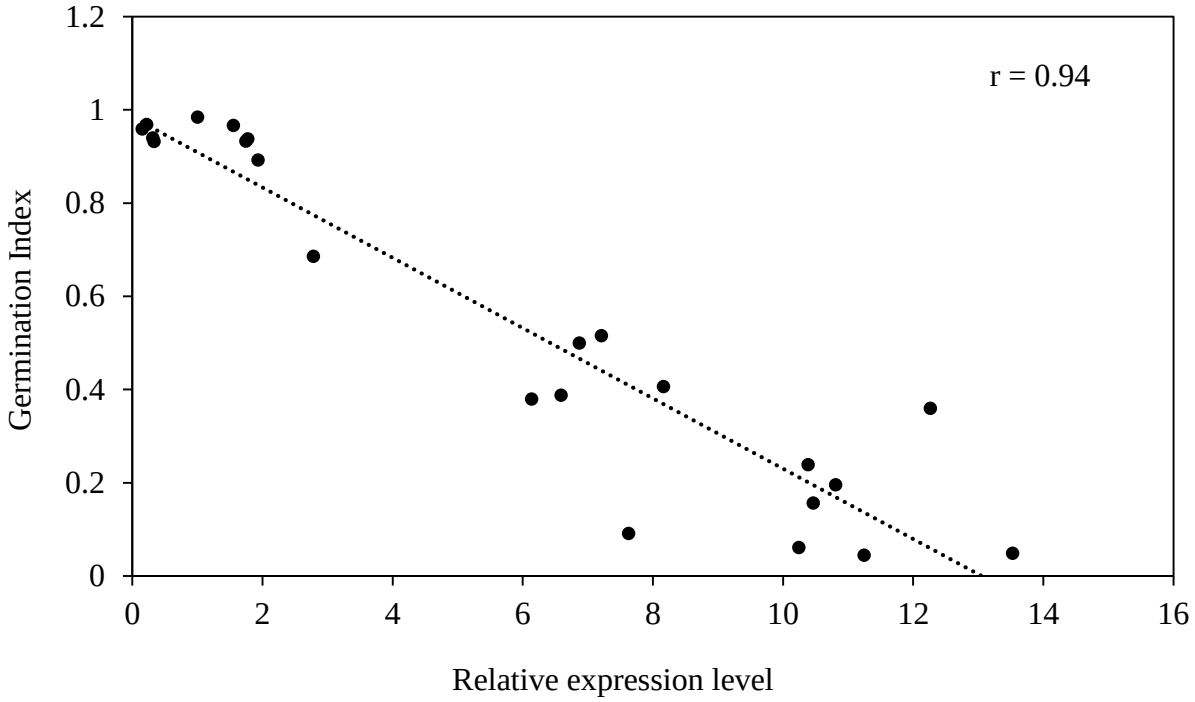

Figure 3.17 Correlation between the expression pattern of *TaABI5* and seed dormancy level of the different wheat genotypes.

3.4 Discussion

This study examined if the expressions of ABA metabolic and signaling genes that determine the level of dormancy in wheat seeds by utilizing 25 different wheat genotypes collected from different parts of the world. Our germination data revealed that the genotypes studied exhibit a wide range of dormancy levels, which allowed us to categorize genotypes as high, intermediate and low level of dormancy (Figure 3.1). Several genes involved in ABA metabolic and signalling pathways have been identified in several plant species (Rodríguez-Gacio *et al.,* 2009), and these genes play important role in the maintenance and release of seed dormancy. In cereal crops, differences in the expression patterns of ABA biosynthetic and catabolic genes, mainly *NCED* and *ABA8'OH*, respectively, affect seed ABA level and dormancy (Tuan *et al.,* 2018). In addition, ABA signalling, which is controlled by a number regulatory factors including the downstream components such as ABI3, ABI4 and ABI5, defines seed sensitivity to ABA and thereby plays a crucial role in regulating seed dormancy and germination (Nambara *et al.*, 2010; Tuan *et al.,* 2018). To increase our understanding of the involvement of *ABA8OH1* and *ABI5* in the regulation of dormancy and preharvest sprouting in wheat, this study performed comparative analysis of genomic nucleotide sequences and total expression level of *ABI5* and *ABA8OH1* genes in wheat genotypes that exhibit a range of variation in the level of seed dormancy.

Although the purpose of this study was to clone full length genomic sequences of *ABA8'OH1* in the A, B and D genomes, the results only displayed sequences homologous to the B genome copy of *TaABA8'OH1* in the different wheat genotypes (Figure 3.2) while amplification and sequencing of the A and D genome copies of the targeted gene resulted in non-target sequences. Earlier reports suggested the presence of high GC content in the genomic sequences of *ABA8'OH1*, and this could have hindered the effort in cloning the full length sequences of all the

homeologs using gene specific primers (Chun-li *et al.,* 2009). Furthermore, no nucleotide variation was observed in the genomic sequence of *TaABA8'OH1B* (Figure 3.8), suggesting that *TaABA8'OH1* is highly conserved with a lower chance of genetic variation at the nucleotide level. This possibly is related to the evolutionary history of *ABA8'OH1* since its phylogenetic analysis revealed high sequence homology among several plant species (Figure 3.5; Zheng *et al.,* 2012).

Several studies in different plant species indicated that members of the *ABA8'OH* (*CYP707A*) gene family are regulated at the transcriptional level, and this regulation plays an important role in determining ABA and dormancy levels (Liu *et al.,* 2009). This study analyzed the expression of *ABA8'OH1* in imbibed embryos of different wheat genotypes that showed different level of seed dormancy. The results of this study showed no clear correlation between the levels of *TaABA8'OH1* expression and seed dormancy across the genotypes studied, however, close association between the two parameters was observed in selected wheat genotypes. Imbibed embryos of wheat genotypes including "AC Domain" and "OS38", which are characterized by high level of seed dormancy/low germination index (Figure 3.1), showed low level of *TaABA8'OH1* expression (Figure 3.6). Likewise, "Grandin", "CIMMYT-11", "RL4452", "ES34" and "CDC Teal", which showed low level of dormancy/high germination index, appeared to have high expression levels of *TaABA8'OH1* (Figure 3.1). Our results suggest that the level of dormancy in these selected wheat genotypes might be controlled by the level of ABA.

Previous studies on the expression patterns of ABA metabolic genes in wheat have also shown that dormant seeds exhibit a low expression level of *TaABA8OH1* as compared to that observed in non-dormant seeds in both dry and imbibed stages (Chono *et al.,* 2013; Son *et al.,* 2016). Similarly, studies in other cereal crops such as barley and rice have shown close correlation between the expression of *ABA8'OH1* and the level of dormancy in seeds (Millar *et al.,* 2006;

Gubler *et al.,* 2008; Du *et al.,* 2015). On the other hand, wheat genotypes with an intermediate level of dormancy showed over 2-fold higher level of *TaABA8OH1* expression as compared to the control i.e. non-dormant genotype "Janz" (Figure 3.6). In addition, dormant wheat genotypes including "AUS1408", "HR5603", "Red RL4137", "Snowbird", "SC8021-V2", and "AC Karma" displayed higher levels of *TaABA8OH1* expression while non-dormant genotypes including "AC Intrepid", "Roblin", "Sunstate", "Otane" and "Janz" showed low levels of *TaABA8OH1* expression. This might be associated with the expression pattern of the ABA biosynthetic genes such as *NCED*s in these lines as ABA level is determined by a coordinated interaction of ABA biosynthesis and catabolism (Nambara *et al.,* 2010). Thus, it would be interesting to investigate ABA level across the genotypes studied. Additionally, wheat genotypes considered in this study represent a diverse genetic background, and this could influence the relationships between the transcriptional regulation of *TaABA8'OH1* and dormancy phenotype.

The second part of the thesis analyzed allelic variation and expression pattern of *TaABI5* in the wheat genotypes described in the section above. Amplification with genome specific primers was able to identify the three homeologues of *TaABI5* in all wheat genotypes studied and the three homeologues exhibited high degree of homology with one another and with the *TaABI5* sequence available in the GenBank database (Table 3.6). Detailed analysis of the sequence of *TaABI5* in the sub genomes of the different wheat genotypes identified single base substitutions at four locations in the A genome copy of *TaABI5* (Figure 3.9), and these base substitutions cause change in amino acids (L and E) (Figure 3.12). Consistently, two amino acid substitutions have been observed in the ABI5 of diploid wheat species *Triticum monococum* (*TmABF*) and *Triticum bioticum* (*TbABF*) (Nakamura *et al.,* 2007). The presence of more than one differential sequence of *TaABI5* due to the allelic variations might indicate that multiple alleles of *TaABI5* may exist in wheat. A previous

study that involved wheat genotypes with varied PHS tolerance reported seven different sequences of *TaABI5* and based on this result the authors suggested that *TaABI5* is a multicopy gene (Zhou *et al.*, 2017).

Studies on ABA transcription factors in Arabidopsis have suggested that ABI3 protein acts upstream of ABI5 in ABA signaling to mediate ABA-dependent inhibition of germination and growth of embryos (Lopez-Molina *et al.*, 2002). The interaction between *ABI3* and *ABI5* in Arabidopsis has been mapped to the C2 and C3 domains of *ABI5* (Nakamura *et al.*, 2001). Since our sequence analysis revealed that the two amino acid substitutions (L and E) of TaABI5 occurr in the C2 domain (Figure 3.13), it is likely that these substitutions affect ABA signaling in wheat seeds. In addition, of the two amino acid substitutions observed, the one that occurred at 126th position (D to E) lies next to the phosphorylation site (Figure 3.13), and as a result it might have a negative or positive influence on the activity of *TaABI5* at the post-transcriptional level. For example, a previous study in Arabidopsis seeds has shown that a new allele of ABI5 due to genetic variation in the C3 domain causes reduced seed sensitivity to ABA during germination (Tezuka *et al.*, 2013).

Although allelic variations were observed in *TaABI5* obtained from the A genome of different genotypes, no such variations were observed in B and D genome copy of *TaABI5*. Similarly, *TmABF*, which shares a high homology with *TaABI5* as shown by our phylogenetic analysis, has been mapped to a seed dormancy QTL located on chromosome 3A (Nakamura *et al.*, 2007), suggesting the importance of the A genome copy of *ABI5* in hexaploid wheat as compared to the B and D genome copies (Figure 3.14).

In addition to the allelic variations, this study investigated the expression of *TaABI5* in the different wheat genotypes. The expression level of *TaABI5* in imbibed embryos of the different

wheat genotypes exhibited a close association with the seed dormancy levels observed (Figure 3.15). Overall, genotypes with a higher level of seed dormancy showed higher levels of *TaABI5* expression while genotypes with lower level of dormancy showed lower levels of *TaABI5* expression. This is further confirmed by correlation coefficient (r) value of 0.94 between *TaABI5* expression and seed dormancy levels among the different wheat genotypes (Figure 3.7). Since increased levels of seed dormancy or PHS tolerance in wheat is reported to be closely associated with ABA sensitivity (Walker-Simmons *et al.,* 1987), our results suggest that *TaABI5* is a crucial regulator of ABA response in wheat seeds, and dormant genotypes with higher expression of *TaABI5* may serve as a genetic pool for PHS resistance. A previous study had also demonstrated the presence of a higher expression level of *TaABF* during imbibition of dormant wheat genotypes, and strong positive correlation ($r \geq 0.78$) was reported between the levels of *TaABF1* expression in embryos and seed dormancy in different wheat genotypes (Rikiishi *et al.,* 2010). In addition, higher expression of *ABI5* and the corresponding protein ABI5 has been found in PHS resistant/dormant genotypes as compared to susceptible/non-dormant genotypes of wheat and sorghum, respectively (Zhou *et al.,* 2017; Rodriguez *et al.,* 2009). These findings suggest that *TaABI5* might play a role in regulating seed germination and dormancy in different crop species. Although a high correlation coefficient value of 0.94 was observed between *TaABI5* expression and seed dormancy levels, genotypes such as HR5603 and CIMMYT-11 showed an expression level of *TaABI5* that was not consistent with their dormancy phenotype (Figure 3.1; Figure 3.15). As the wheat genotypes studied in this project have different backgrounds, it is possible that ABA signaling in these genotypes is controlled by other ABI transcription factors. Alternatively, seed dormancy in these genotypes may be regulated by mechanisms other than ABA signaling. For example, the expression of other ABI transcription factors that are involved in ABI signaling such

as *ABI3* (*TaVp1*) have been shown to be positively associated with the level of seed dormancy in some wheat genotypes (Nakamura *et al.,* 2001).

Previous reports on allelic variation or single nucleotide polymorphism in the coding regions indicated its influence on the promoter activity for gene expression (LeVan *et al.,* 2001). Comparison between nucleotide substitution of guanine (G) by thymine (T) at 365th position in the *TaABI5A* and total expression pattern of *TaABI5* suggest that this base substitution might be responsible for influencing the promoter activity of *TaABI5* and thereby causing low expression level of *TaABI5* in "Roblin", "Sunstate", "Janz", "Kanata" and "Harvest" (Figure 3.15). Consistently, a previous study has also indicated that wheat sub-genomes have variable levels of contribution to the expression of ABA related genes in seeds (Son *et al.,* 2016).

In summary, this study showed that the expression of *TaABA8'OH1* is not closely associated with the dormancy phenotype. This might be due to the difference in the ability of the different genotypes to produce different levels of ABA, which could influence the expression of the ABA catabolic gene *TaABA8'OH1*. On the other hand, the findings of this study demonstrated a strong positive correlation between the levels of *TaABI5* expression and seed dormancy in the different genotypes, suggesting its potential role in regulating PHS tolerance in wheat. Furthermore, the study showed that allelic variations in the sequence of TaABI5 might be responsible in influencing its expression at least in selected genotypes.

4.0 GENERAL DISCUSSION AND CONCLUSIONS

Wheat is an important cereal crop of the global food market and economy as it serves as a raw material for variety of food products. However, production of wheat is negatively affected by a range of factors including preharvest sprouting, which is defined as the germination of seeds in the spike before harvesting under humid conditions, which reduces seed quality and lowers crop yield. It is a major problem to causing financial losses to wheat growers and the food industry in most of the major wheat producing countries in the world. Seed dormancy is the main adaptive trait that is responsible for preharvest sprouting tolerance in seeds of different crop species, and it is regulated mainly by plant hormones. Abscisic acid is one of the major plant hormones that play a crucial role in the control of seed dormancy. Mutational studies in the model plant Arabidopsis were the first to reveal the functions of ABA metabolism and signalling genes in seed dormancy and germination. Among the ABA metabolic genes, those genes encoding the ABA catabolic enzyme ABA8′OH play an important role in regulating ABA levels in seeds and therefore dormancy. With respect to the ABA signalling pathway, genes encoding several downstream transcription factors such as ABI5 act as important mediators of seed response to ABA.

In order to identify ABA related genes as sources of preharvest sprouting tolerance, this thesis project investigated the role of ABA catabolic (*ABA8'OH1*) and (*ABI5*) genes in regulating dormancy and therefore tolerance to preharvest sprouting in wheat using 25 wheat genotypes with different geographical origin and varied seed dormancy level. Using their germination phenotype, these genotypes showed high, intermediate and low level of dormancy. Comparative sequence analysis of the *ABA8'OH1B* gene displayed no nucleotide variation among the wheat genotypes studied and our phylogenetic analysis showed that *TaABA8'OH1* shares high degree of homology with the same gene identified in other plant species. Expression patterns of *TaABA8'OH1*;

however, showed close association with dormancy in only selected genotypes, which includes the dormant genotypes “AC Domain” and “OS38”, non-dormant genotypes “Grandin”, “CIMMYT-11”, “RL4452”, “ES34” and “CDC Teal”. In addition, wheat genotypes with an intermediate level of seed dormancy, which includes “Kenya”, “AC Majestic”, “SC8019-R1”, “Harvest”, “Kanata”, “White RL4137” and “Mckenzie” displayed relatively a high level of *TaABA8'OH1* expression. This study found no correlation between *TaABA8'OH1* expression and seed dormancy levels in some of the lines studied. This might be due to variation in the amount of ABA present in the seeds of the different genotypes, which is also regulated by expression of genes involved in its biosynthesis. Since the wheat genotypes investigated in this study have diverse genetic background, it is also possible that this factor influenced the expression of *TaABA8'OH1* in those lines. Therefore, it is important to examine the level of ABA in the seeds of the different wheat genotypes.

This thesis project also analyzed the sequence and expression patterns of *ABI5* in the different wheat genotypes. A strong correlation ($r = 0.94$) was evident between the levels of *ABI5* expression and seed dormancy of wheat genotypes in almost all lines. Dormant wheat genotypes displayed higher level of *TaABI5* expression level of as compared to the non-dormant genotypes. In addition, intermediate expression levels of *TaABI5* were observed in genotypes having intermediate levels of seed dormancy. Sequence analysis of the A genome copy of *TaABI5* revealed four nucleotide substitutions; however, substitution of guanine (G) by thymine (T) at 365^{th} position in Harvest, Roblin, Sunstate, Janz, Kanata and CDC Teal appears to influence the expression of *TaABI5* as a relatively low expression level of *TaABI5* was observed in these genotypes. Thus, it is possible that this allelic variation reduces ABA sensitivity in the seeds of the

wheat genotypes described above, and this variation might form the basis for designing DNA markers for *ABI5*.

In general, the findings of this thesis provide useful information about the role of *TaABI5* in regulating seed dormancy and therefore tolerance to preharvest sprouting in wheat lines with different genetic backgrounds. However, determination of the physiological role of *TaABI5* in wheat seed dormancy requires additional studies including an examination of the sensitivity of the seeds of the different genotypes to ABA, and modifying the expression level of *TaABI5* and analysis of the resulting mutants of wheat in terms of their seed dormancy level and resistance to preharvest sprouting.

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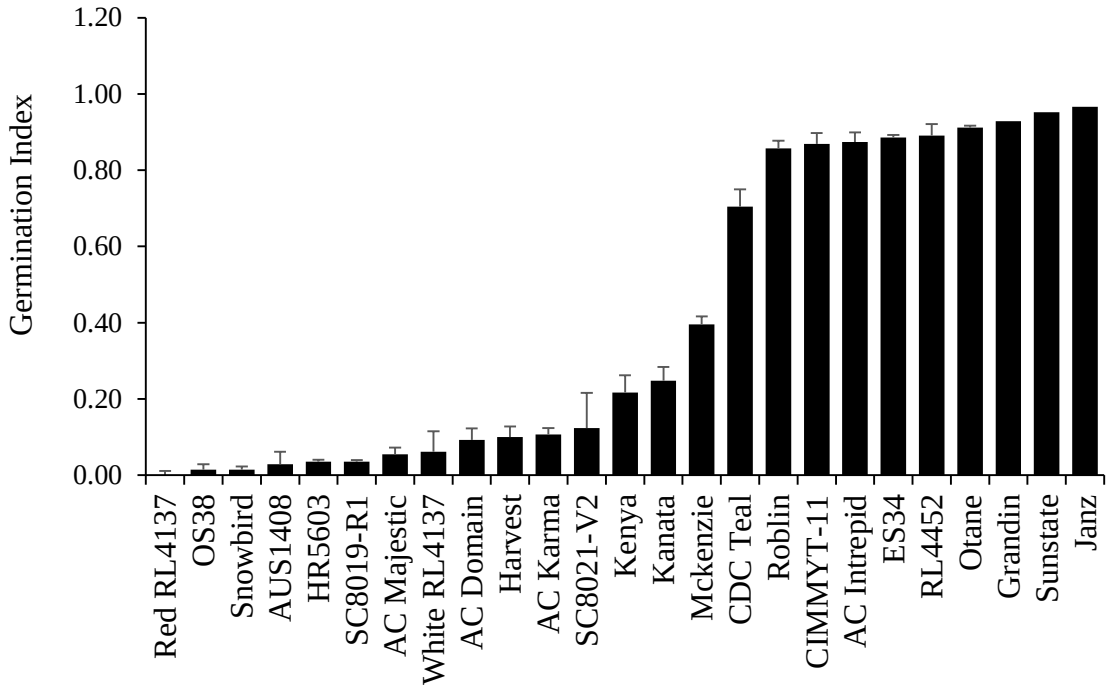
APPENDIX

Appendix 1. Germination index of the different wheat genotypes during imbibition for a period of 7 days/a week. Data are means of three biological replicates ± SE.



Germination index (GI) of each cultivar was calculated based on 7 days of imbibition using the following formula.

$$GI= [(7\times g1) + (6\times g2) + (5\times g3) \ldots\ldots 1\times g7)]/ (7\times n)$$

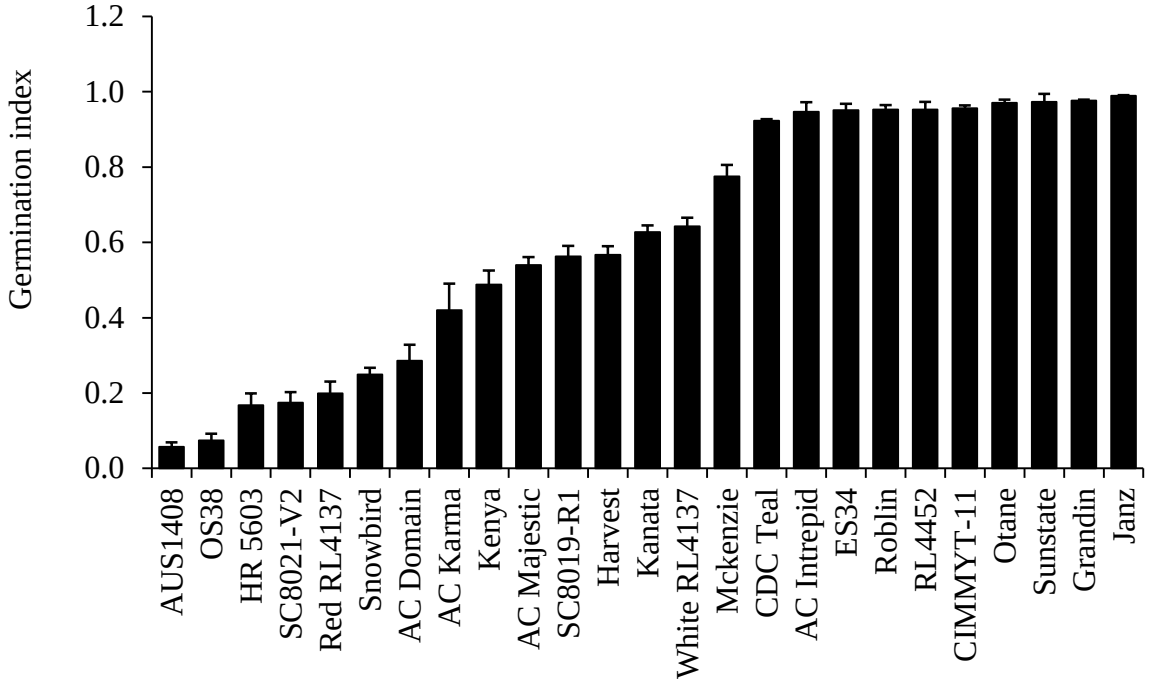
Where g is the number of seeds germinated on the first, second,….., and 7^{th} day of imbibition and n is the total number of seeds plated.

Appendix 2. Analysis of variance for germination index for 2 weeks of different wheat genotypes

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Between groups	24	9.962	0.415	218.624	<0.001
Residual	50	0.0949	0.00190		
Total	74	10.057			

The significance of differences was tested with least significant difference (LSD) at probability < 0.05.

Appendix 3. Germination index for a period of three weeks of the different wheat genotypes during imbibition for a period of three weeks. Data are means of three biological replicates ± SE.



Germination index (GI) of each cultivar was calculated based on 21 days of imbibition using the following formula.

$$GI = [(21 \times g1) + (20 \times g2) + (19 \times g3) \ldots\ldots 1 \times g21)] / (21 \times n)$$

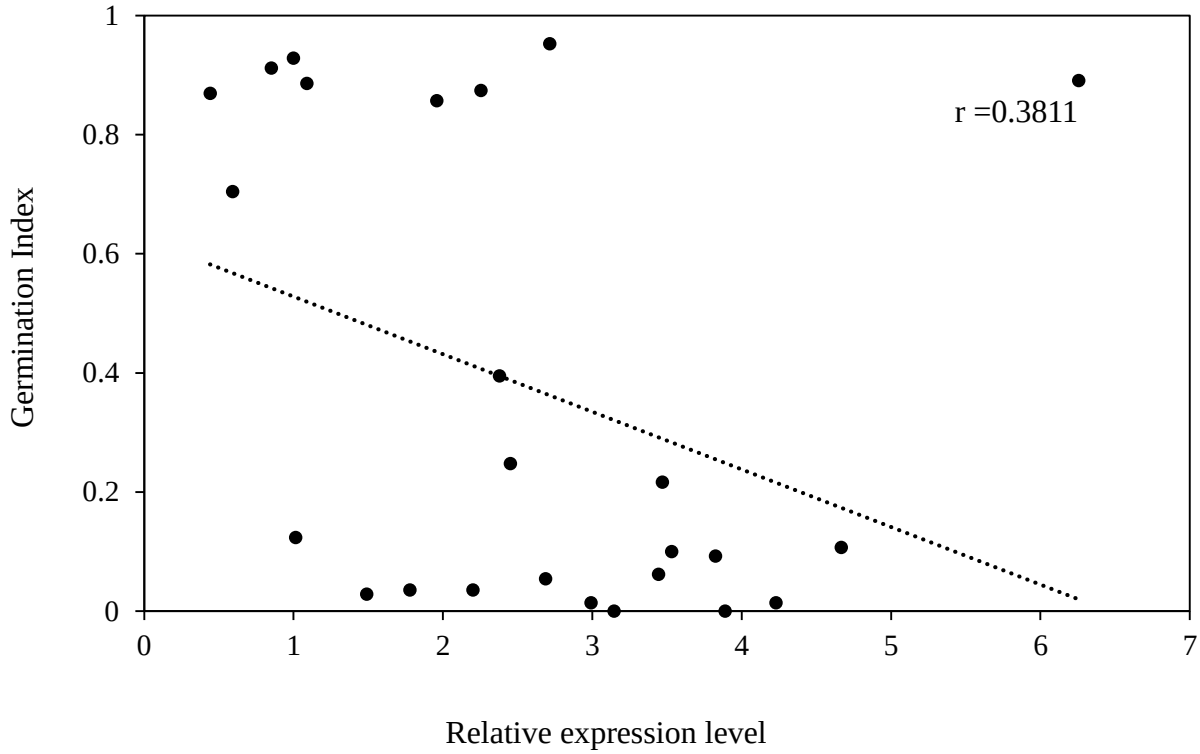
Where g is the number of seeds germinated on the first, second, ….., and 21st day of imbibition and n is the total number of seeds plated.

Appendix 4. Analysis of variance for relative transcript levels of *ABA8'OH1* in the embryos of different wheat genotypes

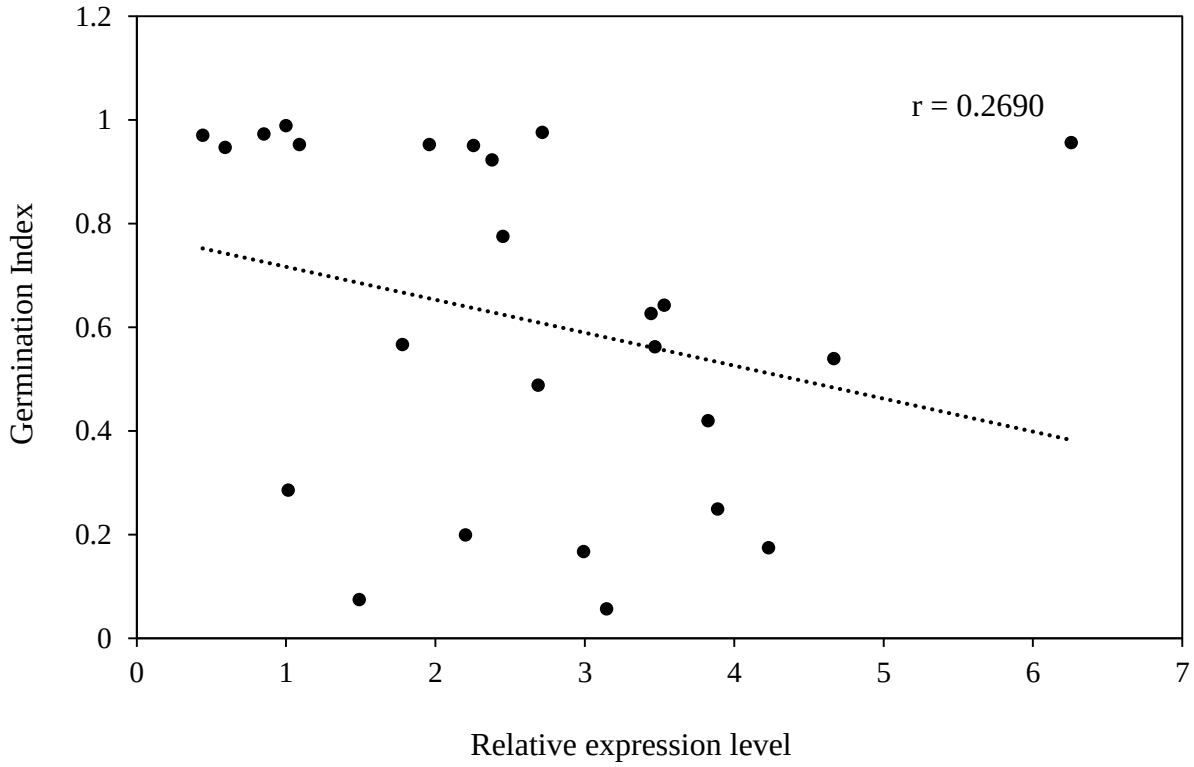
Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Between groups	24	360.349	15.015	2.403	<0.004
Residual	50	312.414	6.248		
Total	74	672.763			

The significance of differences was tested with least significant difference (LSD) at probability < 0.05.

Appendix 5. Correlation between *TaABA8'OH1* expression and seed dormancy levels of the different wheat genotypes (based on GI values from imbibition over 7 days).

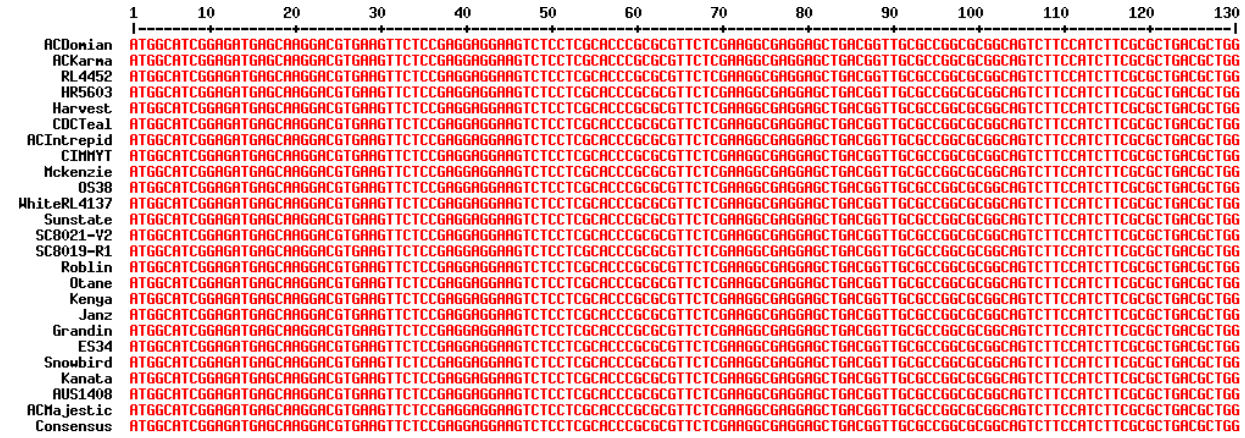


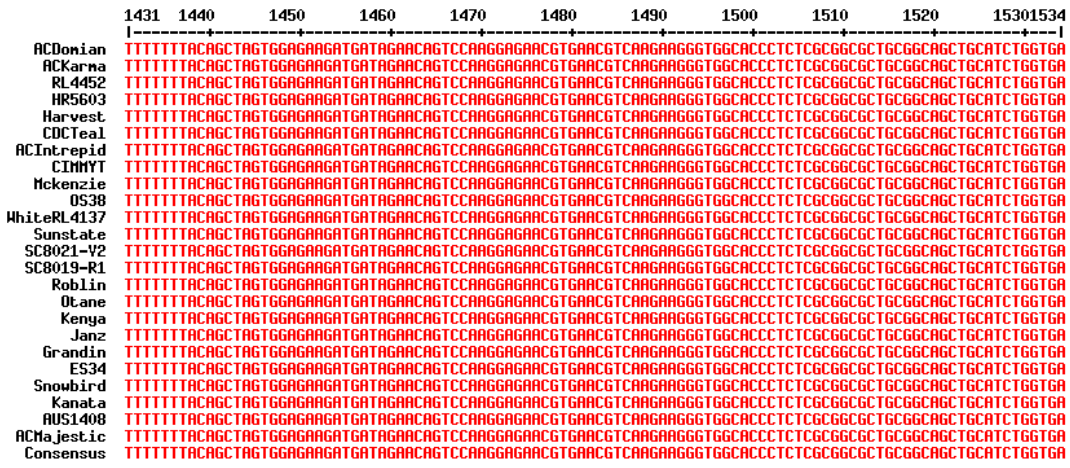
Appendix 6. Correlation between *TaABA8'OH1* expression and seed dormancy levels of the different wheat genotypes (based on GI values from imbibition over three weeks).



Appendix 7. Multiple sequence alignment of the genomic nucleotide sequences of *TaABI5B* originated from the 25 different wheat genotypes.

Appendix 8. Multiple sequence alignment of the genomic nucleotide sequences of *TaABI5D* originated from the 25 different wheat genotypes.





Appendix 9. Exonic and intronic regions of *TaABI5B*. Regions highlighted in grey represent exons while those that are not highlighted indicate introns.

ATGGCGTCGGAGATGAGCAAGGATGTGAAGTTCTCCGAGGAAGAAGTCACCTCGCACCCGCGCG
TTCTCGAAGGTGAGGAGCAGACGGTGGTTGCGCCGGCACGGCAATCGTCCATCTTCGCGCTGAC
GCTGGACGAGTTGCAATACTCCGTGTGCGAGGCGGGGCACAACTTCGGGTCCATGAACATGGAC
GAATTTATGAGCAACATATGGAATGCCGAGGAGTTCCAGGCGGCGACCGGCGGTGGCTTGGTGG
GCATGGAGGTGGCTCCTGTGGTGGGTGCTGGTGGAGGCGGAGGTGGCGGAGATGCAGGAGGAAG
CAACCTAGCCCGGCAGGAGTCGTTCTCCTTGCCTCCCCCGCTGTGCCGGAAGACGGTGGAGGAG
GTGTGGGCTGAGATCAACAGGGAGCCCCGCCCGGTGCATGCCCAGCCTCAGGCCGCGCGACCCT
CACAGCAGCCTCCTGTCCAGCCACCGGTTGTGGCCAACGACCGGCAGGGGACCCTAGGCGAGAT
GACGCTGGAGCAGTTCCTTGTCAAGGCCGGCGTGGTCCGTGGATCTGGCACCGGCGTCCAGGCG
CCTATGCCGGTCGGCATGGTCCATGGACAGATGAACCCCGTGCAGCAGGGGCAGCAGCCTGGCC
CAATGATGTACCCGATGGCACCAGCCAACGGTATATTCCAGGTGATGGGCGACGACATGGGGTT
CATCCCCAACGGGTACGCGGGGATGGCCGTGGTGCCGCCGCCACCACCTCCTCAAGGTGGGGTC
GGTATCGTGAGCCCCGGGTCGTCGGACGGGAGGAGCGCCATGACGCAGGCTGACATGATGAACT
GCATGGGCGACGTAGTGATGATGGAGAATGGTGGCGCCCGAAAACGTGGCGCCCCGAAGGATCA
GTCTCGCGAGAGGAGCATCGAGCGCCGCCACCGCCGCATGATCAAGAACCGTGAGTCAGCCGCA
CGATCGCGTGCCAGGAAGCAGGTACTTACCTTGCTGCCAAAATTGTTGTTTTTATGCAATGCCA
TTTCTGTAACGGTGGTAACAATATTGGTGACACTTGCACTCCCTGTTTCTAAATCCTACAGGCT
TATACCGTGGAGCTTGAAGCTGAACTGAACCACCTCAAGGAGGAGAACGTGCGTCTGAAAGCTG
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TTCTTACCGTTACTAAATTGTTTCATTGTTTTGTTGCAGAAGACAATTCTGCTGACTAAGAAAA
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CCAATCTAATCTTAAAACAGTTAATGTGTGCATGCTGATGAAAACACCTTGTGCCCGTGATCTT
TTTTTCTTACAGCTGGTGGAGCAGTCCAAGGAGAACGTGAAAGCCAAGAAGGGTGGCGTCCTGT
TGCGGCGCTGCGGCAGCTGCATCAGGTGA

Appendix 10. Exonic and intronic regions of *TaABI5D*. Regions highlighted in grey represent exons while those that are not highlighted indicate introns.

ATGGCATCGGAGATGAGCAAGGACGTGAAGTTCTCCGAGGAGGAAGTCTCCTCGCACCCGCGCG
TTCTCGAAGGCGAGGAGCTGACGGTTGCGCCGGCGCGGCAGTCTTCCATCTTCGCGCTGACGCT
GGACGAGCTGCAATACTCGGTGTGCGAGGCAGGGCGCAACTTTGGGTCCATGAACATGGACGAG
TTCATGAGCAACATATGGAATGCCGAGGAGTTCCAAGCGGCCACCGGCGGTGGCTTGGTGGGCA
TGGAGGTGGCTCCTGTGGTGGGTGCTGGTGCAGGCGGAGGTGGCGCAGATGCAGGAGGAAGCAA
CCTAGCCCGGCAGGAGTCGTTCTCCTTGCCTCCCCCGCTGTGCCGGAAGACGGTGGAGGAGGTG
TGGGCTGAGATCAACAGGGAGCCCCGCCAGGTGCATGCTCAGCCTCAGGGCGCGCGAGCCTCAC
AGCAGCCTCCTGTCCAGCCACCGGTTGCGGCCAACGACCGGCAGGGGACCCTAGGCGAGATGAC
GCTGGAGCAGTTTCTTGTCAAGGCCGGCGTGGTCCGTGGATCTGGCGCCGGCGGCCAGGCGCCT
GTGCCGGTCGGCATGGTCCATGCACAGATGAACCCCGTGCAGCAGGGGCAGCAGCCTGGCCCAA
TGATGTACCCGATGGCACCAGCCAACGGCATGTTCCAGGTGATGGGCGACGGCATGGGGTTCGT
CCCCAACGGGTACGCAGGGATGGCCGTGGTGCCGCCACCACCTCCTCCTCAAGGTGGGATGGGT
ATCGTGAGCCCCGGGTCCTCGGACGGGAGGAGCGCCATGACGCAGGCTGACATGATGAACTGCA
TGGGCGACGGAGCGATGATGGAGAACGGCGGCGCCCGGAAACGCGGCGCCCCGGAGGATCAGTC
CTGCGAGAGGAGCATCGAGCGCCGCCACCGCCGCATGATCAAGAACCGTGAGTCAGCCGCACGA
TCGCGTGCCAGGAAGCAGGTACTTACCTTGCTGCCAAAATTGTTGTTTTTTATGCAATGCCATT
TCTGTAACGGTGGTAACAATGTTGGTGACACTTGCACTCCCTGGCTTTCTAAATCCTACAGGCT
TACACCGTGGAGCTTGAAGCTGAACTGAACCACCTCAAGGAGGAGAACGCTCGTCTGAAAGCTG
AGGAGGTACGGAGCTGCTGACCAGTGAAAACCATGAGAAAATTCAGTTATCATATGCTTTTCAG
TTCTTACCGTTACTAAACTGTTTCATTGTTTTGTTGCAGAAGACAATTCTGCTGACCAAGAAAC
AAATGGTATGTACATTTTACTACCATCACCTGGCAATCCTCCTACACTCGCCACCCCTTGCCAA
TTGCTTGTAACAATCCACTGTAATTGCTAAGTCTTTAAATAGTCAATGTGTGCATGCTGATGGA
AACGCCTTGTGGCGGTGATCTTTTTTTTTACAGCTAGTGGAGAAGATGATAGAACAGTCCAAGG
AGAACGTGAACGTCAAGAAGGGTGGCACCCTCTCGCGGCGCTGCGGCAGCTGCATCTGGTGA

Appendix 11. Amino acid sequences of TaABI5B translated from the coding region of *TaABI5B*.

MASEMSKDVKFSEEEVTSHPRVLEGEEQTVVAPARQSSIFALTLDELQYSVCEAGHNFGSMNMD
EFMSNIWNAEEFQAATGGGLVGMEVAPVVGAGGGGGGGDAGGSNLARQESFSLPPPLCRKTVEE
VWAEINREPRPVHAQPQAARPSQQPPVQPPVVANDRQGTLGEMTLEQFLVKAGVVRGSGTGVQA
PMPVGMVHGQMNPVQQGQQPGPMMYPMAPANGIFQVMGDDMGFIPNGYAGMAVVPPPPPPQGGV
GIVSPGSSDGRSAMTQADMMNCMGDVVMMENGGARKRGAPKDQSRERSIERRHRRMIKNRESAA
RSRARKQAYTVELEAELNHLKEENVRLKAEEKTILLTKKKMLVEQSKENVKAKKGGVLLRRCGS
CIR

Appendix 12. Amino acid sequence of TaABI5D translated from the coding region of *TaABI5D*.

MASEMSKDVKFSEEEVSSHPRVLEGEELTVAPARQSSIFALTLDELQYSVCEAGRNFGSMNMDE
FMSNIWNAEEFQAATGGGLVGMEVAPVVGAGAGGGGADAGGSNLARQESFSLPPPLCRKTVEEV
WAEINREPRQVHAQPQGARASQQPPVQPPVAANDRQGTLGEMTLEQFLVKAGVVRGSGAGGQAP
VPVGMVHAQMNPVQQGQQPGPMMYPMAPANGMFQVMGDGMGFVPNGYAGMAVVPPPPPPQGGMG
IVSPGSSDGRSAMTQADMMNCMGDGAMMENGGARKRGAPEDQSCERSIERRHRRMIKNRESAAR
SRARKQAYTVELEAELNHLKEENARLKAEEKTILLTKKQMLVEKMIEQSKENVNVKKGGTLSRR
CGSCIW

Appendix 13. Amino acid sequence alignment of TaABI5Bs from the different wheat genotypes (designated as 1) and original amino acid sequence of TaABI5B (designated as 2).

```
1       MASEMSKDVKFSEEEVTSHPRVLEGEEQTVVAPARQSSIFALTLDELQYSVCEAGHNFGS  60
2       MASEMSKDVKFSEEEVTSHPRVLEGEEQTVVAPARQSSIFALTLDELQYSVCEAGHNFGS  60
        ************************************************************

1       MNMDEFMSNIWNAEEFQAATGGGLVGMEVAPVVGAGGGGGGGDAGGSNLARQESFSLPPP  120
2       MNMDEFMSNIWNAEEFQAATGGGLVGMEVAPVVGAGGGGGGGDAGGSNLARQESFSLPPP  120
        ************************************************************

1       LCRKTVEEVWAEINREPRPVHAQPQAARPSQQPPVQPPVVANDRQGTLGEMTLEQFLVKA  180
2       LCRKTVEEVWAEINREPRPVHAQPQAARPSQQPPVQPPVVANDRQGTLGEMTLEQFLVKA  180
        ************************************************************

1       GVVRGSGTGVQAPMPVGMVHGQMNPVQQGQQPGPMMYPMAPANGIFQVMGDDMGFIPNGY  240
2       GVVRGSGTGVQAPMPVGMVHGQMNPVQQGQQPGPMMYPMAPANGIFQVMGDDMGFIPNGY  240
        ************************************************************

1       AGMAVVPPPPPPQGGVGIVSPGSSDGRSAMTQADMMNCMGDVVMMENGGARKRGAPKDQS  300
2       AGMAVVPPPPPPQGGVGIVSPGSSDGRSAMTQADMMNCMGDVVMMENGGARKRGAPKDQS  300
        ************************************************************

1       RERSIERRHRRMIKNRESAARSRARKQAYTVELEAELNHLKEENVRLKAEEKTILLTKKK  360
2       RERSIERRHRRMIKNRESAARSRARKQAYTVELEAELNHLKEENVRLKAEEKTILLTKKK  360
        ************************************************************

1       MLVEQSKENVKAKKGGVLLRRCGSCIR    387
2       MLVEQSKENVKAKKGGVLLRRCGSCIR    387
        ***************************
```

Appendix 14. Amino acid sequence alignment of TaABI5Ds from the different wheat genotypes (designated as 1) and original amino acid sequence of TaABI5D (designated as 2).

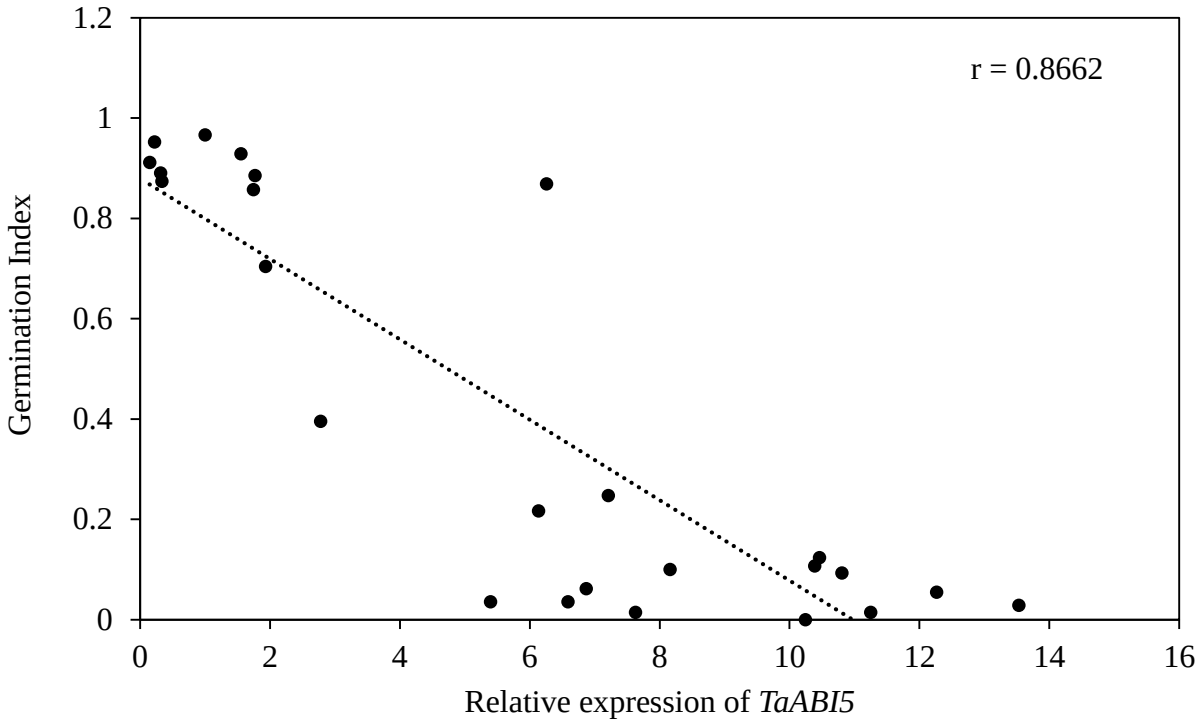
```
1       MASEMSKDVKFSEEEVSSHPRVLEGEELTVAPARQSSIFALTLDELQYSVCEAGRNFGSM  60
2       MASEMSKDVKFSEEEVSSHPRVLEGEELTVAPARQSSIFALTLDELQYSVCEAGRNFGSM  60
        ************************************************************

1       NMDEFMSNIWNAEEFQAATGGGLVGMEVAPVVGAGAGGGGADAGGSNLARQESFSLPPPL  120
2       NMDEFMSNIWNAEEFQAATGGGLVGMEVAPVVGAGAGGGGADAGGSNLARQESFSLPPPL  120
        ************************************************************

1       CRKTVEEVWAEINREPRQVHAQPQGARASQQPPVQPPVAANDRQGTLGEMTLEQFLVKAG  180
2       CRKTVEEVWAEINREPRQVHAQPQGARASQQPPVQPPVAANDRQGTLGEMTLEQFLVKAG  180
        ************************************************************

1       VVRGSGAGGQAPVPVGMVHAQMNPVQQGQQPGPMMYPMAPANGMFQVMGDGMGFVPNGYA  240
2       VVRGSGAGGQAPVPVGMVHAQMNPVQQGQQPGPMMYPMAPANGMFQVMGDGMGFVPNGYA  240
        ************************************************************

1       GMAVVPPPPPPQGGMGIVSPGSSDGRSAMTQADMMNCMGDGAMMENGGARKRGAPEDQSC  300
2       GMAVVPPPPPPQGGMGIVSPGSSDGRSAMTQADMMNCMGDGAMMENGGARKRGAPEDQSC  300
        ************************************************************

1       ERSIERRHRRMIKNRESAARSRARKQAYTVELEAELNHLKEENARLKAEEKTILLTKKQM  360
2       ERSIERRHRRMIKNRESAARSRARKQAYTVELEAELNHLKEENARLKAEEKTILLTKKQM  360
        ************************************************************

1       LVEKMIEQSKENVNVKKGGTLSRRCGSCIW 390
2       LVEKMIEQSKENVNVKKGGTLSRRCGSCIW 390
        ******************************
```

Appendix 15. Analysis of variance for relative transcript levels of *ABI5* in the embryos of different wheat genotypes

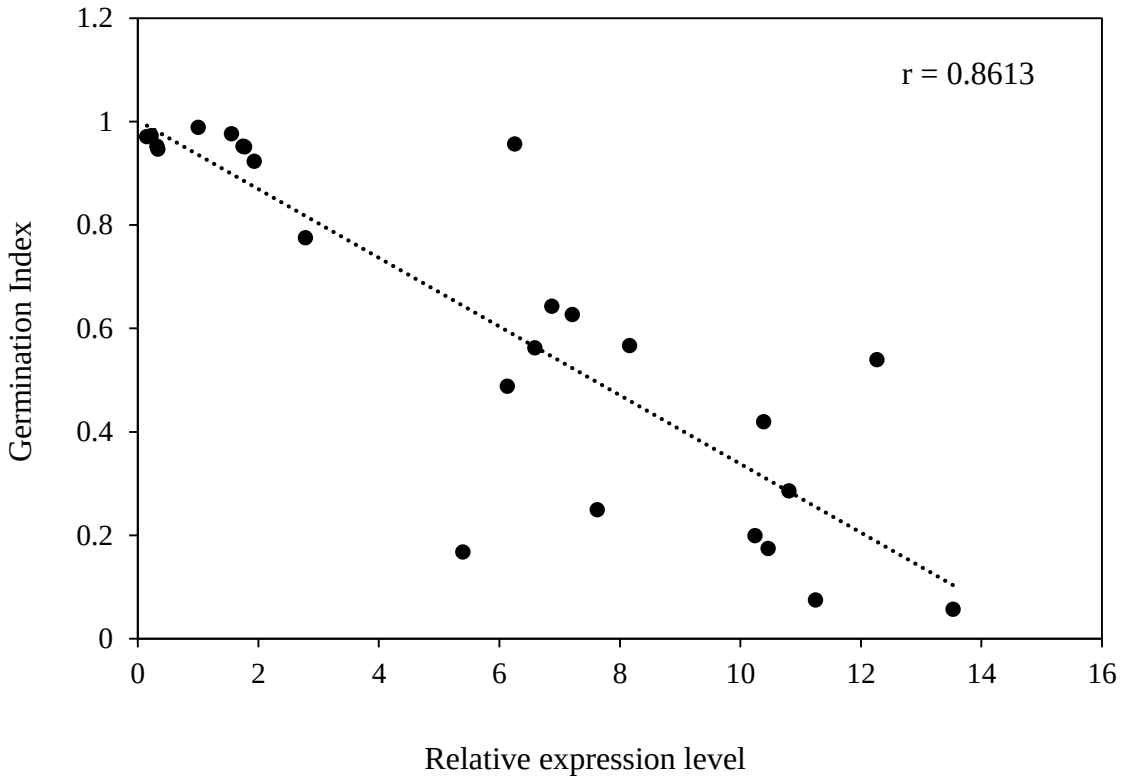
Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Between groups	24	1619.212	67.467	19.528	<0.001
Residual	50	172.748	3.455		
Total	74	1791.959			

The significance of differences was tested with least significant difference (LSD) at probability < 0.05.

Appendix 16. Correlation between *TaABI5* expression and seed dormancy levels of the different wheat genotypes (based on GI values from imbibition over 7 days).



Appendix 17. Correlation between *TaABI5* expression and seed dormancy levels of the different wheat genotypes (based on GI values from imbibition over three weeks).



ABBREVIATIONS

AAO3	abscisic aldehyde oxidases
ABA	abscisic acid
ABA8′OH	ABA 8′ hydroxylase
ABF	ABRE-binding factors
ABI	abscisic acid insensitive
ABRE	ABA-responsive element
ANOVA	analysis of variance
AREB	ABRE-binding proteins
BLAST	basic local alignment search tool
BP	before present
bZIP	basic leucine zipper
cDNA	complimentary deoxyribo nucleic acid
CIMMYT	International Maize and Wheat Improvement Centre
CTAB	cetyltrimethyl ammonium bromide
DAF	days after flowering
DEPC	diethylpyrocarbonate
DNA	deoxynuclic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate
DPA	dihydrophaseic acid
EDTA	ethylene diamine tetraacetic acid
ExPASy	expert protein analysis system

FAO	Food and Agriculture Organization
GA	gibberellin
GI	germination index
HAI	hours after imbibition
HCL	hydrochloric acid
IPP	isopentenyl diphosphate
IPTG	isopropyl β-D-1- thiogalactopyranoside
LEA	late embryogenesis abundant
LiCl	lithium chloride
LSD	least significant difference
MEGA	molecular evolutionary genetic analysis
LB	luria-bertani
NCBI	national centre for biotechnology information
NCED	9-cis epoxycarotenoid dioxygenases
NSY	neoxanthin synthase
PA	phaesic acid
PCR	polymerase chain reaction
PED	peroxisome defective 3
PHS	preharvest sprouting
PP2CS	protein phosphatase 2cs
PVP	polyvinylpyrrolidone
PYR/PYL/RCAR	pyrabactin resistance/pyrabactinlike/regulatory
qPCR	real-time quantitative PCR

QTL	quantitative trait loci
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
RSD	reduced seed dormancy
SDR	short- chain dehydrogenase/reductase
SDS	Sodium dodecyl sulfate
SnRKs	snf1-related protein kinase 2s
TBE	Tris/Borate/EDTA
UV	ultraviolet
Vp	vivipary
ZEP	zeaxanthin epoxidase