The role of abscisic acid and its catabolic genes in the regulation of wheat seed dormancy and germination

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Thesis submitted to the faculty of Graduate Studies of

the University of Manitoba

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Plant Science

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Winnipeg

ABSTRACT

Chitnis, Vijaya R., Ph.D., The University of Manitoba, January 2018. The role of abscisic acid and its catabolic genes in the regulation of wheat seed dormancy and germination Ph.D. supervisor: Dr. Belay T. Ayele

Common wheat is the one of the most important cereal crop in the world. Pre-harvest sprouting (PHS) is one of the major problems affecting wheat yield and quality in Canada and globally. The occurrence of PHS is closely associated with the level of seed dormancy, and abscisic acid (ABA) plays a pivotal role in the regulation of seed dormancy. The catabolism of ABA plays a crucial role for maintaining its homeostasis in plant tissues, including seeds, and this reaction is mediated primarily by ABA 8' hydroxylase, an enzyme encoded by genes designated as *CYP707A*. This study identified the three homeologues of *TaCYP707A1* from Canadian wheat genotypes with contrasting PHS phenotype, and comparison of their genomic sequences indicated the presence of allelic and other variations. Analysis of the total expression of *TaCYP707A1* in different wheat tissues revealed that it is predominantly expressed in seeds during maturation, and genomic contribution of transcripts to the total expression of *TaCYP707A1* are shown to vary with tissues and genotypes. Ectopic expression of *TaCYP707A1* in Arabidopsis demonstrated its function in regulating seed ABA level and dormancy. Furthermore, the spatiotemporal expression patterns of *TaCYP707A1* along with *TaCYP707A2* in the seeds of the wheat genotypes studied suggest that both genes are involved in the regulation of seed ABA and dormancy levels, however, *TaCYP707A1* appears to play a major role. The findings of this thesis also show that the role of ABA in inhibiting the germination of wheat seed is partly mediated by transcriptional regulation of genes involved in the metabolic and signaling pathways of brassinosteroids, ethylene and cytokinin, which in turn suggests changes in the contents and responses of seeds to these particular phytohormones.

ACKNOWLEDGEMENTS

I would first like to express my gratitude to my advisor Dr. Belay T. Ayele, without his advice this task would not have been accomplished. I avail myself of this opportunity to thank my advisory committee members Dr. Mark Jordan, Dr. Dana Schroeder and Dr. Gavin Humphreys for their valuable guidance and feedback. I convey my sincere thanks to Dr. Feng Gao and Ms. Zhen Yao for their help and support during my stay at CTC lab. I also take this opportunity to extend my thanks to past and present members of CTC lab. I express heartfelt thanks to my dear friends at plant science. Thank you for the great times. I am highly indebted to everyone in the Department of Plant Science for their continuous support during my stay.

I sincerely acknowledge the University of Manitoba for the University of Manitoba Graduate Fellowship and the Provincial Government for the Manitoba Graduate Scholarship. I also acknowledge the Natural Sciences and Engineering Research Council of Canada and the Western Grains Research Foundation funding agencies.

This endeavor would not have been completed without the support and encouragement of my mother Bhagyashree and brother Sameer. I would like to extend my gratitude to Suresh, Shailesh, Sharan, Gurudatt, and Surendra for making days in Winnipeg memorable. Finally, I thank Stephanie for her unfailing support and continuous encouragement through the process of thesis writing. This endeavor would not have been accomplished without them.

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FOREWORD

This thesis is written in manuscript style following the University of Manitoba thesis guidelines. The thesis is comprised of a general introduction about the research project and literature review followed by the three main manuscripts as the main body. Each manuscript has an abstract, introduction, materials and methods, results and discussion. The manuscripts are formatted according to the Plant Physiology journal, and they are followed by a general discussion and conclusions, literature cited, and appendices section.

Chapter 1: Introduction

1. INTRODUCTION

Wheat is an important food crop produced across the world, and it is the second most consumed crop next to rice, and the third most produced food crop after rice and corn (FAO, 2012). In Canada, wheat is considered an 'economic fairy to industrial and commercial life', as the economic structure of the Prairie provinces of Alberta, Manitoba and Saskatchewan is dependent on this crop (McCallum and DePauw, 2008). Pre-harvest sprouting, which is defined as germination of physiologically mature seeds on mother plants before harvesting due to wet field conditions (Kolek et al., 1997; Ren et al., 2012), is one of the major problems in wheat growing areas. Pre-harvest sprouting causes downgrading of seed yield and quality, and this in turn leads to economic loss to farmers (Derera, 1989; Barnard and Calitz, 2011). Pre-harvest sprouting is influenced by the level of dormancy in seeds, and the plant hormone abscisic acid (ABA) plays a predominant role in regulation of seed dormancy (Liu et al., 2011).

The level of ABA in plant tissues is regulated by the balance between its biosynthesis and catabolism. ABA catabolism is catalyzed mainly by ABA 8'-hydroxylase, which is encoded by *CYP707A* genes and the expression of these genes can be directly correlated with seed dormancy levels (Kushiro et al., 2004; Nambara and Marion-Poll, 2005; Okamoto et al., 2011). The role of ABA and the expression patterns and physiological function of *CYP707A* genes in seed dormancy and germination has been extensively studied in the model plant Arabidopsis, as well as other dicot species, and the findings have increased the understanding of the molecular mechanisms that underlie dormancy. For example, functional analysis of the Arabidopsis *CYP707A1-4* genes in yeast revealed the *in-vitro* function of these genes in converting bioactive ABA to inactive phaseic acid (Kushiro et al., 2004). Expression analysis of the *CYP707A1* and *CYP707A2* genes of

Arabidopsis showed that the two genes vary in terms of their stage and tissue specific expression patterns. Furthermore, mutational analysis of these two ABA catabolic genes in Arabidopsis has demonstrated their physiological function in regulating seed ABA level and dormancy, however, seeds of *cyp707a1* exhibit higher accumulation of ABA than that of *cyp707a2* mutants (Okamoto et al., 2006). Conversely, constitutive expression of *CYP707A1* in Arabidopsis led to reduction in seed ABA content and dormancy level (Millar et al., 2006).

Given that dormancy level is related to the problem of pre-harvest sprouting in cereal crops, such knowledge is very important for designing tools that can help to reduce the problem. Previous studies in cereal crops, such as barley, have identified and characterized the expression patterns of *CYP707A* genes, and these studies also indicate the importance of the ABA catabolic genes in controlling ABA level and seed dormancy and germination (Chono et al., 2006; Millar et al., 2006). Comparative analysis of the expression patterns of the two barley *CYP707A* genes, *CYP707A1* and *CYP707A2*, in embryos of germinating seeds showed a much higher expression of *CYP707A1* than that of *CYP707A2*. In addition, comparative analysis between dormant and non-dormant barley seeds suggested the importance of *CYP707A1* in regulating ABA levels and dormancy (Millar et al., 2006). A study that involved RNAi mediated knockdown of the barley *CYP707A1* gene indicated that reduced expression of the *CYP707A1* gene leads to an increase in ABA accumulation, as well as depth of seed dormancy (Gubler et al., 2008). In the Japanese wheat cultivar Tamaizumi, a double mutant, due to mutations in the A and D genome copies of *CYP707A1*, is recently reported to exhibit high accumulation of ABA and reduced seed germination (Chono et al., 2013).

In addition to ABA, other plant hormones such as brassinosteroid (BR), ethylene (ET), and cytokinin (CK) have also been shown to be involved in regulation of seed dormancy and

germination. Brassinosteroid is shown to positively regulate gibberellin (GA) signaling (Bouquin et al., 2001), and consequently it promotes GA induced seed germination (Ullah et al., 2002; Assmann, 2005). It has also been reported that ABA and BR interact at the molecular level (Wang et al., 2009), and ABA regulates BR signaling (Yusuf et al., 2017), which implies coregulation of seed dormancy and germination by BR and ABA. Mutation in ET signaling gene *etr1-2* led to higher ABA accumulation and seed dormancy in Arabidopsis, indicating the interaction between ABA and ET in the regulation of dormancy and germination (Chiwocha et al., 2005). The significant role of ethylene in seed dormancy decay is related to its counteracting effect on ABA induced seed dormancy, and promoting endosperm rupture, has been shown in red rice (Gianinetti et al., 2007). A possible antagonistic interaction between ABA and CK has also been reported in Arabidopsis (Wang et al., 2011), and such antagonistic interaction suggests that CK act as a positive regulator of seed germination (Khan, 1968; Saha et al., 1986; Hocart and Letham, 1990). Despite these reports, the molecular bases regulating ABA level and its effect on the metabolism and signaling of other hormones, and thereby seed dormancy and germination, are scarcely studied in cereal crops, especially in hexaploid wheat owing mainly to its complex genetic makeup. This thesis project is therefore aimed:

1) to identify and characterize genome specific homeologs of the ABA catabolic gene *CYP707A1* from dormant and non-dormant Canadian wheat genotypes;

2) to investigate the functionality and expression pattern of the *TaCYP707A1* homeologs with respect to seed dormancy and germination, and;

3) to examine transcriptional changes in the expression of genes involved in the metabolism and signaling of other hormones, mainly BR, ET and CK, due to dormancy loss and in response to ABA.

Chapter 2: Literature Review

2. LITERATURE REVIEW

2.1 Wheat as major food crop

Wheat is the third most produced crop next to rice and corn worldwide according to the 2013 United Nations Food and Agriculture Organization report (FAOSTAT, 2013). Due to its hardy nature, wheat can adapt to various environmental conditions from temperate to tropical climate, and its production covers a large area in many countries from Asia to North America. It is the second most consumed cereal crop next to rice; nearly 35% of the world`s population consumes wheat (FAOSTAT, 2013). The world's most cultivable land is covered by wheat, which accounts for 17% of land area, and is more than any other cereal crop. In addition to being one of the major food crops, wheat is also utilized as animal feed and for industrial uses. Of the total wheat produced in the world, about 65% is consumed as food for humans, 17% is used as feed for animals, and 12% is used in industrial purposes including breweries, bakeries, and other food processing industries (Figure 2.1). The major wheat producing countries in the world are China, India, USA, the Russian Federation, France, and Canada, together contributing about 55% of the world`s total wheat production (FAOSTAT 2013).

2.2 Evolution and domestication of wheat

Bread wheat (*Triticum aestivum* L.) is an allohexaploid that is comprised of three genomes (AA, BB, and DD) carrying 21 pairs of chromosome (2n=42). Wheat was domesticated approximately 10,000 years ago in the fertile crescent of the middle east, and this crop had a very crucial role in human civilization and evolutionary history (Kilian et al., 2010). Wheat originated from three diploid progenitors belonging to genera *Triticum* and *Aegilops* by hybridization and duplication of

genomes. Among the three genomes the 'A-genome' of hexaploid wheat is closely related to wild *Triticum urartu,* and also with cultivated einkorn wheat *Triticum monococcum* (Shewry, 2009). Origin of the 'B-Genome' of hexaploid wheat is still not very clearly known and controversial, however, it is assumed to be closely related to the S-Genome of Sitopsis section from genus *Aegilops*. Because of its close relationship, *Aegilops speltoides* is considered as the B genome progenitor, however more than one species of Sitopsis are referred to as the B genome donor (Sarkar and Stebbins, 1956; Haider, 2013). The tetraploid wheat that evolved at this stage have two prominent forms, one form is *Triticum turgidum* with the genomic composition of AABB, and the other form is *Triticum timopheevii,* which consists of the genome AAGG. However, these forms of tetraploid wheat have significant variation in their genetic components including both the coding and non-coding regions (Gustafson et al., 2009). The origin of these two forms of tetraploid wheat may or may not be by single independent cross between existing wild progenitors (Feldman, 1976, Brown, 1999; Shewry, 2009) The third 'D-genome' is known to come from *Triticum tauschii,* which may have been hybridized with emmer wheat *Triticum turgidum* during the process of domestication (Feldman, 1976; Gustafson et al., 2009). The important traits that wheat possessed, in comparison to other grass species, during the course of evolution and domestication are non-shattering seed and bigger seed size to ensure higher yield and proper germination, enabling farmers to harvest the crop after maturation (Eckardt, 2010). In addition, its adaptability from temperate to tropic environmental conditions made it become the second most consumed food in the world.

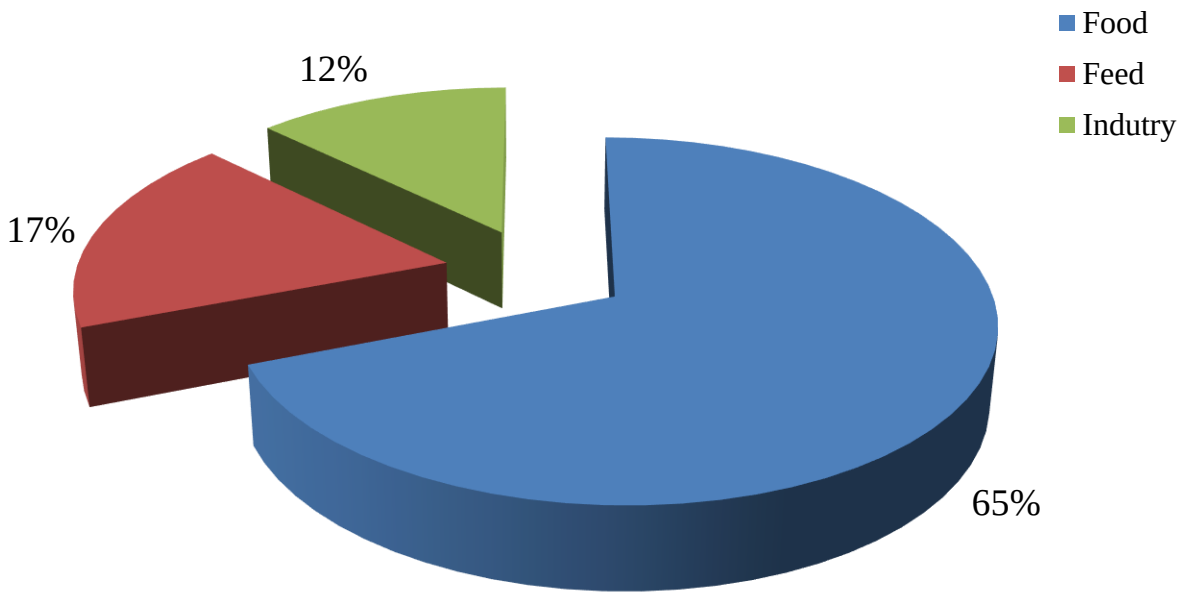


Figure 2.1. Percent utilization of total wheat produced for food, feed, and industry [Source: FAOSTAT (2013)].

2.3 History of wheat cultivation, production, and utilization in Canada

As originally quoted in 1928 by Newman, and further requoted by McCallum and DePauw (2008), wheat was "the economic fairy to the industrial and commercial life of Canada, having built particularly the whole economic structure of the Prairie Provinces". Wheat is the major food source of Canada and is extensively cultivated in the Prairie Provinces of Alberta, Manitoba, and Saskatchewan, which contribute nearly 80-85% of the wheat produced in Canada. It is considered to be one of the most important cash crops next to Canola. Wheat is also grown in other provinces such as British Columbia, Ontario, Quebec, New Brunswick, and Nova Scotia, on a minor scale.

In general, the history of wheat cultivation traces back 10,000 years. It is believed that pastoral nomads spread wheat over the whole world from the fertile crescent, where its cultivation originated (Kilian et al., 2010). Wheat cultivation in western Canada began around two centuries

ago in Selkirk. Seeds of winter wheat brought from Scotland were planted by Scottish settlers in 1812, however, the settlers ended up with poor harvest as the seeds were planted too late. The occurrence of unfavorable conditions such as flooding, pests and disease in subsequent years led to the depletion of the seed stock (Buller, 1919). Nevertheless, a variety referred to as "Red Fife" became popular since it withstood the harsh Canadian weather conditions. Subsequently, various varieties of wheat were imported from other counties including USA, Russia, Australia, Ukraine and Japan and these varieties established well in the Prairies of Canada (Buller, 1919). During World-War-I, wheat production in Canada was significantly reduced due both abiotic and biotic stress factors including drought, pest damage, especially by grass-hoppers, and diseases. By the year of 1920, the most popular variety of Canada 'Marquis' occupied almost 90% of the hard-red spring wheat cultivated area of Canadian Prairies (McInnis, 2004). The developments made with respect to wheat cultivation in Canada led to the formation of the "Canadian Wheat Board" in 1935, which further enhanced the stability of wheat production, and this board played a role in regulating grain prices and thereby supporting farmers (Skogstad, 2005). Furthermore, mechanization in farm operations in the 20th century, along with the advancement in developing superior cultivars, resulted in increased wheat production, which reached 27 million metric tons (MMT) per annum at present (FAOSTAT, 2013). Apart from being a major food source, wheat is also used to feed livestock and as a raw material for several industrial utilizations including in breweries (FAOSTAT, 2013). According to the 2016 report of Statistics Canada, wheat cultivation in Canada covered about 22 million acres (http://www.statcan.gc.ca/tables-tableaux/sum-som/l01/cst01/prim11a-eng.htm).

Canada exports about 20 MMT of wheat annually and it is the second major wheat exporting country, next to United States. Wheat ranks number one among the grain crops exported

from Canada followed by rapeseed and soybean. More than 70% of the wheat produced in Canada is exported to other countries, and this contributes about 6 billion US dollar export value (FAOSTAT, 2013). Major importers of Canadian wheat include China, Japan, Mexico, Italy, and Bangladesh (https://www.grainscanada.gc.ca/statistics-statistiques/cge-ecg/cgem-mecg-eng.htm). Since the major portion of wheat produced in Canada is exported, it is paramount to assure high grain quality to meet the importers demand. To ensure supply of high-quality grain, Canadian wheat varieties are grouped into different classes and the Canadian Grain Commission monitors different grain parameters for quality products.

2.4 Wheat classes and varieties in Canada

Wheat varieties in Canada are classified into unique classes. In each class, varieties are grouped based on their functional properties. The Canadian Grain Commission, which controls the postharvest quality of wheat grains, has classified wheat into nine milling classes in the Canada Western category, and ten milling classes in the Canada Eastern category. This classification helps to provide quality material to end users based on their industry requirements. The nine Canada Western classes are listed (Table 2.1) along with their functional properties. Among the nine wheat classes listed, the Canada Western Red Spring (CWRS) class is the most popular due to its good milling characteristics, which is an important trait for the bakery industries to produce good quality products under different production conditions. Because of better milling character CWRS cultivars have higher demand in the international market (McCallum and DePauw, 2008).

Table 2.1. Wheat classes in western Canada, their characteristics and end use

Wheat class	Characteristic	Uses
Canada Northern Hard Red (CNHR)	Red spring wheat, medium to hard kernels, very good milling quality, medium gluten strength, three milling grades	Hearth breads, flat breads, steamed breads, noodles
Canada Prairie Spring Red (CPSR)	Red spring wheat, medium hard kernels, medium dough strength, two milling grades	Hearth breads, flat breads, steamed breads, noodles
Canada Prairie Spring White (CPSW)	White spring wheat, medium dough strength, two milling grades	Flat breads, noodles, chapatis
Canada Western Amber Durum (CWAD)	Durum wheat, high yield of semolina, excellent pasta making quality, four milling grades	Semolina for pasta, couscous
Canada Western Extra Strong (CWES)	Hard red spring wheat, extra strong gluten, two milling grades	Ideal for blending, used in specialty products when high gluten strengths needed
Canada Western Hard White Spring (CWHWS)	Hard white spring wheat, superior milling quality producing flour with excellent color, three milling grades	Bread and noodle production
Canada Western Red Spring (CWRS)	Hard red spring wheat, superior milling, and baking quality, three milling grades, various granulated protein levels	Production of high volume pan bread, used alone or in blend with other wheat for hearth bread, steamed bred, noodles, flat bread, common wheat pasta
Canada Western Red Winter (CWRW)	Hard red winter wheat, very good milling quality, three milling grades	French breads, flat breads, steamed breads, noodles,
Canada Western Soft White Spring (CWSWS)	Soft white spring wheat, low protein content, three milling grades	Cookies, cakes, pastry, flat breads, noodles, steamed breads, chapatis

Source: Canadian Grain Commission (http://www.grainscanada.gc.ca/wheat-ble/classes/classes-eng.htm).

2.5 Development of wheat cultivars in western Canada

In western Canada wheat cultivars were developed with improved agronomically important traits such as high yield, disease resistance, insect resistance, frost damage, lodging resistance, adaptability to heavy metal soil, herbicide compatibility and stress tolerance (McCaig and DePauw, 1995; DePauw et al., 2007). In addition to major agronomical trait improvement, end use quality of grains is also an important consideration in developing cultivars or in breeding programs. The improvements in end use qualities such as protein content, gluten strength, better milling and dough characteristics and resistance to pre-harvest sprouting, are continuously observed (Paterson et al., 1989; McCaig and DePauw, 1995; Bushuk, 1997).

2.6 Biotic and abiotic stress factors affecting wheat production

Biotic and abiotic stress factors negatively affect crop growth and productivity, leading to substantial yield loss. Drought and heat stress are two major abiotic stress factors which significantly affect crop yield (Fahad et al., 2017). Crop yield loss due to drought is the highest among the abiotic factors, and it is considered as a leading threat for the world food supply, as the changes in rainfall patterns and reduction in the amount of precipitation are causing frequent occurrence of drought (Lobell et al., 2011). A recent report that summarizes data published between 1980 and 2015 indicated that the global reduction of wheat yield due to drought stress alone is up to 20% (Daryanto et al., 2016). The poor growth and development caused by excessive radiation and elevated temperatures also causes significant yield loss in crop plants. A simulation study on the effect of rising temperature on wheat production indicated that every degree Celsius increase in global temperature is expected to cause 6% reduction in production (Asseng et al., 2014). In addition to drought and heat stress, salinity, cold and nutrient stress also have negative

effects on crop production, and all these abiotic factors together are suggested to cause over 50% yield reduction in major crops including wheat (Wang et al., 2003). The occurrence of wet and humid conditions following physiological maturity also leads to pre-harvest sprouting that is known to cause substantial yield and quality loss in wheat (see the next section for detailed discussion of this factor).

In wheat, diseases caused by various pathogens negatively affect its production and yield globally (Kassa et al., 2017). Among these, fusarium head blight and rust are major constraints affecting wheat yield in Canada and globally (Gilbert and Tekauz, 2000; Cuthbert et al., 2007; Ellis et al., 2014; Kassa et al., 2017). The occurrence of fusarium head blight in part of Manitoba, Saskatchewan and Alberta has been reported every year, and, it is also wide-spread in Ontario and Quebec (http://publications.gc.ca/collections/collection_2009/agr/A118-10-16-2005E.pdf). Other plant diseases, including powdery mildew, leaf spot and seedling rot, also affect wheat in Canada. Insect pests such as grasshoppers, cutworm, wheat stem sawfly, wheat midge, aphids and wireworms are widespread in wheat growing provinces of Canada and all affect wheat production (http://publications.gc.ca/collections/collection_2009/agr/A118-10-16-2005E.pdf). The direct yield loss due to pathogens, insect pests and weeds altogether accounts for 20-40% globally (Oerke, 2006)**.**

2.7 Pre-harvest sprouting in wheat

Pre-harvest sprouting (PHS) is precocious germination of physiologically mature grains on the mother plant prior to harvest, due to wet field conditions. A combination of high moisture, low temperature, and availability of oxygen encourages embryo germination, which eventually deteriorates grain quality and yield (Kolek et al., 1997; Ren et al., 2012). Deterioration of grain

and reduction in test seed weight affect the final crop yield. In addition, there is an impact on market value of grain, as PHS downgrades crop quality (Derera, 1989; Barnard and Calitz, 2011). These effects cause economic loss to farmers, and to end user including processing industries and consumers. It is estimated that PHS causes financial loss of $100 million annually in Canada (Clarke et al., 2005; DePauw et al., 2012). Although many factors contribute towards PHS resistance including spike morphology, ease of water uptake and seed physical structure, seed dormancy is considered to be the major genetic component (Liu et al., 2011). Pre-harvest sprouting is common in those cultivars which do not have adequate levels of seed dormancy. On other hand, high level of seed dormancy negatively affects uniformity of seed germination and seedling establishment (Gubler et al., 2005). The two extreme conditions are therefore undesirable for good yield and quality of cereal crops.

2.8 Seed dormancy and germination

Seed dormancy can be defined as the inability of a viable intact seed to germinate under favorable environmental condition (Bewley, 1997; Finkelstein et al., 2008). In natural habitat seeds produced from wild plants have higher dormancy levels to keep seeds viable in prevailing adverse environmental conditions and for better dispersal (Finkelstein et al., 2008). Germination refers to the process that starts with the uptake of water by a dry seed and terminates with the emergence of the embryonic axis through the seed coat (Bewley and Black, 1994).

2.9 Classification of seed dormancy

Baskin and Baskin (2004), proposed a new classification system for seed dormancy, considering both physiological and morphological properties of seed, and this system is modified from Russian

seed physiologist Nikolaeva (2004). The newly developed system classifies seed dormancy into three hierarchical layers, such as class, level, and type, where class contains level and level may contain type. This system includes five classes, namely physiological dormancy (PD), morphological dormancy (MD), morpho-physiological dormancy (MPD), physical dormancy (PY), and combinational dormancy (PY + PD). In each class, there are many levels, and there are many types in each level.

Based on its onset, seed dormancy is classified as primary and secondary (Figure 2.2). Primary dormancy refers to the type of dormancy that occurs prior to dispersal as part of the seed's developmental program and includes all the dormancy classes and their combinations. In other words, primary dormancy can be induced while seeds are on the mother plant either by presence of inhibitors, hormones, or other seed related factors. Whereas, dormancy induced in viable/non-dormant seeds due to unfavorable environmental conditions is known as secondary dormancy (Figure 2.2) (Bewley, 1997; Finkelstein et al., 2008). The interaction of a seed with its surrounding environment is a very complex process, in which factors such as temperature, light, moisture, and nutrition play a crucial role in maintenance and release of seed dormancy (Finch-Savage and Leubner-Metzger, 2006; Finch-Savage et al., 2007).

2.10 Regulation of seed dormancy

Seed related dormancy is inherent, but the induction of dormancy during seed development and its intensity in mature seeds varies greatly with genotype and the environmental conditions experienced by the mother plant during seed development (Bewley, 1997). The degree of dormancy can be correlated with the changes in gene expression, enzyme activity, and hormone metabolism (Carrari et al., 2001; Leubner-Metzger, 2005; Finch-Savage et al., 2007). Previous

studies have also suggested that physiological dormancy can be reversed via altering seed hormonal levels or sensitivity during seed development and germination (Marin et al., 1996; Kushiro et al., 2004; Barrero et al., 2005; Lefebvre et al., 2006; Saito et al., 2006). Plant hormones, such as ABA and gibberellin (GA), play important roles in the process of seed dormancy acquisition, maintenance, and decay (Brady and McCourt, 2003). Lack of dormancy in seeds is associated with increased potential of pre-harvest sprouting in wheat (Nakamura et al., 2010; Liu et al., 2011), while strong dormancy affects germination and seedling establishment. Therefore, understanding the mechanisms of dormancy induction, maintenance and its decay is very important for selecting and/or developing cultivars with intermediate level of dormancy in cereal crops like wheat. The following sections discuss regulation of seed dormancy and germination by different environmental factors and intrinsic factors, mainly plant hormones.

2.11 Environmental factors regulating dormancy and germination

The metabolic and physiological processes occurring in the plant during its growth and development are influenced by ambient temperature. Speed of germination completion is directly related to temperature in non-dormant wheat seeds, whereas the opposite relationship was observed in dormant seeds (George, 1967). Exposure of plants to different temperature regimes during seed development influences the degree of seed dormancy induced in wheat (George, 1967; Reddy et al., 1985). Optimum temperature is required for adequate germination in which the temperature below or above defined optimum temperature will affect the rate of seed germination (Garcia-Huidobro et al., 1982). High temperature during imbibition induces dormancy, due to its effect in enhancing abscisic acid (ABA) biosynthesis and the sensitivity of seeds to ABA. This relationship of high temperature and higher level of seed dormancy was observed in Canadian

wheat cultivars and other genotypes (Nyachiro et al., 2002). In contrast, pretreatment of seeds with low temperature promotes germination by concomitantly stimulating gibberellin biosynthesis and abscisic acid catabolism (Yamauchi et al., 2004).

Adequate moisture is of paramount importance for germination to occur, and speed of germination is inversely related with moisture stress in wheat (Ashraf and Abu-Shakra, 1978). Sensitivity of seed germination to moisture stress has been shown in different crop species including vegetable crops, wheat and sunflower (Ross and Hegarty, 1979; Ghanifathi et al., 2011) Comparative analysis of the effect of moisture stress on germination of *Aegilops geniculata* and cultivars of durum and bread wheat showed genotypic variation in seed germination under moisture stress. However, under high or extreme water stress conditions, seed germination appeared to be affected in most genotypes (Orsenigo et al., 2017).

Light is another environmental factor that affects seed germination. The role of light in seed germination has been shown in different crop species including barley and lettuce (Burger, 1965; Nabors and Lang, 1971; Bewley, 1997). For example, treatment of barley seeds with far red continuous light inhibits germination (Burger, 1965) and imbibition of barley seeds under darkness lead to reduction of ABA accumulation in the embryo by up to 30-50%, thereby enhancing germination as compared to imbibition under white light (Jacobsen et al., 2002). Gene expression analysis in imbibed barley seeds showed that white and blue light promote dormancy by inducing the expression of 9-cis-epoxycarotenoid dioxygenase (*NCED*) genes and ABA content in the embryo of seeds (Gubler et al., 2008; Barrero et al., 2009).

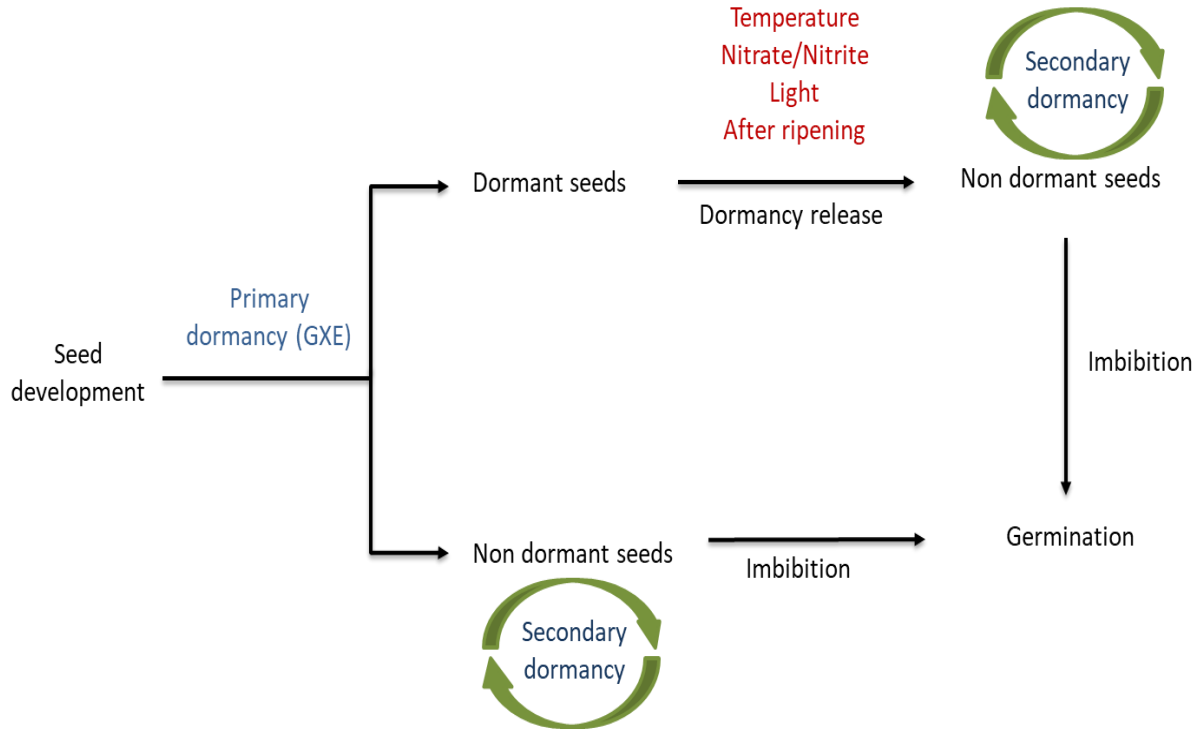


Figure 2.2. Illustration of primary and secondary seed dormancy induction and release cycles by various factors including genetic and environmental (GXE) in seeds (Adopted from Gao and Ayele, 2014).

Seed dormancy can be affected by after-ripening (AR), which refers to a post-harvest practice where mature dried seeds are stored under ambient room temperature. It is a storage duration and storage environment dependent process, where release of dormancy occurs due to increased sensitivity of seeds to conditions that promote germination, and decreased sensitivity to conditions that induce dormancy (Finch-Savage and Leubner-Metzger, 2006; Finch-Savage et al., 2007). Effect of AR in very dry seeds in not significant, therefore, optimum moisture content of seeds is necessary. In contrast, high relative humidity during seed storage negatively affects the efficiency of AR in dormancy decay (Hay et al., 2003; Finch-Savage and Leubner-Metzger, 2006) and seed viability. Release of dormancy by after-ripening has been shown to be associated with

changes in global gene expression patterns, including those involved in DNA replication, nitrogen metabolism, cell wall modification, and ABA and GA biosynthesis and catabolism (Bove et al., 2005; Cadman et al., 2006; Barrero et al., 2009; Gao et al., 2012).

2.12 Regulation of seed dormancy and germination by plant hormones

2.12.1 ABA a major player in seed dormancy regulation

Abscisic acid is the primary mediator of seed dormancy (Finkelstein et al., 2008), which is required for inducing dormancy during embryo maturation, and the accumulation of ABA can be correlated with the degree of primary dormancy (Kermode, 2005). Abscisic acid is an isoprenoid class of plant hormone synthesized from zeaxanthin (Figure 2.3), which is converted to violaxanthin by zeaxanthin epoxidase (ZEP), and this reaction is reversible under high light conditions and is catalyzed by violaxanthin de-epoxidase (VDE). Arabidopsis mutants *aba1* and *aba2* with impaired expression of *ZEP* show precocious germination, confirming the role of ABA biosynthesis in the regulation of seed dormancy and germination (Marin et al., 1996; Barrero et al., 2005). Conversion of violaxanthin to 9'-cis-neoxanthin or 9'cis-violaxanthin is known to be mediated by neoxanthin synthase (*NSY*). No orthologous gene for *NSY* has been identified from Arabidopsis, however, the *NSY* gene has been identified in potato and tomato (Al-Babili et al., 2000; Bouvier et al., 2000). The formation of xanthoxin from 9'-cis-neoxanthin or 9'-cis-violaxanthin is mediated by 9-cis-epoxycarotenoid dioxygenase (NCED), one of the rate limiting steps in abscisic acid biosynthesis. Mutant studies in Arabidopsis have shown the involvement of *NCED* in seed dormancy induction (Chono et al., 2006; Lefebvre et al., 2006; Frey et al., 2012). In Arabidopsis, five genes encoding NCED (*AtNCED* 2, 3, 5, 6, and 9) are cloned. Among the five isoforms, *NCED5*, *NCED6,* and *NCED9* are known to play crucial roles in controlling seed dormancy and germination (Lefebvre

et al., 2006; Frey et al., 2012). In barley, two *NCED* genes have been isolated, namely *HvNCED1* and *HvNCED2* (Millar et al., 2006). The *NCED1* gene of barley is found to be highly expressed in dormant barley embryos, contributing to higher ABA synthesis (Millar et al., 2006). Studies in developing barley seeds have shown that *NCED2* plays a significant role in increasing ABA levels during the early to mid-maturation stage of seed development (Chono et al., 2006). A study with *Brachypodium distachyon* seeds indicated a significant decrease in the expression of *NCED1* in response to after-ripening although its level of expression appeared to be similar between dry dormant and AR seeds, suggesting a role for *BdNCED1* in dormancy (Barrero et al., 2012). Conversion of xanthoxin to abscisic acid aldehyde is mediated by short chain alcohol dehydrogenase or Xanthoxin dehydrogenase (ABA2) (Schwartz et al., 1997; Gonzalez-Guzman et al., 2002). The enzyme abscisic acid aldehyde oxidase (AAO) catalyzes the final step of converting abscisic acid aldehyde to abscisic acid. In Arabidopsis, four isoforms of this gene are reported, of which *AAO3* is reported to be the candidate gene encoding AAO (Seo et al., 2000), and which is reported to have a role in maintaining ABA levels in plants under abiotic stress conditions (Barrero et al., 2006; Melhorn et al., 2007).

2.12.2 ABA catabolism

In addition to its biosynthesis, the catabolism of ABA plays a pivotal role in regulating seed ABA levels. The inactivation of abscisic acid occurs in two ways, conjugation and hydroxylation (Kushiro et al., 2004; Nambara and Marion-Poll, 2005). It is reported that three kinds of hydroxylation reactions occur to form phaseic acid or inactive forms of abscisic acid derivatives. The three hydroxylation reactions are catalyzed by ABA 7'-hydroxylase, 8'-hydroxylase and 9'-hydroxylase, which essentially oxidize the respective methyl group and catalyze the inactivation

reactions (Figure 2.3). The ABA hydroxylases belong to the cytochrome P450 monooxygenase group and are encoded by *CYP707A*s (Krochko et al., 1998; Okamoto et al., 2011). However, among the three hydroxylation pathways, the major form of ABA catabolism in plants is hydroxylation of the 8-methyl group of ABA (Saito et al., 2004; Umezawa et al., 2006).

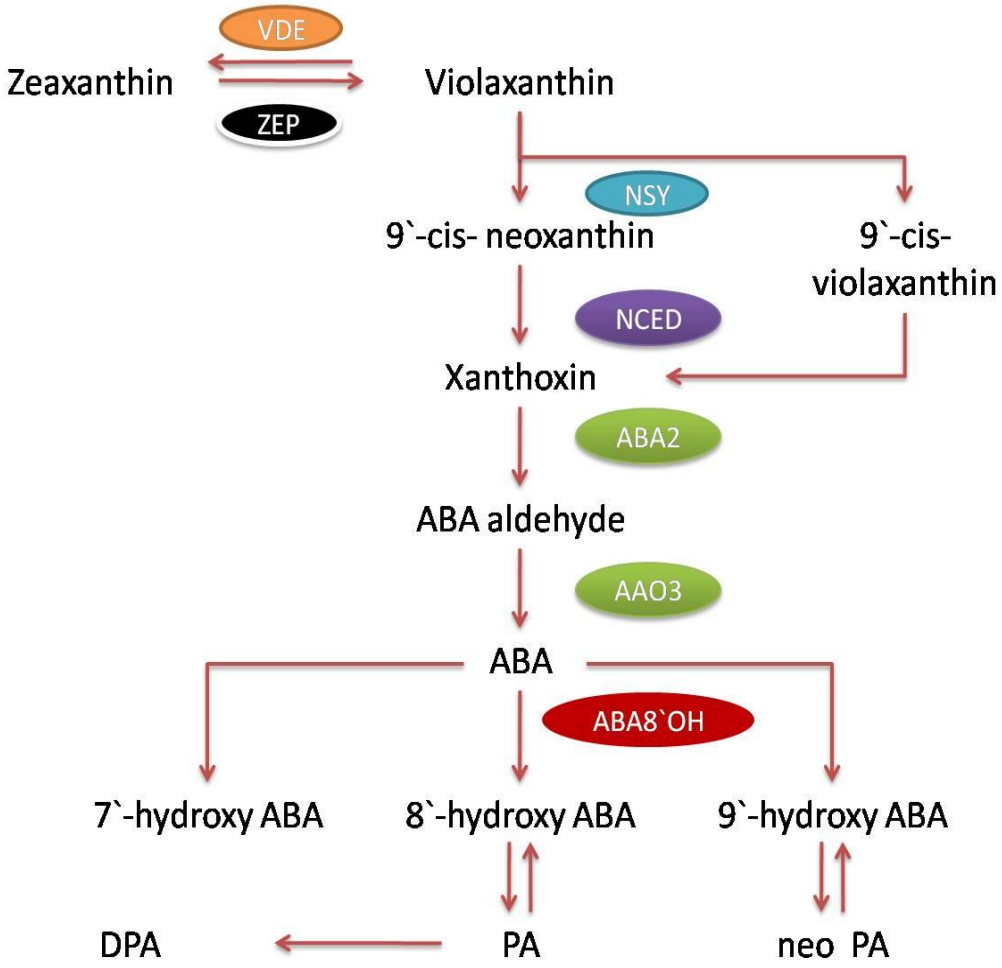


Figure 2.3. Abscisic acid biosynthesis and catabolic pathway in plants. Abbreviations: ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase; NSY, neoxanthin synthase; NCED, 9-cis-epoxycarotenoid dioxygenase; ABA2, Xanthoxin dehydrogenase; AAO3, ABA aldehyde oxidase; ABA8'OH, ABA 8'-hydroxylase; PA, Phaseic acid; DPA, Dihydrophaseic acid.

2.12.3 Importance of ABA 8'-hydroxylases in regulation of seed dormancy

Several studies indicate that the expression of members of the *CYP707A* gene family in plants is regulated in a tissue and stage specific manner. Functional analysis of the Arabidopsis genes encoding ABA 8'-hydroxylases (*CYP707A1-4*) in yeast revealed their role in converting bioactive ABA to inactive phaseic acid (Kushiro et al., 2004). Mutant analysis of these genes suggested the crucial role of *CYP707A2* specifically in decreasing seed ABA level during imbibition, and therefore enhancing germination (Millar et al., 2006). Furthermore, tissue and stage specific analysis of the expression of *CYP707A1* and *CYP707A2* genes revealed that *CYP707A1* is highly expressed in the embryo during the mid-maturation phase, while *CYP707A2* expression is high during late maturity in both embryo and endosperm tissues. Consistently, mutational studies in Arabidopsis showed the presence of higher amount of ABA in the dry seed of *cyp707a1* mutant than in that of *cyp707a2*.

Efforts have also been made to understand the role of *CYP707A* genes encoding ABA8'OH in regulating dormancy in cereal crops. The role of *CYP707A2* in the regulation of dormancy and germination has been shown in rice in which glucose induced delay in germination has been shown to be associated with repression of *CYP707A2* and accumulation of ABA (Zhu et al., 2009; Zhu et al., 2011). In barley, the *CYP707A1* gene has been cloned and its role in maintaining seed ABA content and dormancy characterized. Higher expression of *CYP707A1* during late grain developmental stage was shown to be associated with lower levels of ABA accumulation in barley seeds (Chono et al., 2006). Analysis of *CYP707A1* in the embryo of AR barley seeds indicated its enhanced expression as compared to that observed in the corresponding dormant seeds (Millar et al., 2006), suggesting its pivotal role in dormancy decay. RNAi based knock down of *CYP707A1* in barley resulted in its reduced expression and thereby increased ABA content and depth of

dormancy (Gubler et al., 2008). In wheat, the expression of wheat *CYP707A2* gene appeared to be high during the mid-maturation stage of seed development, suggesting its relation with the induction of seed dormancy (Nakamura et al., 2010). Mutational analysis of *CYP707A1* in the Japanese cultivar, Tamaizumi, indicated that double mutation in the in A and D genome copies of *CYP707A1* leads to high accumulation of ABA and reduced germination in wheat seed (Chono et al., 2013).

2.12.4 Brassinosteroids and its interaction with ABA in seed dormancy regulation

Brassinosteroids (BR) are a class of polyhydroxysteroids, which are involved in the regulation of several plant growth and developmental processes, including seed germination. Biosynthesis of BR begins with conversion of campesterol to campestanol mediated by DEETIOLATED2 (DET2), and the loss of DET2 function in Arabidopsis shows many defects in plant development that can be rescued by the exogenous application of brassinolide (Szekeres et al., 1996; Fujioka et al., 1997). The role of DET2 in converting campesterol to campestanol has been biochemically characterized in the cultured cells of *Catharanthus roseus* (Noguchi et al., 1999). Oxidation of campestanol to brassinolide occurs either via the early C-6 oxidation or late C-6 oxidation pathway, where C-22 hydroxylation takes place before C-6 oxidation involving multiple intermediate steps (Yusuf et al., 2017). This rate limiting step of 22-alpha hydroxylation is mediated by the DWF4 enzyme (Figure 2.4), which belong to the cytochrome P450 protein superfamily (Choe et al., 1998). Chemical inhibition of DWF4 by triadimefon lead to BR deficiency in plants, indicating the pivotal role of DWF4 in BR biosynthesis (Asami et al., 2003). Overexpression of *DWF4* in Arabidopsis results in increased accumulation of downstream

metabolites and the resulting plants show increased vegetative growth and seed yield (Choe et al., 2001).

Brassinosteroid signaling is perceived by the plasma membrane-bound leucine-rich repeat like kinase receptors designated as BRASSINOSTEROID INSENSITIVE1 (BRI1) (Wang et al., 2001), The BRI1 protein activates downstream BR signaling cascade via regulation of cytosolic and nuclear transcription kinases and phosphatases (Li and Chory, 1997). The analysis of repression of germination inhibition by ABA in the BR-insensitive mutant (*bri1-1*) of Arabidopsis, as compared to the wild type, is suggestive of the role of BR signaling and BRI1 in overcoming ABA induced dormancy (Steber and McCourt, 2001). Activated BRI1 phosphorylates BR signaling kinases (BSKs), which in turn activates BRI1 SUPPRESSOR 1 (BSU1), and the activated BSU1 deactivates BRASSINOSTEROID INSENSITIVE 2 (BIN2), a negative regulator of BR signaling, via dephosphorylation (Tang et al., 2008; Kim and Wang, 2010; Clouse, 2011; Yusuf et al., 2017). Inactivation of BIN2 relieves the transcription factor BRASSINAZOLE RESISTANT 1 (BZR1), leading to activation of BR responsive genes (Figure 2.4), via other transcription factors in the nucleus (Yan et al., 2009; Li, 2010; Luo et al., 2010).

The plant hormone BR positively regulates GA signaling (Bouquin et al., 2001), and the signal transduction pathway of BR likely involves heterotrimeric G protein mediated regulation to potentiate GA induced seed germination (Ullah et al., 2002; Assmann, 2005). Consistently, the Arabidopsis G Protein–Coupled Receptor (CGR1), a seven-transmembrane (7TM) receptor, was also shown to have a role in BR and GA signaling in seed germination independent of the heterotrimeric G protein in *gcr1*, *gpa1*, and *agb1* mutants (Chen et al., 2004). The BR and GA signaling integration has been shown to occur through physical interaction between the DELLA protein GAI and BRASSINAZOLE RESISTANT1 (BZR1) (Gallego-Bartolome et al., 2012).

Furthermore, BZR1 was shown to interact with REPRESSOR OF *ga1-3* (RGA) via DELLA mediated signaling and act as a positive regulator of BR signaling and negative regulator of GA signaling (Li et al., 2012). Brassinosteroid has also been reported to interact with ABA at a molecular level, and cross-talk between their signaling cascades occurs after BR perception and before transcriptional activation of BR responses (Wang et al., 2009).

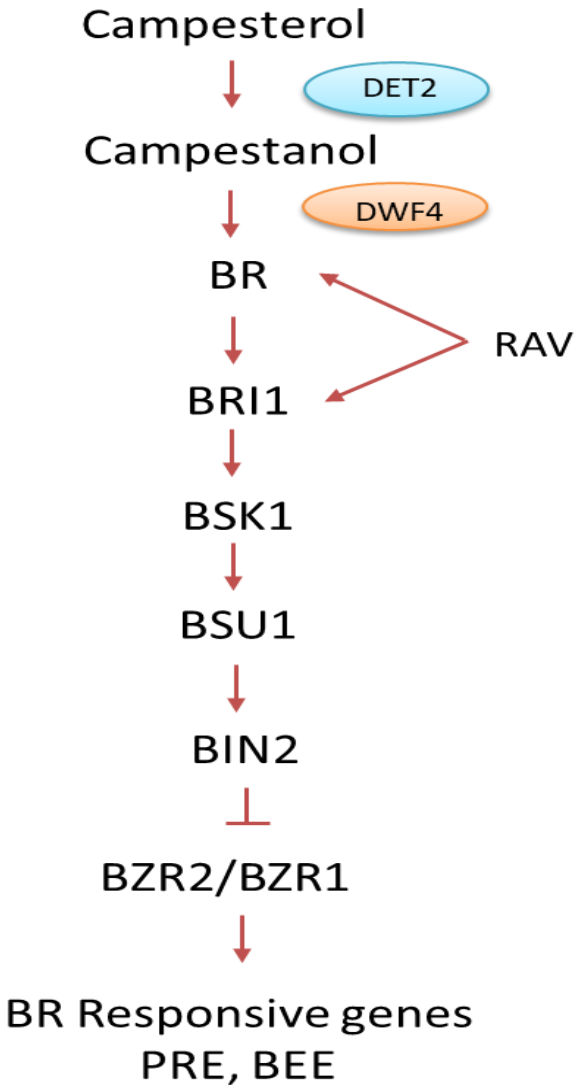


Figure 2.4. Brassinosteroid biosynthesis and signaling pathway in plants. Abbreviations: DET2, Deetiolated 2; DWF4, DWARF; BR, Brassinosteroid; BRI1, BR insensitive 1; RAV, related to ABI3/VP1; BSK, BR signaling kinase; BSU1, BRI1 suppressor; BIN2, BR insensitive 2; BZR, Brassinazole resistant; PRE, paclobutrazol-resistance; BEE, BR enhanced expression.

Germination inhibition induced by exogenous ABA in Arabidopsis seeds expressing *DWF4* has been shown to be reversed by BR (Divi and Krishna, 2010). Studies also indicate that BR and ABA co-regulate many plant developmental processes, including maintenance and release of seed dormancy (Steber and McCourt, 2001; Finkelstein et al., 2008). Furthermore, ABA has been shown to regulate about 35% of BR regulated genes, implying its role in regulating BR signaling (Yusuf et al., 2017).

2.12.5 Ethylene and its interaction with ABA in seed dormancy regulation

Ethylene (ET), a gaseous unsaturated hydrocarbon, has various roles in plant growth and developmental processes from germination to senescence. Establishment of S-adenosylmethionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC) as precursors of ET biosynthesis was a major advance in the elucidation of its biosynthetic pathway (Yang and Hoffman, 1984). The first committed step in ET biosynthesis is the conversion of SAM to ACC by ACC synthase (Yang and Hoffman, 1984), and this step is rate limiting in ET synthesis (Figure 2.5; Kende, 1993). The enzyme ACC oxidase (ACO) catalyzes the conversion of ACC to ethylene (Wang et al., 2002). Genes encoding these enzymes have been successfully cloned from several species (Sato and Theologis, 1989; Hamilton et al., 1991; Spanu et al., 1991).

The identification of several mutants with impaired response to ethylene has led to the cloning and characterization of genes involved in ET signaling and elucidation of the signaling pathway (Wang et al., 2002). Five ET receptors have been identified in Arabidopsis, namely ethylene response 1 (ETR1), ethylene receptor 2 (ETR2), ethylene insensitive (EIN4), ethylene response sensor (ERS1), and ERS2 (Figure 2.5; Hua and Meyerowitz, 1998; Sakai et al., 1998). Ethylene binds to receptors at N-terminal of the transmembrane domain in the presence of copper

co-factor (Hall et al., 2000). In the absence of ET, Raf-like kinase constitutive triple response (CTR1), a negative regulator in ET signaling, is activated (Shakeel et al., 2015), and this leads to negative regulation of the downstream ET response pathways. In presence of ET, CTR1 is inactivated to allow the function of EIN2 as a positive regulator of ET signal transduction. The ET signal transduction occurs downstream from EIN2 to EIN3, which is a nuclear protein that activates a set of transcription factors known as ethylene response factors (ERFs), which in turn leads to the expression of ET responsive genes (Figure 2.5; Wang et al., 2002).

The stimulatory effect of ET on germination has been studied in barley by using ethylene perception inhibitors, and results of this study demonstrated the requirement of ET for optimal germination (Locke et al., 2000). Ethylene promotes seed germination by negatively regulating the action of ABA (Ghassemian et al., 2000). A study on the kinetics of ethylene emission during the germination of red rice seeds revealed that ethylene is not independently involved in speeding up the germination of dormant seeds (Gianinetti et al., 2007), rather, ethylene production increases after the pericarp splits, and this increase in ethylene counteracts ABA induced inhibition of germination and thereby promoting endosperm rupture. Mutational studies showed a higher percentage of seed dormancy in *etr1-2* mutant plants, and this is attributed to a higher accumulation of ABA during seed development (Chiwocha et al., 2005). Studies with *enhanced response ABA3* (*era3*) or *ethylene insensitive 2* (*ein2*) mutants, due to mutations in the corresponding ET signaling components lead to decreased seed sensitivity to ET and thereby enhanced sensitivity of the seeds to ABA (Ghassemian et al., 2000), indicating the role of interaction between ET and ABA signaling in seed germination.

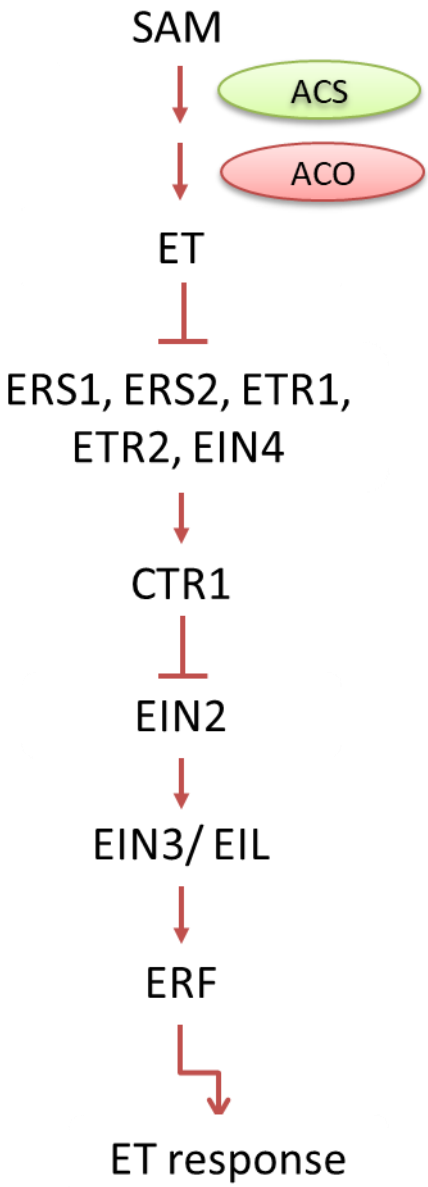


Figure 2.5. Biosynthesis and signaling pathway of ethylene in plants. Abbreviations: SAM, S-adenosyl methionine; ACS, 1-aminocyclopropane-1-carboxylic acid synthase; ACO, 1-aminocyclopropane-1-carboxylic acid oxidase; ET, Ethylene; ERS, ET response sensor; ETR, ET response; EIN, ET insensitive protein; CTR, constitutive triple response; ERF, ET responsive element binding factor.

2.12.6 Cytokinin and its interaction with ABA in seed dormancy regulation

Cytokinin (CK) is another important plant hormone, which has been shown to have a role in promoting seed germination (Khan, 1968). Presence of a high level of CK in germinating maize and rice seeds relative to that found in dry seeds suggests the possible role of this phytohormone in promoting seed germination (Saha et al., 1986; Hocart and Letham, 1990). The first reaction in CK biosynthesis is catalyzed by adenosine phosphate-isopentenyltransferase (IPT), and this step is a rate limiting step in CK synthesis (Figure 2.6; Sakakibara, 2010). Lonely guy (LOG), which encodes a cytokinin riboside 5'-monophosphate phosphoribohydrolase, is another enzyme that plays a crucial role in CK biosynthesis (Kurakawa et al., 2007; Kuroha et al., 2009). Cytokinin is catalyzed by CK oxidase (CKX) which covert CK to adenine and its unsaturated aldehyde by removing the isoprenoid side chain of N^6-(2-isopentenyl) adenine and zeatin (Galuszka et al., 2007). Whereas *cis*-zeatin-o-glucoside (cZOG) catalyzes O-glucosylation of *cis*-zeatin to form a less active conjugate of CK (Kudo et al., 2012) and glucosidase (GLU) converts biologically inactive conjugates to active CK by cleaving cytokinin-O-glucosides (Figure 2.6; Sakakibara, 2010). The CK signal is perceived by its receptors at the plasma membrane and transduced via type-B ARR (To and Kieber, 2008), leading to activation of CK mediated responses (Figure 2.6). A germination promoting role of CK was also proposed in wheat seeds, as CK enhances GA induced *α-amylase* activity (Eastwood et al., 1969). Furthermore, increased expression of the CK biosynthetic gene *isopentenyltransferase 8* (*IPT8*) was observed during germination of the Arabidopsis *germination insensitive to ABA 1* (*gim1*) mutant, suggesting an antagonistic relationship between CK to ABA (Wang et al., 2011). The role of *ABI4* in regulating ABA induced seed dormancy can be related to its mediation of the crosstalk between ABA and CK signaling (Wang et al., 2011; Guan et al., 2014). In *Arabidopsis,* it was demonstrated that *ABI4* inhibits seed

germination by repressing the type-A *Arabidopsis response regulators ARR*s transcripts (Huang et al., 2017). However, the direct role of CK in seed germination and its interaction with ABA during seed germination is still much less understood (El-Showk et al., 2013).

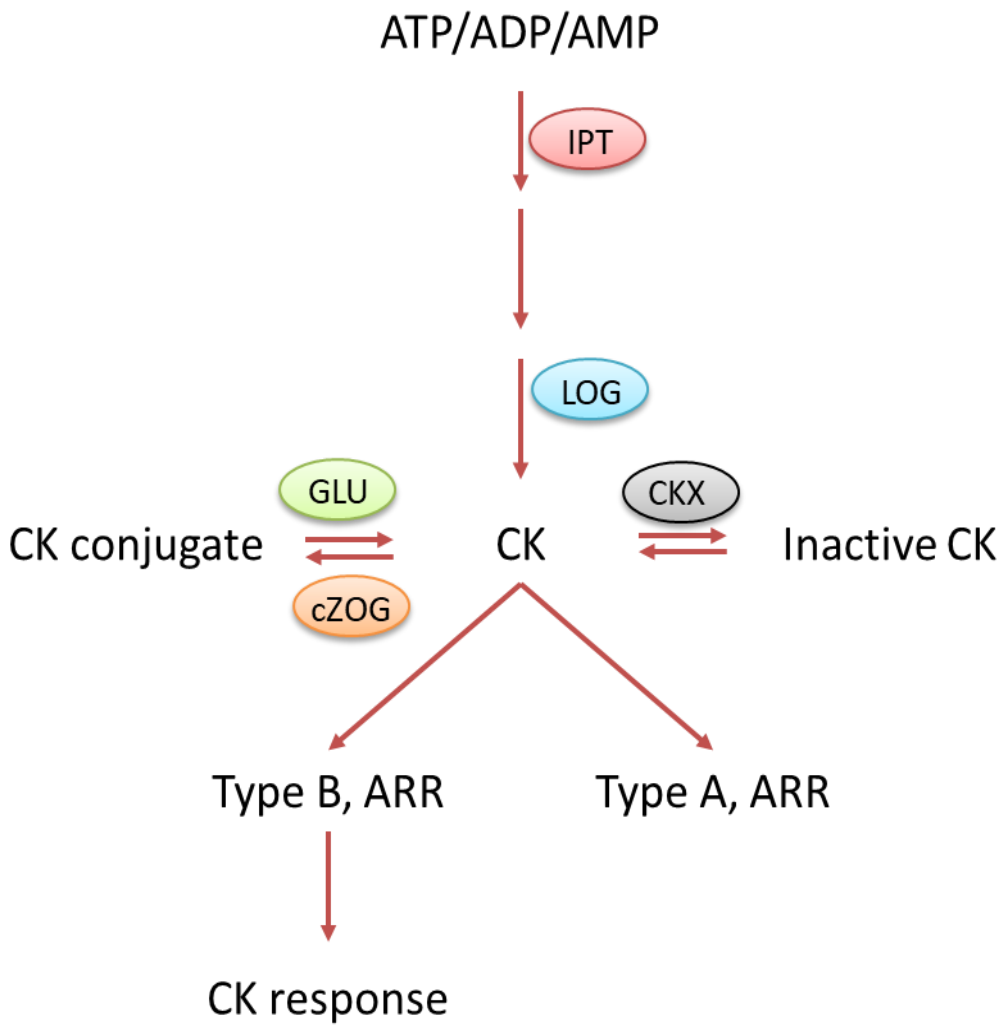


Figure 2.6. Overview of the biosynthesis and signaling pathway of CK in plants. Abbreviations: ATP, Adenosine triphosphate; ADP, Adenosine diphosphate; AMP, Adenosine monophosphate; IPT, Isopentenyltransferases; LOG, Lonely guy; GLU, glucosidase; cZOG, cis zeatin-o-glucoside; CKX, CK oxidase; ARR, Arabidopsis response regulator.

In summary, wheat is an important food crop worldwide, but its productivity is significantly affected by various biotic and abiotic stress factors. Among the major abiotic factors that affect wheat yield and quality is the occurrence of wet and humid conditions before harvest, which cause the germination of seeds on the mother plant (preharvest sprouting). Seeds sprouted prior to harvest exhibit a decrease in yield and end use quality, which ultimately cause economic losses to producers. Preharvest sprouting is influenced by the level of seed dormancy, which is regulated by a number of endogenous factors including ABA. Therefore, understanding the molecular mechanisms underlying the role of ABA in the regulation of dormancy induction, maintenance and release has paramount importance to develop new cultivars with optimum levels of seed dormancy.

Chapter 3: Cloning and characterization of an abscisic acid catabolic gene *TaCYP707A1* from hexaploid wheat (*Triticum aestivum* L.)

Part of this chapter is published as Chitnis VR, Nguyen TN, Ayele BT (2016). A simple and efficient approach to elucidate genomic contribution of transcripts to a target gene in polyploids: the case of hexaploid wheat (*Triticum aestivum* L.). Frontiers in Plant Science 7:1597.

Chitnis VR was involved in designing and performing experiments, analyzing the data, and writing the manuscript. Nguyen TN provided support in sample preparation and performing preliminary experiments.

3. Cloning and characterization of an abscisic acid catabolic gene *TaCYP707A1* from hexaploid wheat (*Triticum aestivum* L.)

3.1 Abstract

Pre-harvest sprouting, which negatively affects wheat yield and quality, is tightly associated with seed dormancy, an adaptive trait that blocks germination under favorable conditions. Dormancy is regulated primarily by abscisic acid (ABA), and the level of ABA in seeds is controlled partly by its catabolism. Using a molecular gene cloning approach, this study identified an ABA metabolic gene, designated as *TaCYP707A1,* and its three homeologues (*TaCYP707A1A, TaCYP707A1B* and *TaCYP707A1D*) from two Canadian wheat genotypes with contrasting seed dormancy phenotypes, and characterized variation in their genomic sequence between the two genotypes. These results indicate that the two genotypes exhibit variation in genomic DNA sequence of *TaCYP707A1* genes, and these variations may underlie their difference in dormancy. Furthermore, the study examines the total expression of *TaCYP707A1* in different tissues of the two wheat genotypes using quantitative polymerase chain reaction (PCR) and elucidated the transcript contribution of each of the three genomes of wheat to its total expression using semi-quantitative PCR with primers amplifying DNA fragments unique to each homeologue of *TaCYP707A1*. Our results showed that the total expression of *TaCYP707A1* and the genomic contribution of transcripts to its total expression varies with genotype and tissue.

3.2 Introduction

Common wheat (*Triticum aestivum* L.) is one of the most important crops world-wide, contributing to approximately 95% of the global wheat production (Dubcovsky and Dvorak, 2007). Common wheat is allopolyploid characterized by a complex genome originated from three diploid species through two rounds of intergeneric hybridization events (He et al., 2003). The first round of intergeneric hybridization involved two diploid species, *T. urartu* as A genome progenitor and a yet-undiscovered or extinct Aegilops species closely related to *Ae. Speltoides* as B genome progenitor (Feldman and Levy, 2012), and produced a tetraploid wheat (*T. turgidum* ssp. durum) with genome composition of AABB (2n = 28). The second round of intergeneric hybridization and polyploidization involved *T. turgidum* (AABB), and *Ae. Tauschii*, as diploid progenitor of the D genome (Feldman and Levy, 2012), and produced the hexaploid common wheat (*T. aestivum*) with genome composition of AABBDD (2n = 42). Each sub genome in both *T. turgidum* and *T. aestivum* consists of seven pairs of homeologous chromosomes and as a result each gene has four and six copies, respectively. This polyploidization/duplication leads to divergence in gene expression that may result in the gain of a new expression state, partitioning of the ancestral function, or complete loss of expression and pseudogenization (Moghe and Shiu, 2014). For example, the most duplicated wheat genes that encode enzymes are active (Hart, 1987), and this might cause an extra genetic dose of such genes. However, an increase in genetic dosage is not necessarily associated with beneficial effects, rather it may cause deleterious effects through inducing functional redundancy or unbalanced gene systems (Feldman and Levy, 2012).

One of the main goals of functional genomics studies in crops is to identify genes of agronomic importance through forward or reverse genetic approaches. Wheat genomic resources that are made available through next-generation sequencing of the whole genome (Brenchley et

al., 2012) and those being generated through the on-going chromosome-based wheat genome sequencing by the International Wheat Genome Sequencing Consortium (https://www.wheatgenome.org/) have important roles in accelerating the identification of novel functional genes. Gene expression analysis is one of the fundamental steps in functional genomics studies as it provides important insights into the physiological function of the target gene(s); it can be performed at genome-wide/large scale or target gene level. Although genome-wide/large scale gene expression analysis has the advantage of measuring the expression level of a large number of genes at the same time, it is expensive and resource intensive. For direct gene discovery, examining the spatial and temporal expression patterns of the targeted genes is an effective and economical approach. However, in polyploid species such as allotetraploid and allohexaploid wheat, each target gene has four or six copies, respectively, which contribute transcripts to its total expression. Elucidating the contribution of each homeologue or genome to the total expression of a given target gene has a paramount significance, for detailed dissection of the molecular mechanisms underlying the regulation of the associated trait of economic importance.

Pre-harvest sprouting is defined as the germination of mature seeds on mother plants due to wet and humid field conditions before harvest (Li et al., 2004). Pre-harvest sprouting affects yield through decreasing test weight of the grain, and deteriorates the milling and baking quality of end-products, and this ultimately causes economic losses to farmers (Derera, 1989; Barnard and Calitz, 2011b). Pre-harvest sprouting is common in those cultivars which have inadequate levels of seed dormancy, which is a major genetic component associated with pre-harvest sprouting in wheat (Nonogaki, 2006; Nonogaki and Nonogaki, 2017). Dormancy is a state at which viable seed fails to germinate under favorable environmental conditions. Shallow dormancy can cause pre-harvest sprouting whereas a high level of seed dormancy prevents germination, which eventually

affects the replanting of newly harvested seeds and quick seedling establishment (Gubler et al., 2005). Considering the above two extreme conditions, developing cultivars with intermediate level of seed dormancy is desirable.

According to Hilhorst, (2011) "primary dormancy indicates the type of dormancy that occurs prior to dispersal as part of the seed's developmental program and includes all the dormancy classes and their combinations". The other type of dormancy, which is induced in non-dormant seeds due to unfavorable environmental conditions, is referred as secondary dormancy (Bewley, 1997; Finkelstein et al., 2008). During the process of primary dormancy release, physiological, biochemical and molecular changes occur within the seed, and this can be associated with changes in hormone metabolism, enzyme activity and gene expression (Carrari et al., 2001; Leubner-Metzger, 2005; Finch-Savage et al., 2007). Hence, studying one of the aforementioned changes during dormancy release/germination provides better understanding of the factors controlling dormancy.

Abscisic acid (ABA) is a plant hormone, which plays a crucial role in regulating various processes of plant growth and development including seed dormancy (Seo and Koshiba, 2002). It has been suggest that the level of ABA can be directly correlated with duration and depth of seed dormancy (Kushiro et al., 2004; Lefebvre et al., 2006). Lower levels of ABA are linked to shallower seed dormancy while a high level of ABA is associated with stronger dormancy (Marin et al., 1996; Barrero et al., 2005). The homeostasis of ABA in plant cells is controlled by its biosynthesis and catabolism. Abscisic acid 8` hydroxylation is considered a major ABA inactivation pathway (Saito et al., 2004; Umezawa et al., 2006), and the enzyme catalyzing this process, abscisic acid 8` hydroxylase, belong to the cytochrome P450 monooxygenase family and is encoded by *CYP707A* genes. The *CYP707A* genes have been identified from various plant

species including the model plant Arabidopsis. In Arabidopsis four isoforms of *CYP707A* genes have been identified, and molecular genetic studies suggest that, the *CYP707A1* and *CYP707A2* family members play important roles in regulating ABA level and the level of dormancy (Kushiro et al., 2004; Saito et al., 2006).

The *CYP707A* genes have also been identified from cereal crops. In barley, two *CYP707A* genes, *CYP707A1* and *CYP707A2*, have been isolated, and between the two genes, *CYP707A1* is reported to be highly expressed during seed germination (Millar et al., 2006) and found to play a role in controlling dormancy (Gubler et al., 2008). While this study was in the process of identifying the *CYP707A1* gene and its homeologs in wheat, Chono et al. (2013) reported the three homeologues of *CYP707A1* from cv. Chinese Spring. Further, mutational analysis in the Japanese wheat cultivar Tamaizumi, provided evidence for the role of *CYP707A1* in regulating seed dormancy and germination via controlling ABA level in seeds (Chono et al., 2013).

Given that seed dormancy is a quantitative trait regulated by a multitude of genetic and environmental factors, studying molecular features underlying the genetic variation in this trait in different wheat lines can help better understand the mechanisms of its regulation. To this end, this chapter of the thesis was aimed at cloning and molecular analysis of the *CYP707A1* gene from two wheat genotypes, AC Domain, which is a pre-harvest sprouting resistant cultivar, and RL4452, which is a pre-harvest sprouting susceptible breeding line. These two lines are used as parental lines for generating pre-harvest sprouting mapping population (Rasul et al., 2009). Furthermore, the expression patterns of these genes in different wheat tissues and the transcript contribution of each genome of hexaploid wheat to the total expression of *CYP707A1* were analyzed in the two wheat genotypes.

3.3 Materials and Methods

3.3.1 Plant Materials

For this study, two wheat genotypes with contrasting sprouting tolerance, AC Domain and RL4452, were used. AC Domain is registered as Canada Western Red Spring (CWRS) marketing class and it is largely grown in western Canada. AC Domain is characterized by its high resistance to pre-harvest sprouting (Townley-Smith and Czarnecki, 2008; Rasul et al., 2009) and it has been widely used as a parent in Western Canadian Spring wheat breeding programs (McCartney et al., 2005). The RL4452 is an unregistered breeding line susceptible to pre-harvest sprouting (Rasul et al., 2009; Rasul et al., 2012). Because of their difference in the level of dormancy, mapping populations resulted from the cross between AC Domain and RL4452 are used for mapping QTLs for pre-harvest sprouting traits.

3.3.2 Growth conditions and tissue collection

Plants were grown in a growth chamber at 22°C/18°C (day/night) under 16/8 h photoperiod. Young leaves were collected from two to four-week-old plants of the two genotypes for extracting genomic DNA and cloning *TaCYP707A1*. For comparative analysis of the expression of *TaCYP707A1* between the tissues of AC Domain and RL4452, flag leaf, peduncle, and internode tissues were harvested from 14-week-old plants. To examine the spatiotemporal expression pattern of *TaCYP707A1*, leaf, seminal and fibrous root (2-week-old stage), leaf and whole root (4-week-old stage), flag leaf, leaf sheath and internode (8-week-old stage; anthesis), and seed (40 DAA; whole seed) tissues were collected from AC Domain plants. All samples were immediately frozen in liquid nitrogen after harvest and stored at -80°C until further use.

3.3.3 Genomic DNA extraction

Genomic DNA was extracted using modified Cetyl Trimethylammonium Bromide (CTAB) method (Porebski et al., 1997). No polyvinylpyrrolidone (PVP) was used in buffer during our extraction and isoamyl alcohol instead of octanal was used along with chloroform during separation steps. About 100 mg young leaf tissue was finely ground in a pestle and mortar with liquid nitrogen and immediately suspended in 4 ml CTAB extraction buffer in a 15 ml centrifuge tube and incubated for 60 min at 60°C. After incubation, 2 ml phenol (pH 7.8) and 2 ml chloroform isoamyl alcohol (24:1) were added and mixed gently, after which the solution was centrifuged at 12000*g* for 10 min. After centrifugation, the supernatant was transferred to fresh 15 ml centrifuge tube containing 4 ml of chloroform: isoamyl alcohol (24:1) and mixed by inverting tube gently followed by centrifugation at 12000*g* for 10 min. The upper aqueous layer was then transferred to 15 ml centrifuge tube and then mixed with ice cold isopropanol (twice the volume of aqueous layer) followed by incubation on ice for about 15 min to facilitate DNA precipitation. The precipitated DNA was pipetted out and transferred to clean 1.5 ml centrifuge and washed with 70% ethanol followed by spinning down at 15000 g for one min. This washing step was repeated twice, after a third wash all supernatant was removed and DNA pellet was dried for about 15 min. The DNA was then resuspended in nuclease free water and treated with 1 µl of 10 mg/ml RNaseA at 65°C for 20 min to get rid of RNA contaminants. The integrity of the DNA was verified with agarose gel and its purity and concentration determined spectrophotometrically.

3.3.4 Cloning and sequence analysis of *TaCYP707A1*

The genomic DNA of AC Domain and RL4452 lines were used to amplify the target gene, *TaCYP707A1*. Two sets of primers were designed to amplify the whole gene. The outer degenerate

forward and reverse primers, designated as *TaCYPA707A1*-F1and R1, were designed from the conserved regions of the ESTs of homologs *CYP7070A1* of Brachypodium, barley, and wheat (Table 3.1), and the second set of nesting forward and reverse primers as reported by Chono et al. (2013) designated as *TaCYP707A1*-F2 and R2 (Table 3.2).

Table 3.1. List of the ESTs of *CYP707A1* homologous sequences along with their accession number used in synthesizing gene specific primers for cloning *TaCYP707A1*.

Accession number	Source
CV769573	Wheat (EST)
CA639805	Wheat (EST)
AK333121	Wheat (cDNA clone)
TC238463	Wheat homologue to UniRef100 from Brachypodium
TC413065	Wheat homologue to UniRef100 form barley

The PCR assay reaction mixture consists of 10 µl of 5x Phusion HF buffer (Thermo Fisher, Waltham, MA, USA), 1.5 µl of DMSO, 1 µl of 10 mM dNTP`s, 1 µl of 10 mM forward and reverse primers, 0.5 µl of 100U Phusion high fidelity DNA polymerase (Thermo Fisher, Waltham, MA, USA), 4 µl of 100 ng template DNA and water to make up the volume to total 50 µl. The obtained reaction mixtures were subjected to the following thermocycling conditions: DNA polymerase activation at 98°C for 45 s, followed by 40 cycles of denaturation at 98°C for 10 s, annealing at 61°C for 30 s and extension at 72°C for 80 s, and final extension at 72°C for 5 min. The amplified products obtained using *TaCYP707A1*-F1 and R1 were subjected to a second round of successive PCR reactions with nesting primers *TaCYP707A1*-F2 to amplify specific target within the first run product. The resulting PCR products were run on 1.5% agarose gel and stained with ethidium bromide. The desired amplicon fragments of about 2kb were excised from the gel and purified using E.N.Z.A. gel extraction kit (Omega Bio-tek, Norcross, GA, USA) following the manufacturer's protocol.

The product obtained from gel purification was subjected A-tailing, as Phusion high fidelity DNA polymerase produces blunt ends. The reaction mixture of A-tailing contains 4 µl of purified blunt end DNA, 2 µl of 10x Taq buffer (containing 20 mM MgCl2, Thermo Fisher, Waltham, MA, USA), 2 µl of 1 mM dATP, 1 µl of Taq DNA polymerase (Thermo Fisher, Waltham, MA, USA) and nuclease-free water to make a total reaction volume of 10 µl. The reaction mixture was incubated at 72°C for 20 min, the obtained product was cloned in to pGEM-T easy vector (Promega, Fitchburg, WI, USA). Transformation to DH5α competent cells (Invitrogen, Carlsbad, CA, USA) was done by heat shock procedure by following the manufacturer`s protocol. Transformed colonies were then screened by blue-white selection and selected colonies were subjected to plasmid isolation (Green and Sambrook, 2012). The purified plasmids were then sequenced with M13 tailed forward and reverse universal primers (Macrogen, Rockville, MD, USA). Additionally, gaps in the full-length gene sequence was filled using internal sequencing primers ISF and ISR (Table 3.2). Sequences were assembled using DNASTAR - Lasergene SeqMan Pro - sequence assembly software (DNASTAR, Madison, WI, USA) and multiple sequence alignment was performed using DNAMan software (Lynnon Corporation, San Ramon, CA, USA).

Table 3.2. List of primers used for cloning and sequence analysis.

Primer name	Sequence (5` to 3`)	Type
TaCYPA1-F1	AGCCTTTCGTTGRCATGGC	Outer primer
TaCYPA1-F2	ATGGCWGCTTTCRTYCTC	Outer primer
TaCYPA1-R1	ACTTCTTTGTGGCGCCGGATCTC	Nested primer
TaCYPA1-R2	GGTYGCCWCTATCGYGCYGTTGAT	Nested primer
TaCYPA1-ISF	GAAGACTGTGAGTGCTTCTTC	Internal sequencing primer
TaCYPA1-ISR	CTTTCCAGCCCTTGGAATC	Internal sequencing primer

Each full-length sequence obtained from assembly was BLASTN searched against the NCBI data base (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) and assigned its genome specificity.

3.3.5 RNA extraction and cDNA synthesis

Total RNA samples from the peduncle and internode tissues were extracted using the GeneJET Plant RNA Purification Mini Kit (Thermo Fisher, Waltham, MA, USA) following the manufacturer's instruction while the total RNA samples from developing seeds and flag leaf tissues were extracted as described before (Nguyen et al., 2016). After determination of the purity and integrity of the total RNA with an Epoch Microplate Spectrophotometer (Biotek, Winooski, VT, USA) and agarose gel electrophoresis, respectively, the total RNA samples were treated with DNase (Ambion, Austin, TX, USA) to eliminate any genomic DNA contamination. The DNase treated total RNA samples were then subjected to cDNA synthesis using iScript Reverse Transcription Supermix Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. The cDNA sample was diluted 20x and then stored in -80°C until further use.

3.3.6 Primers and real time qPCR assay

This study involved the use of the three ABA catabolic genes of hexaploid wheat as target genes [Ta*CYP707A1* (Ta*CYP707A1*A, GenBank ID: AB714577; Ta*CYP707A1B*, GenBank ID: AB714578; Ta*CYP707A1*D, GenBank ID: AB714579]; Chono et al., 2013). The forward and reverse primer sequences that can amplify fragments conserved across all three homeologues of *TaCYP707A1* and thereby detect its collective expression are described in Table 3.3. The wheat β-actin (*Taβ-actin*) gene was used as reference gene for comparative expression analysis of the target

gene in different tissues of the two genotypes and it was amplified using primers described in Table 3.3. The wheat *ubiquitin* gene was used as the reference gene for expression analysis of *TaCYP707A1* in different wheat tissues collected from AC Domain plants at different stages of development (Paolacci et al., 2009; Table 3.3). The real-time qPCR assays for the target and reference genes were performed as described before (Nguyen et al., 2016). Briefly, each qPCR reaction consists of 5 µl of dilute cDNA, 10 µl of SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA), 1.2 µl 5 µM forward primer (300 nM final concentration), 1.2 µl of 5 µM reverse primer (300 nM final concentration) and 2.6 µl diethylpyrocarbonate treated water. The reaction mix was subjected to the following PCR condition using the CFX96 Real-Time PCR system (Bio-Rad, Hercules, CA, USA): DNA polymerase activation at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s and extension at 72°C for 30 s. Transcript levels of the target genes were expressed after normalization with β-actin according to Livak and Schmittgen, (2001). Significant difference between samples was tested using Student's t-test at a probability of $P < 0.05$.

3.3.7 Primers and Semi-quantitative PCR assay

The forward and reverse primers span regions that are polymorphic across the three homeologues of target gene *TaCYP707A1* and thereby amplify fragments unique to each of the three homeologues of the target genes are described in Table 3.3. These primers were used to perform the PCR assays. The PCR assays for Ta*CYP707A1* consist of 5 µl cDNA (50 ng), 2 µl of 10x dream Taq buffer (containing 20 mM MgCl2, Thermo Fisher, Waltham, MA, USA), 0.5 µl of 10 mM dNTPs, 0.5 µl forward primer (5 mM), 0.5 µl reverse primer (5 mM), 0.12 µl of dream Taq DNA polymerase (500 U; Thermo Fisher, Waltham, MA, USA) and nuclease-free water to make

a total reaction volume of 20 µl. The reaction mixtures were then subjected to the following thermocycling conditions: DNA polymerase activation at 95°C for 3 min followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 30 s and extension at 72°C for 25 s, and final extension at 72°C for 5 min.

Table 3.3. Primer sequences used in the determination of the total expression (qPCR) and genomic transcript contribution of *TaCYP707A1*

qPCR			
Gene	Primer sequences		Amplicon size (bp)
	FW (5′ to 3′)	R (5′ to 3′)	
TaCYP707A1	GCCAGGAAGCGGAACAAG	AAGAGGTGCGCCTGAGTA	119
Taβ-actin	GCTGGAAGGTGCTGAGGGA	GCATCGCCGACAGGATGAG	138
Ta-ubiquitin	GCACCTTGGCGGACTACAACATTC	GACACCGAAGACGAGACTTGTGAACC	131

Semi-quantitative PCR					
Gene	Primer sequences		Amplicon size (genome specific bp)		
	FW (5′ to 3′)	R (5′ to 3′)	A	B	D
TaCYP707A1	ATGACCTTCACCCGCAAGG	CCTTTGGGAACGAGGAGGA	56	72	87

3.3.8 Separation of genome specific PCR fragments

The genome specific PCR fragments of *TaCYP707A1* were separated by polyacrylamide gel electrophoresis (PAGE). The 10% gel was prepared by mixing 10 ml of 29% Acrylamide plus 1% N, N'-metheylenebisacrylamide with 16.77 ml of water, 3 ml of 10X Tris/Borate/EDTA (TBE) and 0.22 ml of 10% Ammonium persulfate (APS) and 12 µl of N,N,N',N'-tetramethylethylene diamine (TEMED). The PAGE was run at 100V for 10 h. The gel was then stained in ethidium bromide solution (0.5 µg/ml) for 20-30 min at room temperature before visualization using the Gel Doc XR system (Bio-Rad, Hercules, CA, USA).

3.3.9 Quantitation of band intensity

The area, volume and intensity of each band corresponding to expected size of a given amplicon were analyzed using Quantity One Software (Bio-Rad, Hercules, CA, USA) and the global background subtraction method. The intensities of the band volumes were adjusted by subtracting the intensity of the background volume. The volume of the bands representing each specific PCR fragments was defined by creating a uniform rectangular box around each band to be quantified, and a rectangular box of the same size was also created in the region of the gel picture with no band to be considered as a background.

3.3.10 Genomic contribution of transcripts

The genomic transcript contribution for the target gene *TaCYP707A1*, was expressed as a percentage of their total expression, which is calculated as follows: (adjusted intensity inside the volume of a band corresponding a specific homeologue/the sum of adjusted intensities inside the volumes of bands derived from the three homeologues of a target gene) *100. Significant difference in tissue specific genomic contribution of transcripts between the two wheat genotypes was tested using Student's t-test at a probability of $P < 0.05$.

3.4 Results and discussion

3.4.1 Cloning and characterization of *CYP707A1* from sprouting tolerant and susceptible lines

Amplification of genomic DNA samples with *TaCYP707A1* specific primers designated as *TaCYP707A1*-F2 and R2 (Table 3.2) produced a PCR product of expected size of ~2 kb (Figure 3.1). Multiple transformed colonies containing the target gene isolated from AC Domain and

RL4452 lines were sequenced using vector and gene specific primers. The fragment sequences were assembled into complete genomic DNA sequence and then blast searched against the NCBI GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). Following the blast search, the origin of the genomic DNA sequences from AC Domain and RL4452 were assigned as genome A, B, and D based on the highest sequence homology they exhibited with the NCBI GenBank database sequences (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch).

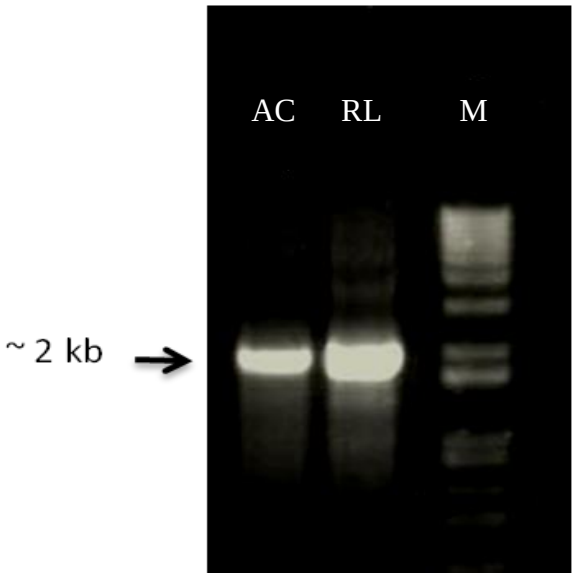


Figure 3.1. PCR products of *TaCYP707A1*. AC, AC Domain; RL, RL4452 wheat lines and M is 1kb+ marker.

Alignment of the AC Domain and RL4452 lines with the previously reported Chinese spring sequence of the target gene from genome A showed 95.6% and 98.5% homology, respectively. Whereas 'B' genome specific sequences from AC Domain and RL4452 showed 95.6% and 94.1% homology with their counterpart from Chinese spring. Furthermore, Chinese spring 'D' genome specific sequence exhibited 95.3% and 95.5% of sequence homology with the

AC Domain and RL4452 sequences of the target gene from genome D, respectively. The high sequence homology (~95%) between AC Domain, RL4452 and Chinese Spring genotypes may indicate that *CYP707A1* is highly conserved across genotypes. The sequence homology between AC Domain and RL4452 for A, B and D genome were 94.8%, 98.1% and 99.5% respectively (Table 3.4). Overall, the complete sequence of *TaCYP707A1* consisted of four introns and five exons in all three genomes. However, the sequence length varies between each genome of AC Domain and RL4452. The sequence length of AC Domain and RL4452 for 'A' genome was 2025b p and 2070 bp, whereas 1981 bp and 1965 bp for 'B' genome, and 2010 bp and 2000 bp for 'D' genome respectively.

Table 3.4. Genomic DNA **s**equence homology matrix of *TaCYP707A1* homeologues from AC Domain, RL4452 and Chinese spring genotypes.

	CS-A	**AC-A**	**RL-A**	**CS-B**	**AC-B**	**RL-B**	**CS-D**	**AC-D**	**RL-D**
CS-A	100%	**95.60%**	**98.50%**	87.40%	90.90%	90.70%	85.90%	92.30%	92.50%
AC-A	**95.60%**	100%	***94.80%***	96.60%	94.70%	94.00%	94.80%	96.60%	96.20%
RL-A	**98.50%**	***94.80%***	100%	92.60%	91.00%	90.80%	91.10%	92.60%	92.30%
CS-B	87.40%	96.60%	92.60%	100%	**95.60%**	**94.10%**	95.40%	99.30%	99.40%
AC-B	90.90%	94.70%	91.00%	**95.60%**	100%	***98.10%***	99.60%	95.70%	95.40%
RL-B	90.70%	94.00%	90.80%	**94.10%**	***98.10%***	100%	98.00%	94.20%	94.10%
CS-D	85.90%	94.80%	91.10%	95.40%	99.60%	98.00%	100%	**95.30%**	**95.50%**
AC-D	92.30%	96.60%	92.60%	99.30%	95.70%	94.20%	**95.30%**	100%	***99.50%***
RL-D	92.50%	96.20%	92.30%	99.40%	95.40%	94.10%	**95.50%**	***99.50%***	100%

Abbreviations: CS-A, Genome A of Chinese Spring; AC-A, Genome A of AC Domain; RL-A, Genome A of RL4452; CS-B, Genome B of Chinese Spring; AC-B, Genome B of AC Domain; RL-B, Genome B of RL4452; CS-D, Genome D of Chinese Spring; AC-D, Genome D of AC Domain; RL-D, Genome D of RL4452. Chinese Spring sequences of genome A (Genbank ID: AB714574), B (Genbank ID: AB714575) and D (Genbank ID: AB714576).

The variation in sequence length between genotypes and genomes is mainly due to insertions or deletions in intronic regions or single nucleotide polymorphism. Although the exons are more conserved across the three genomes and between the genotypes, single nucleotide

polymorphisms have been observed (Appendix 1-3). Given that the *CYP707A1* gene, which encodes for enzyme abscisic acid 8` hydroxylase, is known to be major regulator of ABA level, the sequence variations observed in the intronic and exonic regions of *TaCYP707A* and its homeologues between the two cultivars might underlie their varying depth of dormancy.

3.4.2 Comparative analysis of total expression of *TaCYP707A1* in different tissues between genotypes

The total expression of *TaCYP707A1* in the flag leaf, peduncle, and internode tissues of two hexaploid wheat genotypes used as experimental plant materials in this study, AC Domain and RL4452, was determined using real time quantitative polymerase chain reaction (qPCR) (Figure 3.2a), and gene specific primers designed from the coding regions conserved across the three homeologues of each target gene (Table 3.3). Comparison between tissues in AC Domain revealed that the expression of *TaCYP707A1* was highest in the peduncle and lowest in the flag leaf (Figure 3.2a), suggesting the presence of reduced ABA inactivation in the flag leaf of AC Domain relative to that occurs in the internode and peduncle tissues. The total expression of *TaCYP707A1* in the flag leaf is found to be similar between the two genotypes while its total expression in the internode and peduncle is substantially higher in AC Domain than that observed in RL4452 (Figure 3.2a). These results might suggest the presence of enhanced ABA inactivation in the internode and peduncle tissues of AC Domain than that in RL4452. In the RL4452 genotype, *TaCYP707A1* is expressed at low level across the three tissues.

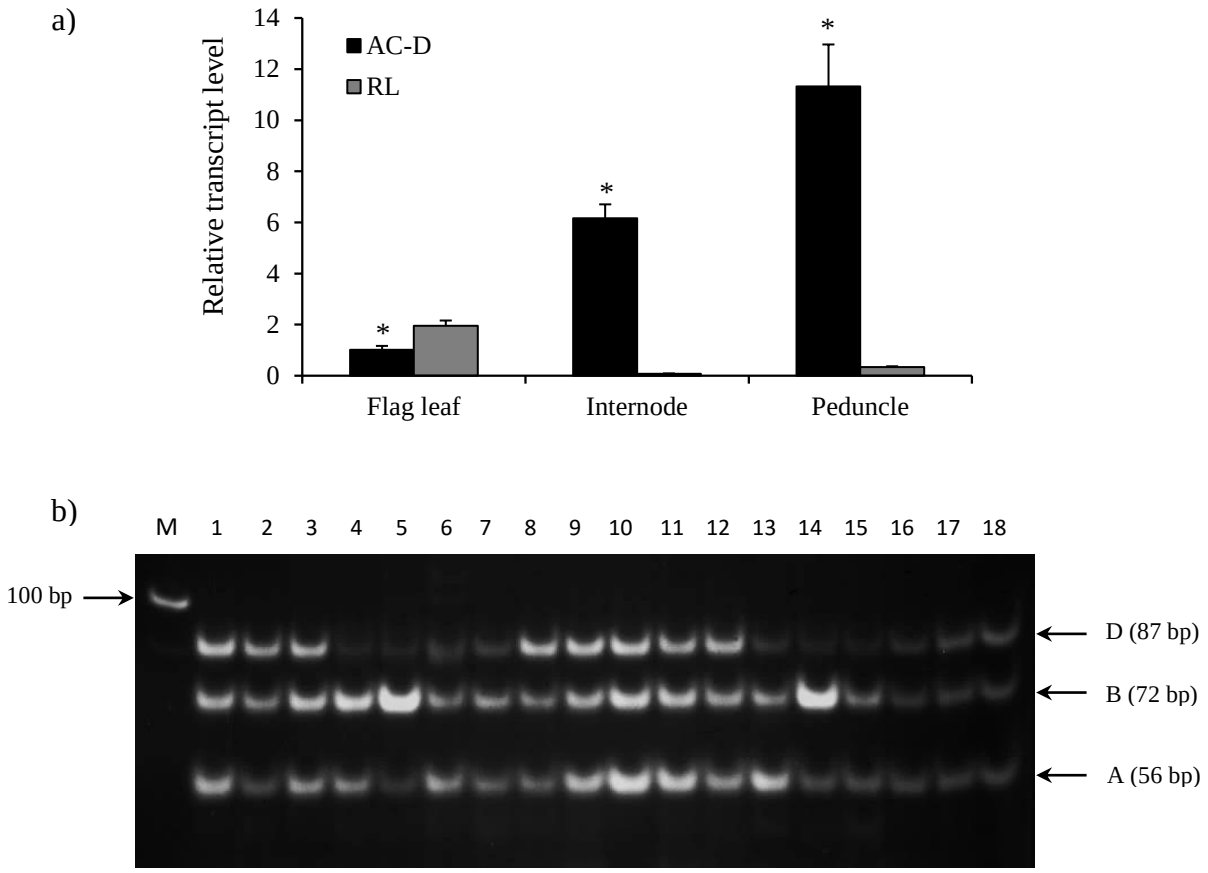


Figure 3.2. Total expression and genomic transcript contribution for *TaCYP707A1* in the flag leaf, internode, and peduncle tissues of hexaploid wheat genotypes AC Domain (AC-D) and RL4452 (RL). Relative transcript abundance of *TaCYP707A1* in the tissues (a). Transcript levels of *TaCYP707A1* were determined using *β-actin* as a reference gene and then expressed relative to that of flag leaf in AC-D, which was set arbitrarily to a value of 1. PCR products representing DNA fragments unique to each homeologue *TaCYP707A1* (b). Data are means ± SE, n=3. Asterisks indicate statistically significant difference in gene expression between the two genotypes ($P<0.05$; Student's t-test). M, Marker lane, lane number 1-9 and 10-18 refers to sample numbers listed in Table 3.5 and Table 3.6, respectively.

3.4.3 Comparative analysis of genomic contribution to total expression of *TaCYP707A1* in different tissues between genotypes

The genomic contributions to the total expression of *TaCYP707A1* were analyzed using gene specific primers that span a region that is polymorphic due to size differences among *TaCYP707A1*A, *TaCYP707A1B* and *TaCYP707A1*D; the forward and reverse primers were

designed to amplify three different amplicons specific to *TaCYP707A1A* (56 bp), *TaCYP707A1B* (72 bp) and *TaCYP707A1D* (87 bp). Separation of the *TaCYP707A1* PCR products using a 10%

Table 3.5. Area, volume and intensity of each DNA band for *TaCYP707A1* homeologues in AC Domain

Tissue Type	Sample #[a]	Replicates	Genome	Area	Volume INT*mm2	Background Vol. INT*mm2	Adjusted Vol. INT*mm2	Total Vol. INT*mm2 (A+B+D)	% Total Volume	Average % Total Volume
Flag leaf	1	1	A	21.73	42716.20	21896.57	20819.63	76839.66	27.09	26.7±0.2
			B	21.73	48562.00	21896.57	26665.44		34.70	35.7±0.5
			D	21.73	51251.16	21896.57	29354.59		38.20	37.7±0.3
	2	2	A	21.73	40493.39	21896.57	18596.82	70895.53	26.23	
			B	21.73	47602.38	21896.57	25705.81		36.26	
			D	21.73	48489.47	21896.57	26592.90		37.51	
	3	3	A	21.73	41321.25	21896.57	19424.68	72739.26	26.70	
			B	21.73	48118.52	21896.57	26221.95		36.05	
			D	21.73	48989.20	21896.57	27092.63		37.25	
Internode	4	1	A	21.73	47775.75	21896.57	25879.19	77914.03	33.22	29.8±3.5
			B	21.73	63368.55	21896.57	41471.98		53.23	55.6±3.0
			D	21.73	32459.44	21896.57	10562.87		13.56	14.5±0.6
	5	2	A	21.73	37900.63	21896.57	16004.06	69834.36	22.92	
			B	21.73	64913.42	21896.57	43016.85		61.60	
			D	21.73	32710.01	21896.57	10813.44		15.48	
	6	3	A	21.73	46889.23	21896.57	24992.66	74829.30	33.40	
			B	21.73	60821.92	21896.57	38925.36		52.02	
			D	21.73	32807.85	21896.57	10911.28		14.58	
Peduncle	7	1	A	21.73	45013.33	21896.57	23116.76	65119.26	35.50	35.9±0.4
			B	21.73	48731.05	21896.57	26834.49		41.21	40.2±0.6
			D	21.73	37064.58	21896.57	15168.01		23.29	23.9±0.8
	8	2	A	21.73	45204.81	21896.57	23308.25	65749.74	35.45	
			B	21.73	47601.69	21896.57	25705.12		39.10	
			D	21.73	38632.94	21896.57	16736.37		25.45	
	9	3	A	21.73	45300.28	21896.57	23403.71	63776.20	36.70	
			B	21.73	47638.35	21896.57	25741.78		40.36	
			D	21.73	36527.28	21896.57	14630.71		22.94	

[a]sample # refers to the lane numbers in Figure 3.3b; INT, intensity.

PAGE resulted in three distinct fragments corresponding to the amplicons derived from each homeologue (Figure 3.2b). As this study was focused on investigating the relative transcript

contribution of each genome to the total expression of the targeted genes, no loading control/check sample or generation of standard curve is required. Amplification of the specific amplicons of the three homeologues of *TaCYP707A1* using the gene specific primers was confirmed using genomic DNA samples (Figure 3.3).

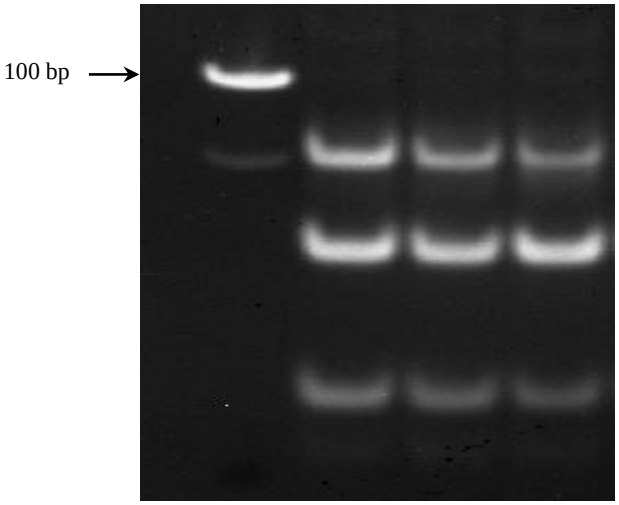


Figure 3.3. PCR products from genomic DNA samples of different concentrations (100 ng, 20 ng, and 4 ng) representing fragments unique to and *TaCYP707A1*.

Determination of the transcript contribution by each genome to the total expression of *TaCYP707A1* was performed through analysis of the area, volume and intensity of the bands representing the amplicons of each homeologue using Quantity One Software (Bio-Rad, Hercules, CA, U.S.A) and the global background subtraction method (Table 3.5, 3.6). Our analysis indicated that all the three genomes exhibit a similar contribution of transcripts to the total expression of *TaCYP707A1* in the flag leaf in both genotypes, although lower contribution of the A genome copy and slightly higher contributions of the B and D genome copies are observed in the AC Domain than in the RL4452 genotype (Figure 3.4). In the internode of both genotypes, genome B appeared to contribute the most transcripts followed by genome A and then by genome D, implying that

TaCYP707A1B accounts for the majority of internode *TaCYP707A1* activity (Figure 3.4). When the two genotypes are compared, the transcript contribution of B genome to the total expression of *TaCYP707A1* in the internode is higher in AC Domain (~56%) than RL4452 (~52%) while the transcript contribution of A genome is higher in RL4452 (35%) than AC Domain (~30%).

Table 3.6. Area, volume and intensity of each DNA band for *TaCYP707A1* homeologues in RL4452

Tissue Type	Sample #[a]	Replicates	Genome	Area	Volume INT*mm2	Background Vol. INT*mm2	Adjusted Vol. INT*mm2	Total Vol. INT*mm2 (A+B+D)	% Total Volume	Average % Total Volume
Flag leaf	10	1	A	21.73	58333.80	21896.57	36437.23	100905.40	36.11	34.9±0.6
			B	21.73	53770.32	21896.57	31873.75		31.59	32.4±0.4
			D	21.73	54490.98	21896.57	32594.42		32.30	32.7±0.4
	11	2	A	21.73	54402.43	21896.57	32505.86	94186.92	34.51	
			B	21.73	53046.20	21896.57	31149.63		33.07	
			D	21.73	52427.99	21896.57	30531.42		32.42	
	12	3	A	21.73	54346.02	21896.57	32449.45	95210.02	34.08	
			B	21.73	52769.63	21896.57	30873.06		32.43	
			D	21.73	53784.09	21896.57	31887.52		33.49	
Internode	13	1	A	21.73	41154.62	21896.57	19258.05	50579.79	38.07	35.1±3.0
			B	21.73	46208.28	21896.57	24311.71		48.07	51.6±3.1
			D	21.73	28906.60	21896.57	7010.03		13.86	13.3±0.3
	14	2	A	21.73	39521.02	21896.57	17624.45	60442.97	29.16	
			B	21.73	56785.56	21896.57	34888.99		57.72	
			D	21.73	29826.10	21896.57	7929.53		13.12	
	15	3	A	21.73	40303.74	21896.57	18407.17	48176.45	38.21	
			B	21.73	45440.45	21896.57	23543.88		48.87	
			D	21.73	28121.97	21896.57	6225.40		12.92	
Peduncle	16	1	A	21.73	34701.65	21896.57	12805.08	31974.18	40.05	38.7±0.96
			B	21.73	32935.34	21896.57	11038.77		34.52	34.2±0.2
			D	21.73	30026.90	21896.57	8130.33		25.43	27.1±1.1
	17	2	A	21.73	33979.56	21896.57	12082.99	32787.99	36.85	
			B	21.73	33070.19	21896.57	11173.62		34.08	
			D	21.73	31427.95	21896.57	9531.38		29.07	
	18	3	A	21.73	34199.86	21896.57	12303.30	31354.33	39.24	
			B	21.73	32564.48	21896.57	10667.91		34.02	
			D	21.73	30279.69	21896.57	8383.12		26.74	

[a]sample # refers to the lane numbers in Figure 3.3b; INT, intensity.s

The D genome appeared to have similar contribution in the internode of both genotypes. The predominance of A genome in transcript contribution in the peduncle appeared to vary mainly with genotype in which the B genome contributes the most in AC Domain (~40%) followed by genome A (~36%) and then by genome D (~24%) while genome A contributes the most (~39%) followed by genome B (~34%) and then by genome D (~27%) in RL4452. These results imply that the activity of TaCYP707A1 in the peduncle is mainly regulated by its B genome copy in AC Domain but by the A genome copy in RL4452. Our results again indicate that the genomic contribution of transcripts to the total expression of *TaCYP707A1* varies with genotype and tissue, reflecting the genetic and spatial regulation of each homeologue in controlling the associated trait, in this case, inactivation of bioactive ABA.

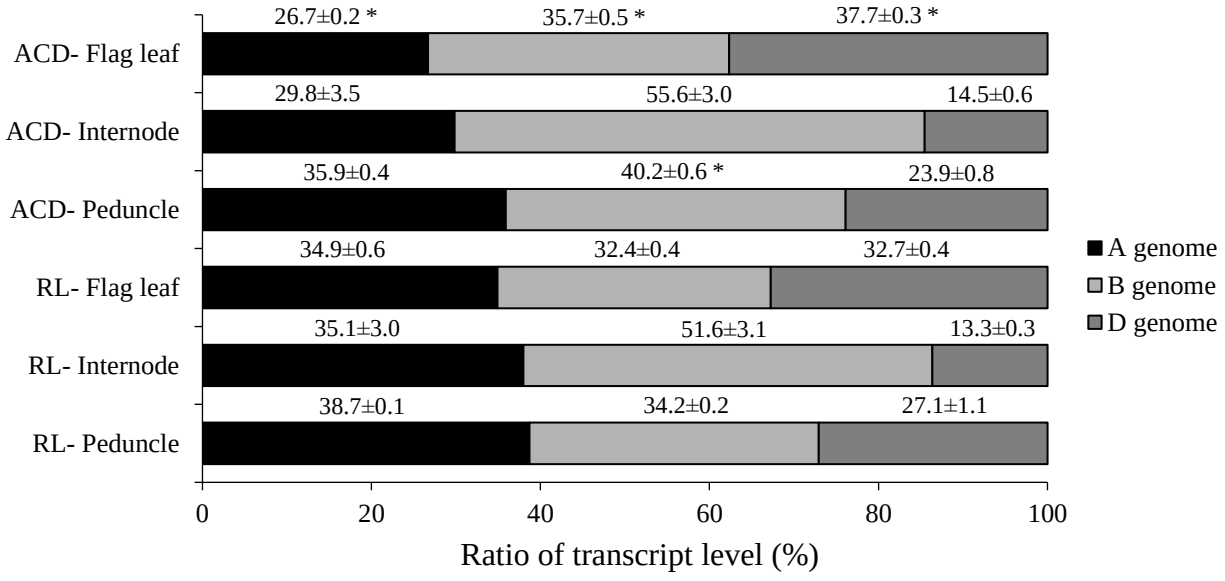


Figure 3.4. Transcript contribution of each genome as percentage to the total expression of *TaCYP707A1* in the flag leaf, internode, and peduncle tissues of hexaploid wheat genotypes AC Domain (AC-D) and RL4452 (RL). Data are means ± SE, n=3. Asterisks indicate statistically significant difference in tissue specific genomic contribution of transcripts between the two genotypes ($P<0.05$; Student's t-test)

3.4.4. Spatiotemporal expression pattern of *CYP707A1* in wheat tissues

To gain insights into the physiological roles of *TaCYP707A1*, its total expression was investigated in different tissues of wheat cv. AC Domain collected at different stages including root and leaf (at 2- and 4-week-old stage), leaf and whole root (4-week-old stage), flag leaf, leaf sheath and internode (8-week-old stage; anthesis) and seed (40 DAA; whole seed). Moderately high expression of *TaCYP707A1* was detected in the root at 4-week-old stage and flag leaf (Figure 3.5a). By 8-week-old stage, when the plant has gone through internode elongation, we analyzed its expression in the root, flag leaf, leaf sheath and internode tissues; the transcripts of *TaCYP707A1* were found to be relatively more abundant in the root and flag leaf. The *TaCYP707A1* gene was found to be expressed predominantly in developing seeds relative to the other tissues studied here, suggesting their significance in regulating ABA level in wheat seeds (Figure 3.5a). Consistent with this result, the expressions of *CYP707A1* was found to be elevated in the developing barley seeds (Chono et al., 2006), and in Arabidopsis seeds at mid-maturation stage (Okamoto et al., 2006).

3.4.5 Spatiotemporal genomic contribution to the total expression of *TaCYP707A1*

Analysis of the contribution of each genome to the transcripts to the total expression of *TaCYP707A1* revealed that genome A contributed the most transcripts in the seed and root tissues followed by genome B and then D, except that both B and D genomes appeared to contribute equally in 4-week-old roots (Figure 3.5b). In the leaf blade and leaf sheath tissues, genome B contributed the most transcripts followed by genome A and then by D, except that genome A and B appeared to contribute equally in the flag leaf. All the three genomes contributed equally in the case of internode tissue. The A and B genomes contribute over 75% of the seed *TaCYP707A1*

transcripts, implying the significance of specific genomes in regulating the activity of seed localized TaCYP707A1.

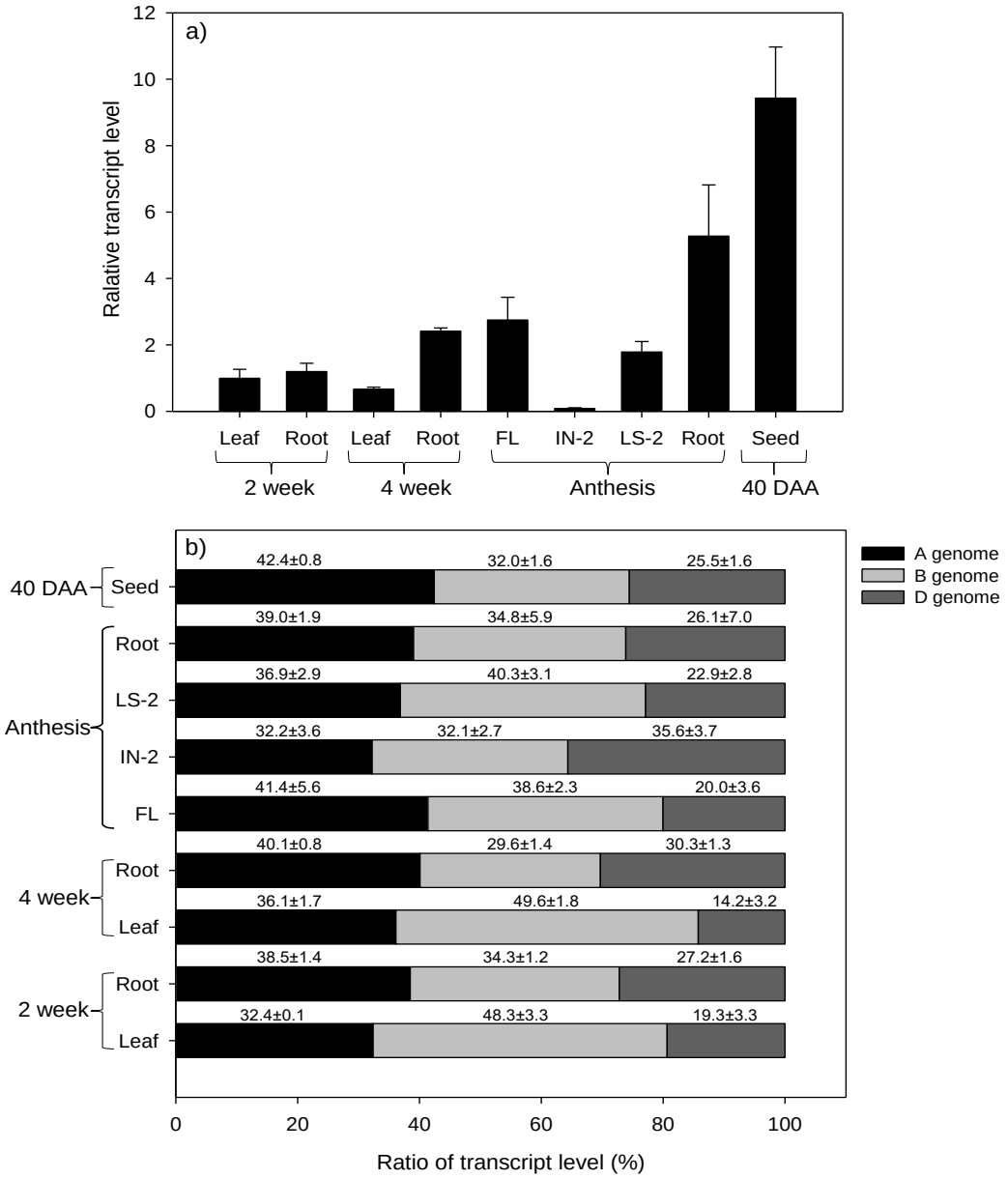


Figure 3.5. Relative transcript level of *TaCYP707A1* in wheat tissues at different stages of development (a). Transcript level of Ta*CYP707A1* was determined using ubiquitin as the reference gene and then expressed relative to that of Ta*CYP707A1* in the leaf tissue at 2-week stage, which was set arbitrarily to a value of 1. Genomic contribution of transcripts to the total transcript abundance of Ta*CYP707A1* (b). Data are means of two to three independent replicates ± SE. FL, flag leaf; IN-2, internode 2 (from the bottom up); LS-2, leaf sheath 2 (leaf sheath wrapping around the second internode); DAA, days after anthesis.

In summary, this study identified *TaCYP707A1* and its three homeologs from Canadian wheat genotypes. Sequence variation in the *TaCYP707A1* homeologs between the AC Domain and RL4452 might underlie the genotypic difference in depth of dormancy. The allelic variations can be studied further with AC Domain X RL4452 mapping population so as to develop molecular markers that can be potentially used for screening and selection of wheat lines with PHS tolerance. The study also characterized expression patterns of *TaCYP707A1*, and the gene is expressed predominantly in developing seeds relative to the other tissues, suggesting its importance in regulating seed ABA level and dormancy. The study also investigated genomic contribution of transcripts to the total expression of *TaCYP707A1* in different tissues of wheat and genotypes using cost-effective PCR assays and conventional reagents and supplies, and such knowledge provides further insights into the physiological roles of *TaCYP707A1* homeologues. The method described here can also be applied to elucidate the genomic contribution of transcripts in other economically important allopolyploid crop species including cotton, oat, and canola.

Chapter 4: Abscisic acid catabolic genes of wheat (*Triticum aestivum* L.): characterization and functionality in seed dormancy

Part of this chapter is published as Son SH, Chitnis VR, Liu A, Gao F, Nguyen TN, Ayele BT (2016) Abscisic acid metabolic genes of wheat (*Triticum aestivum* L.): identification and functionality in seed dormancy and dehydration tolerance. Planta 244: 429-447 (Chitnis VR is co-first author).

Chitnis VR was involved in performing experiments, data analysis and writing the manuscript. Son SH and Liu A contributed in the characterization of the transgenic plants and Gao F in preparing vector constructs.

4. Abscisic acid catabolic genes of wheat (*Triticum aestivum* L.): characterization and functionality in seed dormancy

4.1 Abstract

Abscisic acid (ABA) regulates various plant developmental processes including seed dormancy and germination, and its level in plants is determined partly by its catabolism, which is regulated mainly by ABA 8'-hydroxylation. The present study identified a gene encoding ABA 8'-hydroxylase, designated as *TaCYP707A1*, and its three homeologs (*TaCYP707A1A*, *TaCYP707A1B* and *TaCYP707A1D*) from Canadian wheat cultivars and characterized the functionally of one of the homeologs (*TaCYP707A1B*) in controlling seed ABA level, dormancy, and germination in Arabidopsis. Ectopic expression of *TaCYP707A1B* in wild type or *cyp707a1 cypr707a2* double mutant of Arabidopsis resulted in altered seed ABA level and dormancy. Furthermore, cold treatment, which breaks seed dormancy, resulted in an earlier germination phenotype in both wild type and a *TaCYP707A1B* overexpressing line than that observed in the complemented lines. To gain insights into the physiological roles of *TaCYP707A1*, the study examined its spatiotemporal expression pattern and showed that the gene is highly expressed in developing seeds. Characterization of the expression patterns of *TaCYP707A1* and the other *CYP707A* gene of wheat, *TaCYP707A2*, in different tissues of dormant and non-dormant wheat seeds during seed development and germination suggested their involvement in the regulation of ABA and dormancy level.

4.2 Introduction

Abscisic acid (ABA) regulates a wide range of plant growth and developmental processes such as seed germination and dormancy, and also mediates plant response to environmental stresses such as drought (Nambara et al., 2010). These roles of ABA are partly mediated by its level in the plant tissue, which in turn is controlled by a balance between its biosynthesis and catabolism; the ABA metabolic pathway has been characterized in model plants such as Arabidopsis, and genes encoding the different enzymes in the pathway have been identified (Nambara and Marion-Poll, 2005c). The biosynthesis of ABA starts with the conversion of Zeaxanthin, which is synthesized as a trans-isomer after cyclization and hydroxylation of all trans-lycopene via β-carotene, to all-trans violaxanthin; this reaction is catalyzed by zeaxanthin epoxidases (ZEPs). The all-trans violaxanthin is ultimately converted to C40 9-*cis*-epoxycarotenoids; 9-*cis*-neoxanthin and 9-*cis*-violaxanthin, which in turn are cleaved by 9-*cis*-epoxycarotenoid dioxygenases (NCEDs) to produce xanthoxin. Finally, ABA is produced from xanthoxin via abscisic aldehyde, and these steps are catalyzed by a short-chain dehydrogenase/reductase (SDR) and abscisic aldehyde oxidase, respectively. ABA catabolism takes place either through hydroxylation or conjugation. Hydroxylation at C-8' position, which is catalyzed by ABA 8-hydroxylase (ABA 8'OH) and produces phaseic acid, has been believed to be a predominant ABA inactivation pathway (Cutler and Krochko, 1999). The ABA metabolic steps that are catalyzed by zeaxanthin epoxidase (ZEP), 9-cisepoxycaretenoid dioxygenase (NCED) and ABA 8'-hydroxylase (ABA8'OH) appeared to be rate-limiting (Nambara et al., 2010). Genes encoding these regulatory enzymes have been isolated from several plant species including cereal crops such as maize and barley (Tan et al., 1997; Chono et al., 2006), and these genes form multigene families.

Genes encoding ABA8'OH (*CYP707A*) play important roles in regulating seed ABA level

during seed development and dormancy (Kushiro et al., 2004; Millar et al., 2006; Okamoto et al., 2006). Members of the *CYP707A* gene family are differentially expressed in various tissues; *CYP707A1* transcripts are highly abundant in the silique while *CYP707A2* transcripts are predominant in dry seed and during imbibition (Kushiro et al., 2004). In developing seeds of Arabidopsis, endogenous ABA level peaks at 10 days after flowering (DAF), and this has been shown to be associated with increased expression of *CYP707A1* in both embryo and endosperm tissues. During the late stage of seed development, however, the transcripts of *CYP707A2* are found to be more abundant in both endosperm and embryo tissues (Okamoto et al., 2006). These results suggest that the two genes have temporally distinct roles in regulating seed ABA level during seed development. Imbibition of Arabidopsis seeds leads to enhanced expression of *CYP707A2* and a decrease in seed ABA level (Kushiro et al., 2004; Liu et al., 2009). In addition, seed dormancy release by after-ripening induces the expression of *CYP707A2* (Millar et al., 2006; Okamoto et al., 2006). Consistent with these results, overexpression of *CYP707A1* leads to ~3-fold reduction in ABA level in mature seeds (Millar et al., 2006) while the loss of function mutation in *CYP707A2* results in accumulation of ABA and thereby strong dormancy in both dry and imbibed seeds (Kushiro et al., 2004). The *cyp707a1* and *cyp707a2* double mutant show most severe dormancy among other *cyp707a* double mutants (Okamoto et al., 2006), indicating the significance of the two genes in the regulation of seed ABA level and dormancy. In barley, two genes encoding ABA8'OH have been reported, and *ABA8'OH1* was found to be predominantly expressed in the embryos of imbibing seeds (Millar et al., 2006), and its increased expression is associated with reduction in ABA content and increased germination (Chono et al., 2006). Likewise, repression of *ABA8'OH1* via RNAi-directed silencing led to increased ABA level and dormancy in barley grains (Gubler et al., 2008).

Given that ABA plays important roles in the regulation of seed dormancy, which is tightly associated with pre-harvest sprouting in cereals and the genes encoding ABA8'OH play primary role in regulating ABA level in plant tissues, identification and functional characterization of such genes from economically important crops such as wheat has the potential to provide new tools for enhancing yield and quality. This study isolated *TaCYP707A1* and examined its functionality using heterologous expression in Arabidopsis. To gain more insight into the physiological roles of *TaCYP707A* genes with respect to dormancy induction and maintenance, comparative expression analysis of *TaCYP707A1* and *TaCYP707A2* genes was performed between dormant and non-dormant seeds during seed development and germination. The transcript contribution of each homeologue to the total expression *TaCYP707A1* was also investigated in different tissues and at different stages.

4.3 Materials and Methods

4.3.1 Plant materials and growth conditions

Hexaploid wheat and Arabidopsis plants were used in this study. The Arabidopsis plants materials include the wild-type (Col-0 ecotype) and the *cyp707a1 cyp707a2* double mutant; the Arabidopsis plants were grown in a growth chamber at 22^{o}C/20^{o}C (day/night) under a 16/8 h photoperiod. The wheat genotypes used for this study include AC Domain, which is a registered cultivar in the Canada Western Red Spring (CWRS) wheat market class and exhibits a high level of dormancy (Townley-Smith and Czarnecki, 2008), and RL4452, a breeding line characterized by its low level of dormancy at harvest. All the wheat plants were grown in a greenhouse at 18-22^{o}C/14-18^{o}C (day/night) under a 16/8 h photoperiod. Mature seeds of both genotypes were imbibed to determine the expression of *TaCYP707A1* at whole seed level during germination. For spatiotemporal

comparative expression analysis of *CYP707A1* and *CYP707A2* in developing seeds of AC Domain and RL4452, plants were tagged at anthesis (zero days after anthesis) and the seeds harvested at 20, 30, 40, 50 and 60 days after anthesis. After harvesting, the embryo and endosperm tissues were separated from seeds harvested from the middle region of each spike in liquid nitrogen. All tissues were stored in -80ºC freezer until needed for further use. To perform spatiotemporal comparative expression analysis of the two genes during germination of dormant and non-dormant seeds, freshly harvested seeds of AC Domain and RL4452, and after-ripened seeds of AC Domain were imbibed for 6, 12, 18, and 24 hours. Following imbibition, embryos were separated from endosperm tissue frozen in liquid nitrogen and stored under -80ºC until further use.

4.3.2 Heterologous expression of *TaCYP707A1* in Arabidopsis

Wild-type plants of Arabidopsis (Col-0 ecotype) expressing *CYP707A1B* were generated by using *Agrobacterium tumefaciens* AGL1 strains harboring the CaMV35S::*TaCYP707A1B* construct. Complementation of the *cyp707a1 cyp707a2* double mutant of Arabidopsis in the Col-0 background was performed with the CaMV35S::*TaCYP707A1B* construct, and transformation of the plants was performed using the floral dip method (Clough and Bent, 1998). Transgenic plants expressing the *TaCYP707A1B* homeologue were screened using the hygromycin selection method described previously (Harrison et al., 2006). The putative T0 transformants were maintained in a growth chamber at 22ºC/20ºC (day/night) under a 16/8 h photoperiod. Seeds harvested from the T0 transgenic lines were used to select homozygous lines; T3 homozygous lines were selected for detailed analysis.

4.3.3 Gene expression analysis

4.3.3.1 Total RNA extraction and cDNA synthesis

Total RNA from mature dry and imbibed seeds of Arabidopsis was extracted from three independent replicates using the protocol reported previously (Onate-Sanchez and Vicente-Carbajosa, 2008). For gene expression analysis in wheat, RNA samples from the whole seed and endosperm samples were isolated from three independent replicates as described previously (Yao et al., 2012). The total RNA from embryo tissues was extracted from three independent replicates using the GeneJET Plant RNA Purification Mini Kit (Thermo Fisher, Waltham, MA, USA). To eliminate genomic DNA contamination, the total RNA samples were digested with DNAase (DNA-free Kit; Ambion, Austin, TX, USA) followed by cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) following manufacturer's instructions.

4.3.3.2 Primers

Primers specific to *TaCYP707A1* and *TaCYP707A2* genes were designed from regions conserved across the three homeologues. The primer information for *TaCYP707A1* is described in Chapter 3 Table 3.3, For *TaCYP707A2*, 5′- ACGTCGAGTACAGCCCATT -3′ and 5′- TCGTCGTCGTCGTAGTTGT -3′ were used as forward and reverse primers, respectively. The Arabidopsis *UBIQUITIN 5* gene was used as an internal control for expression analysis of the target genes in Arabidopsis tissues, and it was amplified using previously reported forward (5′- GTGGTGCTAAGAAGAGGAAGA-3′) and reverse (5′- TCAAGCTTCAACTCCTTCTTT-3′) primer sequences (Hwang et al., 2010). The wheat *ubiquitin* gene was used as reference gene for expression analysis of *TaCYP707A1* during imbibition at whole seed level. For spatiotemporal comparative expression analysis of the two genes during seed development and germination, *Taβ-*

actin was used as reference gene. The gene specific primers used to amplify the wheat, *ubiquitin* and *Taβ-actin* genes are listed in Table 3.3 of Chapter 3.

4.3.3.3 Real time qPCR assay

Real-time PCR assays were performed using SsoFast EvaGreen Supermix (Bio-rad, Hercules, CA, USA) and a total reaction volume of 20 µl containing 5 µl of 1:20 diluted cDNA, 10 µl EvaGreen Supermix, 1.2 µl forward primer (5 µM), 1.2 µl reverse primer (5 µM) and 2.6 µl nuclease-free water. Samples were subjected to the following PCR conditions on a CFX96 real-time PCR system (Bio-Rad, Hercules, CA, USA): DNA polymerase activation at 95°C for 3 min followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 10 s and extension at 72°C for 30 s in duplicate in 96-well optical reaction plates (Bio-Rad, Hercules, CA, USA). Relative transcript abundance of the target genes was determined by $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001) using *ubiquitin* or *β-actin* as a reference gene.

4.3.3.4 Genome specific semi-quantitative PCR assay

To determine the transcript contribution of each homeologue to the total expression of *TaCYP707A1*, forward and reverse primers that span polymorphic regions in the 3′ UTR of *TaCYP707A1* were designed as described previously (Chapter3, Table 3.3). Separation of the PCR fragments corresponding to each homologue and quantification of the transcript abundance was performed as described before (Deol et al., 2013).

4.3.4 Germination assay

Seed germination assay for wheat seeds were performed in Petri plate system. Seeds were surface

sterilized with 70% ethanol for a minute followed by 5% sodium hypochlorite treatment for 20 min. The seeds were then rinsed thoroughly five-six times with sterile water to eliminate traces of sodium hypochlorite. Surface sterilized seeds were imbibed between two layers of wet #1 Whatman filter paper in 7ml of sterile water. Twenty seeds per plate were germinated under dark at room temperature. Seeds were then scored as germinated when coleorhiza protrude out of seed coat, at various interval of time.

The germination assay for Arabidopsis was carried out as mentioned above except the use of 0.5% of sodium hypochlorite solution for sterilization and 3 ml of sterile water for imbibition in 9-cm Petri dish (50 seeds per plate, 3 replicates). The Petri dishes were then sealed with parafilm and incubated in a growth cabinet at 23ºC (day/night) under a 16/8 h photoperiod for 48 h. Germination was scored when the radicle ruptured through the endosperm. Seeds imbibed for 24 h were harvested for seed ABA level analysis.

4.3.5 ABA level analysis

Mature air dry and imbibed seed of Arabidopsis were ground into fine powder in liquid nitrogen with mortar and pestle, and then homogenized with 6 ml of 80% (v/v) acetonitrile containing 1% (v/v) acetic acid and the internal standards. After incubation of the homogenate for 1 h at room temperature, the samples were centrifuged at 4 °C for 10 min at 8,000 × g and the supernatants were collected. The pellets were subjected to re-extraction with 6 ml of 80% (v/v) acetonitrile containing 1% (v/v) acetic acid for 1 h at room temperature. The supernatants were pooled and then dried to 1 ml using CentriVap (Labconco, Kansas City, MO, USA). The concentrated extract was loaded onto Oasis HLB cartridge columns (60 mg; Waters, Milford, MA, USA) following conditioning with 3 ml of acetonitrile, methanol and water containing 1% (v/v) acetic acid

sequentially. After wash of the column with 3 ml of water containing 1% (v/v) acetic acid, elution of the extracts was carried out with 6 ml of 80% methanol containing 1% acetic acid, and 2 ml of the eluate was subjected to further analysis for analysis of ABA level. After drying it down completely with CentriVap, the extract was dissolved in 1 ml of methanol and then loaded onto Bond Elute-DEA cartridge columns (100 mg; Agilent, Santa Clara, CA, USA) that had been conditioned with 1 ml of methanol. After washing the column with 2 ml of methanol, ABA was eluted with 2 ml of methanol containing 1 % (v/v) acetic acid. The fraction was then dried completely and brought up with 30 µl of water containing 1 % (v/v) acetic acid for analysis with LC-ESI-MS/MS (Agilent 1260-6430).

4.3.6 Statistical analysis

Significant difference between the different samples was tested using the t Student's test at a probability of $P < 0.05$.

4.4 Results and discussion

4.4.1 Characterization of Arabidopsis expression *TaCYP707A1B*

RT-PCR analysis indicated the expression of *TaCYP707A1* in both dry and imbibed seeds of *TaCYP707A1B*-OE and *TaCYP707A1B*-C lines (Figure 4.1). No expression of *TaCYP707A1* was detected in wild type or *cyp707a1 cyp707a2* double mutant.

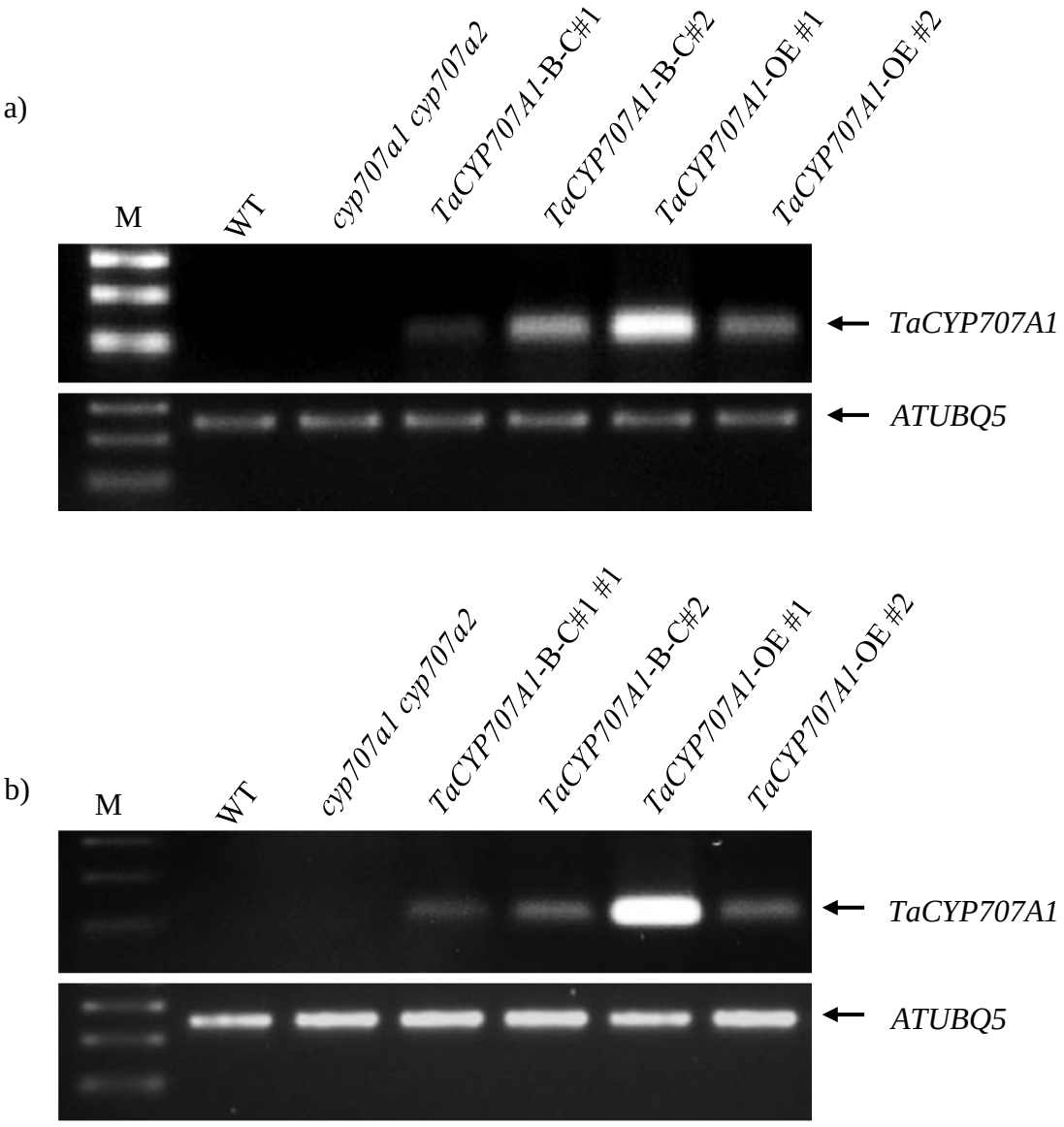


Figure 4.1. Expression of *TaCYP707A1* in dry (a) and imbibed (b) seeds of wild type (WT), *cyp707a1 cyp707a2* double mutant, *TaCYP707A1B*-complemented *cyp707a1 cyp707a2* (*CYP707A1B-C*) and *TaCYP707A1B*-expressing (*CYP707A1B-OE*) Arabidopsis plants. *Ubiquitin5* was used as a reference gene. M is DNA marker.

4.4.2 Functional characterization of *TaCYP707A1*

Mature air-dried seeds of the *cyp70707a1-1 cyp707a2-1* double mutant accumulated a high amount of ABA, which was 40-fold higher than in wild-type seeds (Figure 4.2b), and these seeds did not show any sign of germination following 48 h of imbibition. These results are in agreement with previous reports that show seeds of the *cyp707a1 cyp707a2* double mutant accumulate more ABA and exhibit the most severe dormancy as compared to the other *cyp707a* double mutants (Okamoto et al., 2006). The *cyp707a1a2* double mutant plants complemented with the *TaCYP707A1B* gene exhibited nearly 100% germination over the same imbibition period as wild-type (Figure 4.2a), indicating complementation of the double mutant with *TaCYP707A1B* led to reversal of germination phenotype and seed ABA level to wild-type. Furthermore, overexpression of *TaCYP707A1B* in wild-type Arabidopsis plants resulted in 1.4-fold lower ABA level than that of wild-type seeds and an early germination phenotype; exhibiting 22 to 42 % germination by 30 h after imbibition (HAI), when almost no germination of the wild-type seed was observed. Although no difference in germination was observed between the wild type and *TaCYP707A1B* expressing seeds by 36 HAI (Figure 4.3a), our results suggest that *TaCYP707A1B* is involved in ABA catabolism in wheat and its ectopic expression in Arabidopsis alters seed dormancy level. Consistently, overexpression of the barley *CYP707A1* in Arabidopsis reduces ABA level (~3-fold) in matured dry seeds (Millar et al., 2006). Likewise, increased expression of *CYP707A1* in barley seeds is associated with reduced ABA level and increased germination capacity (Chono et al., 2006), while repression of same gene via RNAi directed silencing led to increased seed ABA level and enhanced dormancy (Gubler et al., 2008). Furthermore double mutation on *TaABA 8'OH1A* and *TaABA 8'OH1D* led to increased seed ABA level and enhanced seed dormancy in wheat (Chono et al., 2013).

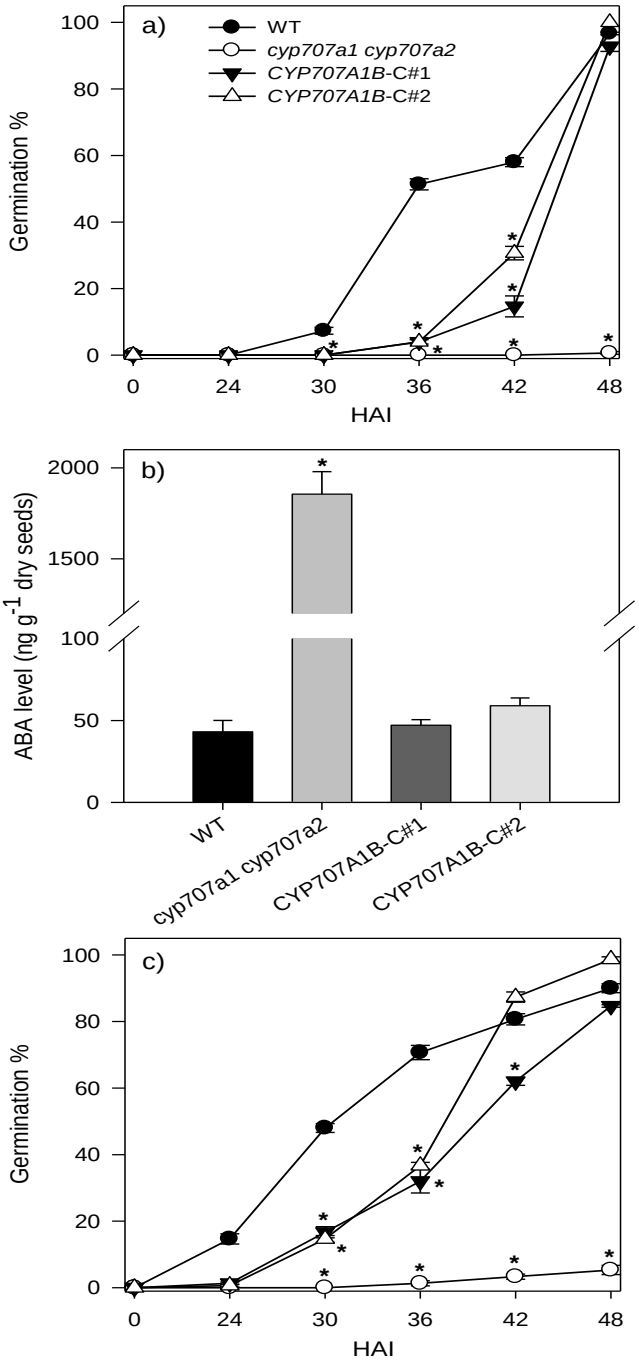


Figure 4.2. Phenotypic analysis of *cyp707a1 cyp707a2* seeds expressing *TaCYP707A1B*. Germination (a) and ABA level in dry seeds (b) freshly harvested from wild-type (WT), *CYP707A1 cyp707a2* double mutant and *TaCYP707A1B*-complemented *cyp707a1 cyp707a2* (*CYP707A1B*-C) plants, and germination kinetics in response to cold (4ºC) treatment for 3 days (c). Data are means of germination and ABA measurements of three independent replicates obtained from plants cultured simultaneously ± SE. Asterisks indicate statistically significant difference as compared to the wild-type control ($P<0.05$; t Student's test). HAI, hours after imbibition.

4.4.3 Seed germination in response to dormancy breaking treatment

Experiments involving cold treatment were performed for *TaCYP707A1B*-C, *TaCYP707A1B*-OE and respective control (wild-type or *cyp707a1 cyp707a2*) seeds. Germination of *cyp70707a1 cyp707a2* seeds appeared not to be affected by the cold treatment which induced early germination phenotype in the control wild-type and *TaCYP707A1B*-C (Figure 4.2a, c). In response to cold treatment approximately ~15% of wild-type seeds germinated within 24 HAI when almost no germination of the *TaCYP707A1B*-C was apparent (Figure 4.2c). The complimented seeds showed germination only after 30 HAI (~16%), and wild-type seeds exhibited superior germination than the *TaCYP707B*-C seeds through 36 HAI. Cold treatment also induced early germination in both wild-type and *TaCYP707B*-OE seeds (Figure 4.3a, c). No difference in germination phenotype was apparent between the cold treated wild-type and *TaCYP707B*-OE seeds throughout the entire imbibition period (Figure 4.3c).

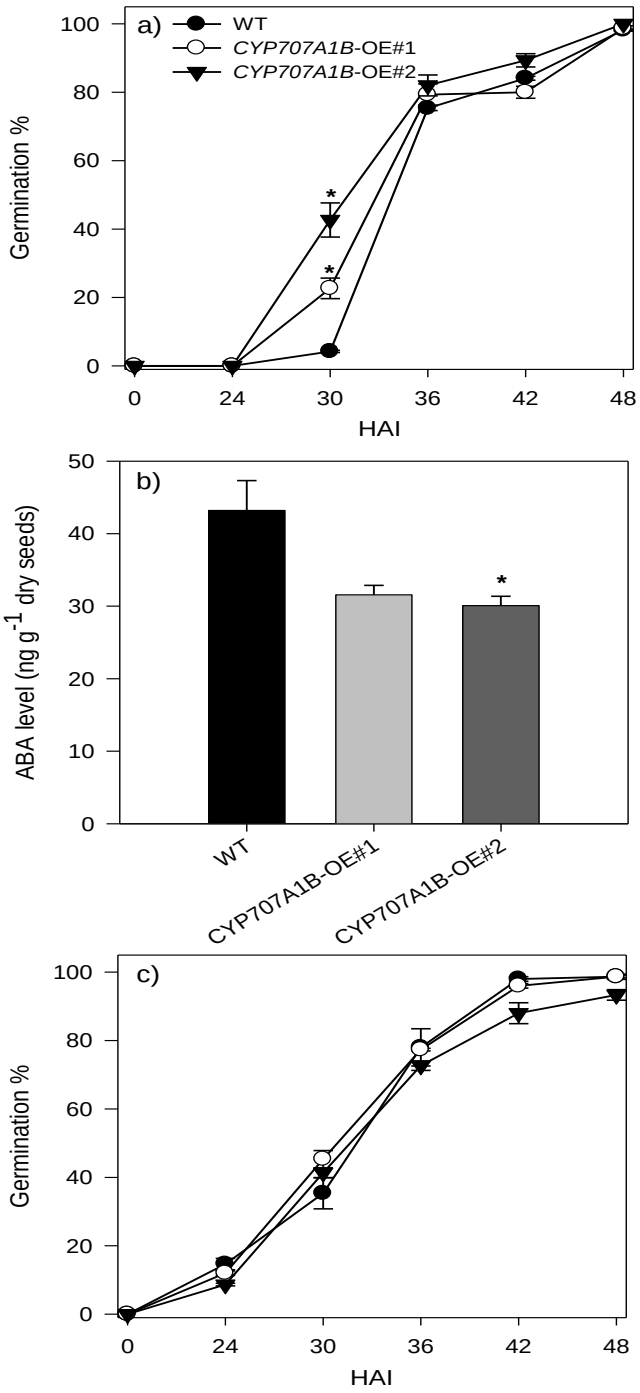


Figure 4.3. Phenotypic analysis of *TaCYP707A1B*-expressing Arabidopsis seeds. Germination (a) and ABA level in dry seeds (b) freshly harvested from wild-type (WT) and *TaCYP707A1B*-expressing (*CYP707A1B*-OE) plants, and germination kinetics in response to cold (4ºC) treatment for 3 days (c). Data are means of germination and ABA measurements of three independent replicates obtained from plants cultured simultaneously ± SE. Asterisks indicate statistically significant difference as compared to the wild-type control ($P<0.05$; t Student's test). HAI, hours after imbibition.

4.4.4 *TaCYP707A1* expression during germination of dormant and non-dormant wheat seeds

The comparative analysis between freshly harvested AC Domain (dormant) and RL4452 (non-dormant) seeds was done through germination phenotype and expression of *CYP707A1*. We found over 93% germination in non-dormant RL4452 seeds in first 24 hours after imbibition, whereas, no germination was evident in case of AC Domain cultivar during same period of time (Figure 4.4a). While all the seeds of RL4452 germinated within 48 HAI, only 5 % of the AC Domain seeds completed germination after 5 days of imbibition (data not shown). Given the difference in germination phenotype of seeds between the two genotypes, the higher expression of *TaCYP707A1* in dry and/or imbibing whole-seeds in the non-dormant genotype (Figure 4.4b) suggest the role of *TaCYP707A1* in regulating seed ABA level and dormancy in wheat, supporting our results from the heterologous system. Since pre-harvest sprouting is closely associated with the level of dormancy manifested in seeds, the *CYP707A1* can be therefore used as tool to develop wheat cultivars with intermediate level of seed dormancy via manipulating its expression level using biotechnological tools.

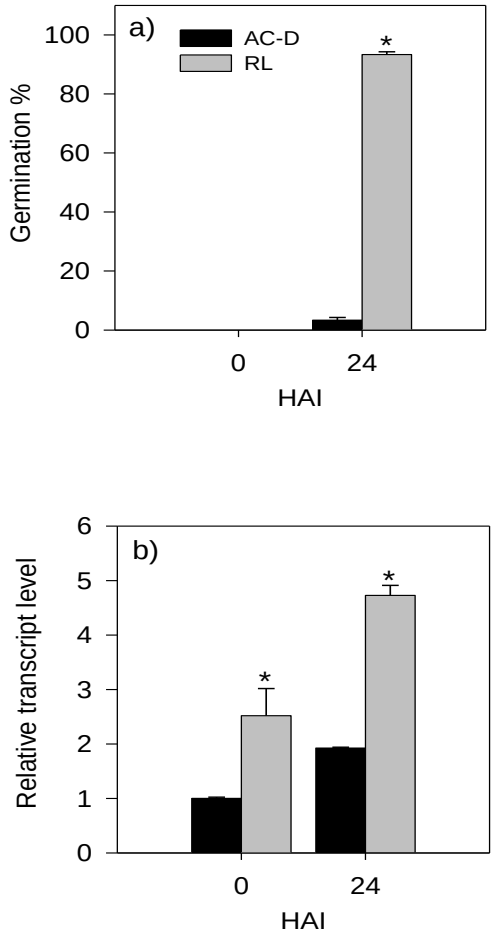


Figure 4.4 Comparison of the germination phenotype and the expression of *TaCYP707A1* gene between dormant and non-dormant seeds. Germination (a), and relative transcript level of *TaCYP707A1* (b) in dry [0 h after imbibition (HAI)] and imbibed (24 HAI) seeds of AC Domain (AC-D, dormant) and RL4452 (RL, non-dormant) wheat genotypes. Transcript level of gene was determined using *ubiquitin* as the reference gene and then expressed relative to that detected in the dry seeds of ‘‘AC Domain,’’ which was set arbitrarily to a value of 1. Data are means of two to three independent replicates ± SE. Asterisks indicate statistically significant difference in germination and transcript abundance between dormant and non-dormant seeds ($P<0.05$; t Student’s test). HAI, hours after imbibition.

4.4.5 Tissue specific expression analysis of *CYP707A1* and *CYP707A2* during seed development in wheat lines with contrasting pre-harvest sprouting tolerance

The expression patterns of *CYP707A1* and *CYP707A2* were also investigated in different tissues of developing seeds of two wheat lines with different levels of pre-harvest sprouting tolerance. The expresion of *CYP707A1* in the embryo of AC Domain showed a slight increase with seed development while that of *CYP707A2* showed a decrease (Figure 4.5a, c). In RL4452, moderate level of expression of *CYP707A1* was observed at the early stage of seed development, but the expression level increased over 5-fold by 30 DAA after which it decreased (~2.5-fold) and remains at this elevated level afterwards. The expression of *CYP707A2* gene in the embryo of RL4452 was lower than that of *CYP707A1* at all stages except that its expression was similar to that of *CYP707A1* by 50 DAA.

Although their expression remained at a low level during the early and mid phases of seed development (20 to 30 DAA), the two genes showed significant induction in their expression in the endosperm tissues of RL4452 as compared to that of AC Domain during the later phases of seed development (Figure 4.5b, d). The observation of higher levels of expression of *CYP707A1* in the embryo during the entire seed development and that of both *CYP707A1* and *CYP707A2* in the endosperm during the late phases of RL4452 than that found in the corresponding tissues of AC Domain suggests the distinct and overlapping tissue specific roles of these genes in lowering the level of ABA in maturing seeds of RL4452, which possess lower level of dormancy at maturity (Figure 4.5; Rasul et al., 2009; Rasul et al., 2012). In agreement with this, higher expression of *CYP707A1* during late maturity in barley seeds has been shown to be correlated with lower ABA accumulation and shallower dormancy, implying direct correlation

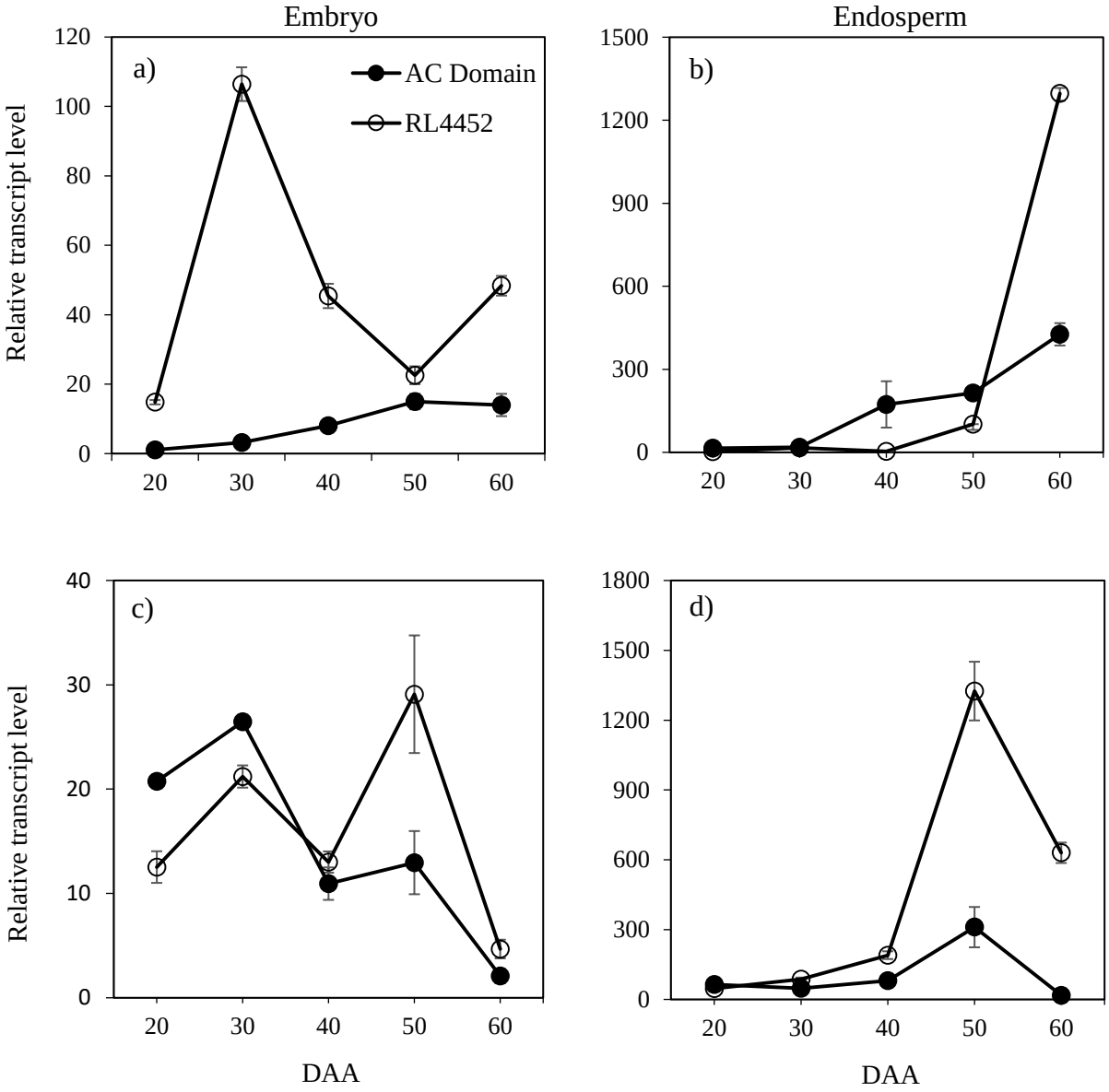


Figure 4.5. Relative transcript level of *CYP707A1* (a, b) and *CYP707A2* (c, d) in the embryos (a, c) and endosperm (b, d) tissues of AC Domain and RL4452 seeds during seed development. Transcript level of both genes was determined using wheat *β-actin* as the reference gene and then expressed relative to that of *CYP707A1* in the AC Domain embryo at 20 days after anthesis (DAA), which was set arbitrarily to a value of 1. Data are means of two to three independent replicates ± SE. DAA, days after anthesis.

between the levels of *CYP707A1* expression and seed dormancy (Chono et al., 2006). The expression of *CYP707A2* also showed an increase by 50 DAA in the embryo of RL4452, and this might suggest its stage specific contribution in the regulation of ABA level. A similar result has been reported in the seeds of Arabidopsis (wild type [Col-0]) in which *CYP707A1* was shown to be highly expressed during the mid seed development stage in the embryo and its expression level decreases afterwards (Okamoto et al., 2006). Although the late phases of seed development in the wild type (Col-0) accession of Arabidopsis is characterized by induction of only *CYP707A2* in both embryo and endosperm tissues (Okamoto et al., 2006), both *CYP707A1* and *CYP707A2* genes of wheat showed either maintenance of elevated expression (*CYP707A1* in the embryo) and slight to marked increases (*CYP707A2* in the embryo at 50 DAA, and *CYP707A1* and *CYP707A2* in the endosperm) in their expression in both embryo and endosperm tissues.

4.4.5.1 Tissue specific genomic contribution of transcripts to the total expression of *TaCYP707A1* during seed development

Transcript contributions from the A, B, and D genomes to the total expression of *CYP707A1* were also examined during seed development. In the embryo of AC Domain, the percentage transcript contribution of the D genome was the highest, that is, between 39% to 53%, suggesting that the *CYP707A1* homeolog from the D genome plays the major role in catabolizing ABA. The transcript contribution by the A genome was the second highest (27% to 38%) followed by the B genome, which contributed from 19% to 27% (Figure 4.6a). In contrast, the three genomes appear to contribute almost equally to the total expression of *CYP707A1* in the endosperm tissue of AC Domain (Figure 4.6b) and this result may imply the importance of all the three homeologous in influencing ABA level in the endosperm. In RL4452 unlike that observed in AC Domain,

transcript contribution by the B genome appeared to be the highest (38% to 44%) followed by the A genome that contributes 30% to 37%

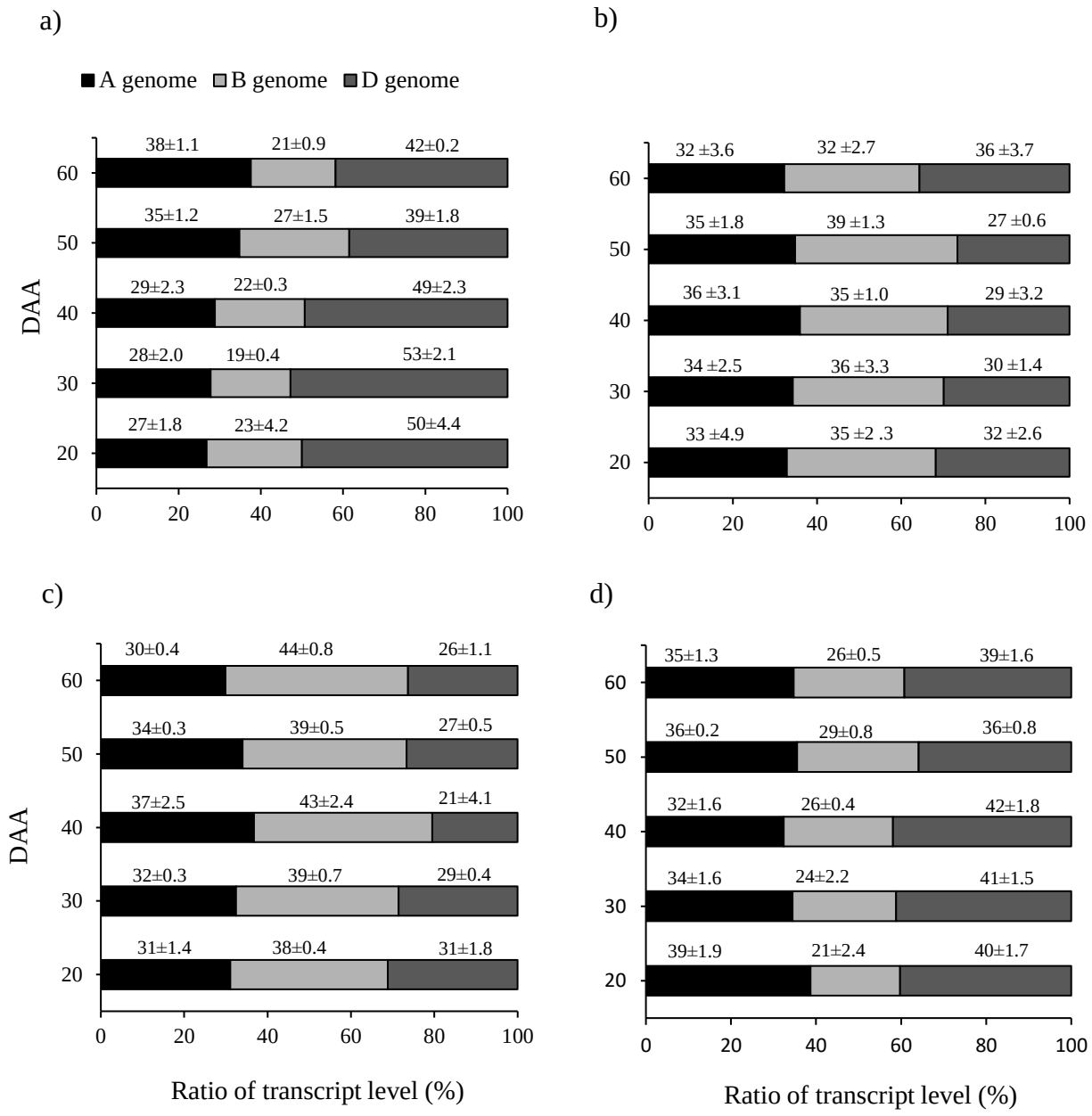


Figure 4.6. Transcript contribution of A, B, and D genomes to the total expression of *CYP707A1* during seed development in AC Domain embryo (a), AC Domain endosperm (b), RL4452 embryo (c) and RL4452 endosperm (d). Data are means of two to three independent replicates ± SE. DAA, days after anthesis.

transcripts, and these results suggest the *CYP707A1* homeologs from the B and A genomes of RL4452 are the major player of ABA catabolism in the embryo tissues. The contribution of the D genome was between 21% to 31% (Figure 4.6c). Differential genomic contribution of transcripts was also observed in the endosperm of RL4452 in which the D genome contribution was the highest (36% to 42%) followed by A genome (32% to 39%), implying the major roles of the two homeologs of *CYP707A1* in the conversion of ABA to its catabolites (Figure 4.6d). The B genome contribution was between 21% to 29%. The data from both genotypes and both tissues also showed that the contribution of transcripts by each genome in each tissue varies with developmental stage. These results overall indicate the genomic contribution of transcripts during seed development varies with genotype, tissue, and stage of development. Variation in genomic contribution of transcripts with tissues and stages has also been reported for other wheat genes such as that encoding sucrose transporter 2 (SUT2) (Deol et al., 2013). Furthermore, genome preferences in gene expression have been reported in other polyploidy crops such as cotton (Adams et al., 2003).

4.4.6 Tissue specific expression analysis of *CYP707A1* and *CYP707A2* during germination in wheat lines with contrasting pre-harvest sprouting tolerance

The expression patterns of both *CYP707A1* and *CYP707A2* genes in both embryo and endosperm appeared to be similar between the two wheat lines. (Figure 4.7). In the embryo, the expression of both genes in both lines increased during early period of imbibition (6 h) after which the expression level declined (3-fold) to a level that was almost maintained until the end of the imbibition period. In the endosperm, however the peak expression of *CYP707A2* occurred earlier (at 12 HAI) than that of *CYP707A1*, which occurred at 18 HAI. Although both genes overall exhibited higher expression in seed tissues of the non-dormant line, RL4452, as compared to

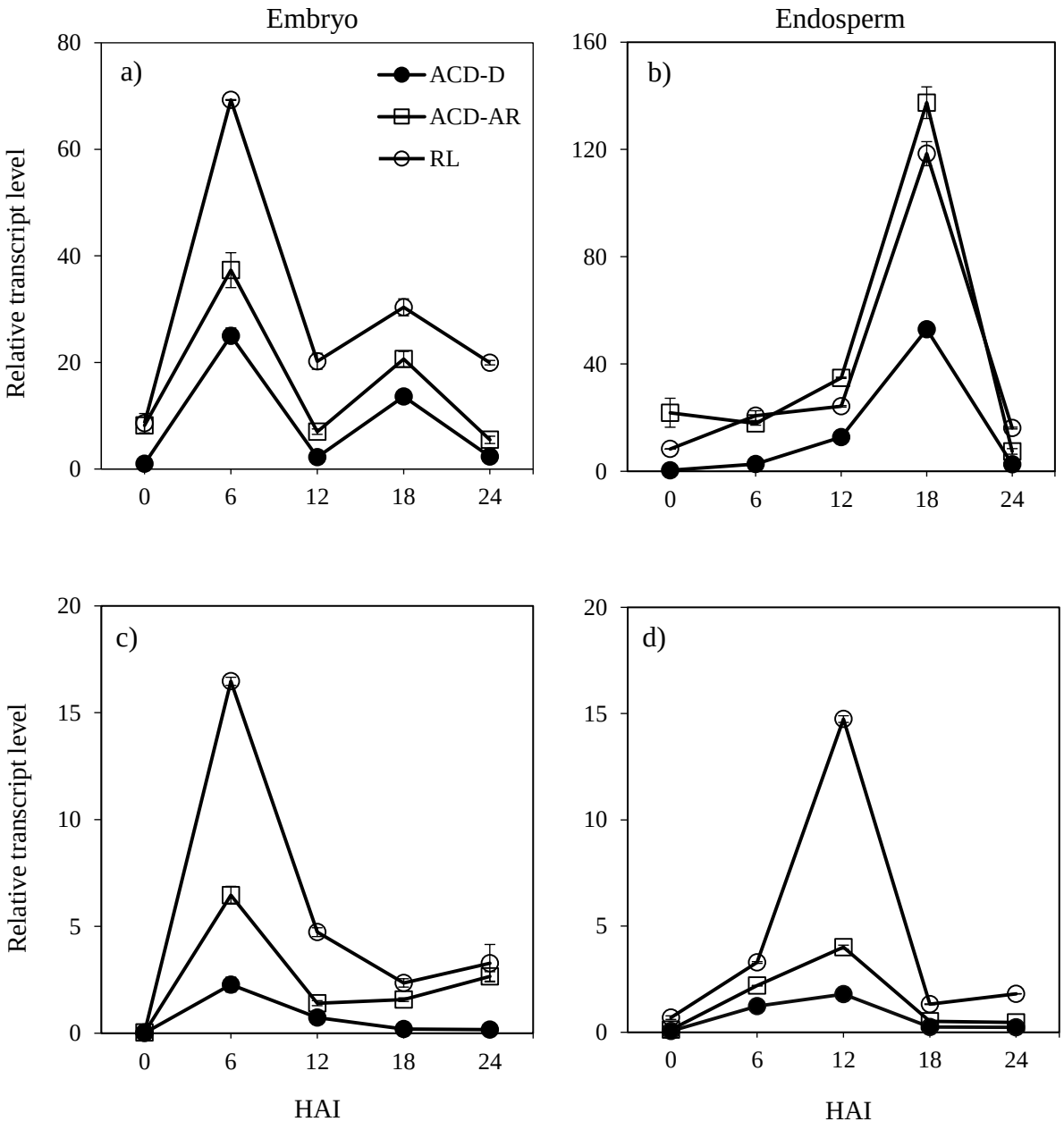


Figure 4.7. Relative transcript level of *CYP707A1* (a, b) and *CYP707A2* (c, d) in the embryos (a, c) and endosperm (b, d) tissues of AC Domain dormant (ACD-D), AC Domain after-ripened (ACD-AR) and RL4452 (RL) seeds during imbibition. Transcript level of both genes was determined using wheat *β-actin* as the reference gene and then expressed relative to that of *CYP707A1* in the embryo of AC Domain seeds at 0 HAI, which was set arbitrarily to a value of 1. Data are means of two to three independent replicates ± SE. HAI, hours after imbibition.

that observed in the corresponding tissues of the dormant line, AC Domain, the expression level of *CYP707A1* in both embryo and endosperm tissues was higher than that of *CYP707A2*.

We further performed after-ripening treatment of the AC Domain seeds and characterized the expression of the two genes during imbibition of these seeds. The after-ripening treatment increased the expression of the both genes in embryo and endospem tissues at most time points during imbibition, though not to the level observed in RL4452 tissues except in the case of *CYP707A1* in the endosperm (Figure 4.7b). However, the *CYP707A1* gene still shows higher expression level in both embryo and endosperm tissues of after-ripened seeds than that of *CYP707A2*. Given that the non-dormant line RL4452 and the after-ripened seeds of AC Domain are able to germinate at 24 HAI, the results suggest the role of the *CYP707A* genes of wheat, mainly that of *CYP707A1*, in regulating seed ABA level and therefore the level of dormancy and germination. Expression and mutant analysis in Arabidopsis, however, indicated *CYP707A2* as important player in regulating ABA level and germination (Kushiro et al., 2004; Okamoto et al., 2006). In agreement with the results shown in this thesis, a study in barley showed enhanced expression of *CYP707A1* and a rapid decrease in ABA content in the embryo of after-ripened seeds as compared to the corresponding dormant seeds (Chono et al., 2006; Millar et al., 2006). Recent studies have shown the relationships between the expression patterns of ABA catabolic genes of wheat, *CYP707A1* and *CYP707A2*, and seed ABA level and germination (Kashiwakura et al., 2016; Izydorczyk et al., 2017) in which the expression of *CYP707A1* and/or *CYP707A2* in both embryo and endosperm tissues contribute to the regulation of seed ABA level and germination. A microarray based analysis of the whole seed tissue showed no difference in expression of an ABA catabolic gene represented on the GeneChip between dormant and after-ripened seeds (Liu et al.,

2013), and this absence of differential expression can be related to the use of whole seed tissue in that particular study.

4.4.6.1 Tissue specific genomic contribution of transcripts to the total expression of *TaCYP707A1* during germination

Genomes A and B contributes the majority of transcripts to the total expression of *CYP707A1* in the embryos of dry dormant and after-ripened seeds of AC Domain (36% to 48%), however, during seed imbibition, genome D appeared to contribute most of the transcripts (over 47%) followed by genome A (27% to 32%) (Figure 4.8 a, b). Genome B contributes the least amount of transcripts (14% to 23%) to the total expression of *CYP707A1* in the embryos of imbibing seeds. In the endosperm, genome A overall contributes the most amount of transcripts before and after imbibition in both dormant and after-ripened seeds of AC Domain (~50% or over) followed by genome D except in the endosperm of dormant dry seeds in which genomes A and D contributed equally (Figure 4.8 d, e). Similar to that observed in the embryo, genome B contributes the least amount of transcripts to the total expression of *CYP707A1* in the endosperm of imbibing dormant and after-ripened seeds of AC Domain (14% to 21%). In the case of RL4452, genomic contributions of transcripts to the total expression of *CYP707A1* in the embryo and endosperm tissues of dry seeds are similar to that observed in the dry after-ripened seeds of AC Domain (Figure 4.8c and f), however, during imbibition, over 45% of *CYP707A1* transcripts in both tissues are contributed by genome D followed by genome A (30% to 32%). Similar to that found in the other seed samples, genome B contributes the least amount of transcripts (< 25%) to the total expression of the gene in both tissues of imbibing RL4452 seeds. Overall the D genome homeologue of *CYP707A1* plays a major role in regulating ABA level in

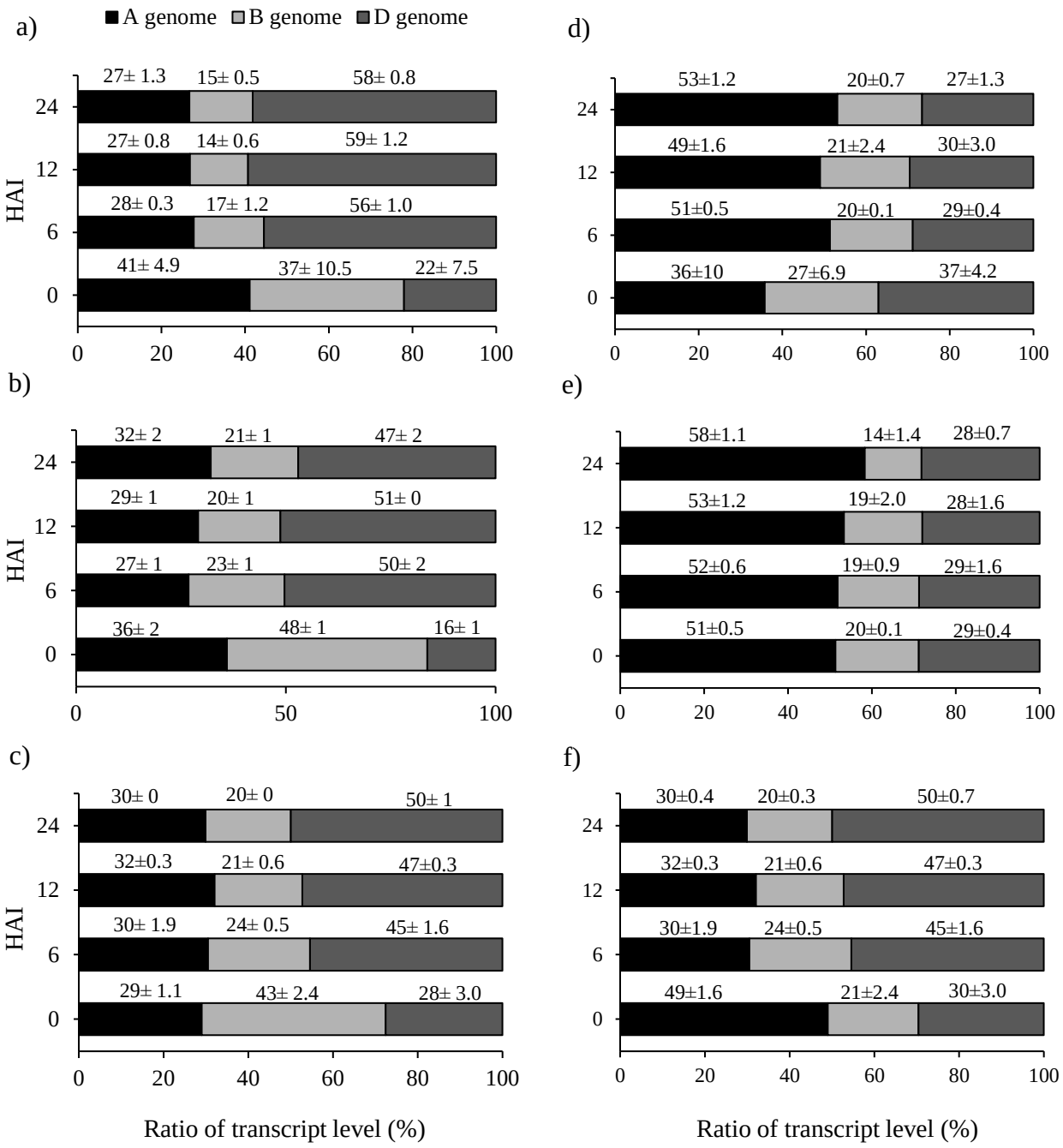


Figure 4.8. Transcript contribution of A, B, and D genomes to the total expression of *CYP707A1* during seed germination in embryo (a, b, and c) and endosperm (d, e, and f) tissues in AC Domain dormant (a, d), AC Domain after ripened (b, e), and RL4452 (c, f) seeds. Data are means of two to three independent replicates ± SE. HAI, hours after imbibition.

the embryo during imbibition irrespective of genotype and dormancy status. In the endosperm, genomic contribution varies with genotype; the A genome contributes the most in AC Domain while the D genome contributes the most in RL4452. Such differential genomic contribution between genotypes and tissues has been shown previously as discussed in section 4.4.5.1.

In summary, the study showed that ectopic expression of one of the homeologues *TaCYP707A1* (*TaCYP707A1B*) in Arabidopsis affected seed ABA level and germination. The observation of higher expression of *TaCYP707A1* and *TaCYP707A2* in the PHS susceptible genotype (RL4452) and after-ripened seeds of the PHS resistant genotype (AC Domain) during seed development and/or germination further suggests role of *TaCYP707A* genes in regulation of ABA level and thereby induction and maintenance of dormancy in wheat seeds. Determining the definitive physiological functions of these and their homeologues with respect to dormancy requires mutational studies in wheat.

Chapter 5: After-ripening induced transcriptional changes of hormonal genes in wheat seeds: the cases of brassinosteroids, ethylene and cytokinin.

This chapter is published as Chitnis VR, Gao F, Yao Z, Jordan MC, Park S, Ayele BT (2014) After-ripening induced transcriptional changes of hormonal genes in wheat seeds: the cases of brassinosteroids, ethylene, cytokinin and salicylic acid. PLOS ONE 9(1): e87543 (Chitnis VR is co-first author).

Chitnis VR was involved in performing experiments and writing the manuscript. Gao F performed data analysis and provided support in the write up; Jordan MC contributed in performing the microarray experiments; Yao Z and Park S provided support in sample preparation.

5. After-ripening induced transcriptional changes of hormonal genes in wheat seeds: the cases of brassinosteroids, ethylene and cytokinin.

5.1 Abstract

Maintenance and release of seed dormancy is regulated by plant hormones; their levels and seed sensitivity being the critical factors. This study reports transcriptional regulation of brassinosteroids (BR), ethylene (ET) and cytokinin (CK) related wheat genes by after-ripening, a period of dry storage that decays dormancy. Changes in the expression of hormonal genes due to seed after-ripening did not occur in the anhydrobiotic state but rather in the hydrated state. After-ripening induced dormancy decay appears to be associated with imbibition mediated increase in the synthesis and signalling of BR, via transcriptional activation of *de-etiolated2*, *dwarf4* and *brassinosteroid signaling kinase*, and repression of *brassinosteroid insensitive 2*. Our analysis is also suggestive of the significance of increased ET production, as reflected by enhanced transcription of *1-aminocyclopropane-1-carboxylic acid oxidase* in after-ripened seeds, and tight regulation of seed response to ET in regulating dormancy decay. Differential transcriptions of *lonely guy*, *zeatin O-glucosyltransferases* and *cytokinin oxidases*, and *pseudo-response regulator* between dormant and after-ripened seeds implicate CK in the regulation of seed dormancy in wheat. These results contribute to further our understanding of the molecular features controlling seed dormancy in wheat.

5.2 Introduction

Seed plays a vital role in the life cycle of plants as it carries genetic information from one generation to another. To this end, seed germination is an important trait playing a critical role for the establishment, growth, and productivity of next generation plants. Dormancy is an adaptive mechanism through which seeds delay their germination even under optimal conditions (Bewley, 1997). In cereal crops such as wheat, intermediate dormancy is desirable as low level of dormancy makes seeds susceptible to pre-harvest sprouting that downgrades grain quality for end-use applications. Whereas, high degree of dormancy has a negative effect on the rate and uniformity of germination; ultimately causing poor seedling establishment. This emphasizes the need to develop cultivars with moderate dormancy, for which dissection of the underlying molecular mechanisms has a paramount significance.

It is well established that the balance between two classical plant hormones, namely abscisic acid (ABA) and gibberellin (GA), is a major regulator of seed dormancy and germination (Finkelstein et al., 2008). However, previous studies mainly with seeds of dicot species have also implicated other plant hormones such as brassinosteroid (BR), ethylene (ET) and cytokinin (CK) in the regulation of these seed physiological processes (Kucera et al., 2005; Wang et al., 2011). Brassinosteroids enhance seed germination mainly by antagonizing the inhibitory effect of ABA. When compared to that of wild type, ABA exerts stronger inhibitory effect on the germination of BR biosynthetic mutant, *det2-1* and BR-insensitive mutant, *bri1-1* seeds of Arabidopsis (Steber and McCourt, 2001). Consistently, inhibition of seed germination by ABA is overcome by overexpression of the BR biosynthetic gene, *DWF4* (Divi and Krishna, 2010). In addition, BRs reverse the non-germination phenotype of severe GA biosynthetic mutants such as *ga1-3* and the GA-insensitive mutant *sleepy1* by a mechanism different from that of GA (Leubner-Metzger,

2001; Steber and McCourt, 2001). However, a recent study indicated the presence of physical interaction between repressor of *ga1-3* (RGA) and brassinazole resistant 1 (BZR1) (Li et al., 2012), which act as negative and positive regulators of GA and BR signaling, respectively; and this might form the molecular basis of interplay between GA and BR in regulating seed dormancy and germination.

Ethylene influences several plant growth and developmental processes, from germination through senescence (Kepczynski and Kepczynska, 1997). Previous studies in sunflower and Arabidopsis have implicated ET in the regulation of seed dormancy (Corbineau et al., 1990) and promoting radicle protrusion by antagonizing ABA (Ghassemian et al., 2000). The repression of *1-aminocyclopropane-1-carboxylic acid oxidase 1* (*ACO1*) by ABA during germination of Arabidopsis seeds, and the presence of high level of *ACO1* transcripts in the seeds of ABA-insensitive mutants (Penfield et al., 2006; Carrera et al., 2008; Linkies et al., 2009) suggest that inhibition of germination by ABA is partly mediated through transcriptional repression of ET synthesis. In agreement with this, mutation in *ETHYLENE RESPONSE 1* (*ETR1*), a gene encoding ET receptor, led to increased seed ABA level and enhanced dormancy (Chiwocha et al., 2005). However, studies with cereal seeds such as barley and red rice have shown that ET is neither involved in the breaking of dormancy nor required for germination, but rather enhances radicle growth following its emergence through the pericarp (Locke et al., 2000; Gianinetti et al., 2007).

The role of CK in regulating seeds dormancy and germination is still unclear. Increased expression of the CK biosynthetic gene, *isopentenyltransferase 8* (*IPT8*), in Arabidopsis confers ABA-insensitivity to *germination insensitive to ABA mutant 1* (*gim1*) seeds, and this is attributable to increased seed CK level and repression of ABA inducible genes including *ABI5*, a key player in seed sensitivity to ABA (Wang et al., 2011). Consistently, CK reverses the inhibitory effect of

ABA on the germination of lettuce seeds (Khan, 1968) and promotes the germination of wheat seeds by stimulating the GA-induced activity of α-amylase (Eastwood et al., 1969). Contrary to these reports, overexpression of CK inactivating genes, *cytokinin oxidase 2* (*CKX2*) and *CKX4*, and loss of function of CK receptors, *Arabidopsis histidine kinase 2* (*AHK2*), *AHK3* and *cytokinin response 1* (*CRE1)/AHK4*, leads to early germination (Riefler et al., 2006).

Studies that shed light on the molecular mechanisms underlying the roles of plant hormones in regulating seed dormancy and germination have been mainly focused on dicot species (Kucera et al., 2005; Linkies and Leubner-Metzger, 2012). Much less is known about these phenomena for seeds of cereal crops such as wheat. Previously, we identified specific molecular switches that mediate the roles of ABA, GA, jasmonate and auxin in regulating seed dormancy release and wheat (Liu et al., 2013). To further our understanding with this respect, the present study explores after-ripening induced transcriptional changes related to BR, ET, and CK in wheat seeds in both dry and hydrated states.

5.3 Materials and Methods

5.3.1 Plant materials

Wheat cultivar AC Domain, which produces dormant seeds at harvest, was used in this study. Immediately after harvest, a portion of seeds was stored at –80°C to maintain dormancy, while another portion was stored at room temperature and ambient relative humidity for a period of 10 months to break dormancy. Germination assays were performed under darkness at 22°C with Petri-plate system between layers of Whatman #1 paper wetted with 7 mL of sterile water. ABA treatment was performed by imbibing AR seeds with 50 µM ABA solution. Seeds were considered germinated when the coleorhiza protruded through the seed coat.

5.3.2 Isolation of mRNAs and microarray analysis

mRNA samples were extracted from three independent biological replicates of dormant and after-ripened seeds at 0, 12 and 24 h after imbibition using the protocol described previously (Gao et al., 2012). Following labeling, the mRNAs isolated from the three independent biological replicates of each sample were subjected to hybridization to the Affymetrix GeneChip Wheat Genome Array (Affymetrix). Scanning of the hybridized microarrays was performed with the Affymetrix GeneChip Operating Software. As each probeset on the Affymetrix GeneChip Wheat Genome Array is defined by 11 probe pairs, the signals obtained from all probe pairs of a given probeset were converted into a single signal value and presented in CEL file format. Adjustment of the total signal intensity in each chip was performed using the 50^{th} percentile of all measurements. The number of probesets with a 'present' detection call was determined by using the Affymetrix Microarray suite (MAS5) statistical algorithm. Normalization of the raw intensity data was performed with Robust Multi-array Average (RMA) methodology after which the data were log (base 2) transformed. The microarray data is available in the NCBI's Gene Expression Omnibus database (GSE32409). Validation of the microarray data was performed with 10 selected hormonal genes (three biological and two technical replicates) using real-time quantitative RT-PCR (qRT-PCR) and the same mRNA samples subjected for microarray analysis as described before (Gao et al., 2012). The sequences of the primers used for real-time qRT-PCR analysis are shown in appendix 10.

5.3.3 Identification of brassinosteroid, ethylene and cytokinin related probesets

Metabolic and signaling genes of BR, ET and CK were identified from the model plant Arabidopsis, and rice and other monocot species as described before (Liu et al., 2013). Briefly,

The Arabidopsis Information Resource database was searched for the target genes, and the resulting nucleotide sequences were used to search for similar sequences in the Rice Annotation Project database (http://rapdb.dna.affrc.go.jp/) using a cut-off E-value of $< 10^{-20}$. The NCBI wheat unigene build #61 (http://www.ncbi.nlm.nih.gov/UniGene/UGOrg.cgi?TAXID=4565) was searched against sequences of the target genes derived from rice and other monocot species. Nucleotide sequences of wheat with a coverage length of ≥ 200 bp and an E-value of $\leq 10^{-50}$ were further subjected to blast search against the wheat 61 K microarray platform database in order to identify their respective probesets on wheat GeneChip using the search tools and criteria described previously (Liu et al., 2013). The candidate probesets annotated by HarvEST Wheat-Chip (http://harvest.ucr.edu) are shown in appendix 4-9.

5.3.4 Expression profile of brassinosteroid, ethylene and cytokinin related probesets

Microarray data were analyzed by the FlexArray software (http://genomequebec.mcgill.ca/Flex-Array) (Blazejczyk et al., 2007) using analysis of variance (ANOVA) as described previously (Gao et al., 2012). Following extraction of the $\log_2$ transformed signal intensities of probesets corresponding to BR, ET and CK metabolism and signalling genes, $\log_2$ and linear fold changes in expression along with the associated *P* values were calculated between dormant and after-ripened seeds before (0 HAI) and during imbibition (12 and 24 HAI) as described before (Liu et al., 2013). Fold changes in expression of ≥1-fold on $\log_2$-scale (≥2-fold on linear-scale) and probability value of $P \leq 0.05$ were used as cut-off values to determine statistically significant differences in expression between comparisons. While the negative fold changes indicate downregulation in the expression of genes, the positive ones reflect upregulation in each comparison under considerations.

5.4 Results and Discussion

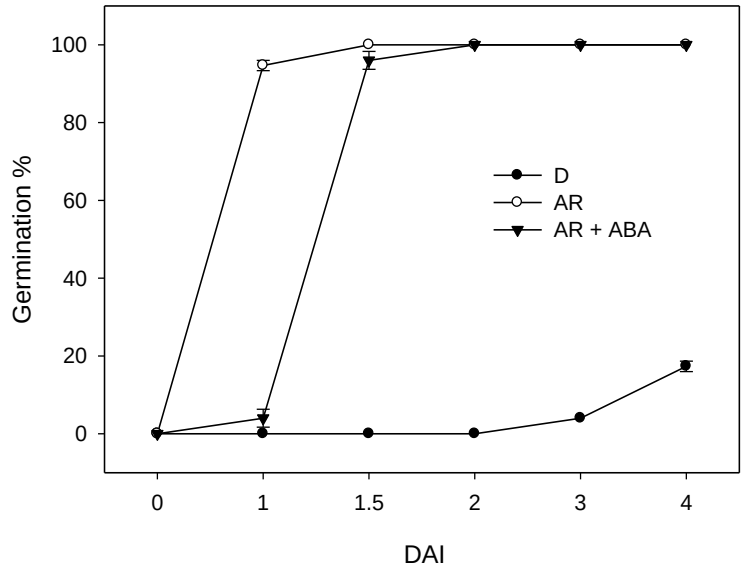
5.4.1 Seed Germination

After-ripening of the dormant wheat seeds led to approximately 95% germination within 24 h of imbibition. However, the corresponding dormant seeds did not exhibit germination within the same period of imbibition, and only 11% of the dormant seeds were able to exhibit coleorhiza beyond the seed coat following 7-day imbibition. Treatment of after-ripened seeds with ABA delayed germination and inhibited seminal root growth (Figure 5.1).

5.4.2 No transcriptional change of hormonal probesets is induced by dry after-ripening

Wheat probesets annotated as the metabolism and signaling genes of BR, ET, and CK were identified using bioinformatics resources based on the criteria described in the Material and Methods section (Appendix 4-9). Comparison of their expression profile between dormant and after-ripened seeds revealed that no transcriptional change is induced by dry after-ripening. Probesets annotated as ABA, GA, jasmonate and auxin metabolism and signaling genes also did not show any changes in expression due to dry after-ripening (Liu et al., 2013). These results suggest that the transcription of hormonal genes is less likely to be an integral part of mechanisms underlying dormancy decay by dry after-ripening (Bazin et al., 2011).

a)



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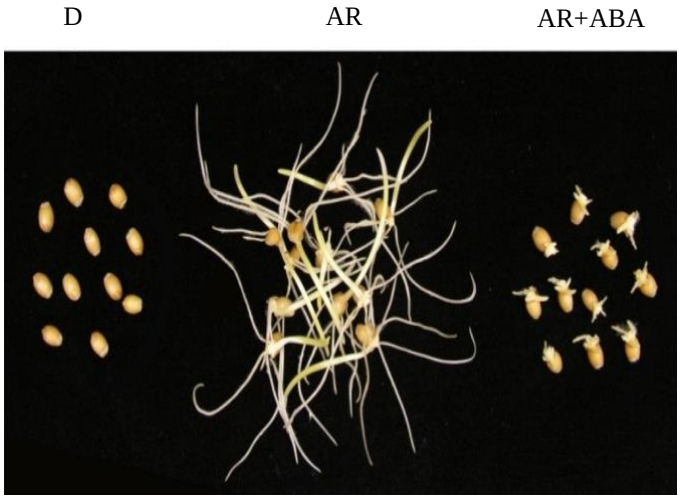

Figure 5.1. Effect of after-ripening on the release of dormancy in wheat seeds**.** Germination percentage of dormant (D) and after-ripened (AR) seeds imbibed in water (D and AR) and ABA (AR) over 4 days (a). Comparison of D and AR seeds following 4-day imbibition in water (D and AR) and ABA (AR). DAI, days after imbibition (b).

5.4.3 After-ripening induces imbibition mediated transcriptional changes of hormonal probesets

Our transcriptomic analysis indicated that imbibition of after-ripened wheat seeds leads to changes in the transcription of specific genes related to the metabolism and signaling of BR, ET, and CK (Appendix 4-9). Such transcriptional changes have also been reported for those genes related to ABA, GA, jasmonate and auxin (Liu et al., 2013).

5.4.4 After-ripening induces transcriptional changes of specific brassinosteroid related probesets

Sixteen probesets annotated as BR metabolic genes are found in the wheat genome GeneChip (Appendix 4), including *de-etiolated2* (*DET2*) encoding steroid 5-alpha- reductase, *constitutive photomorphogenesis and dwarfism* (*CPD*) encoding BR hydroxylase, *dwarf4* (*DWF4*) encoding steroid C-22 hydroxylase and *rotundifolia 3* (*ROT3*) encoding BR C-23 hydroxylase (Figure 2.4). One of the 11 probesets of *DET2*, and a probeset annotated as *DWF4* that encodes an enzyme catalyzing a rate-limiting step in BR biosynthesis (Kim et al., 2006), exhibited upregulation (over 2-fold, $P \leq 0.05$) in imbibed after-ripened relative to dormant seeds (Figure 5.2a, Appendix 11a). It has been shown previously that overexpression of *DWF4*, whose expression is positively correlated with bioactive BR content in plant tissues (Nomura et al., 2001; Shimada et al., 2003), overcomes the inhibitory effect of ABA on germination (Divi and Krishna, 2010), and the ABA sensitivity of *det2* seeds is higher than that of wild type (Steber and McCourt, 2001). Our results, thus, suggest that after-ripening activates BR synthesis during imbibition, and thereby stimulate the breakage of seed dormancy and germination through counteracting the effect of ABA.

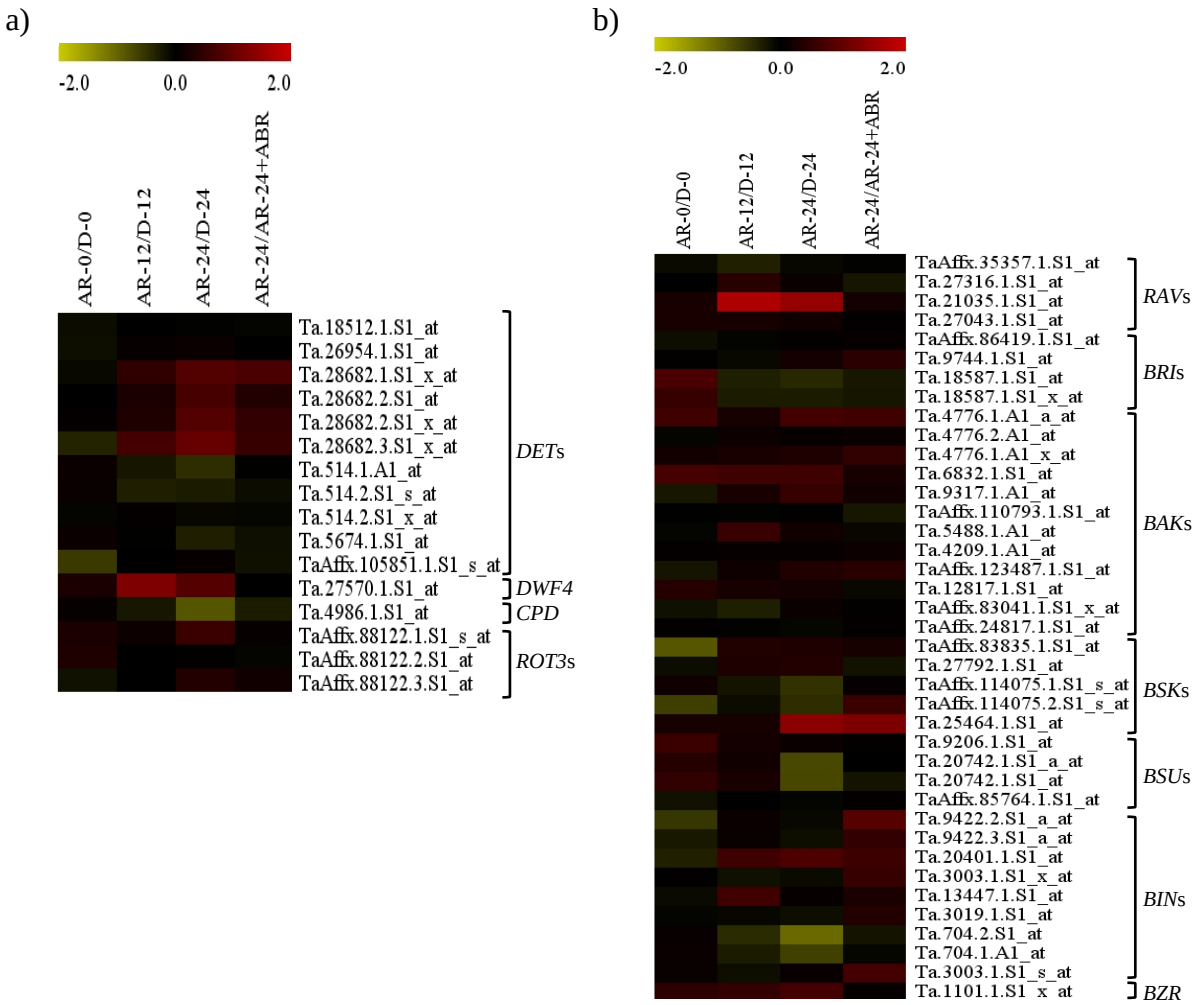

Figure 5.2. Changes in the expression of brassinosteroid (BR) metabolism and signaling genes in response to after-ripening. Fold changes (log_2-scale) in the expression of BR metabolism (a) and signaling (b) related probesets between after-ripened (AR) and dormant (D) seeds in both dry (AR-0/D-0) and imbibed states (AR-12/D-12 and AR-24/D-24), and between AR seeds imbibed without and with ABA (AR-24/AR-24+ABA). The log_2 fold change values are shown by the negative and positive numbers on the bar and the color scale shows upregulation (red) and downregulation (olive green) of the respective probesets. Fold changes (linear-scale) in expression and the associated *P* values are presented in appendix 4, 5. Data are means of 3 independent biological replicates. Abbreviations: *DET2*, *de-etiolated 2*; *DWF4*, *dwarf4*; *CPD*, *constitutive photomorphogenic dwarf*; *ROT3*, *rotundfolia3*; *RAV*, *related to ABI3/VP1*; *BRI*, *brassinosteroid insensitive*; *BAK*, *BRI1-associated receptor kinase*; *BSK*, *BR-signaling kinases*; *BSU*, *BRI suppressor*; *BIN*, *brassinosteroid insensitive*; *BZR*, *brassinazole-resistant*; *MFT*, *mother of FT AND TFL1*. BR metabolic and signaling pathways are shown in Figure 2.4.

A recent report has also indicated the contribution of *GSR*, a member of the GA stimulated transcript (GAST) family, in stimulating BR synthesis (Wang et al., 2009a). After-ripening led to upregulation (over 2-fold, $P \leq 0.05$) of three probesets representing *GSR* during imbibition (Appendix 4). Since the transcripts of probesets annotated as GA biosynthetic (*GA20ox1* and *GA3ox*) and GA responsive genes are activated in imbibing after-ripened wheat seeds (Gao et al., 2012; Liu et al., 2013), our data might suggest the contribution of *GSR* mediated activation of BR synthesis in the regulation of wheat seed dormancy and germination.

Thirty nine probesets annotated as BR signalling genes are represented on Wheat GeneChip (Appendix 5), including *brassinosteroid insensitive 1* (*BRI1*), *regulator of the ATPase of the vacuolar membrane* (*RAV*), *BR signaling kinase* (*BSK*), *BRI1 suppressor 1* (*BSU1*), *BR-insensitive 2* (*BIN2*) and *brassiazole resistant 1* (*BZR1*). A probeset annotated as *BSK2*, which act as a positive regulator of BR signaling, exhibited upregulation in imbibing after-ripened relative to dormant seeds, while another one annotated as *BIN2*, which act as a negative regulator of BR signaling, was downregulated (Figure 5.2b, Appendix 11b). Given that BR signaling has been implicated in the regulation of seed dormancy and germination (Steber and McCourt, 2001), these results suggest transcriptional activation of seed response to BR leading to dormancy decay and germination. Consistently, after-ripening led to imbibition mediated transcriptional activation of probesets annotated as BR responsive genes, *paclobutrazol resistance* (*PRE*) and *BR enhanced expression* (*BEE*) (Figure 5.3), that are involved in cell elongation (Friedrichsen et al., 2002; Zhang et al., 2009), a process necessary for the completion of seed germination. Furthermore, our data showed transcriptional repression of *BSK2* by ABA (Figure 5.2b, Appendix 5), suggesting that one mechanism by which ABA delays wheat seed germination and inhibits seminal root growth is

via repression of BR action. In support of this, ABA suppressed of the transcription of *PRE* during imbibition of after-ripened seeds.

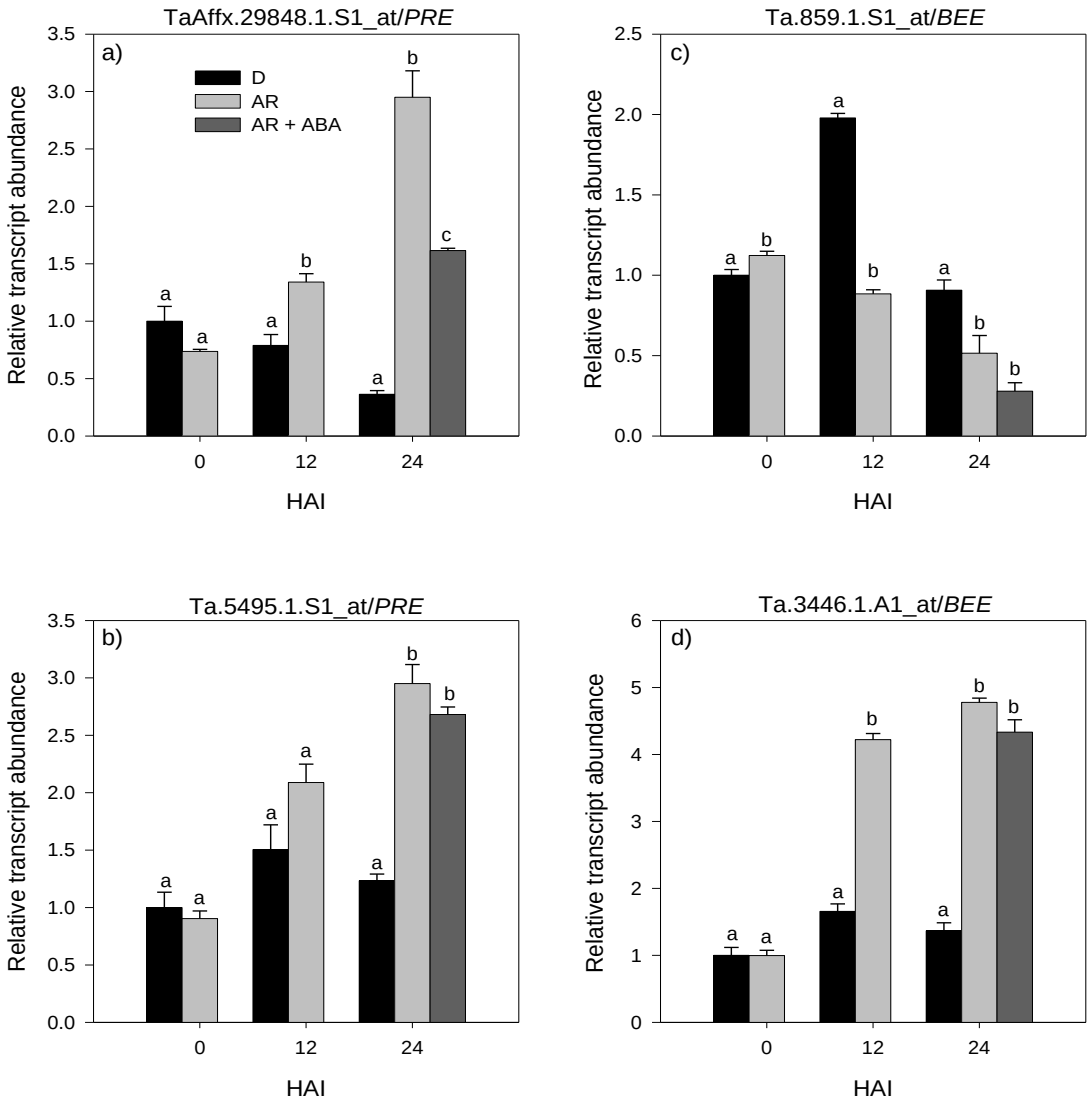

Figure 5.3. Comparison of the expression (log_2-scale) of brassinosteroid (BR) responsive genes. Relative transcript abundance of *paclobutrazol-resistance* (*PRE*) (a, b) and *brassinosteroid enhanced expression* (*BEE*) (c, d) in dormant (D) and after-ripened (AR) seeds before imbibition (D-0 and AR-0) and during imbibition in water (D-12, AR-12, D-24, and AR-24) and ABA (AR-24+ABA). Transcript abundance was expressed relative to that in D-0 seeds, which was arbitrarily set to a value of 1. Data are means of 3 independent biological replicates ± SE. Within each imbibition time point, different letters indicate significant difference in transcript abundance between seed samples at $P \leq 0.05$.

By activating the transcription of BR biosynthetic genes, *ebisudwarf/dwarf2* (*D2*), *dwarf11* (*D11*) and *BR-deficient dwarf1/BR-C6 oxidase* (*BRD1*), and BR signaling gene, *BRI1*; *ABI3/VP1* (*RAV*) *Like1* (*RAVL1*) of rice regulates not only cellular BR homeostasis but also BR sensitivity (Hong et al., 2003; Tanabe et al., 2005; Je et al., 2010). Thus, the upregulation of a probeset annotated as *RAV* in imbibing after-ripened relative to dormant seeds (over 2-fold, $P \leq 0.05$) (Figure 5.2b, Appendix 5) suggests the significance of coordinated regulation of BR synthesis and signaling in dormancy decay and germination of wheat seeds. Furthermore, a recent report has indicated that the antagonistic effect of BR against ABA in Arabidopsis is mediated by *mother of ft and tfl1* (*MFT*), a gene encoding a phosphatidylethanolamine, as BR cannot reverse the ABA hypersensitive and low germination phenotypes of *mft* seeds (Xi and Yu, 2010). However, enhanced expression of *MFT* leads to low germination index, mimicking the inhibitory effect of ABA on germination (Nakamura et al., 2011). Consistent with this, a probeset annotated as *MFT* is downregulated (over 2-fold, $P \leq 0.05$) in imbibing after-ripened relative to dormant seeds (Appendix 5), confirming that *MFT* forms an integral part of dormancy regulatory mechanisms in wheat.

5.4.5 Transcriptional alteration of specific ethylene related probesets by after-ripening

To gain insights into the role of ET in regulating dormancy and germination of wheat seeds, we investigated the differential expression of a total of 78 probesets annotated as ET metabolism and signalling genes (Appendix 6) between after-ripened and dormant seeds. The first committed and rate-limiting step in ethylene biosynthesis is catalyzed by 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS), producing ACC from S-adenosylmethionine, and the ACC is converted to ethylene by 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) (Figure 2.5). No probeset

representing ACS was found to be differentially regulated by after-ripening (Figure 5.4a, Appendix 6). However, ethylene production is controlled by post-transcriptional mechanisms such as stabilization of the ACS protein (Wang et al., 2004), which has been shown to be regulated by BR (Hansen et al., 2009). The induction of BR biosynthetic probesets, *DET* and *DWF4*, in AR seeds might therefore imply the role of after-ripening in enhancing ACS stability through increased BR production. In contrast, four probesets corresponding to *ACO* exhibited upregulation (over 2-fold, $P \leq 0.05$) during imbibition of after-ripened relative to dormant seeds (Figure 5.4a, Appendix 11c).

Given that ET has been implicated in the breakage of seed dormancy in some species such as sunflower (Corbineau et al., 1990) and wild oats (Adkins and Ross, 1981), our data might suggest after-ripening mediated induction of ET synthesis, and thereby seed dormancy release in wheat. Consistently, non-dormant seeds produce more ethylene than their dormant counterparts (Kucera et al., 2005). However, other studies have indicated that this phytohormone is not associated with dormancy decay in cereal seeds such as barley and red rice; but rather with stimulation of the germination of non-dormant seeds (Locke et al., 2000; Gianinetti et al., 2007). Although ABA represses the expression of *ACO1* during germination of Arabidopsis seeds, and seeds of ABA-insensitive mutants contain high level of *ACO1* transcripts (Penfield et al., 2006; Carrera et al., 2008; Linkies et al., 2009), no such effect of ABA was evident in wheat seeds (Figure 5.4a, Appendix 6).

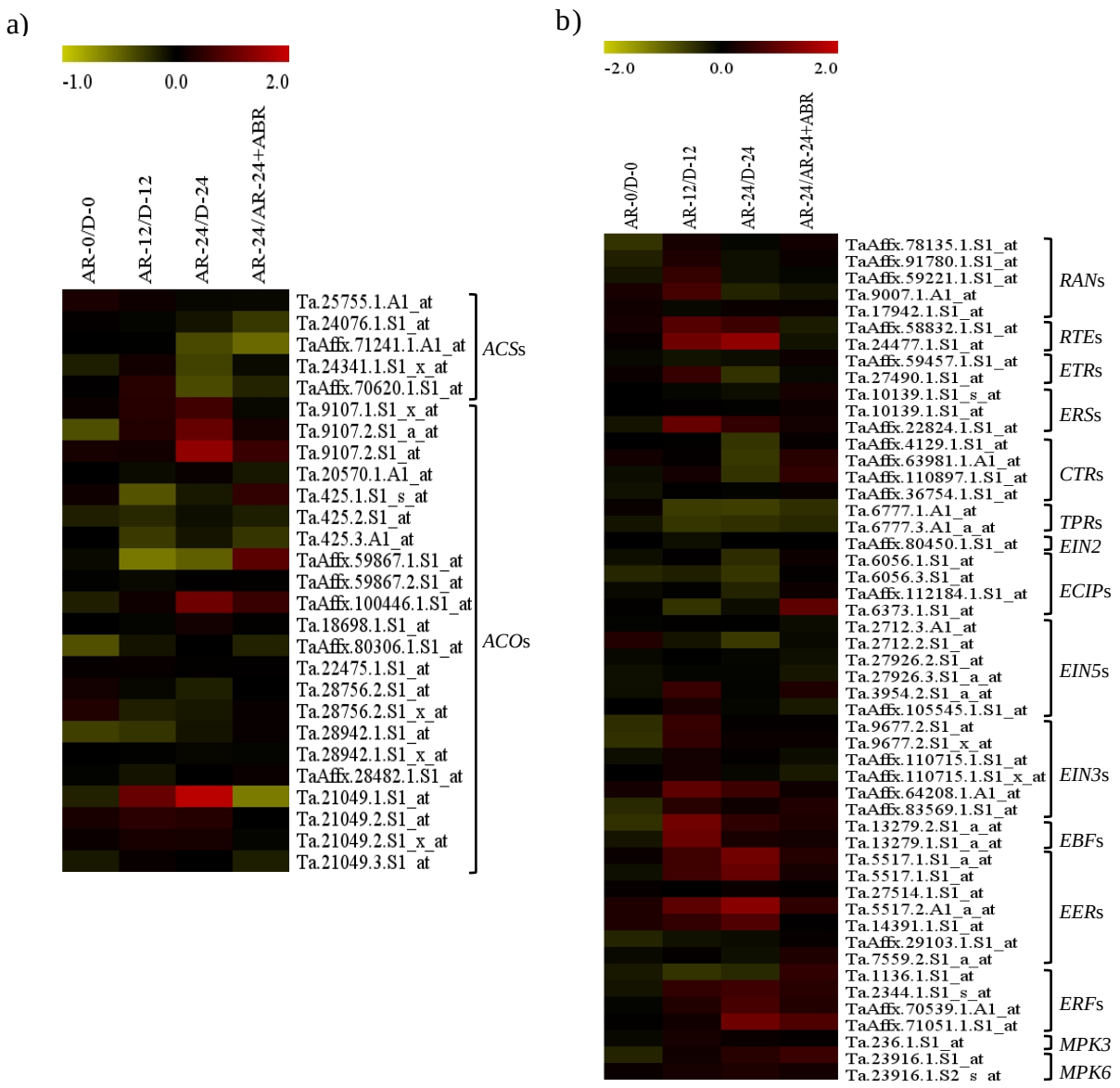

Figure 5.4. Changes in the expression of ethylene (ET) metabolism and signaling genes in response to after-ripening. Fold changes (log_2-scale) in the expression of ET metabolism (a) and signaling (b) related probesets between after-ripened (AR) and dormant (D) seeds in both dry (AR-0/D-0) and imbibed states (AR-12/D-12 and AR-24/D-24), and between AR seeds imbibed without and with ABA (AR-24/AR-24+ABA). The log_2 fold change values are shown by the negative and positive numbers on the bar and the color scale shows upregulation (red) and downregulation (olive green) of the respective probesets. Fold changes (linear- scale) in expression and the associated *P* values are presented in appendix 6 and 7. Data are means of 3 independent biological replicates. Abbreviations: *ACS, 1-aminocyclopropane-1-carboxylic acid synthase*; *ACO, 1-aminocyclopropane-1-carboxylic acid oxidase*; *RAN, responsive-to-antagonist*; *RTE, reversion-to-ethylene sensitivity*; *ETR, ethylene response*; *ERS, ethylene response sensor*; *CTR, constitutive triple response*; *TPR, tetratricopeptide repeat*; *EIN, ethylene insensitive*; *ECIP, EIN2 c-terminus interacting protein*; *EIN5, ethylene insensitive 5*; *EIN3, ethylene insensitive 3*; *EBF, EIN3-binding F box protein*; *EER, enhanced ethylene response*; *ERF, ethylene-responsive element binding factor*; *MPK3, mitogen-activated protein kinase 3*; *MPK6, mitogen-activated protein kinase 6*. ET metabolic and signaling pathways are shown in Figure 2.5.

Our analysis revealed that eight ET signaling probesets, *ethylene response sensor1* (*ERS1*), *reversion-to-ethylene sensitivity 1* (*RTE1*), *EIN3-binding f box protein 1* (*EBF1*), *enhanced ethylene response mutant 3* (*EER3*), *ethylene-responsive element-binding protein 1* (*ERF1*) (Figure 2.5; Appendix 11d), exhibited differential expression between imbibing after-ripened and dormant seeds (over 2-fold, $P \leq 0.05$) (Figure 5.4b, Appendix 7). In Arabidopsis, ET signalling is perceived by a family of receptors including ethylene response1 (ETR1), ETR2, ERS1, ERS2 and ethylene insensitive4 (EIN4) (Bleecker et al., 1998); however, ETR1 and ERS1 play predominant roles (Qu et al., 2007). Given that no ETR1 type receptor is present in wheat (Ma and Wang, 2003), the upregulation of ERS1 (over 2-fold, $P \leq 0.05$) in imbibing after-ripened seeds (Figure 5.4b, Appendix 11d) suggests that transcriptional activation of ET signaling is one of the mechanisms underlying seed dormancy decay by after-ripening. Consistent with this result, probesets representing ET regulated genes such as those encoding endosperm weakening β-glucanase and chitinase B (Leubner-Metzger et al., 1998) are found to be upregulated during imbibition of AR seeds (Gao et al., 2012). After-ripening also triggered imbibition induced upregulation (over 2-fold, $P \leq 0.05$) of probesets representing genes acting as negative regulators of ET signaling (Figure 5.4b). One of these genes is *RTE1*, which inhibits ET signalling via positive regulation of *ETR1* (Resnick et al., 2006; Zhou et al., 2007). However, *RTE1* appears not to play active role in ET signaling as homolog of *ETR1* does not exist in wheat (Ma and Wang, 2003). Transcriptional activation of probesets representing other negative regulators of ET signaling, including *EBF1* and *EER3* was apparent during imbibition of after-ripened seeds, and this might suggest tight transcriptional regulation of ET signaling in wheat seeds.

5.4.6 After-ripening and transcriptional change of specific cytokinin related probesets

Based on our search criteria, the wheat genome GeneChip consists of 27 probesets annotated as genes involved in CK biosynthesis, *isopentenyl transferases* (*IPT*) and *LONELY GUY* (*LOG*); CK inactivation, *CK oxidases* (*CKX*); CK conjugation, *zeatin O-glucosyltransferases* (*ZOG*); and hydrolysis of CK conjugates, *β-glucosidases* (*GLU*) (Figure 2.6). A probeset representing *LOG* and three probesets representing *GLU* were upregulated (over 2-fold; $P \leq 0.05$; Appendix 11e) in imbibed after-ripened relative to dormant seeds (Figure 5.5a, Appendix 8). However, a probeset annotated as *CKX* and two probesets of *ZOG* also exhibited higher expression in after-ripened than dormant seeds during imbibition (Appendix 11e). Consistently, seeds from Arabidopsis plants overexpressing *AtCKX2* or *AtCKX4* confer early germination (Riefler et al., 2006) and seed CK content declines during imbibition of non-dormant sorghum seeds (Dewar et al., 1998). Given that CK has been implicated in the breakage of seed dormancy in several species (Kucera et al., 2005), our data taken together imply the importance of tight regulation of seed CK level in controlling wheat seed dormancy and germination. Of the 40 probesets annotated as CK signaling genes (Appendix 9), only a probeset annotated as *APRR9*, encoding a pseudo response regulator was differentially expressed between imbibed after-ripened and dormant seeds; exhibiting over 2-fold downregulation in after-ripened relative to dormant seeds (Figure 5.5b; Appendix 11f). Given that mutants of CK receptors, central players of CK signaling, exhibit early germination (Riefler et al., 2006) and members of the Arabidopsis pseudo-response regulators, including APRR9, play a role in regulating physiological processes (Matsushika et al., 2000), our results may imply the likely involvement of *APRR* genes in regulating seed dormancy.

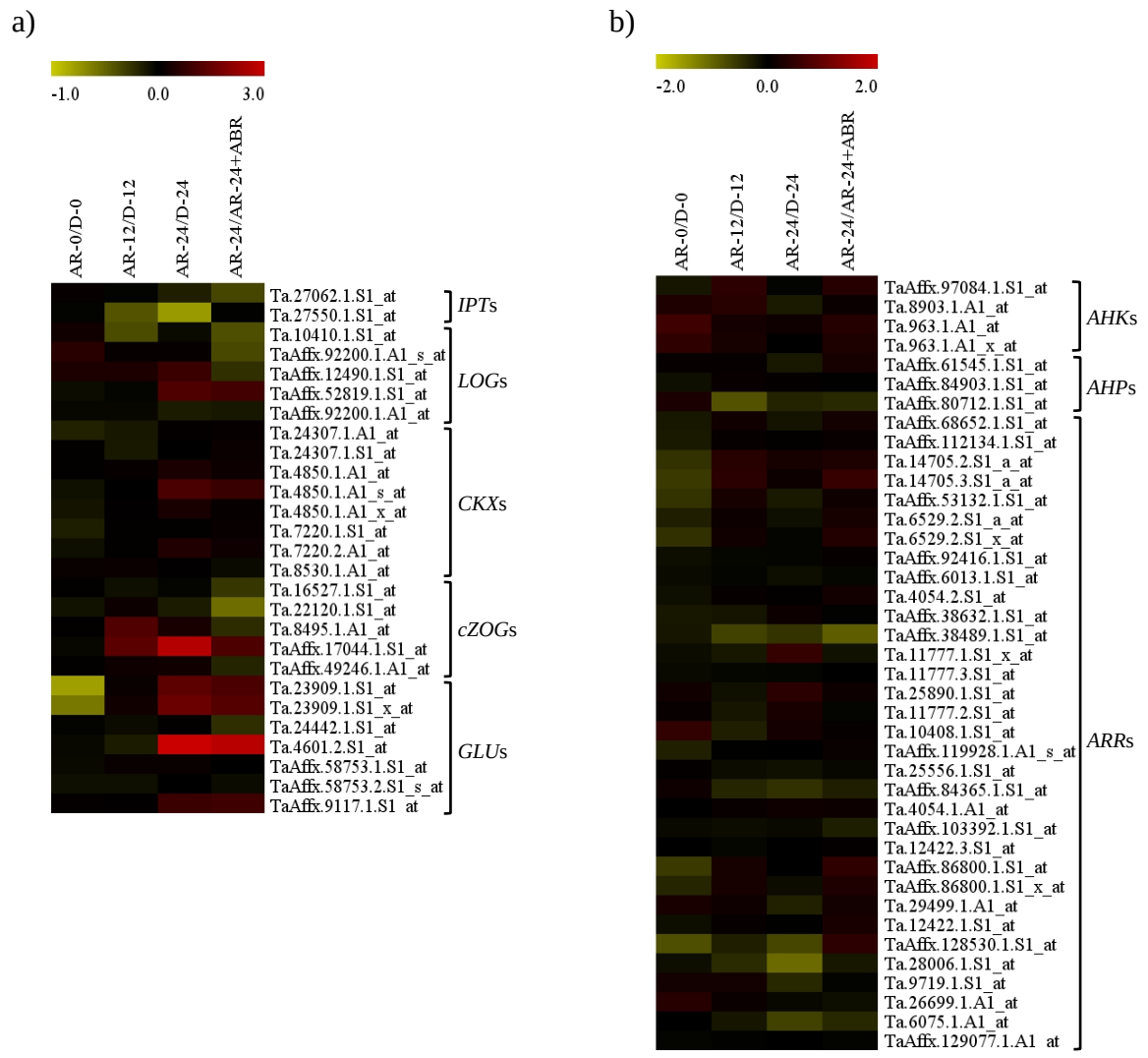

Figure 5.5. Changes in the expression of cytokinin (CK) metabolism and signaling genes in response to after-ripening. Fold changes (log_2-scale) in the expression of CK metabolism (a) and signaling (b) related probesets between after-ripened (AR) and dormant (D) seeds in both dry (AR-0/D-0) and imbibed states (AR-12/D-12 and AR-24/D-24), and between AR seeds imbibed without and with ABA (AR-24/AR-24+ABA). The log_2 fold change values are shown by the negative and positive numbers on the bar and the color scale shows upregulation (red) and downregulation (olive green) of the respective probesets. Fold changes (linear- scale) in expression and the associated *P* values are presented in appendix 8, 9. Data are means of 3 independent biological replicates. Abbreviations: DMAPP, dimethylallyl diphosphate; AMP, adenosine monophosphate; *IPT*, *isopentenyltransferase*; *LOG*, *lonely guy*; *CKX*, *cytokinin oxidase*; *cZOG*, *cis zeatin-o-glucoside*; *GLU*, *glucosidase*; *AHK*, *Arabidopsis histidine kinase*; *AHP*, *Arabidopsis histidine-containing phosphotransmitter*; *ARR*, *Arabidopsis response regulator*. CK metabolic and signaling pathways are shown in Figure 2.6.

Validation of the microarray data using real time qRT-PCR with 9 selected hormonal genes revealed a high degree of positive correlation between the array and qPCR data (Figure 5.6), indicating the reliability of our microarray data.

In summary, our analysis indicates imbibition induced activation and repression of specific transcriptional switches related to BR, ET and CK, and these results suggest that seed dormancy decay by after-ripening and the subsequent germination of wheat seeds is partly mediated by changes in seed content of and response to these plant hormones. Furthermore, our results suggest that the role of ABA in suppressing the germination of wheat seeds is mediated at least partly by transcriptional repression or activation of molecular elements involved in the metabolic and signaling pathways of BR, ET and CK. Given that plant hormones are important regulators of seed dormancy and germination, the present study offers further insights into the molecular mechanisms underlying their interaction in the regulation of seed dormancy release and germination in wheat. Such knowledge is critical to develop wheat cultivars with improved tolerance to pre-harvest sprouting, one of the recurrent problems in the production of quality wheat.

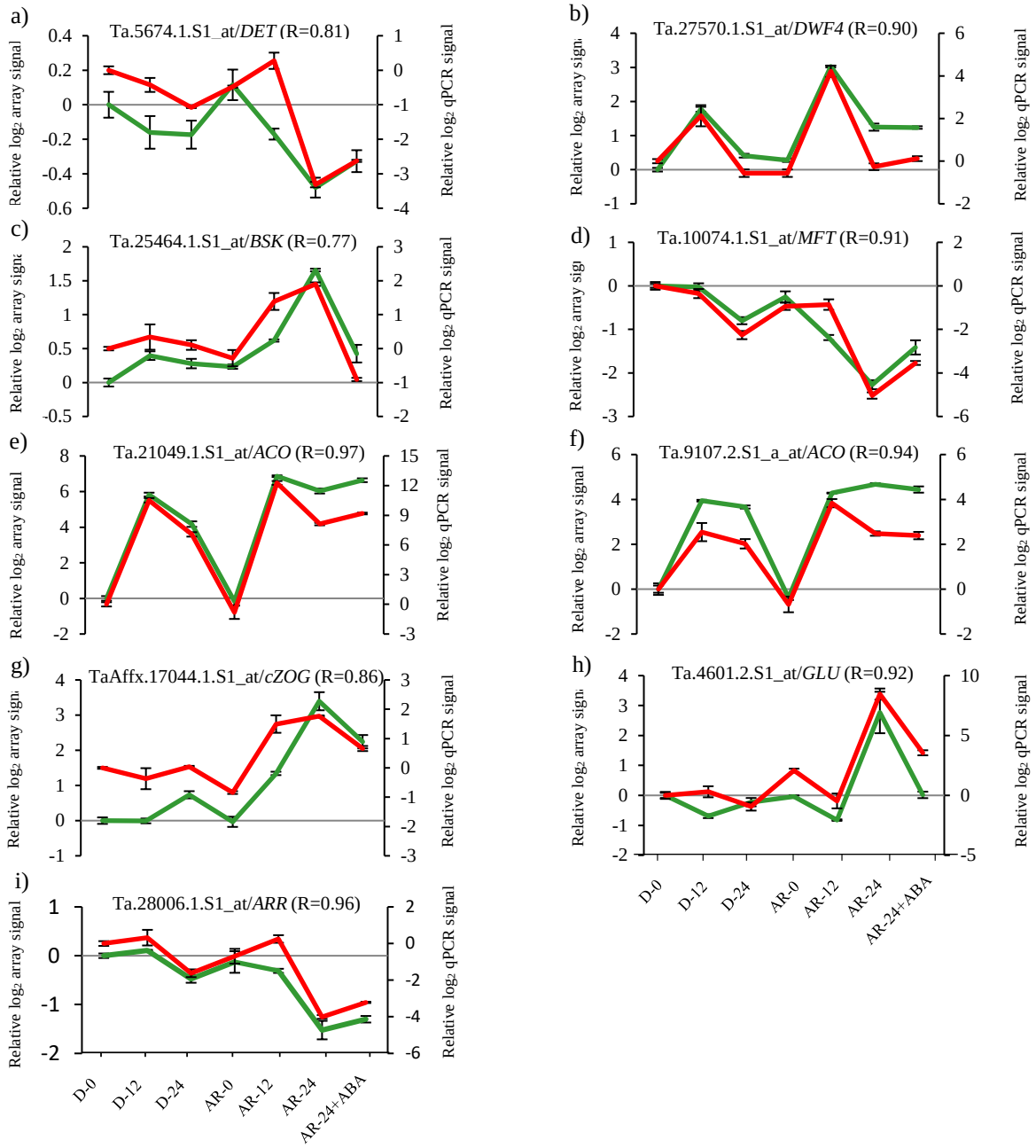

Figure 5.6. Validation of the microarray data with real-time qRT-PCR. Comparison of the microarray and qPCR data for selected probestes representing genes related to brassinosteroid (a-d), ethylene (e, f), cytokinin (g-i) using real time quantitative RT-PCR. Green curves in each graph represent DNA microarray data (left y-axis) while the red curves represent qPCR data (right y-axis) for both dormant (D) and after-ripened (AR) samples before imbibition (D-0 and AR-0), and after imbibition in water (D-12, D-24, AR-12 and AR-24) and ABA (AR-24+ABA). Log_2 signal intensities for each probeset in both microarray and qPCR experiments were expressed relative to that derived from D-0 sample, which was arbirarily set to a value of 0. Data are means of 3 independent biological (and two technical) replicates ± SE. The probeset ID, the corresponding gene name and the pearson correlation coefficient (R) between the microarray and qPCR data are indicated at the top of each graph. Abbreviations: *DET*, *de-etiolated*; *DWF4*, *dwarf4; BSK*, *BR-signaling kinases*; *MFT*, *mother of FT AND TFL1*; *ACO*, *1-aminocyclopropane-1-carboxylic acid oxidase 1*; *cZOG*, *cis zeatin-o-glucoside*; *GLU*, *glucosidase*. *ARR*, *Arabidopsis response regulator*.

Chapter 6: General Discussion and Conclusion

6. GENERAL DISCUSSION AND CONCLUSIONS

Wheat is one of the major crops cultivated extensively across the world for food, feed, and industrial purposes. Pre-harvest sprouting (PHS) is one of the major problems that reduces wheat yield and deteriorates seed quality, causing economic loss to farmers in wheat growing areas. It is associated with the level of dormancy, an adaptive trait that prevents germination under suitable conditions. Dormancy is regulated by the plant hormone abscisic acid (ABA); ABA level is regulated by a balance between its biosynthesis and catabolism. Studies with model plants indicate that the expression levels of genes encoding the rate limiting ABA biosynthetic and ABA catabolic enzymes, 9-cis-epoxycarotenoid dioxygenase (NCED) and ABA-8`hydroxylase (ABA8′OH; encoded by *CYP707A*s), respectively, have direct correlation with seed dormancy levels. Mutational analysis of the *NCED* and *CYP707A* genes demonstrated the definitive role of specific members of these genes in the regulation of ABA levels and seed dormancy. Although, the homologs of these genes have been identified and their role in regulating ABA content and dormancy characterized in some crop species, this has been scarcely studied in wheat. The aim of this thesis was to identify ABA catabolic genes from wheat and study their role in wheat seed dormancy and germination. The study also examined ABA induced transcriptional regulation of the metabolism and signaling of other hormones during the germination of wheat seeds.

The first part of this thesis identified three homeologous *CYP707A1* gene and characterizes their spatiotemporal expression pattern in two wheat genotypes, AC Domain (resistant to PHS) and RL4452 (susceptible to PHS), with contrasting PHS tolerance. Comparative genomic DNA sequence analysis of the homeologs identified between the two wheat genotypes indicated the presence of genotypic variation, mainly due to insertions and deletions in the intron region of the gene, and these differences might underlie the genotypic variation in depth of dormancy between

two wheat genotypes. Further sequence analysis of *TaCYP707A1* in the mapping population derived from a cross between the two genotypes may reveal genetic variations such as single nucleotide polymorphisms (SNPs), which can be used to develop molecular markers for screening and selection of wheat lines with intermediate level of seed dormancy. Expression analysis of *TaCYP707A1* in different tissues during plant development indicated variations in expression level between tissues and stages. *TaCYP707A1* is found to be highly expressed in seeds. In addition, the study examined genome specific contributions (from A, B, and D genomes) to the total expression of *TaCYP707A1*, and the findings showed that genome specific contribution of transcripts differs with genotype and tissue, and this may imply distinct roles of each homeologous in ABA catabolism. In addition to regulation of dormancy, ABA is involved in the regulation of various other plant growth developmental processes such as stomatal movement, root growth and shoot growth under stress conditions. Thus, the role of *TaCYP707A1* and its homeologues in other developmental processes and plant stress tolerance need to be elucidated.

The second part of the thesis investigated the role of ABA catabolic gene *TaCYP707A1* in regulating ABA level and dormancy using heterologous expression of one of the homeologs, *TaCYP707A1B,* in Arabidopsis using wild type and *cyp707a1 cyp707a2* double mutant plants. Expression of *TaCYP707A1B* in *cyp707a1 cyp707a2* plants rescued ABA level in seeds to wild type level and induced an earlier germination phenotype. These findings were also confirmed by ectopic expression of *TaCYP707A1B* in wild-type Arabidopsis plants, which also resulted in an early germination phenotype. Characterization of the other two homeologues of *TaCYP707A1*, from A genome and the D genome, through heterologous expression might provide important information about the contribution of each homeologue to *TaCYP707A1*'s role in dormancy and

germination. This can be complemented by enzyme kinetics analysis of the recombinant proteins of the three homeologs of *TaCYP707A1*.

Furthermore, comparative expression analysis of *TaCYP707A1* and its isoform *TaCYP707A2* was performed between the two wheat genotypes with contrasting sprouting tolerance, AC Domain (dormant) and RL4452 (less dormant). The comparative analysis during imbibition also included after-ripened AC Domain seeds. The results of this study indicated genotypic variation in the expression of the two genes in seed tissues during developmental and imbibition events in which RL4452 seeds have higher expression of the two *CYP707A* genes than that of AC Domain. After-ripening of AC Domain seeds also caused variation in the expression of the two genes during imbibition in which after-ripened seeds displayed higher expression of the two *CYP707A* genes than the dormant seeds, suggesting the importance of the two genes in the regulation of ABA level in seed tissues, as well as in dormancy and germination. However, the expression level of *TaCYP707A1* is overall higher than that of *TaCYP707A2,* suggesting its major role in ABA and dormancy level regulation.

The genome specific contribution to the total expression of *TaCYP707A1* analysis in different seed tissues during seed development and germination suggest that each homeologue, tissue, and genotype specific contribution towards ABA 8' hydroxylase activity or the catabolism of ABA. Determination of the physiological role of this ABA catabolic gene in wheat needs further characterization. For example, characterizing its expression and genomic contributions in AC Domain X RL4452 mapping population lines can provide more insights into its role with respect to dormancy. Given that seed dormancy is a quantitative trait regulated not only by genetic but also by environment factors, using seed samples collected from mapping population lines grown under different environmental conditions is important.

The last part of the thesis was aimed at investigating transcriptional changes in brassinosteroid, ethylene, and cytokinin metabolic, and signaling genes due to after-ripening, which was effective in the release of dormancy in wheat. Treatment of the non-dormant (after-ripened) seeds with ABA caused delay of germination. As a result of this, the study also examined how ABA treatment affects the expression of these genes during imbibition of non-dormant seeds. After-ripening of dormant seed upregulated the expression of genes encoding brassinosteroid biosynthesis enzymes such as *Deetiolated 2* (*DET2*) and *dwarf 4* (*DWF4*), and a gene that positively regulates brassinosteroid signaling, *brassinosteroid signaling kinases 2* (*BSK2*), but downregulated that of *brassinosteroid insensitive2* (*BIN2*), a negative regulator of brassinosteroid signaling, implying the role of brassinosteroid in dormancy decay. However, ABA treatment of after-ripened seeds resulted in downregulation of *BSK2*, suggesting that repression of BR action contributes to the suppression of germination by ABA. The study also showed upregulation of genes encoding the ethylene biosynthesis enzyme, *1-aminocyclopropane-1-carboxylic acid oxidase* (*ACO*), and one of the receptors of ethylene signal, *ethylene response sensor1* (*ERS1*), by after-ripening, and these results imply the induction of ethylene synthesis and signaling contribute to dormancy loss. The downregulation of *ERS* by ABA suggests that dormancy in wheat is influenced by ethylene-ABA interaction. The effect of after-ripening was also observed on the expression of cytokinin related genes in which genes encoding cytokinin biosynthetic enzymes such as *lonely guy* (*LOG*), and cytokinin inactivation enzymes such as *cytokinin oxidase* (*CKX*) exhibited upregulation in after-ripened seeds, implying that maintenance of cytokinin homeostasis is required for dormancy release. Functional analysis of the genes that showed differential expression in response to after-ripening and understanding how these genes are regulated by ABA is essential to clarify their roles in the control of dormancy in wheat.

In conclusion, this study identified the ABA inactivating gene, *TaCYP707A1*, from Canadian wheat varieties and characterized its functionality with respect to seed dormancy. Furthermore, it provided insights into molecular elements that mediate the interaction between ABA and other plant hormones implicated in regulating dormancy. The findings overall enhance our understanding of the molecular mechanisms underlying the regulation of seed dormancy in wheat, which is necessary to reduce the effects of pre-harvest sprouting on wheat production.

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Appendix 1. Sequence alignment of 'A' genome copy of *TaCYP707A1*. CS-A, Genome A of Chinese Spring; AC-A, Genome A of AC Domain; RL-A, Genome A of RL4452. Sequences of genome A copy from Chinese Spring (Genbank ID: AB714574). Coding regions are highlighted in grey.

```
CS-A            CCACCACCACCACCCATCTCCTCTCCTCTCCCCCCCTCGTCTTCCTCCTCCGACTCCTTC
AC-A            ------------------------------------------------------------
RL-A            ------------------------------------------------------------

CS-A            GGGGCTCCTATCCTTTGCAGCTGACGGAACCGACGAAAGCCTTTCGTTGACATGGCTGCT
AC-A            ----------------------------------------------------ATGGCTGCT
RL-A            ----------------------------------------------------ATGGCTGCT

CS-A            TTCGTTCTCTTCCTCTGCTTGCTCGTGCCGCTCGTCCTCGCGTGCGCCGTCCGCGCCAGG
AC-A            TTCGTCCTCTTCCTCTGCTTGCTCGTGCCGCTCGTCCTCGCGTGCGCCGTCCGCGCCAGG
RL-A            TTCGTCCTCTTCCTCTGCTTGCTCGTGCCGCTCGTCCTCGCGTGCGCCATCCGCGCCAG-

CS-A            AAGAAGGGCGCCGGCGGGAGGTCGTCGTCGGGCGGCGGCGGCGGCAAGAAGGGCGGCAGC
AC-A            AAGAAGGGCGCCGGCGGGAGGTCGTCGTCGGGCGGCGGCGGCGGCAAGAAGGGCGGCAGC
RL-A            --GAAGGGCGCCGGCGGCCGTTCGTCGTCGGGCGGCGGC---------AAGAAAGGCAGC

CS-A            AGCAGCCTGCCGCTGCCGCCGGGGTCCATGGGGTGGCCGTACGTGGGCGAGACCACGCAG
AC-A            AGCAGCCTGCCGCTGCCGCCGGGGTCCATGGGGTGGCCGTACGTGGGCGAGACCACGCAG
RL-A            AGCAGCCTGCCGCTGCCGCCCGGGTCGATGGGGTGGCCGTACGTGGGCGAGACCACGCAG

CS-A            CTCTACTCCTCCAAGAACCCCAACGTCTTCTTCGCCAGGAAGCGCAACAAGTACGGGCCC
AC-A            CTCTACTCCTCCAAGAACCCCAACGTCTTCTTCGCCAGGAAGCGCAACAAGTACGGGCCC
RL-A            CTGTACTCCTCCAAGAACCCCAACGTCTTCTTCGCCAGGAAGCGGAACAAGTACGGGCCC

CS-A            ATCTTCAAGACGCACATCCTCGGGTGCCCCTGCGTCATGGTGTCCAGCCCCGAGGCCGCC
AC-A            ATCTTCAAGACGCACATCCTCGGGTGCCCCTGCGTCATGGTGTCCAGCCCCGAGGCCGCC
RL-A            ATCTTCAAGACGCATATCCTCGGGTGCCCCTGCGTCATGGTGTCCAGCCCCGAGGCCGCG

CS-A            AAGTTCGTGCTCGTCACTCAGGCGCACCTCTTCAAGCCTACCTTCCCGGCCAGCAAGGAG
AC-A            AAGTTCGTGCTCGTCACTCAGGCGCACCTCTTCAAGCCTACCTTCCCGGCCAGCAAGGAG
RL-A            AAGTTCGTGCTTGTTACTCAGGCGCACCTCTTCAAGCCTACCTTCCCGGCCAGCAAGGAG

CS-A            CGGATGCTGGGCCCCCAGGCCATCTTCTTCCAGCAGGGGGACTACCACGCCCACCTCCGC
AC-A            CGGATGCTGGGCCCCCAGGCCATCTTCTTCCAGCAGGGGGACTACCACGCCCACCTCCGC
RL-A            CGGATGCTGGGCCCCCAGGCCATCTTCTTCCAGCAGGGGGACTACCATGCCCACCTCCGC

CS-A            CGTCTCGTCTCCCGCGCCTTCTCCCCCGAGGCCATCCGCGGCTCCGTCCCCGCCATCGAG
AC-A            CGTCTCGTCTCCCGCGCCTTCTCCCCCGAGGCCATCCGCGGCTCCGTCCCCGCCATCGAG
RL-A            CGTCTCGTCTCACGCGCCTTCTCTCCCGAGGCCATCCGCGGTTCCGTCCCTGCCATCGAG
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CS-A            GCTATCGCCCTCCGCTCCCTCGGCTCCTGGGAAGACCTGCAAGTCAACACCTTCCAGGAG
AC-A             GCTATCGCCCTCCGCTCCCTCGGCTCCTGGGAAGACCTGCAAGTCAACACCTTCCAGGAG
RL-A             GCTATCGCCCTCCGCTCCCTCGGCTCCTGGGAAGACCTGCAAGTCAACACCTTCCAAGAG

CS-A            ATGAAGACTGTGAGTGCTTCTTCTTCTTCCTCCTCTTCCATTCCCCGCCACTTGCTCTGC
AC-A             ATGAAGACTGTGAGTGCTTCTTCTTCTTCCTCCTCTTCCATTCCCCGCCACTTGCTCTGC
RL-A             ATGAAGACTGTGAGTGCTTCTTCTTCTTCCTCCTCTTCCATTCCCCGCCACTTGCTCTGC

CS-A            TTTCCTCTGCTCTGCTCTAGTGCTAAATGATTGGAGCTCGAGGCTGATC-----------
AC-A             TTTCCTCTGCTCTGCTCTAGTGCTAAATTATTGGAGCTCGAGGCTGATC-----------
RL-A             TTTCCTCTGCTCTGCTCTAGTGCTAAATTATTGGAGCTCGAGGCTGATCGTTGTGTTGGT

CS-A            ------------------------------------------------------------
AC-A             ------------------------------------------------------------
RL-A             GTTATGGCGCAGTACGCTCTGAATGTGGCATTGCTGTCCATCTTCGGGGAGGAGGAGATG

CS-A            -----------------GTTGTGTTGGTGTTATGGCGCAGTACGCTCTGAATGTGGCATT
AC-A             -----------------GTTGTGTTGGTGTTATGGCGCAGTACGCTCTGAATGTGGCATT
RL-A             CAGTACATCGAGGAGCTGTTGTGTTGGTGTTATGGCGCAGTACGCTCTGAATGTGGCATT

CS-A            GCTGTCCATCTTCGGGGAGGAGGAGATGCAGTACATCGAGGAGCTGAAGCAGTGCTACCT
AC-A             GCTGTCCATCTTCGGGGAGGAGGAGATGCAGTACATCGAGGAGCTGAAGCAGTGCTACTT
RL-A             GCTGTCCATCTTCGGGGAGGAGGAGATGCAGTACATCGAGGAGCTGAAGCAGTGCTACTT

CS-A            GACGCTGGAGAAGGGGTACAACTCGATGCCGGTGAACCTGCCGGGCACGCTGTTCCACAA
AC-A             GACGCTGGAGAAGGGGTACAACTCGATGCCGGTGAACCTGCCGGGCACGCTGTTCCACAA
RL-A             GACGCTGGAGAAGGGGTACAACTCGATGCCGGTGAACCTGCCGGGCACGCTGTTCCACAA

CS-A            GGCCATGAAGGCCCGGAAGCGGCTGGGCGCCATTGTGGCCCACATCATCTCGGCCCGGCG
AC-A             GGCCATGAAGGCCCGGAAGCGGCTGGGCGCCATTGTGGCCCACATCATCTCGGCCCGGCG
RL-A             GGCCATGAAGGCCCGGAAGCGGCTGGGCGCCATTGTGGCCCACATCATCTCGGCCCGGCG

CS-A            CGAGCGCGAGCGCGGGAGCGACCTCCTGGGCTCCTTCATGGACGGCCGCGAGGCGCTCAC
AC-A             CGAGCGCGAGCGCGGGAGCGACCTCCTGGGCTCCTTCATGGACGGCCGCGAGGCGCTCAC
RL-A             CGAGCGCGAGCGCGGGAGCGACCTCCTGGGCTCCTTCATGGACGGCCGCGAGGCGCTCAC

CS-A            CGACGACCAGATCGCCGACAACGCCATCGGCGTCATCTTCGCCGCGCGCGACACCACCGC
AC-A             CGACGACCAGATCGCCGACAACGCCATCGGTGTCATCTTCGCCGCGCGCGACACCACCGC
RL-A             CGACGACCAGATCGCCGACAACGCCATCGGTGTCATCTTCGCCGCGCGCGACACCACCGC

CS-A            CAGCGTGCTCACGTGGATGGTCAAGTTCCTCGGCGACAACCCCGCCGTCCTCAAAGCCGT
AC-A             CAGCGTGCTCACGTGGATGGTCAAGTTCCTCGGCGACAACCCCGCCGTCCTCAAAGCCGT
RL-A             CAGCGTGCTCACGTGGATGGTCAAGTTCCTCGGCGACAACCCCGCCGTCCTCAAAGCCGT
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CS-A            CACCGTAAGTCGCCGTCATTTCATGAACCCAGCTGACCCGCTAGGCACCCGATCGAAAAG
AC-A            CACCGTAAGTCGCCGTCATTTCATGAACCCAGCTGACCCGCTAGGCACCCGATCGAAAAG
RL-A            CACCGTAAGTCGCCGTCATTTCATGAACCCAGCTGACCCGCTAGGCACCCGATCGAAAAG

CS-A            CAGCGACTGACCCGTTCATCATACAATCAACAGGAAGAGCACGCCGAGATCGCGAGGGAG
AC-A            CAGCGACTGACTCGTTCATCATACAATCAACAGGAAGAGCACGCCGAGATCGCGAGGGAG
RL-A            CAGCGACTGACTCGTTCATCATACAATCAACAGGAAGAGCACGCCGAGATCGCGAGGGAG

CS-A            AAGGCGTTGTCCGGCGAGGCGCTGTCGTGGGCCGACACGCGGCGGATGCGGTTGACGGGC
AC-A            AAGGCGCTGTCCGGCGAGGCGCTGTCGTGGGCCGACACGCGGCGGATGCGGTTGACGGGC
RL-A            AAGGCGCTGTCCGGCGAGGCGCTGTCGTGGGCCGACACGCGGCGGATGCGGTTGACGGGC

CS-A            CGGGTGATCCAGGAGACGATGCGGGTGGCGTCCATCCTCTCCTTCACCTTCAGGGAGGCC
AC-A            CGGGTGATCCAGGAGACGATGCGGGTGGCGTCCATCCTCTCCTTCACCTTCAGGGAGGCC
RL-A            CGGGTGATCCAGGAGACGATGCGGGTGGCGTCCATCCTCTCCTTCACCTTCAGGGAGGCC

CS-A            GTGGAGGACGTGGAGTACCAAGGTGAGCAGAGCAGAGGCATCAATC----GCTTTGGTCG
AC-A            GTGGAGGACGTGGAGTACCAAGGTGAGCAGAGCAGAGACATCAATC----GCTTTGGTCG
RL-A            GTGGAGGACGTGGAGTACCAAGGTGAGCAGAGCAGAGGCATCAATC----GCTTTGGTCG

CS-A            TTTGAGGCAGCGCAGTGCTGTGCTCCGCTGTCCT-TCTCGGAGCACAGCAGTGCGCTGCC
AC-A            TTTGTGGCAGCGCAGTGCTGTACTCCGCTGT-CCCTCTCGGAGTACAGCAGTGAGCTGCC
RL-A            TTTGAGGCAGCGCAGTGCTGTGCTCCGCTGT-CT-TCTCGGAGCACAGCAGTGCGCTGCC

CS-A            TGCCCGCCTGC-------CTGCCTGCGCATGAACTGGCTCG-----GAAAGGACGCGCTC
AC-A            TGCCTGCCTGCGCATGAACTGGCTCGGAAAGGACGCGCTCCTAACCGAACGAACGAAATA
RL-A            TGCCCGCCTGC-------CTGCCTGCGCATGAACTGGCTCG-----GAAAGGACGCGCTC

CS-A            CTAACTCAAGGGAAACTCGCAACTCACCTCGACTTGCTCTCCTCTGTGCGTGCAGGGTAC
AC-A            GAC---CAACTCAAACTCGCAACTCACCTCGACTTGCTCTCCTCTGTGCGTGCAGGGTAC
RL-A            CTAACTCAAGGGAAACTCGCGACTCACCTCGACTTGCTCTCCTCTGTGCGTGCAGGGTAC

CS-A            CTGATTCCCAAGGGCTGGAAAGTGCTTCCCCTGTTCCGGAACATCCACCACAACCCCGAC
AC-A            CTGATTCCCAAGGGCTGGAAAGTGCTTCCCCTGTTCCGGAACATCCACCACAACCCCGAC
RL-A            CTGATTCCCAAGGGCTGGAAAGTGCTTCCCCTGTTCCGGAACATCCACCACAACCCCGAC

CS-A            CACTTCCCCTCCCCCGAAAAGTTCGACCCTTCACGATTCGAGGTCAGCATCATCATTCAC
AC-A            CACTTCCCCTCCCCTGAAAAGTTCGATCCTTCACGATTCGAGGTCAGCATCA----TCAC
RL-A            CACTTCCCCTCCCCCGAAAAGTTCGACCCTTCACGATTCGAGGTCAGCATCATCATTCAC

CS-A            AGCCCTCTGTTTGACGAGTCTCTTCTTCGATTTCGATTGATCGTTATCTGATTATTATAC
AC-A            AGCCCTCTGTTTGACGAG--TCTGCTTCGA-TTCGATTGATCATTATCTG---ATTATAC
RL-A            AGCCCTCTGTTTGACGAGTCTCTTCTTCGATTTCGATTGATCGTTATCTGATTATTATAC
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CS-A            GTCTTGGTTCGTGACTGCAGGTGGCCCCCAAGCCCAACACGTTCATGCCGTTCGGGAACG
AC-A            GTTTTGGTTGCTGACTGCAGGTGGCCCCCAAGCCCAACACGTTCATGCCGTTCGGGAACG
RL-A            GTCTTGGTTCGTGACTGCAGGTGGCCCCCAAGCCCAACACGTTCATGCCGTTCGGGAACG

CS-A            GGACCCACTCGTGCCCCGGCAACGAGCTGGCCAGGCTGGAGATGCTCGTCCTCTGCCACC
AC-A            GGACCCACTCGTGCCCCGGCAACGAGCTGGCCAAGCTGGAGATGCTCGTCCTCTGCCACC
RL-A            GGACCCACTCGTGCCCCGGCAACGAGCTGGCCAGGCTGGAGATGCTCGTCCTCTGCCACC

CS-A            ACCTCGCCACCAAGTACAGATGGTCCACCTCCAAGTCCGAGAGCGGCGTCCAGTTCGGCC
AC-A            ACCTCGCCACCAAGTACAGATGGTCCACCTCCAAGTCCGAGAGCGGCGTCCAGTTCGGCC
RL-A            ACCTCGCCACCAAGTACAGATGGTCCACCTCCAAGTCCGAGAGCGGCGTCCAGTTCGGCC

CS-A            CCTTCGCCCTCCCCATCAACGGCCTCCCCATGACCTTCACCCGCAAG---GACAAGAACA
AC-A            CCTTCGCCCTCCCCATCAACGGCCTCCCCATGACCTTCACCCGCAAGGACGACAAGAACA
RL-A            CCTTCGCCCTCCCCATCAACGGCCTCCCCATGACCTTCACCCGCAAG---GACAAGAACA

CS-A            AAGCCTGACTCCTCCTCGTTCCCAAAGGAAAATCAACGGCACGATAGAGGCGACGCCGTG
AC-A            AAGCCTGAGCCATCCATCCATCCATCCATCCATCGCCTCCTCCTCGTTCCCAAAGGGAAA
RL-A            AAGCCTGACTCCTCCTCGTTCCCAAAGGAAAATCAACGGCACGATAGTGGCGAC------

CS-A            GCACATGCGAGCGAGCTGGACCGGCTCCGTCCGCGTCCGCGGCGGTCGCCGTCGGAGGGG
AC-A            TCAACAGCACGATAGAGGCAAC----------------------------------------
RL-A            ------------------------------------------------------------

CS-A            ACACTTGCAGCCGTGGGCGCCGCGGCGGGAGCGGCAGCGCGCTGGCGGAGGCCGATCGGA
AC-A            ------------------------------------------------------------
RL-A            ------------------------------------------------------------

CS-A            GAGAGCACGGGCCGTCAAAAGTCCTCTGTACAGATTCTTGAGCCAGGACCATGACGGCCG
AC-A            ------------------------------------------------------------
RL-A            ------------------------------------------------------------

CS-A            GGCGAGACGAGACCAAGAAAAATCCCGCTCTTGAAAACACACACAGACGTAGGCATTCTC
AC-A            ------------------------------------------------------------
RL-A            ------------------------------------------------------------

CS-A            AGCTAGGGAGATCCGGCGCCACAAAGAAGT
AC-A            ------------------------------
RL-A            ------------------------------
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Appendix 2. Sequence alignment of 'B' genome of *TaCYP707A1***.** CS-B, Genome B of Chinese Spring; AC-B, Genome B of AC Domain; RL-B, Genome B of RL4452. Sequences of genome B copy from Chinese Spring (GenBank ID: AB714575). Coding regions are highlighted in grey.

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CS-B            ACCACCACCACCCGTCTCCTCTCCTCTCCTCTCC-TCCTCGTCTTCCTCCTTCGGCCTCC
AC-B            ------------------------------------------------------------
RL-B            ------------------------------------------------------------

CS-B            TCCTTTGCAGCTAACGGAACCGAAGAAAGCCTTTCGTTGACATGGCTGCTTTCATCCTCT
AC-B            ----------------------------------------ATGGCTGCTTTCGTTCTCT
RL-B            ----------------------------------------TTGGCTGCTTTCGTTCTCT

CS-B            TCCTCTGCTTGCTCGTGCCGCTCGTCCTCGCGTGCGCCGTCCGCGCCAGGAAGGGCGCCG
AC-B            TCCTCTGCTTGCTCGTGCCGCTCGTCCTCGCGTGCGCCATCCGCGCCAGGAAGGGCGCCG
RL-B            TCCTCTGCTTGCTCGTGCCGCTCGTCCTCGCGTGCGCCATCCGCGCCAGGAAGGGCGCCG

CS-B            CCGGGCGGGCGTCGTCGGGCGGCGGCGGCAAAAAGGGCGGCACCAGCCTGCCGCTGCCGC
AC-B            GCGGCCGTTCGTCGTCGGGCGGCGGCAAGAAA---GGCAGCAGCAGCCTGCCGCTGCCGC
RL-B            GCGGCCGTTCGTCGTCGGGCGGCGGCAAGAAA---GGCAGCAGCAGCCTGCCGCTGCCGC

CS-B            CGGGGTCGATGGGGTGGCCGTACGTGGGCGAGACCACGCAGCTCTACTCCTCCAAGAACC
AC-B            CCGGGTCGATGGGGTGGCCGTACGTGGGCGAGACCACGCAGCTGTACTCCTCCAAGAACC
RL-B            CCGGGTCGATGGGGTGGCCGTACGTGGGCGAGACCACGCAGCTGTACTCCTCCAAGAACC

CS-B            CCAACGTCTTCTTCGCCAGGAAGCGGAACAAGTACGGGCCCATCTTCAAGACGCACATCC
AC-B            CCAACGTCTTCTTCGCCAGGAAGCGGAACAAGTACGGGCCCATCTTCAAGACGCATATCC
RL-B            CCAACGTCTTCTTCGCCAGGAAGCGGAACAAGTACGGGCCCATCTTCAAGACGCATATCC

CS-B            TCGGGTGCCCCTGCGTCATGGTGTCCAGCCCGGAGGCCGCCAAGTTCGTGCTCGTCACTC
AC-B            TCGGGTGCCCCTGCGTCATGGTGTCCAGCCCCGAGGCCGCGAAGTTCGTGCTTGTTACTC
RL-B            TCGGGTGCCCCTGCGTCATGGTGTCCAGCCCCGAGGCCGCGAAGTTCGTGCTTGTTACTC

CS-B            AGGCGCACCTCTTCAAGCCTACCTTCCCGGCCAGCAAGGAGCGGATGCTGGGCCCCCAGG
AC-B            AGGCGCACCTCTTCAAGCCTACCTTCCCGGCCAGCAAGGAGCGGATGCTGGGTCCCCAGG
RL-B            AGGCGCACCTCTTCAAGCCTACCTTCCCGGCCAGCAAGGAGCGGATGCTGGGTCCCCAGG

CS-B            CCATCTTCTTCCAGCAGGGGGACTACCATGCCCACCTCCGCCGTCTCGTCTCACGCGCCT
AC-B            CCATCTTCTTCCAGCAGGGGGACTACCACGCTCACCTCCGCCGTCTGGTCTCCCGCGCCT
RL-B            CCATCTTCTTCCAGCAGGGGGACTACCACGCTCACCTCCGCCGTCTGGTCTCCCGCGCCT

CS-B            TCTCTCCCGAGGCCATCCGCGGTTCCGTCCCTGCCATCGAGGCTATCGCCCTCCGCTCCC
AC-B            TCTCCCCCGAGGCCATCCGCGGCTCCGTCCCCGCCATCGAGGCTATCGCCCTCCGCTCCC
RL-B            TCTCCCCCGAGGCCATCCGCGGCTCCGTCCCCGCCATCGAGGCTATCGCCCTCCGCTCCC
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CS-B            TCGGCTCCTGGGAAGACCTGCAAGTCAACACCTTCCAAGAGATGAAGACTGTGAGTGCTT
AC-B            TCGGCTCCTGGGAAGACCTTCAAGTCAACACCTTCCAAGAGATGAAGACTGTGAGTG---
RL-B            TCGGCTCCTGGGAAGACCTTCAAGTCAACACCTTCCAAGAGATGAAGACTGTGAGTG---

CS-B            CTTCTTCTTCCTCTTCCATTCCCGCTTGCTCTGCTTTCCTCTGCTCTGCTCTACTGCTAA
AC-B            ---CTTCTTCCTCTTCCATTCCCACTTGCTCTGCTTCCCTCTGCTCTGCTCTACTGCTAA
RL-B            ---CTTCTTCCTCTTCCATTCCCACTTGCTCTGCTTCCCTCTGCTCTGCTCTACTGCTAA

CS-B            ATGATTGGAGCTCGAGGCTGATCCTTCTCTTGGTGTCGTGGCGCAGTACGCTCTGAATGT
AC-B            ATAATTGGAGCTTGAGACTGATCCTTCTGTTGGTGTCGTGGCGCAGTACGCTCTGAATGT
RL-B            ATAATTGGAGCTTGAGACTGATCCTTCTGTTGGTGTCGTGGCGCAGTACGCTCTGAATGT

CS-B            GGCATTGCTGTCCATCTTCGGCGAGGAGGAGATGCAGTACATCGAGGAGCTGAAGCAGTG
AC-B            GGCATTGCTGTCCATCTTCGGGGAGGAGGAGATGCAGTACATCGAGGAGCTGAAGCAGTG
RL-B            GGCATTGCTGTCCATCTTCGGGGAGGAGGAAATGCAGTACATCGAGGAGCTGAAGCAGTG

CS-B            CTACCTGACGCTGGAGAAGGGGTACAACTCGATGCCGGTGAACCTGCCGGGCACGCTGTT
AC-B            CTACCTGACGCTGGAGAAGGGGTATAACTCGATGCCGGTGAACCTGCCGGGCACGCTGTT
RL-B            CTACCTGACGCTGGAGAAGGGGTATAACTCGATGCCGGTGAACCTGCCGGGCACGCTGTT

CS-B            CCACAAGGCCATGAAGGCCCGAAAGCGGCTGGGCGCCATTGTGGCCCACATCATCTCGGC
AC-B            CCACAAGGCCATGAAGGCCCGGAAGCGGCTGGGCGCCATTGTGGCCCACATCATCTCGGC
RL-B            CCACAAGGCCATGAAGGCCCGGAAGCGGCTGGGCGCCATTGTGGCCCACATCATCTCGGC

CS-B            CCGGCGCGAGCGCGAGCGCGGGAGCGACCTCCTGGGCTCCTTCATGGACGGCCGCGAGGC
AC-B            CCGGCGCGAGCGTGAGCGCGGGAGCGACCTCCTGGGCTCCTTCATGGACGGCCGCGAGGC
RL-B            CCGGCGCGAGCGTGAGCGCGGGAGCGACCTCCTGGGCTCCTTCATGGACGGCCGCGAGGC

CS-B            GCTCACCGACGACCAGATCGCCGACAACGCCATCGGCGTCATCTTCGCCGCGCGCGACAC
AC-B            GCTCACCGACGACCAGATCGCCGACAACGCCATCGGCGTCATCTTCGCCGCGCGCGACAC
RL-B            GCTCACCGACGACCAGATCGCCGACAACGCCATCGGCGTCATCTTCGCCGCGCGCGACAC

CS-B            CACCGCCAGCGTGCTCACGTGGATGGTCAAGTTCCTCGGCGACAACCCCGCCGTCCTCAA
AC-B            CACCGCCAGCGTGCTCACGTGGATGGTCAAGTTCCTCGGCGACAACCCCGCCGTGCTCAA
RL-B            CACCGCCAGCGTGCTCACGTGGATGGTCAAGTTCCTCGGCGACAACCCCGCCGTGCTCAA

CS-B            AGCCGTCACCGTAAGTCGCCATCAACCAGCTGACCCGCTTGGTACCCGATCGAAAAGCAG
AC-B            AGCCGTCACCGTAAGTCGCCATCAACCAGCTGACCCGCTTGGTACCCGATCGAAAAGCAG
RL-B            AGCCGTCACCGTAAGTCGCCATCAACCAGCTGACCCGCTTGGTACCCGATCGAAAAGCAG

CS-B            TGGCTGACCCGTGCGTCGTACAATTAACAGGAAGAGCACGCTGAGATCGCGAGGGAGAAG
AC-B            CGACTGACCCGTGCATCCAACAATTAACAGGAAGAGCATGCCGAGATCGCGAGGGAGAAG
RL-B            CGACTGACCCGTGCATCCAACAATTAACAGGAAGAGCATGCCGAGATCGCGAGGGAGAAG
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CS-B            GCGTTGTCCGGCGAGCCACTGTCGTGGGCCGACACGCGGCGGATGCGGATGACGGGCCGG
AC-B            GCGTTGTCCGGCGAGCCGCTGTCGTGGGCCGACACGCGGCGGATGCGGATGACGGGCCGG
RL-B            GCGTTGTCCGGCGAGCCGCTGTCGTGGGCCGACACGCGGCGGATGCGGATGACGGGCCGG

CS-B            GTGATCCAGGAGACGATGCGGGTGGCGTCCATCCTCTCCTTCACCTTCAGGGAGGCCGTG
AC-B            GTGATCCAGGAGACGATGCGGGTGGCGTCCATCCTCTCCTTCACCTTCAGGGAGGCCGTG
RL-B            GTGATCCAGGAGACGATGCGGGTGGCGTCCATCCTCTCCTTCACCTTCAGGGAGGCCGTG

CS-B            GAGGACGTGGAGTACCAAGGTGAGCAGAGCAGAGACATCAAT--------CGCTTTGGTC
AC-B            GAGGACGTGGAGTACCAAGGTGAGACATCAATCAACTTCGCGCGCGCGCGCGCTTTGGTC
RL-B            GAGGACGTGGAGTACCAAGGTGAGACATCAATCAACTTCGCGCGCGCGCGCGCTTTGGTC

CS-B            GTTTGTGGCAGCGCAGTGCTGTACTCCGCTGTCCCTCTCGGAGTACAGCAGTGAGCTGCC
AC-B            GTTTGCGGCAGCGCAGCGCCGTACTGTGCTGTCCCTCTCGGAGTACAGCAGTGCGCTGCC
RL-B            GTTTGCGGCAGCGCAGCGCCGTACTGTGCTGTCCCTCTCGGAGTACAGCAGTGCGCTGCC

CS-B            TGCCTGCCTGCGCATGAACTGGCTCGGAAAGGACGCGCTCCTAACCGAACGAACGAAATA
AC-B            TGCCTGCCTGCGCATGAACTGGCTCGGAAAGGACGTGCTCCTAACCGAACGGG---AATA
RL-B            TGCCTGCCTGCGCATGAACTGGCTCGGAAAGGACGTGCTCCTAACCGAACGGG---AATA

CS-B            GACCAACTCAAACTCGCAACTCACCTCGACTTGCTCTCCTCTGTGCGTGCAGGGTACCTG
AC-B            GACCAACTCGAACTCGCAACTCACCTCGACTCGCTCTCTTCTGTGCGTGCAGGGTACCTG
RL-B            GACCAACTCGAACTCGCAACTCACCTCGACTCGCTCTCTTCTGTGCGTGCAGGGTACCTG

CS-B            ATTCCCAAGGGCTGGAAAGTGCTTCCCCTGTTCCGGAACATCCACCACAACCCCGACCAC
AC-B            ATTCCCAAGGGCTGGAAAGTGCTTCCCCTGTTCCGGAACATCCACCCACACCCCGACCAC
RL-B            ATTCCCAAGGGCTGGAAAGTGCTTCCCCTGTTCCGGAACATCCACCACAACCCCGACCAC

CS-B            TTCCCCTCCCCTGAAAAGTTCGATCCTTCACGATTCG--AGGTCAGCATCATCACAGCCC
AC-B            TTCCCCTCCCCCGAAAAGTTCGATCCTTCACGATTCG--AGGTCAGCATCATAACAACCC
RL-B            TTCCCCTCCCCCGAAAAGTTCGATCCTTCACGATTCG--AGGTCAGCATCATAACAACCC

CS-B            TCTGTTTGACGAGTCTGCTTCGATTCGATTGATCATTATCTGATTATACGTTTTGGTTGC
AC-B            TCTATTTGACGAGCCTGCTTCGATTCGATTGATCATTATCTGATTATACGTTTTGGTTCG
RL-B            TCTATTTGACGAGCCTGCTTCGATTCGATTGATCATTATCTGATTATACGTTTTGGTTCG

CS-B            TGACTGC--AGGTGGCCCCCAAGCCCAACACGTTCATGCCGTTCGGGAACGGGACCCACT
AC-B            TGACTGC--AGGTGGCCCCCAAGCCCAACACGTTCATGCCGTTCGGGAACGGGACCCACT
RL-B            TGACTGC--AGGTGGCCCCCAAGCCCAACACGTTCATGCCGTTCGGGAACGGGACCCACT

CS-B            CGTGCCCCGGCAACGAGCTGGCCAAGCTGGAGATGCTCGTCCTCTGCCACCACCTCGCCA
AC-B            CGTGCCCCGGCAACGAGCTGGCCAAGCTGGAGATGCTCGTCCTCTGCCACCACCTCGCCA
RL-B            CGTGCCCCGGCAACGAGCTGGCCAAGCTGGAGATGCTCGTCCTCTGCCACCACCTCGCCA
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CS-B            CCAAGTACAGATGGTCCACCTCCAAGTCCGAGAGCGGCGTCCAGTTCGGCCCCTTCGCCC
AC-B             CCAAGTACAGATGGTCCACCTCCAAGTCCGAGAGCGGCGTCCAGTTCGGCCCCTTCGCCC
RL-B             CCAAGTACAGATGGTCCACCTCCAAGTCCGAGAGCGGCGTCCAGTTCGGCCCCTTCGCCC

CS-B            TCCCCATCAACGGCCTCCCCATGACCTTCACCCGCAAGGACGACAAGAACAAAGCCTGAG
AC-B             TCCCCATCAACGGCCTCCCCATGACCTTCACCCGCAAG---GACAAGAACAAAGCCTGAG
RL-B             TCCCCATCAACGGCCTCCCCATGACCTTCACCCGCAAG---GACGAGAACAAAGCCTGAC

CS-B            CCATCCATCCATCCATCCATCCATCGCCTCCTCCTCGTTCCCAAAGGGAAATCAACAGCG
AC-B             CCATCCATCCATCACCTCCTCC-TCGTTCCCAAAGGAAAATCAACAGCGCGATAGTGGCA
RL-B             TCCTCCTCGTTCCCAAAGGAAA-AT-----CAACAGCACGATAGAGGCGAC---------

CS-B            CGATAGAGGCGACGGCGTGGCACATGCGAGCGAGCTGGATCGGCTCCGTCCGTGTCCGTG
AC-B             AC----------------------------------------------------------
RL-B             ----------------------------------------------------------

CS-B            CCGGTCGCCGTCGGAGGGGACACTTGTAGCCGTGGGCGCCGCGGCGGGAGCGGGAGCGGG
AC-B             ----------------------------------------------------------
RL-B             ----------------------------------------------------------

CS-B            CTGGCGGAGGCCGATCGGAGAGAGCACGGGCCGTCAAAGTCCTTTGTACAGATTCTTCAG
AC-B             ----------------------------------------------------------
RL-B             ----------------------------------------------------------

CS-B            CCAGGACCATGACGGCTGGGCGAGACAAGACCAAGAAAAATCCCACTCTTCAAAA-ACAC
AC-B             ----------------------------------------------------------
RL-B             ----------------------------------------------------------

CS-B            ACACACGTAGGCATTCTCAGCTAGGGAGATCCGGCGCCACAAAGAAGT
AC-B             -------------------------------------------------
RL-B             -------------------------------------------------
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Appendix 3. Sequence alignment of 'D' genome of *TaCYP707A1*. CS-D, Genome D of Chinese Spring; AC-D, Genome D of AC Domain; RL-D, Genome D of RL4452. Sequences of genome B copy from Chinese Spring (Genbank ID: AB714576). Coding regions are highlighted in grey.

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CS-D            ----CCACCACCACCCA--TCTCCTCTCCTCGTCTTCCTCCTCGGACTCCTTCGGGCTCA
AC-D            ------------------------------------------------------------
RL-D            ------------------------------------------------------------

CS-D            TCCTTTGCACCTAACAGAAGCGAAGAGAGCCTTTCGTTGGCATGGCAGCTTTCATCCTCT
AC-D            ------------------------------GAATTCGATTATGGCTGCTTTCGTTCTCT
RL-D            ----------------------------------------ATGGCAGCTTTCGTTCTCT

CS-D            TCCTCTGCTTGCTCGTGCCGCTCGTCCTCGCGTGCGCCATCCGCGCCAGGAAGGGCGCCG
AC-D            TCCTCTGCTTGCTCGTGCCGCTCGTCCTCGCGTGCGCCGTCCGCGCCAGGAAGGGCGCCG
RL-D            TCCTCTGCTTGCTCGTGCCGCTCGTCCTCGCGTGCGCCGTCCGCGCCAGGAAGGGCGCCG

CS-D            GCGGCCGTTCGTCGTCGGGCGGCGGCAAGAAA---GGCAGCAGCAGCCTGCCGCTGCCGC
AC-D            CCGGGCGGGCGTCGTCGGGCGGCGGCGGCAAAAAGGGCGGCACCAGCCTGCCGCTGCCGC
RL-D            CCGGGCGGGCGTCGTCGGGCGGCGGCGGCAAAAAGGGCGGCACCAGCCTGCCGCTGCCGC

CS-D            CCGGGTCGATGGGGTGGCCGTACGTGGGCGAGACCACGCAGCTGTACTCCTCCAAGAACC
AC-D            CGGGGTCGATGGGGTGGCCGTACGTGGGCGAGACCACGCAGCTCTACTCCTCCAAGAACC
RL-D            CGGGGTCGATGGGGTGGCCGTACGTGGGCGAGACCACGCAGCTCTACTCCTCCAAGAACC

CS-D            CCAACGTCTTCTTCGCCAGGAAGCGGAACAAGTACGGGCCCATCTTCAAGACGCATATCC
AC-D            CCAACGTCTTCTTCGCCAGGAAGCGGAACAAGTACGGGCCCATCTTCAAGACGCACATCC
RL-D            CCAACGTCTTCTTCGCCAGGAAGCGGAACAAGTACGGGCCCATCTTCAAGACGCACATCC

CS-D            TCGGGTGCCCCTGCGTCATGGTGTCCAGCCCCGAGGCCGCGAAGTTCGTGCTTGTTACTC
AC-D            TCGGGTGCCCCTGCGTCATGGTGTCCAGCCCGGAGGCCGCCAAGTTCGTGCTCGTCACTC
RL-D            TCGGGTGCCCCTGCGTCATGGTGTCCAGCCCGGAGGCCGCCAAGTTCGTGCTCGTCACTC

CS-D            AGGCGCACCTCTTCAAGCCTACCTTCCCGGCCAGCAAGGAGCGGATGCTGGGTCCCCAGG
AC-D            AGGCGCACCTCTTCAAGCCTACCTTCCCGGCCAGCAAGGAGCGGATGCTGGGCCCCCAGG
RL-D            AGGCGCACCTCTTCAAGCCTACCTTCCCGGCCAGCAAGGAGCGGATGCTGGGCCCCCAGG

CS-D            CCATCTTCTTCCAGCAGGGGGACTACCACGCTCACCTCCGCCGTCTGGTCTCCCGCGCCT
AC-D            CCATCTTCTTCCAGCAGGGGGACTACCATGCCCACCTCCGCCGTCTCGTCTCACGCGCCT
RL-D            CCATCTTCTTCCAGCAGGGGGACTACCATGCCCACCTCCGCCGTCTCGTCTCACGCGCCT

CS-D            TCTCCCCCGAGGCCATCCGCGGCTCCGTCCCCGCCATCGAGGCTATCGCCCTCCGCTCCC
AC-D            TCTCTCCCGAGGCCATCCGCGGTTCCGTCCCTGCCATCGAGGCTATCGCCCTCCGCTCCC
RL-D            TCTCTCCCGAGGCCATCCGCGGTTCCGTCCCTGCCATCGAGGCTATCGCCCTCCGCTCCC
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CS-D            TCGGCTCCTGGGAAGACCTTCAAGTCAACACCTTCCAAGAGATGAAGACTGTGAGTG---
AC-D            TCGGCTCCTGGGAAGACCTGCAAGTCAACACCTTCCAAGAGATGAAGACTGTGAGTGCTT
RL-D            TCGGCTCCTGGGAAGACCTGCAAGTCAACACCTTCCAAGAGATGAAGACTGTGAGTGCTT

CS-D            ---CTTCTTCCTCTTCCATTCCCACTTGCTCTGCTTCCCTCTGCTCTGCTCTACTGCTAA
AC-D            CTTCTTCTTCCTCTTCCATTCCCGCTTGCTCTGCTTTCCTCTGCTCTGCTCTACTGCTAA
RL-D            CTTCTTCTTCCTCTTCCATTCCCGCTTGCTCTGCTTTCCTCTGCTCTGCTCTACTGCTAA

CS-D            ATAATTGGAGCTTGAGACTGATCCTTCTGTTGGTGTCGTGGCGCAGTACGCTCTGAATGT
AC-D            ATGATTGGAGCTCGAGGCTGATCCTTCTCTTGGTGTCGTGGCGCAGTACGCTCTGAATGT
RL-D            ATGATTGGAGCTCGAGGCTGATCCTTCTCTTGGTGTCGTGGCGCAGTACGCTCTGAATGT

CS-D            GGCATTGCTGTCCATCTTCGGGGAGGAGGAGATGCAGTACATCGAGGAGCTGAAGCAGTG
AC-D            GGCATTGCTGTCCATCTTCGGCGAGGAGGAGATGCAGTACATCGAGGAGCTGAAGCAGTG
RL-D            GGCATTGCTGTCCATCTTCGGCGAGGAGGAGATGCAGTACATCGAGGAGCTGAAGCAGTG

CS-D            CTACCTGACGCTGGAGAAGGGGTATAACTCGATGCCGGTGAACCTGCCGGGCACGCTGTT
AC-D            CTACCTGACGCTGGAGAAGGGGTACAACTCGATGCCGGTGAACCTGCCGGGCACGCTGTT
RL-D            CTACCTGACGCTGGAGAAGGGGTACAACTCGATGCCGGTGAACCTGCCGGGCACGCTGTT

CS-D            CCACAAGGCCATGAAGGCCCGGAAGCGGCTGGGCGCCATTGTGGCCCACATCATCTCGGC
AC-D            CCACAAGGCCATGAAGGCCCGAAAGCGGCTGGGCGCCATTGTGGCCCACATCATCTCGGC
RL-D            CCACAAGGCCATGAAGGCCCGAAAGCGGCTGGGCGCCATTGTGGCCCACATCATCTCGGC

CS-D            CCGGCGCGAGCGTGAGCGCGGGAGCGACCTCCTGGGCTCCTTCATGGACGGCCGCGAGGC
AC-D            CCGGCGCGAGCGCGAGCGCGGGAGCGACCTCCTGGGCTCCTTCATGGACGGCCGCGAGGC
RL-D            CCGGCGCGAGCGCGAGCGCGGGAGCGACCTCCTGGGCTCCTTCATGGACGGCCGCGAGGC

CS-D            GCTCACCGACGACCAGATCGCCGACAACGCCATCGGCGTCATCTTCGCCGCGCGCGACAC
AC-D            GCTCACCGACGACCAGATCGCCGACAACGCCATCGGCGTCATCTTCGCCGCGCGCGACAC
RL-D            GCTCACCGACGACCAGATCGCCGACAACGCCATCGGCGTCATCTTCGCCGCGCGCGACAC

CS-D            CACCGCCAGCGTGCTCACGTGGATGGTCAAGTTCCTCGGCGACAACCCCGCCGTGCTCAA
AC-D            CACCGCCAGCGTGCTCACGTGGATGGTCAAGTTCCTCGGCGACAACCCCGCCGTCCTCAA
RL-D            CACCGCCAGCGTGCTCACGTGGATGGTCAAGTTCCTCGGCGACAACCCCGCCGTCCTCAA

CS-D            AGCCGTCACCGTAAGTCGCCATCAACCAGCTGACCCGCTTGGTACCCGATCGAAAAGCAG
AC-D            AGCCGTCACCGTAAGTCGCCATCAACCAGCTGACCCGCTTGGTACCCGATCGAAAAGCAG
RL-D            AGCCGTCACCGTAAGTCGCCATCAACCAGCTGACCCGCTTGGTACCCGATCGAAAAGCAG

CS-D            CGACTGACCCGTGCATCCAACAATTAACAGGAAGAGCATGCCGAGATCGCGAGGGAGAAG
AC-D            TGGCTGACCCGTGCGTCGTACAATTAACAGGAAGAGCACGCTGAGATCGCGAGGGAGAAG
RL-D            TGGCTGACCCGTGCGTCGTACAATTAACAGGAAGAGCACGCTGAGATCGCGAGGGAGAAG
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CS-D            GCGTTGTCCGGCGAGCCGCTGTCGTGGGCCGACACGCGGCGGATGCGGATGACGGGCCGG
AC-D            GCGTTGTCCGGCGAGCCACTGTCGTGGGCCGACACGCGGCGGATGCGGATGACGGGCCGG
RL-D            GCGTTGTCCGGCGAGCCACTGTCGTGGGCCGACACGCGGCGGATGCGGATGACGGGCCGG

CS-D            GTGATCCAGGAGACGATGCGGGTGGCGTCCATCCTCTCCTTCACCTTCAGGGAGGCCGTG
AC-D            GTGATCCAGGAGACGATGCGGGTGGCGTCCATCCTCTCCTTCACCTTCAGGGAGGCCGTG
RL-D            GTGATCCAGGAGACGATGCGGGTGGCGTCCATCCTCTCCTTCACCTTCAGGGAGGCCGTG

CS-D            GAGGACGTGGAGTACCAAGGT----------GAGACATCAATCAACTTCGCGCGCGCGCG
AC-D            GAGGACGTGGAGTACCAAGGTGAGCAGAGCAGAGACATCAAT------------------
RL-D            GAGGACGTGGAGTACCAAGGTGAGCAGAGCAGAGACATCAAT------------------

CS-D            CGCTTTGGTCGTTTGCGGCAGCGCAGCGCCGTACTGTGCTGTCCCTCTCGGAGTACAGCA
AC-D            CGCTTTGGTCGTTTGTGGCAGCGCAGTGCTGTACTCCGCTGTCCCTCTCGGAGTACAGCA
RL-D            CGCTTTGGTCGTTTGTGGCAGCGCAGTGCTGTACTCCGCTGTCCCTCTCGGAGTACAGCA

CS-D            GTGCGCTGCCTGCCTGCCTGCGCATGAACTGGCTCGGAAAGGACGTGCTCCTAACCGAAC
AC-D            GTGAGCTGCCTGCCTGCCTGCGCATGAACTGGCTCGGAAAGGACGCGCTCCTAACCGAAC
RL-D            GTGAGCTGCCTGCCTGCCTG-GCATGAACTGGCTCGGAAAGGACGCGCTCCTAACCGAAC

CS-D            GGG---AATAGACCAACTCGAACTCGCAACTCACCTCGACTCGCTCTCTTCTGTGCGTGC
AC-D            GAACGAAATAGACCAACTCAAACTCGCAACTCACCTCGACTTGCTCTCCTCTGTGCGTGC
RL-D            GAACGAAATAGACCAACTCAAACTCGCAACTCACCTCGACTTGCTCTCCTCTGTGCGTGC

CS-D            AGGGTACCTGATTCCCAAGGGCTGGAAAGTGCTTCCCCTGTTCCGGAACATCCACCACAA
AC-D            AGGGTACCTGATTCCCAAGGGCTGGAAAGTGCTTCCCCTGTTCCGGAACATCCACCACAA
RL-D            AGGGTACCTGATTCCCAAGGGCTGGAAAGTGCTTCCCCTGTTCCGGAACATCCACCACAA

CS-D            CCCCG----------------------ACCACTTCCCCTCCCCCGAAAAGTTCGATCCTT
AC-D            CCCCG----------------------ACCACTTCCCCTCCCCTGAAAAGTTCGATCCTT
RL-D            CCCCG----------------------ACCACTTCCCCTCCCCTGAAAAGTTCGATCCTT

CS-D            CACGATTCGAGGTCAGCATCATA----ACAACCCTCTATTTGACGAGCCTGCTTCGATTC
AC-D            CACGATTCGAGGTCAGCATCATC----ACAGCCCTCTGTTTGACGAGTCTGCTTCGATTC
RL-D            CACGATTCGAGGTCAGCATCATC----ACAGCCCTCTGTTTGACGAGTCTGCTTCGATTC

CS-D            GATTGATCATTATCTGATTATACGTTTTGGTTCGTGACTGCAGGTGGCCCCCAAGCCCAA
AC-D            GATTGATCATTATCTGATTATACGTTTTGGTTGCTGACTGCAGGTGGCCCCCAAGCCCAA
RL-D            GATTGATCATTATCTGATTATACGTTTTGGTTGCTGACTGCAGGTGGCCCCCAAGCCCAA

CS-D            CACGTTCATGCCGTTCGGGAACGGGACCCACTCGTGCCCCGGCAACGAGCTGGCCAAGCT
AC-D            CACGTTCATGCCGTTCGGGAACGGGACCCACTCGTGCCCCGGCAACGAGCTGGCCAAGCT
RL-D            CACGTTCATGCCGTTCGGGAACGGGACCCACTCGTGCCCCGGCAACGAGCTGGCCAAGCT
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CS-D            GGAGATGCTCGTCCTCTGCCACCACCTCGCCACCAAGTACAGATGGTCCACCTCCAAGTC
AC-D            GGAGATGCTCGTCCTCTGCCACCACCTCGCCACCAAGTACAGATGGTCCACCTCCAAGTC
RL-D            GGAGATGCTCGTCCTCTGCCACCACCTCGCCACCAAGTACAGATGGTCCACCTCCAAGTC

CS-D            CGAGAGCGGCGTCCAGTTCGGCCCCTTCGCCCTCCCCATCAACGGCCTCCCCATGACCTT
AC-D            CGAGAGCGGCGTCCAGTTCGGCCCCTTCGCCCTCCCCATCAACGGCCTCCCCATGACCTT
RL-D            CGAGAGCGGCGTCCAGTTCGGCCCCTTCGCCCTCCCCATCAACGGCCTCCCCGTGACCTT

CS-D            CACCCGCAAG---GACA---AGAACAAAGCCTGAG------------CCA----------
AC-D            CACCCGCAAG---GACGACAAGAACAAAGCCTGAG------------CCATCCATCCATC
RL-D            CACCCGCGAG---GACGACGAGAACGAAGCCTGAG------------CCGTCCGTCCATC

CS-D            --TCCATCCATCACCTCCTCCTCGTTCCC-AAAGGAAAATCAACAGCACGATAGTGGCAA
AC-D            CATCCATCCATCGCCTCCTCCTCGTTCCC-AAAGGGAAATCAACAGCACGATAGAGGCAA
RL-D            CGTCCATCCATCGCCTCCTCCTCGTTCCCGAAAGGGAAATCAACCGCGCGATAGAGGCAA

CS-D            CGGCGTGGCACAT----GCGAGCTGGATCGGCTCCGTCCGTGTCCGTGGCGGTCGCCGTC
AC-D            C------------------------------------------------------------
RL-D            C------------------------------------------------------------

CS-D            GGAGGGGACACTTGTAGCTGTGGGCGCCGCGGCGGGAGCGAGAGCGCGCTGGCGGAGGCC
AC-D            ------------------------------------------------------------
RL-D            ------------------------------------------------------------

CS-D            GATCGGAGAGAGCACGGGCCGTCAAAGTCCTTTGTACATATTCTTCAGCCAGGACCATGA
AC-D            ------------------------------------------------------------
RL-D            ------------------------------------------------------------

CS-D            CGGCCGGGCGAGACAAGACCAAGAAAAATCCCACTCTTCAAAACACACACAGACGTAGGC
AC-D            ------------------------------------------------------------
RL-D            ------------------------------------------------------------

CS-D            ATTCTCAGCTAGGGAGATCCGGCGCCACAAAGAAGT
AC-D            -----------------------------------
RL-D            -----------------------------------
```

Appendix 4. Differential expression of brassinosteroid metabolism related probesets during seed germination.

		AR-0/D-0			AR-12/D-12			AR-24/D-24			AR-24/AR-24+ABA		
Gene	Probeset	Log2 FC	P-value	Linear FC	Log2 FC	P-value	Linear FC	Log2 FC	P-value	Linear FC	Log2 FC	P-value	Linear FC
DET	Ta.18512.1.S1_at	-0.12	0.34	-1.09	0.02	0.85	1.01	-0.02	0.76	-1.01	-0.05	0.60	-1.04
DET	Ta.26954.1.S1_at	-0.13	0.31	-1.10	0.07	0.38	1.05	0.10	0.64	1.07	0.02	0.91	1.02
DET	Ta.28682.1.S1_x_at	-0.08	0.74	-1.06	0.48	0.00	1.40	0.81	0.00	1.75	0.74	0.01	1.67
DET	Ta.28682.2.S1_at	0.02	0.89	1.01	0.26	0.01	1.20	0.70	0.00	1.62	0.33	0.05	1.26
DET	Ta.28682.2.S1_x_at	0.06	0.66	1.04	0.30	0.00	1.23	0.81	0.00	1.75	0.51	0.00	1.42
DET	Ta.28682.3.S1_x_at	-0.35	0.01	-1.28	0.68	0.00	1.60	1.02	0.00	2.02	0.55	0.11	1.46
DET	Ta.514.1.A1_at	0.11	0.40	1.08	-0.21	0.08	-1.16	-0.44	0.07	-1.36	-0.01	0.92	-1.01
DET	Ta.514.2.S1_s_at	0.10	0.25	1.07	-0.30	0.02	-1.23	-0.26	0.00	-1.20	-0.12	0.29	-1.09
DET	Ta.514.2.S1_x_at	-0.05	0.52	-1.04	0.06	0.35	1.04	-0.08	0.48	-1.05	-0.06	0.65	-1.04
DET	Ta.5674.1.S1_at	0.12	0.38	1.08	-0.01	0.93	-1.01	-0.31	0.04	-1.24	-0.15	0.15	-1.11
DET	TaAffx.105851.1.S1_s_at	-0.56	0.01	-1.47	0.02	0.93	1.01	0.08	0.72	1.06	-0.16	0.50	-1.12
***CYP90B1*(*DWF4*)**	Ta.27570.1.S1_at	0.28	0.02	1.21	1.25	0.00	2.38	0.84	0.00	1.79	0.01	0.92	1.01
***CYP90A1*(*CPD*)**	Ta.4986.1.S1_at	0.08	0.05	1.06	-0.24	0.00	-1.18	-0.83	0.01	-1.78	-0.27	0.18	-1.21
***CYP90D1*(*ROT3*)**	TaAffx.88122.1.S1_s_at	0.27	0.23	1.21	0.13	0.15	1.09	0.58	0.06	1.49	0.07	0.72	1.05
***CYP90D1*(*ROT3*)**	TaAffx.88122.2.S1_at	0.30	0.00	1.23	0.02	0.91	1.01	0.04	0.58	1.03	-0.05	0.58	-1.04
***CYP90D1*(*ROT3*)**	TaAffx.88122.3.S1_at	-0.17	0.36	-1.13	-0.01	0.83	-1.00	0.36	0.17	1.28	0.17	0.46	1.12

Appendix 5. Differential expression of brassinosteroid signaling related probesets during seed germination.

		AR-0/D-0			AR-12/D-12			AR-24/D-24			AR-24/AR-24+ABA		
Gene	Probeset	Log2 FC	P-value	Linear FC	Log2 FC	P-value	Linear FC	Log2 FC	P-value	Linear FC	Log2 FC	P-value	Linear FC
RAV	TaAffx.35357.1.S1_at	-0.10	0.41	-1.07	-0.31	0.00	-1.24	-0.07	0.67	-1.05	-0.02	0.89	-1.02
RAV	Ta.27316.1.S1_at	0.00	1.00	-1.00	0.41	0.12	1.33	0.11	0.46	1.08	-0.21	0.17	-1.16
RAV	Ta.21035.1.S1_at	0.25	0.13	1.19	1.72	0.00	3.29	1.47	0.01	2.76	0.20	0.49	1.15
RAV	Ta.27043.1.S1_at	0.23	0.11	1.18	0.22	0.18	1.17	0.17	0.24	1.12	0.02	0.77	1.02
BRI	TaAffx.86419.1.S1_at	-0.13	0.34	-1.10	-0.03	0.83	-1.02	0.05	0.83	1.04	0.08	0.77	1.06
BRI	Ta.9744.1.S1_at	0.04	0.82	1.03	-0.08	0.47	-1.06	0.22	0.09	1.16	0.43	0.00	1.35
BRI	Ta.18587.1.S1_at	0.74	0.15	1.67	-0.29	0.02	-1.23	-0.39	0.31	-1.31	-0.23	0.27	-1.17
BRI	Ta.18587.1.S1_x_at	0.54	0.17	1.46	-0.26	0.07	-1.20	-0.27	0.42	-1.21	-0.21	0.23	-1.15
BAK	Ta.4776.1.A1_a_at	0.63	0.01	1.55	0.24	0.05	1.18	0.68	0.01	1.60	0.63	0.00	1.55
BAK	Ta.4776.2.A1_at	-0.04	0.41	-1.03	0.13	0.24	1.09	0.07	0.55	1.05	0.11	0.48	1.08
BAK	Ta.4776.1.A1_x_at	0.18	0.17	1.14	0.25	0.09	1.19	0.31	0.17	1.24	0.49	0.06	1.41
BAK	Ta.6832.1.S1_at	0.70	0.01	1.63	0.61	0.03	1.53	0.61	0.01	1.53	0.25	0.12	1.19
BAK	Ta.9317.1.A1_at	-0.22	0.34	-1.17	0.25	0.20	1.19	0.54	0.11	1.46	0.17	0.53	1.13
BAK	TaAffx.110793.1.S1_at	-0.02	0.94	-1.01	-0.02	0.87	-1.02	-0.01	0.89	-1.01	-0.24	0.14	-1.18
BAK	Ta.5488.1.A1_at	-0.04	0.84	-1.03	0.56	0.01	1.48	0.18	0.17	1.13	-0.08	0.41	-1.05
BAK	Ta.4209.1.A1_at	0.05	0.73	1.03	0.07	0.35	1.05	0.07	0.46	1.05	0.14	0.29	1.10
BAK	TaAffx.123487.1.S1_at	-0.20	0.42	-1.15	0.15	0.26	1.11	0.32	0.03	1.25	0.40	0.07	1.32
BAK	Ta.12817.1.S1_at	0.37	0.01	1.29	0.22	0.08	1.17	0.19	0.33	1.14	-0.08	0.66	-1.06
BAK	TaAffx.83041.1.S1_x_at	-0.16	0.41	-1.12	-0.29	0.01	-1.22	0.11	0.05	1.08	0.05	0.78	1.03
BAK	TaAffx.24817.1.S1_at	0.06	0.55	1.04	-0.02	0.79	-1.01	-0.06	0.71	-1.04	0.05	0.51	1.03
BSK	TaAffx.83835.1.S1_at	-0.85	0.08	-1.80	0.33	0.01	1.26	0.30	0.41	1.23	0.23	0.53	1.17
BSK	Ta.27792.1.S1_at	-0.12	0.52	-1.09	0.31	0.00	1.24	0.34	0.13	1.27	-0.18	0.38	-1.13
BSK	TaAffx.114075.1.S1_s_at	0.17	0.42	1.12	-0.18	0.01	-1.13	-0.49	0.00	-1.40	0.07	0.45	1.05
BSK	TaAffx.114075.2.S1_s_at	-0.61	0.10	-1.53	-0.11	0.17	-1.08	-0.44	0.27	-1.36	0.59	0.27	1.50
BSK	Ta.25464.1.S1_at	0.23	0.02	1.18	0.22	0.03	1.17	1.38	0.00	2.60	1.23	0.00	2.35
BSU	Ta.9206.1.S1_at	0.58	0.00	1.49	0.21	0.16	1.16	0.12	0.36	1.09	0.03	0.82	1.02
BSU	Ta.20742.1.S1_a_at	0.37	0.14	1.30	0.18	0.39	1.13	-0.71	0.00	-1.64	0.00	0.98	-1.00
BSU	Ta.20742.1.S1_at	0.49	0.06	1.40	0.25	0.21	1.19	-0.70	0.01	-1.63	-0.19	0.28	-1.14
BSU	TaAffx.85764.1.S1_at	-0.17	0.29	-1.13	0.01	0.91	1.01	-0.05	0.05	-1.03	0.04	0.72	1.03
BIN	Ta.9422.2.S1_a_at	-0.54	0.19	-1.45	0.11	0.44	1.08	-0.08	0.79	-1.06	0.86	0.05	1.82
BIN	Ta.9422.3.S1_a_at	-0.24	0.45	-1.18	0.10	0.48	1.07	-0.13	0.49	-1.09	0.51	0.05	1.42
BIN	Ta.20401.1.S1_at	-0.32	0.29	-1.25	0.62	0.00	1.54	0.75	0.04	1.69	0.60	0.10	1.51
BIN	Ta.3003.1.S1_x_at	0.03	0.87	1.02	-0.16	0.04	-1.12	-0.10	0.49	-1.07	0.54	0.02	1.45
BIN	Ta.13447.1.S1_at	-0.10	0.65	-1.07	0.64	0.02	1.56	0.07	0.57	1.05	0.27	0.10	1.21
BIN	Ta.3019.1.S1_at	-0.05	0.43	-1.04	-0.07	0.34	-1.05	-0.13	0.16	-1.10	0.34	0.01	1.27
BIN	Ta.704.2.S1_at	0.09	0.17	1.06	-0.43	0.00	-1.34	-1.03	0.00	-2.05	-0.19	0.26	-1.14
BIN	Ta.704.1.A1_at	0.12	0.22	1.09	-0.27	0.01	-1.20	-0.63	0.01	-1.55	-0.06	0.70	-1.04
BIN	Ta.3003.1.S1_s_at	0.10	0.61	1.07	-0.14	0.13	-1.10	0.09	0.61	1.06	0.67	0.01	1.60
BZR	Ta.1101.1.S1_x_at	0.43	0.01	1.34	0.52	0.00	1.43	0.66	0.01	1.58	0.07	0.69	1.05

Appendix 6. Differential expression of ethylene metabolic metabolism related probesets during seed germination.

		AR-0/D-0			AR-12/D-12			AR-24/D-24			AR-24/AR-24+ABA		
Gene	Probeset	Log2 FC	P-value	Linear FC	Log2 FC	P-value	Linear FC	Log2 FC	P-value	Linear FC	Log2 FC	P-value	Linear FC
ACS	Ta.25755.1.A1_at	0.27	0.24	1.21	0.15	0.54	1.11	-0.03	0.63	-1.02	-0.04	0.53	-1.03
ACS	Ta.24076.1.S1_at	0.06	0.44	1.04	-0.02	0.41	-1.02	-0.10	0.59	-1.07	-0.27	0.24	-1.21
ACS	TaAffx.71241.1.A1_at	0.02	0.83	1.01	-0.01	0.83	-1.00	-0.36	0.05	-1.28	-0.52	0.01	-1.43
ACS	Ta.24341.1.S1_x_at	-0.14	0.39	-1.10	0.18	0.01	1.14	-0.32	0.18	-1.25	-0.05	0.82	-1.03
ACS	TaAffx.70620.1.S1_at	0.03	0.94	1.02	0.39	0.00	1.31	-0.36	0.04	-1.29	-0.17	0.26	-1.13
ACO	Ta.9107.1.S1_x_at	0.10	0.75	1.07	0.38	0.00	1.30	0.64	0.00	1.55	-0.04	0.57	-1.03
ACO	Ta.9107.2.S1_a_at	-0.39	0.23	-1.31	0.34	0.00	1.27	1.01	0.00	2.02	0.23	0.19	1.18
ACO	Ta.9107.2.S1_at	0.22	0.05	1.17	0.18	0.25	1.14	1.44	0.00	2.72	0.57	0.06	1.49
ACO	Ta.20570.1.A1_at	0.01	0.95	1.01	-0.06	0.41	-1.04	0.10	0.30	1.07	-0.11	0.22	-1.08
ACO	Ta.425.1.S1_s_at	0.15	0.16	1.11	-0.41	0.00	-1.33	-0.12	0.75	-1.09	0.49	0.26	1.41
ACO	Ta.425.2.S1_at	-0.16	0.17	-1.11	-0.20	0.25	-1.15	-0.07	0.24	-1.05	-0.14	0.04	-1.10
ACO	Ta.425.3.A1_at	0.02	0.78	1.01	-0.29	0.17	-1.22	-0.10	0.42	-1.07	-0.26	0.07	-1.20
ACO	TaAffx.59867.1.S1_at	-0.05	0.81	-1.03	-0.59	0.01	-1.51	-0.47	0.25	-1.38	0.91	0.07	1.88
ACO	TaAffx.59867.2.S1_at	-0.01	0.95	-1.01	-0.05	0.65	-1.03	0.03	0.86	1.02	0.03	0.81	1.02
ACO	TaAffx.100446.1.S1_at	-0.15	0.25	-1.11	0.16	0.13	1.12	1.09	0.01	2.13	0.58	0.11	1.49
ACO	Ta.18698.1.S1_at	0.02	0.84	1.02	-0.02	0.88	-1.02	0.19	0.35	1.14	0.05	0.78	1.03
ACO	TaAffx.80306.1.S1_at	-0.39	0.07	-1.31	-0.09	0.30	-1.07	0.01	0.96	1.01	-0.17	0.12	-1.12
ACO	Ta.22475.1.S1_at	0.07	0.23	1.05	0.09	0.49	1.06	0.05	0.52	1.03	0.03	0.64	1.02
ACO	Ta.28756.2.S1_at	0.19	0.10	1.14	-0.03	0.80	-1.02	-0.15	0.34	-1.11	0.00	1.00	-1.00
ACO	Ta.28756.2.S1_x_at	0.33	0.02	1.26	-0.15	0.23	-1.11	-0.12	0.32	-1.08	0.09	0.62	1.06
ACO	Ta.28942.1.S1_at	-0.31	0.00	-1.24	-0.26	0.18	-1.19	-0.09	0.46	-1.06	0.09	0.35	1.07
ACO	Ta.28942.1.S1_x_at	0.00	0.98	1.00	-0.01	0.94	-1.01	-0.04	0.65	-1.02	-0.02	0.69	-1.02
ACO	TaAffx.28482.1.S1_at	-0.02	0.82	-1.01	-0.10	0.39	-1.07	0.02	0.81	1.01	0.11	0.38	1.08
ACO	Ta.21049.1.S1_at	-0.16	0.56	-1.12	1.03	0.00	2.04	1.86	0.00	3.62	-0.61	0.02	-1.52
ACO	Ta.21049.2.S1_at	0.25	0.11	1.19	0.43	0.00	1.35	0.37	0.20	1.29	0.02	0.94	1.01
ACO	Ta.21049.2.S1_x_at	0.12	0.20	1.09	0.25	0.01	1.19	0.23	0.15	1.17	-0.02	0.90	-1.01
ACO	Ta.21049.3.S1_at	-0.12	0.20	-1.09	0.09	0.04	1.06	0.00	0.98	-1.00	-0.14	0.22	-1.11

Appendix 7. Differential expression of ethylene signaling related probesets during seed germination.

		AR-0/D-0			AR-12/D-12			AR-24/D-24			AR-24/AR-24+ABA		
Gene	Probeset	Log2 FC	P-value	Linear	Log2 FC	P-value	Linear	Log2 FC	P-value	Linear	Log2 FC	P-value	Linear FC
RAN	TaAffx.78135.1.S1_at	-0.50	0.01	-1.42	0.24	0.02	1.18	-0.06	0.61	-1.04	0.18	0.37	1.13
RAN	TaAffx.91780.1.S1_at	-0.32	0.04	-1.25	0.31	0.00	1.24	-0.15	0.35	-1.11	0.12	0.47	1.09
RAN	TaAffx.59221.1.S1_at	-0.20	0.39	-1.15	0.53	0.01	1.44	-0.15	0.23	-1.11	-0.06	0.53	-1.04
RAN	Ta.9007.1.A1_at	0.25	0.14	1.19	0.69	0.01	1.61	-0.35	0.04	-1.27	-0.20	0.15	-1.15
RAN	Ta.17942.1.S1_at	0.18	0.13	1.13	-0.08	0.20	-1.06	0.10	0.13	1.07	0.10	0.14	1.07
RTE	TaAffx.58832.1.S1_at	0.21	0.26	1.16	0.84	0.00	1.79	0.61	0.01	1.53	-0.27	0.06	-1.20
RTE	Ta.24477.1.S1_at	0.09	0.34	1.06	1.10	0.00	2.14	1.42	0.00	2.68	-0.17	0.13	-1.13
ETR	TaAffx.59457.1.S1_at	-0.08	0.42	-1.06	-0.18	0.37	-1.13	-0.12	0.01	-1.09	0.14	0.11	1.10
ETR	Ta.27490.1.S1_at	0.11	0.48	1.08	0.55	0.01	1.46	-0.49	0.10	-1.41	-0.09	0.49	-1.06
ERS	Ta.10139.1.S1_s_at	0.02	0.92	1.01	-0.06	0.52	-1.04	-0.13	0.42	-1.09	0.22	0.16	1.17
ERS	Ta.10139.1.S1_at	0.00	0.99	1.00	0.03	0.66	1.02	-0.02	0.86	-1.02	0.13	0.41	1.09
ERS	TaAffx.22824.1.S1_at	-0.18	0.59	-1.14	1.01	0.00	2.01	0.51	0.03	1.42	0.20	0.36	1.15
CTR	TaAffx.4129.1.S1_at	-0.01	0.99	-1.00	0.03	0.22	1.02	-0.51	0.01	-1.43	0.08	0.30	1.06
CTR	TaAffx.63981.1.A1_at	0.18	0.32	1.13	0.05	0.53	1.04	-0.54	0.01	-1.46	0.39	0.03	1.31
CTR	TaAffx.110897.1.S1_at	-0.12	0.50	-1.09	0.20	0.13	1.15	-0.50	0.02	-1.41	0.49	0.01	1.40
CTR	TaAffx.36754.1.S1_at	-0.17	0.03	-1.13	0.03	0.64	1.02	-0.03	0.89	-1.02	0.10	0.60	1.07
TPR	Ta.6777.1.A1_at	0.09	0.40	1.07	-0.58	0.00	-1.50	-0.61	0.00	-1.53	-0.45	0.00	-1.37
TPR	Ta.6777.3.A1_a_at	-0.19	0.22	-1.14	-0.52	0.00	-1.44	-0.46	0.01	-1.38	-0.39	0.00	-1.31
EIN2	TaAffx.80450.1.S1_at	-0.01	0.97	-1.00	-0.15	0.05	-1.11	-0.01	0.95	-1.01	0.01	0.93	1.01
ECIP	Ta.6056.1.S1_at	-0.14	0.49	-1.10	0.03	0.63	1.02	-0.43	0.04	-1.35	0.14	0.48	1.10
ECIP	Ta.6056.3.S1_at	-0.38	0.26	-1.30	-0.31	0.12	-1.24	-0.52	0.05	-1.44	0.05	0.77	1.04
ECIP	TaAffx.112184.1.S1_at	-0.09	0.59	-1.07	-0.02	0.77	-1.01	-0.34	0.03	-1.27	0.13	0.35	1.10
ECIP	Ta.6373.1.S1_at	0.04	0.91	1.03	-0.51	0.00	-1.42	-0.12	0.46	-1.09	0.94	0.00	1.91
EIN5	Ta.2712.3.A1_at	-0.04	0.80	-1.03	0.04	0.58	1.03	-0.01	0.95	-1.01	-0.12	0.41	-1.09
EIN5	Ta.2712.2.S1_at	0.35	0.05	1.28	-0.19	0.09	-1.14	-0.57	0.03	-1.49	-0.09	0.62	-1.07
EIN5	Ta.27926.2.S1_at	-0.08	0.74	-1.06	-0.01	0.77	-1.01	-0.06	0.54	-1.04	-0.15	0.16	-1.11
EIN5	Ta.27926.3.S1_a_at	-0.15	0.10	-1.11	-0.04	0.55	-1.03	-0.04	0.81	-1.03	-0.21	0.25	-1.16
EIN5	Ta.3954.2.S1_a_at	-0.13	0.64	-1.09	0.57	0.00	1.48	-0.04	0.80	-1.03	0.30	0.00	1.23
EIN5	TaAffx.105545.1.S1_at	0.00	1.00	1.00	0.26	0.16	1.20	-0.06	0.57	-1.04	-0.25	0.07	-1.19
EIN3	Ta.9677.2.S1_at	-0.44	0.07	-1.36	0.54	0.00	1.45	0.06	0.85	1.04	0.06	0.83	1.04
EIN3	Ta.9677.2.S1_x_at	-0.47	0.08	-1.39	0.50	0.01	1.41	0.12	0.62	1.09	0.12	0.65	1.09
EIN3	TaAffx.110715.1.S1_at	-0.13	0.62	-1.09	0.19	0.01	1.14	0.06	0.75	1.04	-0.12	0.53	-1.09
EIN3	TaAffx.110715.1.S1_x_at	0.01	0.94	1.00	0.22	0.13	1.16	-0.07	0.47	-1.05	-0.25	0.15	-1.19
EIN3	TaAffx.64208.1.A1_at	0.21	0.33	1.16	0.94	0.00	1.92	0.60	0.02	1.51	0.15	0.29	1.11
EIN3	TaAffx.83569.1.S1_at	-0.41	0.14	-1.33	0.39	0.01	1.31	0.15	0.39	1.11	0.33	0.38	1.25
EBF	Ta.13279.2.S1_a_at	-0.48	0.16	-1.40	1.12	0.00	2.17	0.47	0.13	1.39	0.29	0.39	1.22
EBF	Ta.13279.1.S1_a_at	-0.20	0.04	-1.15	1.06	0.00	2.08	0.25	0.06	1.19	0.20	0.17	1.15
EER	Ta.5517.1.S1_a_at	0.10	0.15	1.07	0.64	0.00	1.55	1.13	0.00	2.18	0.37	0.05	1.29
EER	Ta.5517.1.S1_at	-0.16	0.59	-1.11	0.63	0.00	1.55	1.02	0.00	2.02	0.23	0.19	1.17
EER	Ta.27514.1.S1_at	0.06	0.79	1.04	-0.01	0.97	-1.00	0.11	0.28	1.08	0.06	0.46	1.04
EER	Ta.5517.2.A1_a_at	0.31	0.06	1.24	0.92	0.00	1.89	1.35	0.00	2.55	0.47	0.17	1.38
EER	Ta.14391.1.S1_at	0.32	0.05	1.24	0.51	0.00	1.43	0.80	0.00	1.75	0.03	0.83	1.02
EER	TaAffx.29103.1.S1_at	-0.36	0.05	-1.28	-0.17	0.20	-1.12	-0.11	0.65	-1.08	0.07	0.80	1.05
EER	Ta.7559.2.S1_a_at	-0.09	0.73	-1.07	0.05	0.75	1.04	-0.15	0.44	-1.11	0.31	0.18	1.24
ERF	Ta.1136.1.S1_at	-0.24	0.11	-1.18	-0.50	0.00	-1.42	-0.41	0.03	-1.33	0.48	0.13	1.40
ERF	Ta.2344.1.S1_s_at	-0.19	0.25	-1.14	0.49	0.00	1.40	0.62	0.03	1.54	0.39	0.13	1.31
ERF	TaAffx.70539.1.A1_at	-0.05	0.69	-1.03	0.33	0.01	1.26	0.71	0.04	1.64	0.34	0.26	1.26
ERF	TaAffx.71051.1.S1_at	0.03	0.91	1.02	0.17	0.31	1.13	1.09	0.00	2.13	0.78	0.00	1.72
MPK3	Ta.236.1.S1_at	-0.08	0.55	-1.06	0.22	0.07	1.17	0.10	0.49	1.07	0.06	0.68	1.04
MPK6	Ta.23916.1.S1_at	-0.34	0.37	-1.27	0.17	0.05	1.13	0.40	0.18	1.32	0.59	0.12	1.50
MPK6	Ta.23916.1.S2_s_at	0.11	0.09	1.08	0.23	0.00	1.18	0.30	0.04	1.23	0.21	0.05	1.16

Appendix 8. Differential expression of cytokinin metabolism related probesets during seed germination.

		AR-0/D-0			AR-12/D-12			AR-24/D-24			AR-24/AR-24+ABA		
Gene	Probeset	Log2 FC	P-value	Linear FC	Log2 FC	P-value	Linear FC	Log2 FC	P-value	Linear FC	Log2 FC	P-value	Linear FC
IPT	Ta.27062.1.S1_at	0.12	0.52	1.08	-0.02	0.58	-1.01	-0.15	0.49	-1.11	-0.34	0.11	-1.27
IPT	Ta.27550.1.S1_at	-0.01	0.94	-1.01	-0.41	0.01	-1.33	-0.75	0.00	-1.69	-0.01	0.76	-1.01
LOG	Ta.10410.1.S1_at	0.27	0.22	1.20	-0.38	0.06	-1.30	-0.05	0.61	-1.03	-0.39	0.00	-1.31
LOG	TaAffx.92200.1.A1_s_at	0.60	0.02	1.52	0.11	0.51	1.08	0.11	0.70	1.08	-0.35	0.07	-1.28
LOG	TaAffx.12490.1.S1_at	0.43	0.07	1.35	0.43	0.12	1.35	0.86	0.00	1.82	-0.23	0.06	-1.18
LOG	TaAffx.52819.1.S1_at	-0.06	0.61	-1.04	-0.03	0.75	-1.02	1.17	0.17	2.24	0.98	0.25	1.97
LOG	TaAffx.92200.1.A1_at	-0.04	0.83	-1.03	-0.03	0.55	-1.02	-0.13	0.50	-1.10	-0.11	0.49	-1.08
CKX	Ta.24307.1.A1_at	-0.16	0.29	-1.12	-0.12	0.13	-1.08	0.09	0.17	1.06	0.10	0.13	1.07
CKX	Ta.24307.1.S1_at	0.06	0.59	1.04	-0.12	0.04	-1.09	0.01	0.89	1.01	0.17	0.02	1.12
CKX	Ta.4850.1.A1_at	0.06	0.86	1.04	0.10	0.23	1.07	0.41	0.00	1.33	0.20	0.07	1.15
CKX	Ta.4850.1.A1_s_at	-0.08	0.73	-1.06	0.01	0.88	1.01	1.12	0.00	2.17	0.85	0.01	1.80
CKX	Ta.4850.1.A1_x_at	-0.09	0.54	-1.07	0.04	0.43	1.03	0.39	0.03	1.31	0.12	0.38	1.09
CKX	Ta.7220.1.S1_at	-0.14	0.16	-1.10	0.07	0.57	1.05	0.07	0.59	1.05	0.13	0.41	1.10
CKX	Ta.7220.2.A1_at	-0.08	0.71	-1.05	0.05	0.63	1.03	0.49	0.11	1.40	0.22	0.38	1.16
CKX	Ta.8530.1.A1_at	0.15	0.61	1.11	0.16	0.12	1.12	0.04	0.70	1.03	-0.05	0.27	-1.04
cZOG	Ta.16527.1.S1_at	0.06	0.46	1.04	-0.08	0.52	-1.05	-0.02	0.80	-1.01	-0.26	0.04	-1.20
cZOG	Ta.22120.1.S1_at	-0.09	0.23	-1.06	0.21	0.06	1.15	-0.13	0.57	-1.09	-0.54	0.07	-1.45
cZOG	Ta.8495.1.A1_at	0.07	0.37	1.05	1.20	0.00	2.30	0.37	0.27	1.29	-0.22	0.49	-1.16
cZOG	TaAffx.17044.1.S1_at	-0.03	0.85	-1.02	1.35	0.00	2.55	2.66	0.00	6.32	1.15	0.02	2.22
cZOG	TaAffx.49246.1.A1_at	0.05	0.83	1.03	0.24	0.00	1.18	0.26	0.29	1.19	-0.18	0.04	-1.13
GLU	Ta.23909.1.S1_at	-0.78	0.01	-1.72	0.14	0.54	1.10	1.37	0.00	2.58	1.11	0.00	2.15
GLU	Ta.23909.1.S1_x_at	-0.59	0.03	-1.50	0.27	0.28	1.20	1.58	0.00	2.99	1.25	0.01	2.38
GLU	Ta.24442.1.S1_at	-0.01	0.94	-1.01	-0.06	0.54	-1.04	0.06	0.48	1.04	-0.22	0.06	-1.17
GLU	Ta.4601.2.S1_at	-0.04	0.68	-1.03	-0.13	0.12	-1.09	2.99	0.01	7.97	2.75	0.02	6.74
GLU	TaAffx.58753.1.S1_at	-0.05	0.37	-1.03	0.14	0.02	1.10	0.14	0.04	1.10	0.00	0.97	1.00
GLU	TaAffx.58753.2.S1_s_at	-0.07	0.18	-1.05	-0.07	0.56	-1.05	0.02	0.88	1.01	-0.06	0.65	-1.04
GLU	TaAffx.9117.1.S1_at	0.08	0.79	1.06	0.04	0.75	1.03	0.88	0.04	1.84	0.94	0.03	1.92

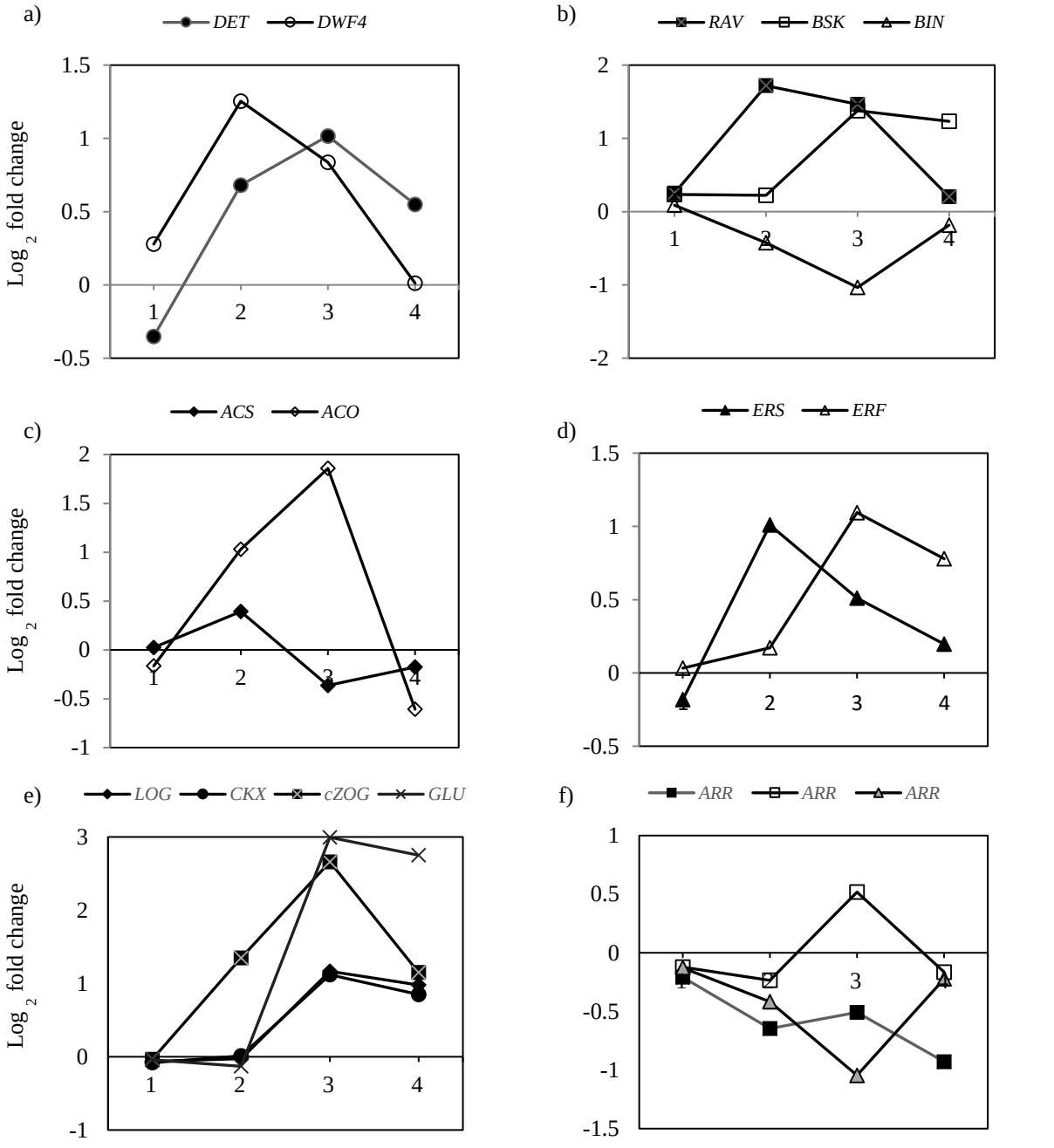
Appendix 9. Differential expression of cytokinin signaling related probesets during seed germination.

		AR-0/D-0			AR-12/D-12			AR-24/D-24			AR-24/AR-24+ABA		
Gene	Probeset	Log2 FC	P-value	Linear FC	Log2 FC	P-value	Linear FC	Log2 FC	P-value	Linear FC	Log2 FC	P-value	Linear FC
AHK	TaAffx.97084.1.S1_at	-0.22	0.52	-1.16	0.44	0.14	1.36	-0.03	0.84	-1.02	0.38	0.08	1.30
AHK	Ta.8903.1.A1_at	0.30	0.24	1.23	0.39	0.06	1.31	-0.25	0.18	-1.19	0.11	0.47	1.08
AHK	Ta.963.1.A1_at	0.62	0.03	1.54	0.21	0.31	1.16	0.15	0.44	1.11	0.37	0.09	1.30
AHK	Ta.963.1.A1_x_at	0.48	0.21	1.39	0.23	0.24	1.17	0.02	0.90	1.02	0.30	0.14	1.23
AHP	TaAffx.61545.1.S1_at	0.07	0.67	1.05	0.07	0.53	1.05	-0.24	0.12	-1.18	0.23	0.13	1.17
AHP	TaAffx.84903.1.S1_at	-0.14	0.13	-1.10	0.09	0.49	1.06	0.06	0.35	1.04	-0.01	0.94	-1.01
AHP	TaAffx.80712.1.S1_at	0.26	0.29	1.20	-0.81	0.02	-1.76	-0.34	0.00	-1.27	-0.41	0.02	-1.33
ARR	TaAffx.68652.1.S1_at	-0.23	0.52	-1.17	0.17	0.39	1.12	-0.19	0.26	-1.14	0.20	0.33	1.15
ARR	TaAffx.112134.1.S1_at	-0.25	0.13	-1.19	0.08	0.51	1.06	0.04	0.71	1.03	0.09	0.05	1.06
ARR	Ta.14705.2.S1_a_at	-0.49	0.19	-1.40	0.40	0.02	1.32	0.22	0.18	1.17	0.29	0.17	1.22
ARR	Ta.14705.3.S1_a_at	-0.57	0.16	-1.48	0.43	0.01	1.35	0.13	0.62	1.09	0.54	0.23	1.46
ARR	TaAffx.53132.1.S1_at	-0.51	0.04	-1.42	0.24	0.00	1.18	-0.25	0.34	-1.19	0.15	0.65	1.11
ARR	Ta.6529.2.S1_a_at	-0.31	0.55	-1.24	0.14	0.12	1.10	-0.15	0.45	-1.11	0.22	0.29	1.17
ARR	Ta.6529.2.S1_x_at	-0.47	0.34	-1.39	0.17	0.02	1.13	-0.05	0.77	-1.04	0.36	0.15	1.28
ARR	TaAffx.92416.1.S1_at	-0.13	0.50	-1.10	-0.06	0.33	-1.04	-0.05	0.74	-1.04	0.06	0.62	1.05
ARR	TaAffx.6013.1.S1_at	-0.10	0.59	-1.07	-0.05	0.66	-1.03	-0.13	0.32	-1.10	-0.06	0.16	-1.04
ARR	Ta.4054.2.S1_at	-0.14	0.52	-1.10	0.07	0.66	1.05	0.05	0.58	1.03	0.19	0.32	1.14
ARR	TaAffx.38632.1.S1_at	-0.23	0.14	-1.17	-0.20	0.20	-1.15	0.14	0.41	1.10	-0.02	0.83	-1.01
ARR	TaAffx.38489.1.S1_at	-0.21	0.07	-1.16	-0.65	0.00	-1.57	-0.51	0.01	-1.42	-0.93	0.01	-1.90
ARR	Ta.11777.1.S1_x_at	-0.12	0.13	-1.09	-0.23	0.03	-1.18	0.52	0.02	1.43	-0.17	0.32	-1.12
ARR	Ta.11777.3.S1_at	-0.09	0.23	-1.06	-0.06	0.65	-1.04	-0.04	0.82	-1.03	0.02	0.93	1.01
ARR	Ta.25890.1.S1_at	0.18	0.16	1.13	-0.16	0.03	-1.12	0.43	0.00	1.35	0.13	0.13	1.09
ARR	Ta.11777.2.S1_at	0.09	0.36	1.06	-0.22	0.01	-1.16	0.26	0.01	1.19	-0.05	0.63	-1.03
ARR	Ta.10408.1.S1_at	0.49	0.06	1.41	-0.31	0.07	-1.24	0.21	0.20	1.16	0.07	0.73	1.05
ARR	TaAffx.119928.1.A1_s_at	-0.32	0.13	-1.25	-0.01	0.92	-1.01	0.02	0.86	1.01	0.10	0.45	1.07
ARR	Ta.25556.1.S1_at	0.06	0.68	1.04	-0.12	0.00	-1.09	-0.16	0.05	-1.12	-0.07	0.32	-1.05
ARR	TaAffx.84365.1.S1_at	0.14	0.26	1.11	-0.37	0.01	-1.29	-0.48	0.04	-1.40	-0.30	0.10	-1.23
ARR	Ta.4054.1.A1_at	0.00	1.00	1.00	0.09	0.45	1.06	0.14	0.69	1.10	0.13	0.62	1.10
ARR	TaAffx.103392.1.S1_at	-0.09	0.63	-1.07	-0.12	0.06	-1.09	-0.09	0.35	-1.06	-0.29	0.13	-1.22
ARR	Ta.12422.3.S1_at	0.00	0.99	1.00	-0.06	0.45	-1.04	0.02	0.69	1.01	0.06	0.29	1.04
ARR	TaAffx.86800.1.S1_at	-0.57	0.16	-1.49	0.23	0.07	1.17	-0.01	0.98	-1.00	0.47	0.21	1.38
ARR	TaAffx.86800.1.S1_x_at	-0.36	0.20	-1.29	0.22	0.06	1.16	-0.12	0.55	-1.09	0.32	0.31	1.25
ARR	Ta.29499.1.A1_at	0.25	0.35	1.19	0.14	0.30	1.10	-0.33	0.25	-1.25	0.19	0.29	1.14
ARR	Ta.12422.1.S1_at	-0.13	0.53	-1.09	0.08	0.46	1.06	-0.01	0.96	-1.01	0.25	0.06	1.19
ARR	TaAffx.128530.1.S1_at	-0.78	0.11	-1.71	-0.30	0.00	-1.23	-0.70	0.01	-1.62	0.45	0.32	1.37
ARR	Ta.28006.1.S1_at	-0.13	0.60	-1.09	-0.42	0.00	-1.34	-1.05	0.01	-2.06	-0.22	0.33	-1.17
ARR	Ta.9719.1.S1_at	0.19	0.07	1.14	0.19	0.14	1.14	-0.39	0.00	-1.31	-0.03	0.76	-1.02
ARR	Ta.26699.1.A1_at	0.38	0.01	1.30	0.11	0.34	1.08	-0.08	0.18	-1.06	-0.13	0.15	-1.10
ARR	Ta.6075.1.A1_at	0.01	0.98	1.00	-0.21	0.10	-1.16	-0.65	0.04	-1.57	-0.39	0.06	-1.31
ARR	TaAffx.129077.1.A1_at	-0.04	0.74	-1.03	-0.02	0.79	-1.01	0.02	0.75	1.02	-0.03	0.55	-1.02

Appendix 10. Primer sequences used for real-time quantitative RT-PCR analysis

Probeset ID	Corresponding gene	Hormone type	Forward Primer	Reverse Primer
Ta.27570.1.S1_at	*DWF4*	Brassinosteroid	TTGAAGAACTGCGGGAGGAG	GCCGCAAGGTCTCGTTTATAAC
Ta.5674.1.S1_at	*DET*	Brassinosteroid	GTGTCTGGCGGACTGTCTTTG	CGAGCTGATGGGCTGTAGTGA
Ta.25464.1.S1_at	*BSK*	Brassinosteroid	TGGGCAGAAGGCTTATGAGTTG	GTCCAGCCATCCAGCAGAAAC
Ta.10074.1.S1_at	*MFT*	Brassinosteroid	CCTCTACACCCTCGTGATGA	GCACCACCACCTCACCTTTA
Ta.21049.1.S1_at	*ACO*	Ethylene	CTGTCGGTGGCGACGTTCTA	GCAAACTTGGTGCCCTGGTAG
Ta.9107.2.S1_a_at	*ACO*	Ethylene	GGCGCACGTACAGCTTCATG	CATCGCCTCCTTCTACAACCC
TaAffx.17044.1.S1_at	*cZOG*	Cytokinine	GCTGCCTGGCAAGCAGATC	TGAACTCGGACATCAGCTTGG
Ta.4601.2.S1_at	*GLU*	Cytokinine	CGATTCGGCCTCGTCTACAT	AACTGTCACCGTTGCGTTGTTA

Appendix 11. Fold changes (log2-scale) in the expression of BR metabolism (a) and signaling (b); ethylene metabolism (c) and signaling (d); cytokinin metabolism (e) and signaling (f) related probesets between after-ripened (AR) and dormant (D) seeds. Numbers on x- axis indicates 1 =AR-0/D-0 HAI, 2 = AR-12/ D-12 HAI, 3 = AR-24/ D-24 HAI, 4 = AR-24/ AR- 24HAI+ABA, HAI= hours after imbibition.



Appendix 12. List of abbreviations

ACS	1-aminocyclopropane-1-carboxylic acid synthase
ACO1	1-aminocyclopropane-1-carboxylic acid oxidase 1
NCED	9-cis-epoxycarotenoid dioxygenase
ABA8'OH	ABA 8'-hydroxylase
ABA	Abscisic acid
AAO	Abscisic acid aldehyde oxidase
ACO	1-aminocyclopropane-1-carboxylic acid oxidase
MAS5	Affymetrix microarray suite
AHK	Arabidopsis histidine kinase
AHP	Arabidopsis histidine-containing phosphotransmitter
ABA2	Alcohol dehydrogenase or Xanthoxin dehydrogenase
AR	After ripened
AHK2	Arabidopsis histidine kinase2
AHK3	Arabidopsis histidine kinase3
ARR	Arabidopsis response regulator
BAK	BRI1-associated receptor kinase
BIN	Brassinosteroid insensitive
BEE	Brassinosteroids enhanced expression
BZR1	Brassiazole resistant 1
BR	Brassinosteroids
BRI	Brassinosteroids insensitive
BSK	Brassinosteroids signaling kinases
BSU	BRI suppressor
BZR	Brassinazole-resistant
CNHR	Canada Northern Hard Red
CPSR	Canada Prairie Spring Red
CPSW	Canada Prairie Spring White
CWAD	Canada Western Amber Durum
CWES	Strong Canada Western Extra
CWHWS	Canada Western Hard White Spring
CWRS	Canada Western Red Spring
CWRS	Canada Western Red Spring
CWRS	Canada Western Red Spring
CWRW	Canada Western Red Winter
CWSWS	Canada Western Soft White Spring

CTAB	Cetyl Trimethylammonium Bromide
CKX	Cytokinin oxidase
CPD	Constitutive photomorphogenesis and dwarfism
CTR	Constitutive triple response
CK	Cytokinin
CKX2	Cytokinin oxidase2
CRE1/AHK4	Cytokinin response1
cZOG	*cis* zeatin-o-glucoside
DAA	Days after anthesis
DET2	De-etiolated2
DPA	Dihydrophaseic acid
DWF4	Dwarf4
EBF	EIN3-binding F box protein
ECIP	EIN2 c-terminus interacting protein
EER	Enhanced ethylene response
EIN	Ethylene insensitive
EIN3	Ethylene insensitive 3
EIN5	Ethylene insensitive 5
ERF	Ethylene-responsive element binding factor
ERS	Ethylene response sensor
ESTs	Expressed Sequence Tags
ET	Ethylene
ETR	Ethylene response
FAO	United nation food and agriculture organization
FAOSTAT	United nation food and agriculture organization statistics
CGR1	G Protein–Coupled receptor
GIM1	Germination insensitive to ABA mutant 1
GA	Gibberellin
GLU	Glucosidase
HAI	Hours after imbibition
IPT	Isopentenyltransferase
LOG	Lonely guy
MMT	Million metric tons
MD	Morphological dormancy
MPD	Morpho-physiological dormancy
MFT	Mother of ft and tfl1
MPK	Mitogen-activated protein kinase 3
NSY	Neoxanthin synthase

PA	Phaseic acid
PRE	Paclobutrazol resistance
PAGE	Polyacrylamide gel electrophoresis
PY	Physical dormancy
PD	Physiological dormancy
PCR	Polymerase chain reaction
PHS	Pre-harvest sprouting
QTLs	Quantitative Trait Loci
RAN	Responsive-to-antagonist
RAV	Related to ABI3/VP1
RGA	REPRESSOR OF ga1-3
RMA	Robust Multi-array Average
ROT3	Rotundfolia3
RTE	Reversion-to-ethylene sensitivity
SNPs	Single nucleotide polymorphism
7TM	Seven-transmembrane
TPR	Tetratricopeptide repeat
VDE	Violaxanthin de-epoxidase
ZEP	Zeaxanthin epoxidase